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Interactions between feather-degrading bacteria and an avian host: zebra finches (*Taeniopygia guttata*)

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Interactions between feather-degrading bacteria and their avian host: zebra finches (*Taeniopygia guttata*)

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de la Faculté de Biologie et de Médecine

R. Wittek

Prof. Riccardo Wittek

It is not only fine feathers that make fine birds

-Aesop

(The Jay and the Peacock)

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Chapter 1

Introduction

Interactions between organisms are pervasive in nature (Herre *et al.* 1999). Organisms associate intra and / or inter-specifically, either by cooperating, or by engaging in conflict to exploit their interacting partner. Intra-specific conflict and competition in some organisms increases their reproductive activity (Barnes & Kuklinski 2005). Males of many species compete intra-specifically for mating opportunities with more and better females (Huber 2005). Individuals cooperate within their species to forage in a group, enhancing their individual benefit (Pekar *et al.* 2005) and their fitness. Fitness is defined as the survival and/or reproductive success of individuals (Clayton 1997). On the other hand, inter-species interactions are a major cause of adaptive radiation (Pellmyr & Leebens-Mack 2000) and affect the abundance, distribution, phenotype, and genotypic diversity of individuals (Strauss & Irwin 2004). Inter-species interactions can be classified based on their trophic levels in food chain, such as predator – prey interactions (Clark 2004). Another approach classifies inter-specific interactions, called *symbioses*, based on the fitness of the interacting partners. The interacting partners of symbiosis are termed *hosts* (those with larger body size) and *symbionts* (those with smaller body size) (Saffo 1993). Most symbionts occur as either *parasites*, which increase their own fitness at the cost of fitness of the host; *mutualists*, which increase theirs as well as their hosts' fitness; or *commensals*, where either ones' fitness is enhanced without any effect on the fitness of another (Ebert & Herre 1996; Cheney & Cote 2005; Jog & Watve 2005).

Symbionts are transmitted either horizontally or vertically through generations, mediated by inter- and intra-specific interactions of their hosts (Jog & Watve 2005). Parasitic symbionts enhance their fitness by increasing transmission, aided by their virulence (Frank 1992), causing a significant reduction in the body condition and fitness of hosts. Horizontally transmitted parasites generally have higher virulence, increasing host mortality. Although host mortality could reduce the parasites' fitness, such a fitness cost is balanced by parasites' increased horizontal transmission, suggesting a link in the fitness of hosts and symbionts. Models indicate that changes in transmission dynamics of symbionts can vary their virulence (Ganusov & Antia

2003) and that a trade-off between the resource depletion and new host encounter rate may shape the symbiont's infection strategy (Fenton & Rands 2004). The infection strategy of symbionts has been found to depend on the host physiology (Redman *et al.* 2001), which governs the outcome of the symbiotic relationship. For an symbiotic interaction between two species, symbionts have been observed to be mutualists, commensals, and parasites depending on the hosts' physiological condition (Cheney & Cote 2005; McCreadie *et al.* 2005), indicating that such interactions are context dependent. For some symbioses, it is difficult to measure the effect of symbionts on hosts' fitness (Nuttal 1997; Lindstrom *et al.* 2003), whereas for some others, symbionts that reduce host fitness also allow them certain benefits (Fry *et al.* 2004). This suggests that there are many symbionts, whose role either varies with environmental factors or cannot be determined. Hence, it is often difficult to define a single role for some symbiotic interactions based solely on the effect of interactions on the fitness of hosts and symbionts.

Like other vertebrates, birds play host to various symbionts. Birds harbor diverse parasitic symbionts such as microbial pathogens and feather mites (Moller 1991; Hubalek *et al.* 1995). Parasites alter the body condition and behavior of birds, reducing their fitness (Boots & Knell 2002; Lindstrom *et al.* 2003). On the other hand, mutualistic symbionts residing in the gut of some birds play an important role in their nutrition by helping degrade certain substrates (Dominguezbello *et al.* 1993). Other symbionts benefit the birds by acting as growth promoters and interact competitively with pathogenic bacteria (Moreno *et al.* 2003). The presence of such microbes is critically important in avian growth and development.

