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Natural variation in plumage bacterial assemblages in two wild breeding passerines

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary of the following papers, which are referred to in the text by the Roman numerals:

I. Saag, P., Tilgar, V., Mänd, R., Kilgas, P., Mägi, M. 2011. Plumage bacterial assemblages in a breeding wild passerine: relationships with ecological factors and body condition. Microbial Ecology, 61: 740–749.

II. Saag, P., Mänd, R., Tilgar, V., Kilgas, P., Mägi, M., Rasmann, E. 2011. Plumage bacterial load is related to species, sex, biometrics and fledging success in co-occurring cavity-breeding passerines. Acta Ornithologica, 46(2): 191–201.

III. Saag, P., Kilgas, P., Mägi, M., Tilgar, V., Mänd, R. 2012. Inter-annual and body topographic consistency in the plumage bacterial load of Great tits. Journal of Field Ornithology, 83(1): 94–100.

IV. Kilgas, P., Saag, P., Mägi, M., Tilgar, V., Mänd, R. Plumage bacterial load increases rapidly during nest-building in a passerine bird. Journal of Ornithology, in press, doi:10.1007/s10336–011–0801–3.

V. Kilgas, P., Saag, P., Mägi, M., Edenberg, M., Tilgar, V., Mänd, R. Variation in assemblages of feather bacteria in relation to plumage coloration in female great tits (*Parus major*). Condor, accepted for publication.

Original publications are reproduced with the permission of the publishers.

I contributed to planning the work and collecting field data. I solely devised appropriate molecular and microbiological methods and performed all laboratory procedures and analyses, except plumage coloration analyses for paper V. I was primarily responsible for data analyses in papers I–III. I wrote the first drafts of papers I-III and contributed to the completion of all papers.

1. INTRODUCTION

Wild animals harbor a diverse community of bacteria and fungi (reviewed in Clayton 1999). Considering the important impact that symbiotic and parasitic microorganisms can have on their hosts (Hackstein & Van Alen 1996, Nuttall 1997), investigating microbe-host interactions may help to explain behavioral and reproductive variation within and between host populations. However, detailed research into the bacterial loads of wild animals and the influence of bacteria on hosts has been limited by the requirement for very complex methods. Recently, however, molecular and microbiological techniques have developed rapidly, and avian ecologists have adopted these methods with great enthusiasm (e.g. Burtt & Ichida 1999, Lucas et al. 2005, Shawkey et al. 2005). The number of papers indexed in the Thomson Reuters Web of Science dealing with feather bacteria increased approximately seven-fold between the last decade of 20th century and the first decade of the new century, clearly illustrating the explosion of interest in the topic. The evidence generated by this recent work suggests that plumage bacteria play an important role in shaping the life histories of wild birds (Clayton & Moore 1997, Burtt & Ichida 1999, Muza et al. 2000, Gunderson et al. 2009).

Birds make significant efforts to maintain plumage function and to control ectoparasite load. Preening and other forms of grooming are critical for limiting the abundance of feather lice and other arthropods (Hart 1997). Behaviors such as anting, dusting, sunning, and the inclusion of green vegetation in nesting material may also defend against ectoparasites and bacteria (Clayton 1999). Moreover, laboratory studies indicate that uropygial oil protects plumage – either chemically (Shawkey et al. 2003a, Ruiz-Rodriguez et al. 2009) or physically (Reneerkens et al. 2008) – against damage by feather-degrading bacilli (see also Møller et al. 2009).

Bird plumage can host various assemblages of bacteria and fungi, several of which are capable of degrading feather keratin (Sangali & Brandelli 2000, Lucas et al. 2003b, Riffel et al. 2003, Shawkey et al. 2003a). Bacterial damage to feathers may have important fitness consequences for wild birds. Plumage deterioration may result in decreased thermal insulation (Brush 1965) and aerodynamic efficiency (Swaddle et al. 1996). In the long-term, these negative effects might reduce parental survival and reproductive success; the latter via changes in parental condition (Burtt & Ichida 1999, Muza et al. 2000) or indirectly through the trade-off between reproductive effort and self-preening behavior (Burtt & Ichida 1999, Merilä & Hemborg 2000, Muza et al. 2000). Furthermore, feather-degrading bacteria could also affect plumage-based communication between birds by changing feather coloration. Given that plumage coloration is believed to reflect individual quality (Hamilton & Zuk 1982), bacterial-induced changes in this trait may reduce individual reproductive success by influencing social dominance and mate choice (Shawkey et al. 2007, Gunderson et al. 2009, Shawkey et al. 2009a, Ruiz.-de-Castaneda et al. 2012). At

the same time, many bacteria detected on feathers produce antimicrobial substances (Riley & Wertz 2002, Peralta-Sanchez et al. 2010) and may therefore play a role in protecting eggs from infections with pathogenic bacteria (Gunderson et al. 2008, Shawkey et al. 2009b, Peralta-Sanchez et al. 2010). However, certain feather-inhabiting bacteria may also act as pathogens (Bruce & Drysdale 1994). Little is known about interspecific antagonism and dominance within the bacterial communities inhabiting feathers, although such processes may play an important role in determining individual bird fitness. For example, when fast-growing generalist bacterial species (dominants) are present in a community, they might depress overall bacterial diversity (Martin-Platero et al. 2006, Soler et al. 2008, Peralta-Sanchez et al. 2010).

Feather-degrading bacteria probably associate with all species of wild birds (Burtt 2009), and within-species many, if not all, individuals harbor such bacteria in their plumage (Gunderson et al. 2009, Peele et al. 2009). However, to date, relatively few studies have examined the factors that shape feather-degrading bacterial assemblages in avian plumage (see Muza et al. 2000, Shawkey et al. 2003a, Burtt & Ichida 2004, Cristol et al. 2005, Lucas et al. 2005, Saranathan & Burtt 2007). The variability of keratinolytic bacterial assemblages colonizing bird plumage is most likely related to avian feeding behavior (e.g., ground versus canopy birds – Burtt & Ichida 1999) and soil characteristics (Lucas et al. 2003b). As bacterial communities differ between habitats depending on soil parameters, it has been suggested that the structure of bacterial assemblages in plumage is habitat dependent, and that this might result in differences even between individuals of the same bird species (Burtt & Ichida 1999, 2004, Bisson et al. 2007). On the other hand, as characteristics of habitat might determine the exposure of birds to a particular set of bacteria, the evolution of habitat-specific defense mechanisms might be promoted (Ruiz-Rodriguez et al. 2009). Habitat differences and feeding behavior in combination with specific defense mechanisms against feather-degrading bacteria might also result in different bacterial loads on different body parts (Burtt & Ichida 1999). However, there is shortage of published evidence concerning the habitat-dependent fitness consequences of feather degrading bacteria. Similarly, only a few studies have described seasonal changes in plumage bacteria assemblages (Burtt & Ichida 1999), e.g. during the pre-breeding and breeding periods (Bisson et al. 2009).

To summarize the state of current knowledge, it appears fair to conclude that while many valuable studies have been conducted (Table 1), there remains a shortage of information about many aspects of feather bacteria ecology. The largest gaps in our knowledge are in understanding how plumage bacterial assemblages are related to a bird's sex, body condition and breeding effort, as well as to habitat and season. Such knowledge is crucial for planning experiments to determine and understand the causality of previously reported relationships: whether and how feather-bacteria influence a bird's body condition, breeding success and survival. In order to generalize current findings, more comparable data from different study systems and bird species are required.

Table 1. Previously described relationships between ecological and life-history traits and feather-degrading (FDB) and other plumage-inhabiting bacteria in birds.