Microbial symbionts also occur on the plumage of birds (Muza *et al.* 2000), living either freely or by attaching themselves to the feathers (Lucas *et al.* 2005). Such interactions between birds and bacteria is likely to be an ancient association, as some avian fossils show the presence of bacteria on feathers of early birds (Davis & Briggs 1995). Some of the present day feather based bacteria are capable of degrading feather keratin *in vitro* (Burtt & Ichida 1999) and this capability is spread across diverse bacterial taxa (Lucas *et al.* 2003). Such bacteria are present both on intact

avian plumage and in soil (Burtt & Ichida 1999; Lucas *et al.* 2003). Recent data suggests that a particular keratinolytic bacterium does not effect feather degradation when present on live birds (Cristol *et al.* 2005). However, there is no data on the activity and role of other keratinolytic bacteria on live birds. To understand and characterize the role of such symbionts in their interactions with birds, we need to test and catalogue the effect of these bacteria on hosts' body condition, behavior, and fitness. We also need to study the response of hosts to these symbionts and whether any of the hosts' morphological and / or physiological traits affect the activity of such symbionts.

Such a study is presented in this thesis. Chapter 2 of this thesis studies the effect of different loads of keratinolytic bacteria (called EB: Ecto-Bacteria), on the body condition of their hosts: zebra finches (*Taeniopygia guttata*). Bacterial symbionts rarely occur in pure cultures on birds and other hosts and rather occur in communities (Lucas *et al.* 2005). Therefore, this study observes the effect of differential loads of the EB community (a selection of ten cultivated EB isolates) on some morphological and physiological traits of hosts, which are indicative of their body condition. Since body condition of an individual correlates with its fitness (Blums *et al.* 2005), alterations in the body condition of birds could change their fitness. The expressions of some of the observed morphological and physiological traits depend on the body condition of the hosts and are called condition-dependent traits. Some of these condition-dependent traits are sexually selected traits that are implicated in mate choice of the hosts. In this study, we test our hypothesis that differential loads of such ecto-bacteria would alter the body condition of their hosts.

Chapter 3 focuses on observing the effect of the load of EB community on male mate choice behavior of zebra finches. Male zebra finches choose mates based on the expression of some of the females' condition-dependent traits, called females' sexually selected traits. In our set-up, Chapter 3 tests the hypothesis that differential loads of microbial flora of zebra finches would alter their mate choice behavior. We predict that an increase in the load of symbionts on a group of females would reduce

the expression of their sexually selected traits and skew the male preference for females towards females with lower bacterial load and a better body condition.

Changes in the morphology and behavior of hosts are correlated with variations in the presence, load, and activity of their symbionts (Fitze & Richner 2002; Moreno *et al.* 2003; Lucas *et al.* 2005). Birds vary morphologically between sexes and species in their plumage coloration. Recent data documents differential degradation of melanized and non-melanized feathers by keratinolytic bacteria in broth, or in highly humid conditions (Burtt & Ichida 2004; Goldstein *et al.* 2004). These studies also speculate a role for such bacteria in the evolution of plumage coloration (Grande *et al.* 2004). Such *in vitro* studies neither simulate *in situ* conditions of feather degradation on live birds, nor demonstrate any correlation between bacterial growth and observed feather degradation. Such deficiencies are addressed in our *in vitro* experiments in Chapter 4, which observe the growth of EB while studying feather degradation in broth. In this chapter, I investigate the effect of melanization of feathers on their degradability and corresponding EB growth, in set-ups with and without broth. In addition, I test whether our EB isolates live freely on feathers or whether they attach themselves to feathers, of live birds. Microbial attachment to substrates is an energy dependent activity and attachment of EB isolates to feathers could suggest that EB are active on live birds.

Many of the avian plumage symbionts are pathogens. In the study presented in chapter 5, using a traceable EB isolate, we investigate the behavioral mechanisms that aid rates and routes of infection and transmission of microbial symbionts. Investigating a link between social and sexual behaviors of the hosts and the transmission of their symbionts within a population is important in epidemiology. With the rise of new and emerging avian diseases, some of which are zoonotic in nature, such a study could have significant medical and epidemiological implications.

Chapter 2

Ecto-bacteria affect the expression of bill hue, plasma carotenoids, & preen glands in zebra finches (*Taeniopygia guttata*)

The manuscript is in preparation for submission to *Ecology Letters*.

Introduction:

Microbial parasites can have a detrimental effect on their hosts' morphology (Zuk *et al.* 1998), such as reduction in body mass and /or other morphological traits, leading to a reduced social rank (Duckworth *et al.* 2004). Expression of such morphological traits depends on the body condition of the individual and hence they are called condition-dependent morphological traits. Some microbes also occur as opportunistic parasites, causing disease when present in greater loads (MacPherson & Uhr 2004), due to a dysfunctional immune system of the host (Guarner & Malagelada 2003), and /or due to environmental factors (Lacoste *et al.* 2001; Dror *et al.* 2005). Body condition and the expression of condition-dependent morphological traits of hosts are negatively correlated with parasitic bacterial loads (Thompson *et al.* 1997; Hamilton & Poulin 1999). A reduction in hosts' body condition, with an increase in their microbial load, is brought about by a trade-off between the hosts' investment in immune function and in displays used in signalling (Jacot *et al.* 2004). Together, this suggests that the role and virulence of such microbes depend on host physiology, microbial loads, and environmental stress.

Birds are host to diverse ecto-bacteria (Muza *et al.* 2000), some of which are pathogens and cause a reduction in their body condition (Bayer *et al.* 1976). Some bacteria can degrade feather keratin *in vitro* by producing Keratinase and are present as the normal ecto-flora of birds (Burtt & Ichida 1999). Such bacteria, since found in both soil and avian plumage (feathers on live birds), can degrade molted feathers lying on soil and could degrade feathers on live birds. The effect of many of these bacteria on hosts' body condition is unknown (Lucas *et al.* 2003). Feathers contain small peptides of avian Φ keratin, whereas the larger peptides of avian Φ keratin are present in their beaks (Farner Donald S. 1993). Beaks consists of a compact layer of keratinized epidermal cells, molded around the bony core of each mandible and are frequently colored using melanins, lipochromes, or carotenoids (Welty 1964).

Carotenoids in birds are present either in circulating or in stored forms, wherein circulating plasma carotenoids help in boosting the immune system (McGraw & Ardia 2003). For many birds, stored red carotenoids, which make the bills and plumage redder, are less abundant than yellow carotenoids and cause red ornamentation to be costlier (Hill 1996) and more attractive (McGraw & Ardia 2003). The redness of bills in male zebra finches (*Taeniopygia guttata*) changes during their breeding cycle. Male bill redness is least intense at the end of the breeding cycle and is a condition-dependent trait (Burley *et al.* 1992; Birkhead *et al.* 1998). Bill color in zebra finches is a sexually selected trait (McGraw & Ardia 2003). Environmental, physiological, and reproductive constraints alter the optimal phenotypic expression of bill color in zebra finches (Burley *et al.* 1992; Zann 1996). In some birds, parasite loads of hosts reduce their bill coloration (Figuerola *et al.* 2005), but there is yet no evidence of alteration of bill coloration due to the presence of non-parasitic symbionts.

Birds also use their beaks to preen themselves. Preening involves spreading secretions from the uropygial gland on plumage, to maintain plumage condition (Montalti *et al.* 2005). The uropygial gland secretes uropygial oil, or preen oil (Farner Donald S. 1982), which has some anti-microbial properties against certain bacteria (Bandyopadhyay & Bhattacharyya 1996, 1999; Shawkey *et al.* 2003). Removal of this gland leads to a conspicuous change in the normal microbial flora of the plumage (Bandyopadhyay & Bhattacharyya 1996). Uropygial gland weight changes with the age (Sandilands *et al.* 2004b) and body condition of birds (Farner Donald S. 1982), whereas preen oil composition changes with their diet, sex, and age and such changes could change their plumage odors (Sandilands *et al.* 2004a). This suggests that uropygial gland morphology and preen oil composition are condition-dependent traits, though it is not known whether microbial loads of birds change the morphology and activity of uropygial glands.

Given that the presence of pathogens in hosts is negatively correlated with hosts' body condition, which is mediated by a trade-off between the hosts' investment in immunity and in growth and reproduction, we want to understand the effect of

certain keratinolytic microbial symbionts on hosts' body condition and immune state to help understand the role of such symbionts. In this study, we experimentally enhanced the load of keratinolytic bacterial symbionts (called EB: Ecto-Bacteria) on feathers of zebra finches (*Taeniopygia guttata*), to investigate the effect of EB on the hosts' body condition and on the trade-off between the hosts' investment in morphological displays and in immune competence. We propose that an increase in the ecto-microbial load of birds can effect a change in the expression of some of their condition-dependent morphological traits and immune competence.