Source of variation	Studied bacterial parameter	Effect direction	Reference
Inter-individual differences			
Sex	FDB abundance	various effects	Gunderson et al. *J. Avian Biol.* 2009
	FDB and/or total bacterial abundance	females > males	Lucas et al. *Mol. Ecol.* 2005; Møller et al. *Fun. Ecol.* 2009; Czirjak et al. *Microb. Ecol.* 2010
Brood size	Total bacterial abundance	positive correlation	Lucas et al. *Mol. Ecol.* 2006
	FDB diversity	positive correlation	Lucas et al. *Mol. Ecol.* 2005
	Total bacterial diversity	effect on FDB assemblage structure	Lucas et al. *Mol. Ecol.* 2005
Body mass	FDB abundance	various effects	Gunderson et al. *J. Avian Biol.* 2009
Preening gland size	FDB abundance	positive correlation	Møller et al. *Fun. Ecol.* 2009
Intra-individual differences			
Body parts	FDB abundance	venter > dorsum	Burtt & Ichida *Auk* 1999
Feather regions	Total bacterial abundance	distal > proximal	Muza et al. *Wilson Bull.* 2000
Feather color	Feather resistance to FDB	no color effect	Mahler et al. *Ibis* 2010
	Feather resistance to FDB	black > white	Goldstein et al. *Auk* 2004; Gunderson et al. *J. Avian Biol.* 2008; Ruiz-de-Castaneda et al. *Biol. J. Linn. Soc.* 2012
	Feather resistance to FDB	light > dark	Grande et al. *Ardeola* 2004
	FDB abundance	color effect	Gunderson et al. *J. Avian Biol.* 2009; Burtt et al. *Biol. Lett.* 2011
Feather hue	FDB abundance	negative correlation	Shawkey et al. *Naturwissenschaften* 2008
Feather chroma	FDB abundance	positive correlation	Shawkey et al. *Am. Nat.* 2007

Table 1 (Continued). Previously described relationships between ecological and life-history traits and feather-degrading (FDB) and other plumage-inhabiting bacteria in birds.

Source of variation	Studied bacterial parameter	Effect direction	Reference
Behavioral activities			
Anting	Total bacterial abundance	no effect	Revis & Waller *Auk* 2004
Molt	Total bacterial abundance	no effect	Giraudeau et al. *J. Avian Biol.* 2010
Preening	FDB abundance	negative effect on FDB growth	Shawkey et al. *J. Avian Biol.* 2003
Foraging habit	FDB abundance	ground feeding > canopy birds	Burtt & Ichida *Auk* 1999
Migration	Total bacterial diversity	migratory > sedentary	Bisson et al. *Microb. Ecol.* 2009
Environmental variables			
Sunlight intensity	Total bacterial abundance	negative correlation	Saranathan & Burtt *Wilson Journal of Ornithology* 2007
Habitat	FDB diversity	various effects	Bisson et al. *Microb. Ecol.* 2007
	FDB abundance	salt marsh > freshwater marsh	Peele et al. *Auk* 2009
	FDB activity	humid > arid	Burtt & Ichida *Condor* 2004
Season	FDB abundance	winter > summer	Burtt & Ichida *Auk* 1999
Other Factors			
Breeding pair	FDB and/or total bacterial abundance	intrapair correlation	Lucas et al. *Mol. Ecol.* 2005; Gunderson et al. *J. Avian Biol.* 2009
Population differences	FDB and total bacterial abundance	positive correlation with breeding colony size	Møller et al. *Fun. Ecol.* 2009; Czirjak et al. *Microb. Ecol.* 2010
Species differences	Total bacterial diversity	various effects	Shawkey et al. *Waterbirds* 2006
Antibacterial substances (AS) in preening oil	FDB abundance and keratinolysis	AS inhibits FDB and keratinolysis, produced by symbiotic bacteria	Martin-Platero et al. *Appl. Environ. Microbiol.* 2006; Martin-Vivaldi et al. *J. Avian Biol.* 2009; Ruiz-Rodriguez et al. *J. Exp. Biol.* 2009; Martin-Vivaldi et al. *Proc. Roy. Soc.* 2010
Captivity	FDB activity	no effect	Cristol et al. *Auk* 2005

The general aim of my thesis was to explore the extent, pattern and correlates of natural variation in numbers and species richness of bacterial assemblages inhabiting the plumage of two co-occurring cavity-breeding passerines, Great Tits (*Parus major*) and Pied Flycatchers (*Ficedula hypoleuca*), which share habitat and nesting site requirements, but differ markedly in certain other life-history traits. Specifically, I studied the following questions:

(i) Do plumage bacterial assemblages differ (both in terms of density and species richness) between these two bird species breeding in the same area and using the same habitats and nest sites? Similar bacterial patterns in comparable bird species would suggest that the factors shaping bacterial communities (e.g., sex, habitat, season, parental effort etc.) act in similar ways in different bird species. On the other hand, inter-specific differences would indicate that some bacterial characteristics are species-specific;

(ii) Do plumage bacterial assemblages differ between body regions in the studied bird species? I expected that more bacteria would be recorded on the ventral than the dorsal regions, because the ventral part usually comes into greater contact with potential contamination sources, including the ground (Burtt & Ichida 1999). I also expected intra-individual differences in plumage bacterial load between different body parts to be consistent through time;

(iii) Do plumage bacterial assemblages differ between habitats? I expected that bacterial load would be higher in more heterogeneous (e.g., deciduous forest) than more homogeneous habitats (e.g., coniferous forest);

(iv) Do plumage bacterial assemblages vary through the breeding season? I expected bacterial load to increase during the breeding season due to the seasonal increase in air temperature (Burtt & Ichida 1999, Peele et al. 2009), the increasing time of exposure to contamination sources, and potentially due to the cumulative effects of reproductive effort on individual condition (Lucas et al. 2005);

(v) Do plumage bacterial assemblages vary between years? I expected that different climatic conditions in different years would influence bacterial abundance to some extent. At the same time, if bacterial abundance reflects individual quality (e.g., resistance to bacterial infestation), consistent differences between individuals might be expected despite annual variability.

(vi) Do plumage bacterial assemblages differ between sexes? I expected females to harbor relatively higher loads of bacteria on their plumage than males, because of the more diverse reproductive activities undertaken by females (Lucas et al. 2005);

(vii) Are plumage bacterial assemblages related to parental body condition and reproductive output? I expected both relationships to be negative, due to possible negative influence that plumage bacteria may exert on host (see above);

(viii) Are bacterial assemblages related to plumage coloration? I expected high bacterial load to be associated with less bright/colorful plumage, because bacteria may damage feather structure (Shawkey et al. 2007, Gunderson et al. 2009, Shawkey et al. 2009a, Ruiz-de-Castaneda et al. 2012).

2. MATERIAL AND METHODS

2.1. Study system

The Great Tit (*Parus major*) is a small (18–19 g) insectivorous passerine, which is common throughout the palearctic region (Cramp & Perrins 1993). Great Tits mainly forage in the tree canopy during the breeding period, feeding mostly on Lepidoptera and sawfly larvae and spiders (Cramp & Perrins 1993, Gosler 1993). The species is only partly migratory in the study region (Vilbaste 1994), and if it migrates, then it does not usually travel very long distances. Many individuals spend the winter in the vicinity of their breeding area, usually close to human settlements (Perrins 1979, Gosler 1993, Vilbaste 1994). Great Tits inhabit various types of woodland, but prefer deciduous forests for breeding (Mänd et al. 2005). The species is a facultative double brooder, and in the study area 40–70% of females lay a second clutch during the breeding season (Mägi & Mänd 2004). In the study area, nest building starts at the end of April. The first breeding period lasts approximately from the end of April to the middle of June, while the second breeding period lasts from the end of June to the end of July. First clutches usually contain 9–12 eggs (Hõrak et al. 1995), while second clutches are generally smaller (Mägi & Mänd 2004).

The Pied Flycatcher (*Ficedula hypoleuca*) is a small (12–13 g) insectivorous migratory passerine, that occurs throughout much of northern and eastern Europe (Lundberg & Alatalo 1992). Pied Flycatcher diet consists of various arthropods, and birds forage both in trees and on the ground (Lundberg & Alatalo 1992). Pied Flycathers share the same habitats as Great Tits. Unlike Great Tits however, Pied Flycatchers do not lay two clutches. In the study area Flycatchers start to breed at least two weeks later than Great Tits, with breeding lasting from the end of May to the end of June. Pied Flycatcher clutches normally contain 6–7 eggs.

Besides sharing habitat preferences, both species breed in tree-holes and will also readily accept nest-boxes. Both species are also short-lived, with more than half of individuals breeding only once (Perrins 1979, Lundberg & Alatalo 1992). Hence, there are many similarities in the ecology of the studied species, but also several differences (see above).

All field studies were conducted in northern Europe, near Kilingi-Nõmme (58^{o} 7'N, 25^{o} 5'E), SW Estonia, in 2007 (only Great Tit) and 2008 (both species). The study area covers approximately 50 km^2 and contains a mosaic of two forest types – coniferous and deciduous (Figure 1).

Birds bred in wooden nest boxes with a cavity of 11 × 11 × 30 cm and an entrance diameter of 3.5 – 4.0 cm. Nest boxes were mounted on tree trunks at a height of 1.5 – 2.0 m and were distributed in both (coniferous and deciduous) habitats. The distance between neighboring nest boxes was 50–60 m. Nest boxes were cleaned to remove old nest material before the beginning of the breeding season.

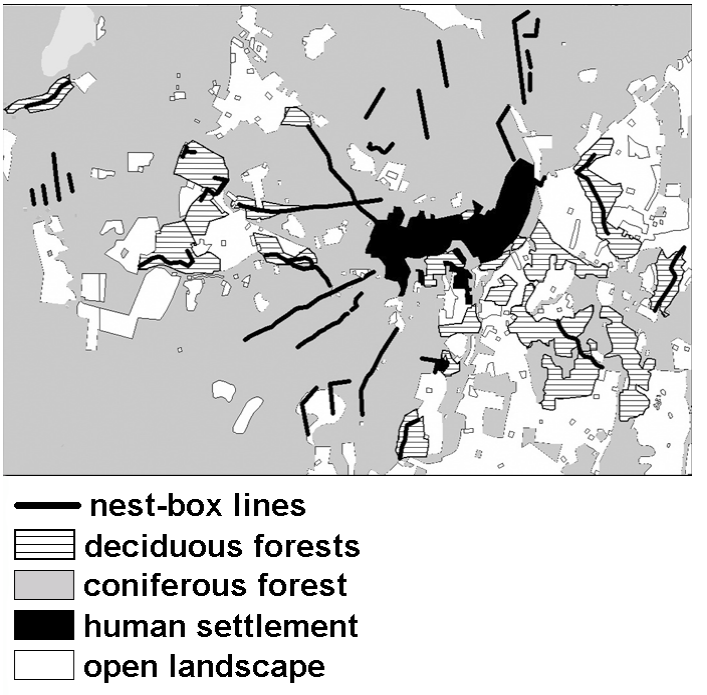

Figure 1. Schematic map of the study area.

2.2. Collecting field data and samples

All nest boxes were checked to record the laying date of the first egg, the clutch size and the hatching dates of both the first and second broods (in Great Tits). These two brood categories were clearly distinguishable since there is no overlap between the laying dates of the first and second clutches. Second broods were also confirmed by ringing data, as at least one adult from each breeding pair had already been captured in a particular year (see below). The number of fledglings per nest was recorded. Adults were captured using automatic traps at their nests during the second half of the nestling period. In 2007 (but not in 2008), female Great Tits were also captured during the pre-laying (nest-building) stage (at night when roosting in nest boxes, see paper **IV** for details). Male Great Tits were more distrustful of the traps, compared with females; therefore, the male sample size was much smaller and unequally distributed between breeding attempts. In the case of Pied Flycatchers, there was no significant difference between sexes in terms of trapping efficiency. Trapped adults were individually marked with numbered metal rings. Birds were weighed with a Pesola spring balance to a precision of 0.1 g, and tarsus measurements taken to the nearest 0.1 mm and wing length to the nearest 1 mm using digital callipers.

A fresh pair of examination gloves was used each time a new bird was handled. Within 30 sec after capture, about 5 ventral feathers were plucked from the right side of each bird's chest, using forceps cleaned in 96% ethanol, and placed in dry clean disposable microtubes to assess the number of bacteria on feathers. In 2008, but not in 2007, the same number of dorsal feathers (from the center of the back) was also collected from Great Tits. Feathers from the different body regions were placed in separate tubes. In both years, another sample of five feathers (from the same body regions) was collected from each bird to determine the species richness of feather-degrading bacterial assemblages. See papers **I**, **III** and **IV** for details.

2.3. Density measures of bacteria on feathers

Two distinguishable ecological types of bacteria occur in bird plumages: free-living and attached bacteria. Studies of bacterial communities in soil, water and sediment have demonstrated that free-living bacteria are usually more labile, while attachment provides a more stable environment and protection against grazing, chemical antibiotics or physical stress (see Ozawa & Yamaguchi 1986, Lucas et al. 2003a, Selje & Simon 2003 for references).

Free-living bacteria were washed out from the feathers using PBS (phosphate buffered saline) solution. To remove attached bacteria, feathers were sonicated in detergent solution in order to break down biofilms (Lucas et al. 2003a). Free-living and attached bacteria samples were stored and analyzed separately. Direct counts were performed with a flow cytometry machine (BD LSR II) that was calibrated to detect only bacterium sized tagged particles. DNA-binding dye SYBR Green was used for tagging. The number of feathers in each sample was recorded in order to calculate bacterial density per feather (as Lucas et al. 2005). See paper **I** for details.

2.4. Feather-degrading bacterial species richness

At the laboratory, feather-degrading bacterial assemblages were enriched (following Lucas et al. 2005). The ribosomal intergenic spacer analysis (RISA) method was used to analyze the structure of feather-degrading assemblages obtained in the enrichment cultures. Each RISA band is assumed to correspond to one bacterial species and is referred to as a phylotype (following Muyzer et al. 1993, Stach et al. 2003, to point out that RISA bands are anonymous). Thus, the band profile reflects bacterial assemblage structure, while the number of bands corresponds to the bacterial assemblage richness (Ranjard et al. 2000). In this thesis, term phylotype is used for referring RISA results, otherwise term species is used.

This method is fairly coarse (and therefore easily applicable and cheap) and does not allow ecological characteristics (free-living or attached) to be assigned

to particular bacterial phylotypes. Also, due to the specific limitations of RISA analysis, a certain portion of true species richness might be overlooked. However, general estimates of diversity should still be reliable (Ranjard et al. 2000). See paper **I** for details.

2.5. Feather color measures

Three additional feathers were plucked from each Great Tit individual from a standard position in the yellow breast plumage. Breast color was characterized by measuring chroma values using a spectrophotometer (Ocean Optics USB2000 with Ocean Optics DH2000 lamp). Chroma corresponds to color purity on a scale from 0 to 100, with 100 representing pure color. Chroma was measured in the visible range of 400–700 nm (Senar et al. 2008; Broggi and Senar 2009). The three feathers were put on top of each other and three measurements were obtained from each individual. The mean was used in analyses. Within-individual repeatability of the three chroma measurements was high ($r = 0.86$; according to Lessells & Boag 1987). See paper **V** for details.