Material and Methods:

Experimental set-up:

Thirty-six captive zebra finches (18 males and 18 females), banded using colored plastic rings, were used for the experiment. Birds were housed in same sex pairs in cages with food, water, and sand available *ad libitum*. The temperature and relative humidity in the cages were maintained at 27º C and 60% (± S.D.). Light and dark periods were set at 18 and 6 hours, respectively. Tarsal lengths of all birds were measured before the start of the experiment with vernier callipers and their weights were measured before and during the experiment using an electronic balance (Mettler). The experiment consisted of a Treatment phase of 60 days, followed by 40 days of Recuperation phase. The birds were divided into three Treatment groups: a test group 'B' (n = 16) and two control groups: 'T' (n = 14) and 'C' (n = 6). There were no significant differences in the body mass or uropygial gland volumes [calculated by multiplying the three dimensions (length, breadth, and height) of the gland with vernier calipers] of birds between the three groups (One-Way ANOVA; Body Mass, $F_{2, 35} = 0.21$, $p = 0.81$; Tarsus Lengths, $F_{2, 35} = 0.13$, $p = 0.87$; Uropygial gland volumes, $F_{2, 35} = 0.24$, $p = 0.78$).

Treatment phase:

Each test bird in group B was treated on their wing, breast, and tail feathers with 1 ml of a bacterial suspension [cell density: 10^5 cells/ml; estimated using Direct

Microscopic Count (Atlas 1995)] with a sterile paint brush. The bacterial suspension was composed of a community of 10 different keratinolytic bacteria [called EB: Ecto-Bacteria;(Lucas *et al.* 2003)], with all isolates present in similar cell densities. The EB treatments were aimed at increasing the bacterial loads of group B birds within an ecologically significant range. Therefore, the cell density of the EB suspension was made similar to that of pond water ($< 10^6$ cells/ml) frequented by wild birds (Diler *et al.* 2000; Al-Harbi 2003). For controls, group T birds had their wing, breast, and tail feathers treated with 1 ml of sterile PBS with a sterile brush and group C individuals had similar feathers ruffled with a dry sterile brush. The birds were treated once every seven days, for 60 days.

Bacterial sampling:

To test the effect of EB treatments on the microbial loads of birds, the bacterial load on the plumage of all birds was determined on the 30^{th} day of the Treatment phase. We developed a sampling technique in which a print of two adjacent wing and two adjacent tail feathers of every bird was impressed on separate sterile agarose slabs. An area of 64 mm^2 of the agarose imprint of every feather (four samples per bird) was removed and aseptically transferred into a micro-centrifuge tube, containing 200 µl of sterile PBS. After 10 seconds of vigorous shaking, each tube was plated out on Casein Starch Agar (CSA), in two dilutions (Atlas 1995). The CSA plates were incubated at 25° C for 24 hours, following which the numbers of colonies on all plates were enumerated (Atlas 1995). Comparing the microbial counts of the adjacent feathers, we found the method to be highly repeatable (Wing feathers: $r^2 = 0.94$, $n = 35$, $p < 0.001$; Tail feathers: $r^2 = 0.97$, $n = 35$, $p < 0.001$).

Recuperation phase:

The Recuperation phase started on the 60^{th} day of the experiment and involved housing the birds in their cages without any treatments for an additional 40 days. Before stopping the treatments on the 60^{th} day of the experiment, males and females were randomly exposed to each other for 90 minutes, albeit in separate cages with wire gauze to separate them.

Observations of Body weight, Uropygial gland volumes and Bill colors:

To test the effect of EB treatments on morphological traits of birds, we measured their body weight and uropygial gland volume, on days 0, 30, and 60 from the start of the experiment. Furthermore, we digitally photographed the bills of all birds, at a fixed distance from a neutral light, on days 30, 61, and 100 from the start of the experiment. Bill hue, a measure of the intensity of redness of bill redness, for all birds was calculated as the mean of 5 RGB (Red, Green and Blue) hue values, from five random locations on the bill using Adobe Photoshop 5.0 (Fitze & Richner 2002). Bill hue is negatively correlated with bill redness (Bright *et al.* 2004).

Observations of plasma carotenoid levels and cellular immune response:

To test the effect of EB treatments on the immune competence of zebra finches, we assessed the cellular immunity and plasma carotenoid levels in all the birds, on the 30^{th} day of the experiment. We measured the level of plasma carotenoids (circulating carotenoids) of all birds by HPLC, using a reverse phase C-18 column (Blount *et al.* 2003). Cellular immune response was tested on the 30^{th} day of the experiment using PHA, an antigen which induces the T-cell proliferative response of the organism (Smits & Williams 1999). Before and after the test, the thickness of treatment sites on the left and right wings of all birds was measured, with a spessimeter. The test site on the left wing of the bird was injected intra-dermally with a 20 µl of PHA solution (concentration 5mg/ml, made using sterile PBS), whereas the control site on the right wing was injected similarly but with an equal volume of sterile PBS. Twenty-four hours later, we measured the thickness of test and control sites. The T-cell proliferative response was calculated as the difference between the change in thickness of the PHA-injected test and PBS-injected control site, in each bird.