2.6. Statistical analysis

Statistical analysis was performed using program Statistica (Statsoft, Tulsa, Oklahoma). Bacterial density estimates were log-transformed prior to analysis to satisfy the assumption of normality. General linear models (GLMs) were used to model variation in bacterial density. Akaike information criterion (AIC) was used for model selection in study **II**, otherwise predictors were removed from the initial models using a backward stepwise procedure, when non-significant ($P > 0.05$). See original papers for more details.

3. RESULTS

3.1. Species differences

The density of attached bacteria on feathers was lower in Pied Flycatchers than Great Tits (**II**). Free-living bacterial density and the mean number of feather-degrading bacterial phylotypes per bird did not differ significantly between the two bird species. In total, 16 bacterial phylotypes were detected from 110 Great Tit individuals and 12 bacterial phylotypes were found from 42 Pied Flycatchers (**II**).

3.2. The effect of body topography

In Great Tits, the densities of free-living, but not attached, bacteria on dorsal and ventral feathers were positively correlated. Correlation between the richness of feather-degrading bacterial communities in these body areas was also positive, but marginally non-significant (**III**). At the same time, the densities of both attached and free-living bacteria were significantly higher on dorsal than on ventral feathers in Great Tits, while the richness of feather-degrading bacterial communities on dorsal and ventral feathers did not differ significantly (**III**).

3.3. Habitat differences

In Great Tits, the densities of both free-living and attached bacteria were significantly higher in individuals breeding in deciduous habitat compared with those breeding in coniferous habitat (**I, IV**). A habitat difference in the number of phylotypes was only apparent during the nest-building stage – individuals carried fewer phylotypes in deciduous than in coniferous habitat (**I**). By contrast, no significant effect of habitat type on the plumage bacterial community was detected in the Pied Flycatcher (**II**).

3.4. Seasonal and annual changes

The density of both free-living and attached bacteria on Great Tit feathers was higher during the nest-building period than during either the first-brood or second-brood stage (**I**). Moreover, the precise stage of nest-building also had a significant effect on the density of attached bacteria on Great Tit feathers, with the density tending to increase towards the final stage of nest-building (**IV**). The decline in bacterial density between the nest-building stage and the first brood was confirmed by repeated measures analysis of the same individuals in the case of attached but not free-living bacteria (**I**). Later in the season, the density of attached bacteria increased significantly from the first to second brood (**I**).

The density of attached bacteria on feathers was generally higher in 2008 than in 2007 (**I**, **III**). In six females, captured in the two consecutive years, there was a significant positive correlation between the densities of attached bacteria in the different years (**III**).

3.5. Sex differences

Males of both Great Tits and Pied Flycatchers combined had fewer attached bacteria on their plumage than females (**II**).The difference remained when data from Pied Flycatchers were analyzed separately, and males also had fewer bacterial phylotypes on their feathers than females on average (**II**). Male Great Tits, however, supported on average more bacterial phylotypes than females, and the difference was more pronounced during second broods than during first broods (**I**). No significant sex differences were found in the density of free-living bacteria. Both free-living and attached bacterial densities were correlated within breeding pair members.

3.6. Bacteria, female condition and breeding output

In Great Tits, a positive relationship between free-living bacterial density and female mass was found during both the first and second breeding periods (**I**), while the density of attached bacteria was negatively related to female mass only during the first breeding period (**I**). No significant effect of body mass was found on feather-degrading bacterial phylotypic richness. In Pied Flycatchers, there was a significant positive correlation between the density of attached bacteria and the tarsus length of adult birds, and a negative correlation between the number of bacterial phylotypes and body mass (**II**). No significant effect of body mass was found on free-living bacterial density.

There was a negative relationship between the density of attached bacteria and the number of fledglings in Great Tits but not in Pied Flycatchers (**II**). There was no such relationship with clutch size (**II**).

3.7. Bacteria and plumage coloration

In Great Tits, plumage chroma during the nestling-feeding period was negatively correlated with the phylotypic richness of feather-degrading bacteria, but not with densities of attached or free-living bacteria (**V**). The change in chroma between the pre-laying and chick-rearing periods was correlated with the change in attached bacterial density during same time period. This means that in individuals whose attached bacterial densities increased over time, chroma simultaneously decreased (**V**).

4. DISCUSSION

4.1. Species differences

The total number of bacterial phylotypes observed in Great Tits (18 phylotypes from 290 individuals, **I**) and Pied Flycatchers (12 phylotypes from 42 individuals, **II**) was of approximately the same magnitude as recorded in other studied wild bird species: in House Finches *Carpodacus mexicanus* (13 phylotypes from 29 individuals) and in Eastern Bluebirds *Sialia sialis* (15 phylotypes from 4 individuals) (Shawkey et al. 2003a, Shawkey et al. 2005). However, the mean richness of bacterial phylotypes per individual (the sample consisting of five ventral feathers) was relatively low both in Great Tits (2.3 ± 1.6, **I**) and Pied Flycatchers (2.0 ± 1.0, **II**), compared with that recorded from European Starlings *Sturnus vulgaris* (7 ± 3) using exactly the same method (Lucas et al. 2005). Given the relatively high abundance of feather-degrading bacteria in soil (Lucas et al. 2003b), differences in foraging behavior could be a possible explanation for such interspecific differences in bacterial diversity. Great Tits and Pied Flycatchers mainly forage in the canopy, while starlings are mainly ground-foragers. Similarly, other authors have found feather-degrading bacteria to be more abundant in the plumage of ground-feeding birds compared with canopy birds (Burtt & Ichida 1999). However, one must consider that climatic and local soil or habitat parameters might also influence microbial communities, making it more difficult to compare different studies.

While the mean richness of bacterial phylotypes did not differ between the studied species, there was a significant difference in the density of attached bacteria: Pied Flycatchers harbored significantly fewer bacteria than Great Tits. However, on average Pied Flycatchers spend more time foraging on the ground than Great Tits (Haartman 1954, Alatalo & Alatalo 1979). Hence, the explanation of interspecific differences based on different foraging strategies does not seem to apply in a comparison of these two species. A possible alternative explanation is that Great Tit nests are much larger than those of Pied Flycatchers, contain more materials collected from the ground, and probably also harbor more feather-degrading bacteria. Also, it has previously been noted that migratory birds generally carry fewer bacteria on their feathers than sedentary birds (Bisson et al. 2009). The finding that the Pied Flycatcher, which is a long-distant migrant, has a lower average bacterial load than the mostly sedentary Great Tit, corresponds well with this general pattern. A third possible reason for the observed inter-specific differences in bacterial load is that the plumage coloration of these two species is very different, and feather color may influence the associated bacterial assemblage (Goldstein et al. 2004, Gunderson et al. 2008, Peralta-Sanchez et al. 2010, Peralta-Sanchez et al. 2011, see also Ruiz-de-Castaneda et al. 2012). However, the reasons underlying bacterial community differences seem to go beyond coloration, because Goodenough and Stallwood (2010) recently found that bacterial loads in nests differ significantly

even between bird species with as close biology and plumage coloration as Blue and Great Tits (*Cyanistes caeruleus et Parus major*), breeding in the same area.

Although it is impossible to draw firm conclusions based on a comparison of two species or from the scarce existing literature, the results presented here clearly indicate that bird species living in the same area and using the same habitats and nest sites may still harbor a significantly different bacterial load on their feathers. These findings are therefore consistent with the conclusion of Goodenough and Stallwood (2010) that despite substantial intraspecific variation in bacterial microflora in birds, there are significant interspecific differences even when host species are closely related, ecologically similar, sympatric, and construct very similar nests. Hence, plumage bacterial communities are not solely determined by the soil and habitat characteristics; certain species-specific factors are also important.

4.2. The effect of body topography

A significant correlation was found between the densities of free-living bacteria on the ventral and dorsal feathers of individual Great Tits (**III**). This matches the finding of Gunderson et al. (2009) who found a strong correlation between bacterial intensities on different body parts of the same Eastern Bluebird individuals. Such results are unsurprising, given that bacterial loads are known to be correlated even between the different parents within breeding pairs (Lucas et al. 2005, Gunderson et al. 2009, **I**). The most plausible explanation for this phenomenon is that pair-mates infect each other with bacteria via their common nest and brood (**I**). Hence, if bacteria can easily pass between frequently interacting individuals, it seems reasonable to expect that they can easily spread from one body region to another. It is noteworthy that the correlation between the densities of attached bacteria in different body regions was weaker than the relationship between free-living bacterial densities and was not significant (**III**). This corresponds to the finding (**I**) that correlation in the levels of contamination between members of a breeding pair is also much weaker for attached than free-living bacteria. Thus it seems that attached bacteria do not have the same propensity to spread as free-living bacteria.

A rather unexpected result of the study (**III)** was that densities of feather bacteria associated with Great Tits were higher on the dorsal than the ventral plumage. This is at odds with the prediction made in the Introduction. Significant body topographic variation in feather bacterial loads in birds has been also detected by earlier studies (Burtt & Ichida 1999, Bisson et al. 2007). However, the only study (Burtt & Ichida 1999) that examined the direction of this difference in several bird species (but not in Great Tits) reported that bacterial loads tend to be higher on ventral than dorsal feathers. Burtt and Ichida's (1999) result is somewhat more intuitive than that reported here from Great Tits, as the main source of bacteria is presumed to be the soil, and ventral

feathers certainly come into close contact with the ground and other contaminated substrates. Moreover, the fact that sunlight inhibits bacterial growth (Saranathan & Burtt 2007) should also decrease dorsal bacterial densities. However, the yellow chest in Great Tits is probably used to signal to conspecifics (Hõrak et al. 2001), and dirt accumulation can reduce plumage coloration (Surmacki & Nowakowski 2007). It has been shown that ornamented bird species devote more time to sanitation behaviours compared with non-ornamented species (Walther & Clayton 2005). Consequently, it is possible that Great Tits preen their chests more carefully than their backs. It is also relevant to note that Burtt and Ichida (1999) only examined the presence or absence of feather-degrading bacilli (mainly *Bacillus licheniformis*), which represent only a fraction of the entire diversity of feather bacteria. In contrast, non-selective methods were used in study **III** to estimate the total abundance of all types of feather-inhabiting bacteria. Thus, it is possible that the difference between ventral and dorsal body parts described by Burtt and Ichida (1999) may not hold universally for all ecological types of bacteria, and that other factors besides contact with the ground may be responsible for body topographic variation in the abundance of feather bacteria. The results described here also deliver a methodological message of great importance: in order to ensure comparability, the collection of feather samples must be standardized; otherwise the uneven distribution of bacteria in plumage could generate highly biased results.

4.3. Habitat differences

Habitat-related differences in bacterial density (**I**, **IV**) and phylotypic richness (**I**) in Great Tit plumage showed contrasting patterns: while the number of phylotypes per bird was higher in coniferous habitat, bacterial densities were higher in deciduous habitat. Hence, the habitat-related pattern of variation in feather bacterial densities conforms to the prediction made in the Introduction that birds inhabiting more diverse habitats harbor more bacteria on their feathers. However, habitat-related differences in the phylotypic richness of bacteria did not conform to this pattern.

A negative relationship between species diversity and abundance variables has previously been described for plant communities at relatively high productivity levels (Adams 2009). In bacterial communities, such a relationship can most plausibly be explained by interspecific antagonism and dominance. For example, many bacterial species produce antibacterial chemicals that suppress the growth of other bacteria (Riley & Wertz 2002, Peralta-Sanchez et al. 2010). When fast-breeding (or fast-growing) generalist species (dominants) are present in a community, they might depress overall bacterial diversity (Martin-Platero et al. 2006, Soler et al. 2008, Peralta-Sanchez et al. 2010). Although deciduous forests are generally much more diverse habitats than managed conifers and contain more diverse microhabitats in which birds might

become contaminated with bacteria, rapid infestation with dominant bacteria may inhibit colonization by other bacterial species.

The results of the study (**I**) indicate that the structure of bacterial communities may vary significantly between habitats even at small geographical scales. In this context it is noteworthy that the physiological condition of breeding Great Tits in the same study area was found to be worse in deciduous than in coniferous forest (Kilgas et al. 2006, 2007, Mägi et al. 2009). Hence, the possibility cannot be ruled out that the relatively high bacterial load associated with deciduous habitat may represent one of the factors contributing to the habitat difference in adult condition.

No significant effect of habitat type on plumage bacterial community was detected in the Pied Flycatcher (**II**). However, this may be a result of the significantly lower sample sizes collected for this species, compared with Great Tits (**II**).

4.4. Seasonal changes

One prediction in the Introduction was that the warm and humid environment of the nest and a reduced preening effort due to the increased need to devote more time to feeding offspring would cause a seasonal increase in bacterial abundance on feathers. This prediction was not supported by the results of the study. In fact, female Great Tits supported significantly more bacteria (both attached and free-living) in their plumage during the nest-building period than during the first and second broods (**I**).

Such a pre-laying peak in bacterial density may be caused by different mechanisms. First, it may be related to nest-building behavior, which brings birds into increased contact with the ground and nest materials (as suggested in **I**), while possibly leaving them little time for self-preening. Indeed, Great Tits spend considerable time on the ground during the nest-building phase to collect moss, dry grass, hair, wool etc. (Cramp & Perrins 1993). This explanation assumes that the density of bacteria in plumage increases rapidly during the short period of nest building. However, an alternative explanation is derived from the fact that Great Tits roost in cavities (nest boxes in our study area) during the winter. It is therefore possible that damp nest-boxes containing old nest material harbor a diverse bacterial community and leave tits highly infested in the spring (**I**).

The results of the study (**IV**) provide support to the first explanation. The pre-laying peak in bacterial density appears to be related to nest-building behavior: the densities of attached feather bacteria in female Great Tits increased during a fairly short period between nest initiation and nest completion. A similar trend, though not statistically significant, was found in the case of free-living bacteria. Both of these trends appeared to be very similar within both habitat types. It is noteworthy that the number of free-living

bacteria tended to increase when the nests were only 'half ready' – just after the first animal hairs appeared in the nests. However, the number of attached bacteria tended to increase once nests were completed (**IV**). This appears to be a logical result, as attached bacteria presumably take some time to become attached.

Thus these results indicate that the high pre-laying density of bacteria in the plumages of Great Tits is not a relic from an earlier period, but appears to be related to nest-building activity. Besides the reasons already listed above, it is perhaps noteworthy that the start of egg-laying in tits is often induced by a sudden spell of warm weather (pers. obs.), and birds may need to hurry with nest-building to such a degree that they have to significantly reduce self-preening during that time. Thus, increase of plumage bacterial load during the short period of nest-building may be one mechanism mediating the costs of nest-building on individuals. Recent studies have shown that nest building in Great Tits and Blue Tits is indeed an energy-demanding endeavor, the outcome of which may be related to individual quality (Tomás et al. 2006, Broggi & Senar 2009). Bacterial abundance in the plumage may decline in subsequent breeding stages due to regular preening activities and reduced contact with the soil.