We analyze all data using One-Way ANOVAs. Statistical tests were done using JMP 5.0.1a.

Results:

The treatments of group B birds with EB suspension had a significant effect on their bacterial loads. Bacterial sampling of the wing and tail feathers revealed that test birds in group B had significantly higher bacterial loads, than control birds in groups C and T ($F_{2, 33} = 4.10$, $p = 0.02$; Mean number of bacterial colonies per treatment group ± S.E.: B = 536.3 ± 79.6, C = 329.1 ± 138, T = 209.69 ± 82.4). This data was tested for homogeneity of variance across groups using Bartlett's test and was found to be equal across all the three Treatment groups (F ratio = 1.8, $df = 2$, $p = 0.16$), showing that the requirements for One-Way ANOVA were met. Bacterial loads for all feathers were found to be within the observed ecto-bacterial loads of wild birds (Burtt & Ichida 1999). There was no significant effect of the treatments on the body mass of birds (30^{th} day: $F_{2, 31} = 0.59$, $p = 0.56$; 60^{th} day: $F_{2, 34} = 0.89$, $p = 0.41$).

We found no significant effect of the treatments regimes on the bill hues of birds between the three Treatment groups, on the 30^{th} day of the experiment ($F_{2, 35} = 1.94$, $p = 0.15$). However in males, EB treatments had a significant effect on bill hues, on both the 30^{th} and the 61^{st} day (30^{th} day: $F_{2, 17} = 4.46$, $p = 0.03$; 61^{st} day: $F_{2, 16} = 30.96$, $p < 0.001$). Group B males had higher bill hues than males in control groups, in both observations. At the end of the Recuperation phase (40 days after stopping EB treatments), there was no effect of EB treatments on male bill hues (Fig. 1; $F_{2, 17} = 1.83$, $p = 0.19$). On exposure to females, control males (group C and T) decreased their bill hues significantly more than test (group B) males [(C = T) > B, $F_{2, 16} = 6.63$, $p = 0.009$, Tukey post-hoc for $p < 0.05$; (Fig. 2)], indicating that the bills of control males were redder as compared to test males. No significant effect of the EB treatment was observed on female bill hues (30^{th} day: $F_{2, 17} = 1.69$, $p = 0.21$; 61^{st} day: $F_{2, 17} = 0.03$, $p = 0.96$).

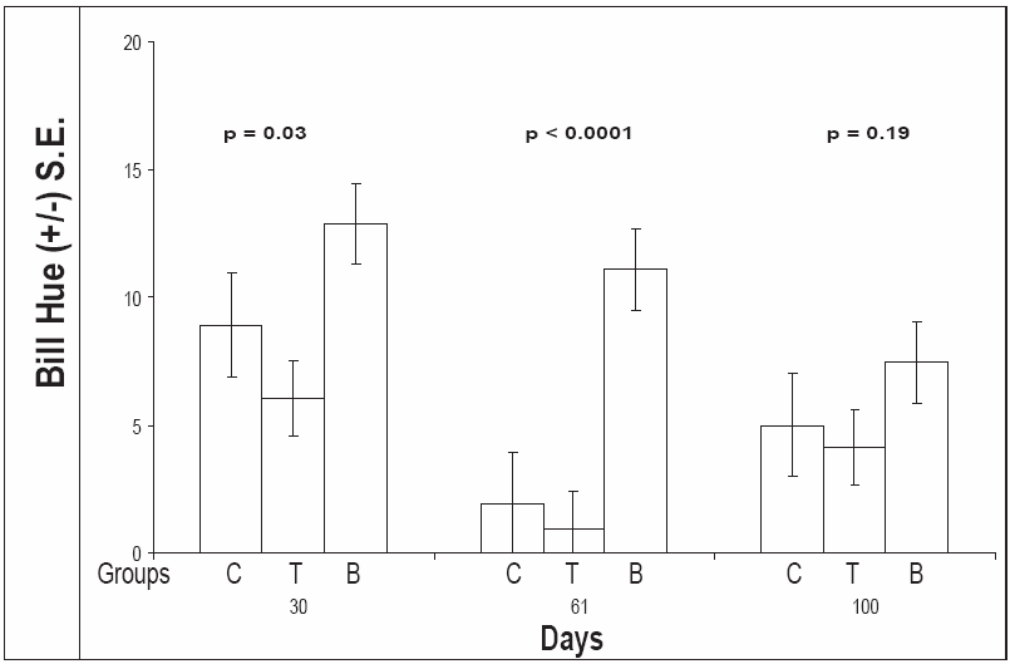

Figure 1: Bill hues of all males by Treatment group over the length of the experiment.