These results indicate that the density of bacteria in plumage is variable and can fluctuate rapidly during the breeding season. This is also the first study to demonstrate that bacterial load in plumage increases significantly throughout the nest-building process in a free-living arboreal bird. Previously, fast changes in bacterial densities within a single breeding phase have been reported from chick-rearing European Starlings, where free-living, but not attached, bacterial densities increased in response to brood-size manipulation (Lucas et al. 2005).

However, more consistently with the initial prediction, there was also an increase in attached bacterial load between the first and second breeding attempts in the Great Tit (**I**). This can most plausibly be explained by the extended exposure of plumage to various kinds of microbes, while favorable climatic conditions prevailing in midsummer during the second broods might also enhance bacterial growth and density. Moreover, bacterial density may increase during the season as a result of the cumulative negative effects of reproductive effort on individual condition and preening activities during multiple breeding attempts.

4.5. Annual variation

The density of attached bacteria was significantly higher in 2008 than in 2007 (**I**). The most plausible reason for the difference between years was the considerably higher mean ambient temperature and level of precipitation in the early spring of 2008 (according to the Estonian Meteorological and Hydrological Institute), which probably favored bacterial growth (Burtt & Ichida 1999, 2004, Peele et al. 2009). Hence, the results of the study (**III**) support the predictions made in the Introduction.

A strong correlation was found between attached bacterial densities within the same individual females in two successive years (**III**). These results indicate that knowing the bacterial density on feathers of an individual in one particular year does not allow the absolute bacterial density on its feathers in the next year to be estimated. Rather, it allows the rank or magnitude of bacterial load compared with other individuals to be predicted, because this appears to remain fairly constant between years. Hence, bacterial loads of individuals are not merely the result of unpredictable contingencies, but reflect something about individual birds that remains constant over time. Unfortunately, owing to the small sample sizes, these data do not allow us to determine if this is related to individual differences in body condition (Clayton 1999), preening behavior (Walther & Clayton 2005), uropygial oil production or its composition (Martin-Vivaldi et al. 2009, Møller et al. 2009), properties of nesting areas, or to some other factor.

4.6. Sex differences

Comparing the different Great Tit parents within individual breeding pairs showed that bacterial density was correlated within-pair, but females had a tendency on average to support fewer bacterial phylotypes than males (**I**). As within-pair differences in parental physical activity are expected to be smaller than differences between pairs (Verhulst & Tinbergen 1997), and there is also an expected trade-off between parental provisioning effort and self-preening behavior, one would expect within-pair bacterial densities to be correlated (see also Lucas et al. 2005). An alternative explanation could be that breeding partners share the same breeding territory, are thus exposed to similar bacterial assemblages and may even infect each other with bacteria via their common nest and brood (**I**). Nonetheless, females of both studied species carried more attached bacteria on their feathers than males (**II**). In Pied Flycatchers, but not in Great Tits, females also carried more bacterial phylotypes per individual than males (**II**). Similarly, a higher bacterial load in females, compared with males, was found in European Starlings (Lucas et al. 2005) and Barn Swallows *Hirundo rustica* (Møller et al. 2009, Czirjak et al. 2010). This sex difference is again predictable, because females carry out more diverse activities during the different stages of reproduction (see Introduction). Furthermore, female Great

Tits and Pied Flycatchers in this study area roost in nest boxes, while males roost in the tree canopy. Therefore, females may come into more frequent contact with bacteria-contaminated nest material than males do. Secondly, given that on average male parents invest less effort in their broods than females do (Cramp & Perrins 1993, Verhulst & Tinbergen 1997, Sanz et al. 2000, Sisask et al. 2010), they can devote more time to self-preening.

However, the reasons for significant differences between partners in bacterial phylotypic richness are less clear. Unlike Pied Flycatchers, the number of bacterial phylotypes in the plumage of Great Tits was higher in males than in females (**I**). It seems counterintuitive that male Great Tits support more bacterial phylotypes than females (**I**), for the reasons listed above. However, it may be that because female Great Tits come into greater contact with soil and bacteria than males do, the dominant bacterial species suppress bacterial diversity in their plumage to a greater extent. While males can devote more time to self-preening than females, the application of preen waxes may inhibit the growth and density of some bacteria on feathers, but may not greatly reduce the species diversity of the microbial assemblages (Shawkey et al. 2003a). It is also noteworthy that the difference between the sexes in the mean phylotypic richness of bacteria was larger during second broods than during first broods (**I**). This was presumably related either to the higher ambient temperature during the second broods promoting bacterial development, or to the increased time available for preening in males due to the relatively small size of second broods. The latter is also supported by the finding that males indeed invest relatively less into smaller broods (Verhulst & Tinbergen 1997). However, it then remains unclear why a similar sex × breeding stage interaction was not revealed in bacterial density.

Together with the few results available from other studies, the results described here suggest that the abundance of feather bacteria in different passerines tends to be generally higher in females than in males. However, as repeatedly shown in this thesis, a greater density of bacteria on feathers does not necessarily also mean a higher number of bacterial phylotypes.

4.7. Bacteria, female condition and breeding parameters

As expected, a significant negative correlation was found between the phylotypic richness of plumage bacteria and adult body mass in Pied Flycatchers (**II**). There was also a negative correlation between attached bacterial density and female mass during the first broods in Great Tits (**I**). However, free-living bacterial density in the latter species was positively correlated with female mass (**I**). The first two findings are thus consistent with the assumption of less contaminated birds also being high-quality individuals that exhibit good body condition (see Introduction). Several studies have

reported similar negative associations between parameters of plumage bacterial assemblages and adult body condition in other species. For example, Gunderson et al. (2009) reported a negative correlation between plumage bacteria intensity and body condition of females in Eastern Bluebirds.

However, there is some inconsistency between the results of different correlative studies. For example, in the same study by Gunderson and colleagues (2009), the body condition of males was in fact positively correlated with bacteria intensity. The counterintuitive positive correlation between free-living bacteria and female mass in Great Tits (**I**) may indicate that female breeders with high body mass (which are usually high-quality individuals; e.g., Merilä & Wiggins 1997) spend more time and energy searching for calcium-rich snail shells for their eggs and food for their nestlings (Tinbergen & Dietz 1994), compared with poor-quality individuals, and are therefore more frequently in contact with possible sources of micro-organisms. Thus, although one certainly cannot draw general conclusions from a small number of correlative studies, the available evidence suggests that the negative relationship between bacteria and body condition may not be universal, and its precise manifestation may depend on various factors, including species-specific factors.

In Great Tits, a negative correlation also emerged between the density of attached bacteria in the plumage of parents and the number of fledged young (**II**). There was no such relationship with clutch size, suggesting that the negative relationship reflects processes occurring during the incubation and/or fledging stage. It may be that birds with high bacterial load are low-quality individuals that did not adjust their brood size to reflect their own reproductive potential and the availability of resources (e.g. Slagsvold & Lifjeld 1990); and such individuals are therefore unable to care adequately for all of their offspring. Indeed, the finding that attached bacterial density and female mass are negatively correlated in Great Tits (**I**) and other species (see discussion and references above) are all in good correspondence with this hypothesis. Secondly, the parents of the broods with high nestling mortality may have increased their parental provisioning effort in order to avoid starvation of their young, and therefore had less time for self-preening. Indeed, it has been previously shown in the same study area, that poor growth of Great Tit nestlings is associated with higher feeding frequency by parents (Mägi et al. 2009). Moreover, Lucas et al. (2005) demonstrated the existence of a trade-off between parental effort and self maintenance experimentally: European Starlings with enlarged broods had more free-living bacteria on their feathers than birds with reduced broods. A third potential explanation for the negative relationship between bacterial load in adults and the number of fledglings is that mortality of nestlings causes an increase in the number of bacteria in the nest (e.g., via the decay of corpses in the nest), and that adult birds that are in contact with such a nest accumulate higher levels of contamination. However, this explanation has to be considered unlikely, because no association between bacterial loads of adults and mortality of nestlings was detected up to the time of adult sampling (**I**).

4.8. Bacteria and plumage coloration

Feather chroma of female Great Tits tended to be negatively related to the phylotypic richness of feather-degrading bacteria during the chick-rearing period, but not during the period before egg-laying (**V**). At the same time, no associations between chroma and bacterial densities were found in either of the breeding stages. However, the seasonal change in attached bacterial densities was negatively correlated with seasonal change in chroma (**V**), implying that the increase in bacterial load was accompanied by a decrease in chroma.

To my knowledge, this is the first study on female birds reporting associations between carotenoid-based coloration and plumage bacteria. The only previous study found that male House Finches with redder plumage had lower feather-degrading bacterial loads (Shawkey et al. 2009a). The chroma of the yellow chest plumage of female Great Tits may signal some aspect of individual quality (Senar et al. 2008; Broggi and Senar 2009). Previous studies have found that plumage chroma is more sensitive to developmental perturbations and body condition than hue (Shawkey et al. 2003b, Senar et al. 2008). Chroma in Great Tits has been found to be unrelated to the carotenoid content of the feather, indicating that other mechanisms may be responsible for the variation in this trait (Senar et al. 2008).

Due to the correlative nature of the study (**V**) it remains unclear whether plumage bacteria directly affect plumage color or if these two variables are correlated with each other through some other factor. For example, it has previously been found that in Eastern Bluebirds feather degrading bacteria can directly affect structural plumage coloration (Shawkey et al. 2007, Gunderson et al. 2009). It is thus possible that feather-degrading bacteria affect the feather microstructure that is also involved in generating the carotenoid-based coloration (Shawkey & Hill 2005). It is also possible that feather-degrading bacteria could damage the carotenoid structure (as suggested by McGraw & Hill 2004).

Alternatively, it has been suggested that moulting may decrease the bacterial load on feathers (Burtt & Ichida 1999, but see Giraudeau et al. 2010). At the same time the chroma of new feathers may differ from old feathers reflecting nutritional conditions during the molt (e.g., Hill 2000). As Great Tits start moulting at the end of the breeding season, this process can sometimes overlap with the timing of late broods (e.g., Orell & Ojanen 1980). In the year when plumage coloration was analysed, females were captured before egg-laying, resulting in the desertion of initial nests (**V)** and presumably also in some delay to egg-laying in replacement nests. Therefore, the possibility cannot be excluded that the negative association between seasonal change in the density of attached bacteria and plumage color was caused by the fact that some individuals started moulting earlier than others and this affected both traits simultaneously.

In several species plumage coloration can also change between molts due to bleaching, dirt accumulation and abrasion. Such changes can also vary individually, so that in some birds plumage coloration increases, while in others it decreases or does not change with time (McGraw & Hill 2004). It is possible that some individuals are able to take better care of their plumage, thus affecting both plumage coloration and bacterial communities living on feathers. In conclusion, whatever the reasons may be, the results of this study showed that the yellow chest color in Great Tits is related to feather-degrading bacterial phylotypic richness and that there are parallel seasonal changes in plumage color and bacterial load.

4.9. Conclusions

This thesis revealed that bacterial assemblages on the feathers of breeding birds are correlated with many avian life-history traits: (i) bacterial load in deciduous habitat (which in this study area appears to be be less suitable for breeding than coniferous habitat judging from the physiological condition of adults and fledglings; Kilgas et al. 2006, 2007, Mägi et al. 2009) is higher than that in coniferous habitat (**I**); (ii) females (which carry out a greater diversity of activities during the breeding season) of both studied species carry more bacteria on their feathers than males (**I**, **II**); (iii) bacterial abundance and assemblage richness are correlated with bird body mass (**I**, **II**) and feather color intensity (**V**); and last but not least, (iv) a rapid increase in bacterial abundance occurs during the nest-building period (**IV**). Among other things, these findings suggest that a correlation is present between breeding effort and self-maintenance. Because of the correlative nature of studies in this thesis, one cannot ascertain whether this is a causal trade-off or just correlation, caused or mediated by other factors. However, taking into account earlier findings reported in literature – some of which were based on experimental manipulation (Lucas et al. 2005) (Table 1), or provided indirect evidence that feather-degrading bacteria might actively degrade feathers on living birds (Shawkey et al. 2007, Gunderson et al. 2009, Shawkey et al. 2009a) – the occurrence of such trade-offs seems likely.

Nonetheless, the generality of these findings remains unclear. Between-species and body topographic comparisons showed that certain species-specific factors play an important role, and that, consequently, formerly described patterns are unlikely to apply universally for all bird species and ecological types of bacteria (**II**, **III**). Further study is needed, involving more bird species from different regions and habitats, the use of manipulative approaches, the consideration of a wider range of variables, larger sample sizes, and consideration of bacterial species composition in samples.

SUMMARY

Microorganisms have been shown to play an important role in shaping the life-histories of animals. It has recently been suggested that feather-degrading bacteria influence the trade-off between parental effort and self-preening behavior in birds and may thus affect the individual fitness of birds. However, in order to design appropriate experiments to test this assumption, it is first necessary to collect more information on the basic parameters of these bacterial communities, such as patterns of natural variation in their density and composition, and environmental sources of infestation for birds.

In this thesis, a complex study design was used, coupling molecular and microbiological techniques with field-based collection of ecological and life-history data on two cavity-breeding passerines. The study was conducted in wild breeding populations of Great Tits (*Parus major*) and Pied Flycatchers (*Ficedula hypoleuca*) in a unique study area consisting of a mosaic of two very different habitat types in Estonia, northern Europe. While the species are similar in terms of their nest site requirements, they differ in certain other traits. Environmental and life-history correlates of natural variation in the abundance of free-living and attached bacteria and in the species diversity of feather-degrading bacteria inhabiting bird plumage were explored. Associations of bacterial infestation of feathers with bird body topography, plumage coloration, individual condition and reproductive output was also investigated. The density and species richness of bacterial assemblages was measured using flow cytometry and ribosomal intergenic spacer analysis (RISA).

The density of plumage bacteria was significantly higher in Great Tits than in Pied Flycatchers, providing evidence that the level of bacterial contamination differs even between co-occurring host species that share habitat, nest site and general foraging preferences (paper **II**).

The densities of both types of bacteria were higher on the dorsal than on the ventral feathers of Great Tits. The densities of free-living, but not attached, bacteria on the two body regions were highly positively correlated (paper **III**). This result highlights the importance of following a standard sample collection methodology in order to ensure comparability of data.

In Great Tits, the number of bacterial phylotypes per bird was higher in coniferous habitat, while bacterial densities were higher in deciduous habitat (paper **I**). This demonstrates that the structure of bacterial communities may vary significantly between habitats even at small geographical scales.

This is the first study to demonstrate that the density of attached bacteria on feathers may increase rapidly during the nest building process, and thereafter decline (papers **I** and **IV**). The density of free-living bacteria exhibited a similar pattern. The density of bacteria in bird plumage varied significantly between years; however, attached bacterial densities on the same individuals in successive years were correlated (paper **III**).

Bacterial species richness in Great Tits was sex dependent, with more diverse bacterial assemblages present on males than on females (paper **I**). At the same time, bacterial densities were higher in females than in males in both species (papers **I** and **II**). The latter finding supports the hypothesis that bacterial abundance on the plumage of the different sexes is related to the different activities carried out by males and females during the breeding season.

In Great Tits, free-living bacterial density was positively correlated with female mass; conversely, there was a negative correlation between attached bacterial density and female mass during the period of peak reproductive effort (paper **I**). In Pied Flycatchers, a negative correlation between parental body mass and the richness of feather-degrading bacterial phylotypes was found (paper **II**). In Great Tits, higher densities of bacteria in the plumage of parent birds were also associated with the production of fewer fledglings (paper **II**). These results indicate that feather-bacterial assemblages may be related to bird condition and reproductive effort.

During the chick-rearing period, feather chroma was negatively related to feather-degrading bacterial species richness in Great Tit females. Moreover, the seasonal change in the density of attached bacteria was accompanied by an opposite change in feather chroma (paper **V**). This is the first study to demonstrate the existence of associations between carotenoid-based coloration and bacterial assemblages in the plumage of female birds.

In conclusion, the findings contained in this thesis will supposedly improve our understanding of how bacterial assemblages on the feathers of breeding birds interact with environmental variables and host life history parameters. Such knowledge is crucial for future attempts to describe and understand the causality of such relationships.

SUMMARY IN ESTONIAN

Sulestiku bakterikoosluste pesitsusaegne varieeruvus kahel vabaltelaval värvuliseliigil

Mikroorganismid mängivad loomade elukäigu kujundamisel tähtsat rolli. Hiljuti on väidetud, et sulgilagundavad bakterid põhjustavad lõivsuhet lindude sigimispingutuse ja sulestiku eest hoolitsemise vahel ja võivad seega avaldada mõju lindude kohasusele. Selleks aga, et osata planeerida korrektseid eksperimente nimetatud väite kontrollimiseks, tuleb esmalt koguda rohkem informatsiooni sulestiku bakterikoosluste põhiparameetrite – arvukuse ja liigilise koosseisu – loodusliku varieeruvuse seaduspärasuste ning lindude bakteritega nakatumise peamiste allikate kohta.

Käesolevas töös kasutati kompleksset uurimistöö ülesehitust, ühendades omavahel molekulaarsete ja mikrobioloogiliste meetodite rakendamise ja ulatusliku ökoloogilise ja elukäigu-andmestiku kogumise kahe suluspesitseva värvulise – rasvatihase (*Parus major*) ja must-kärbsenäpi (*Ficedula hypoleuca*) – looduslikest populatsioonidest, mis paiknevad ühisel, kahe väga erineva elupaiga mosaiiki sisaldaval uurimisalal. Neid liike ühendavad sarnased pesapaigavaliku eelistused, kuid nad erinevad teineteisest mitmete muude omaduste poolest. Uuriti lindude sulgedel elavate vabaltelavate ja kinnitunud bakterite arvukuse ja liigilise mitmekesisuse seoseid elukeskkonna omaduste ja elukäigu parameetritega. Samuti pöörati tähelepanu sulebakterite koosluste omaduste seostele linnu kehapiirkonna, sulestiku värvuse, isendi konditsiooni ja sigimisedukusega. Bakterite asustustiheduse ja bakterikoosluste liigilise mitmekesisuse määramiseks kasutati vastavalt läbivoolutsütomeetriat ja ribosomaalse geenidevahelise speisserjärjestuse analüüsi (RISA).

Sulestikubakterite arvukus leiti rasvatihasel olevat tunduvat kõrgem kui must-kärbsenäpil. See näitab, et sulestiku bakteritega asustatuse tihedus võib erineda isegi koosesinevate, samu elupaiku, pesapaiku ja toitumisharjumusi jagavate linnuliikide vahel (artikkel **II**).

Mõlema eelmainitud bakteritüübi arvukus oli rasvatihase seljapoolel kõrgem kui kõhupoolel. Vabaltelavate (kuid mitte kinnitunud) bakterite arvukuses oli kahe kehapiirkonna vahel tugev positiivne seos (artikkel **III**). See leid rõhutab proovide kogumise metoodika standardiseerimise tähtsust.

Rasvatihastel leiti keskmine sulebakterite mitmekesisus isendi kohta olevat suhteliselt kõrgem okasmetsas, kuid bakterite arvukus oli kõrgem hoopiski lehtmetsas. See tulemus näitab, et sulestikubakterite koosluste omadused võivad erineda elupaikade vahel isegi väga väikesel geograafilisel skaalal.

Käesolevas uurimuses näidati esimest korda, et kinnitunud bakterite arvukus lindude sulestikus võib pesa ehitamise jooksul kiirelt tõusta ja hiljem uuesti langeda (artiklid **I** ja **IV**). Sarnased muutused leidsid aset ka vabaltelavate bakterite arvukuses. Bakterite arvukus sulestikus varieerus märkimisväärselt ka

aastate vahel, ehkki kinnitunud bakterite arvukused samadel isenditel eri aastatel olid omavahel korrelatsioonis (artikkel **III**).

Bakterite liigiline mitmekesisus rasvatihaste sulgedel oli soost sõltuv, kusjuures isastel olid bakterikooslused keskmiselt mitmekesisemad kui emastel lindudel (artikkel **I**). Samas aga oli bakterite arvukus kõrgem emaste sulgedel, kusjuures see seos kehtis nii rasvatihasel kui ka must-kärbsenäpil (artiklid **I** ja **II**). Neist leidudest viimane on kooskõlas hüpoteesiga, mille kohaselt bakterite arvukus eri soost lindude sulgedel on seotud sugupoolte erineva tööjaotuse ja sellest tingitud käitumisega sigimisperioodil.

Vabaltelavate bakterite arvukus rasvatihaste sulgedel oli positiivses seoses emaslinnu massiga, samas aga kinnitunud bakterite arvukus seostus sigimispingutuse tipp-perioodil emase massiga negatiivselt (artikkel **I**). Must-kärbsenäppidel leiti negatiivne seos linnu kehamassi ja sulebakterite liigilise mitmekesisuse vahel (artikkel **II**). Rasvatihastel oli bakterite arvukus kõrgem ka neil lindudel, kellel lennuvõimestus vähem järglasi (artikkel **II**). Need tulemused viitavad, et sulestiku bakterikooslused võivad sõltudalindude konditsioonist ja sigimispingutuse suurusest.

Poegade kasvatamise perioodil oli emaste rasvatihaste sulestiku eredus negatiivses seoses sulgi lagundavate bakterite liigilise mitmekesisusega. Veelgi enam, kinnitunud bakterite arvukuse muutusega pesitsushooaja jooksul kaasnes vastupidise suunaga muutus sulestiku ereduses (artikkel **V**). Tegu on esimese uurimusega, kus karotinoididel põhineva sulestikuvärvuse seos sulestiku bakteriflooraga leidis kinnitust emaslindude puhul.

Kokkuvõttes aitavad käesolevas töös kirjeldatud tulemused paremini mõista, kuidas sulestikku asustavad bakterikooslused on seotud elukeskonna omadustega ja lindude elukäigu parameetritega. Need teadmised on hädavajalikud edasiste uuringute planeerimiseks, et välja selgitada ja mõista kirjeldatud seoste taga peituvaid põhjuslikke mehhanisme.

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Saag, P.; Kilgas, P.; Mägi, M.; Tilgar, V.; Mänd, R. (2012). Inter-annual and body topographic consistency in the plumage bacterial load of Great Tits. Journal of Field Ornithology, 83(1): 94–100.

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Kilgas, P., Saag, P., Mägi, M., Edenberg, M., Tilgar, V., Mänd, R. (2012). Variation in assemblages of feather bacteria in relation to plumage coloration in female great tits (*Parus major*). Condor, xx–xx. [ilmumas]

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