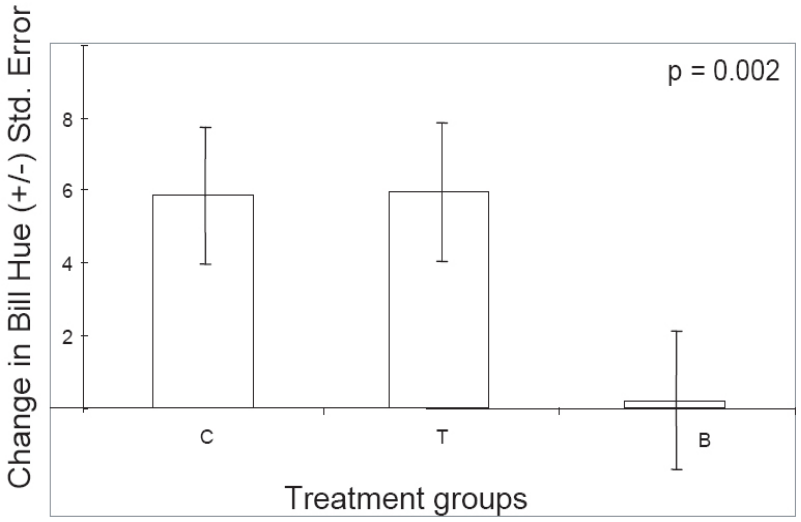

Figure 2: Change in bill hues of all males by Treatment groups on exposure to females.

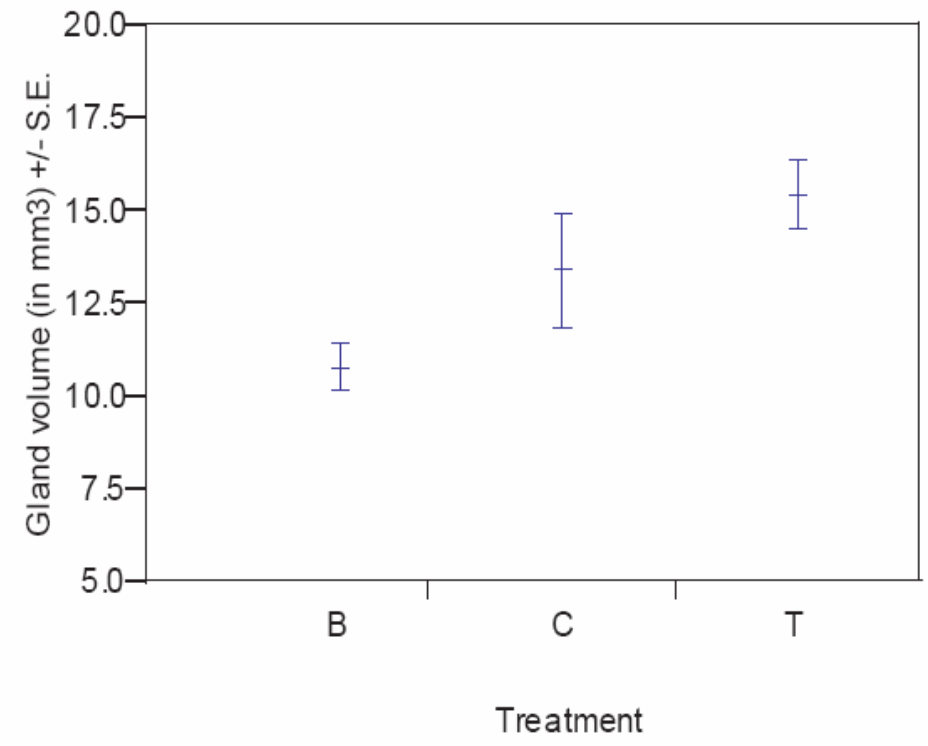

Figure 3: Gland volumes of all birds by Treatment groups on the 30th day of the experiment.

We found uropygial gland volumes to be significantly altered by EB treatments. Group B birds had significantly smaller uropygial gland volumes, compared to control birds (Fig. 3; 30th day: $F_{2, 32} = 5.72$, $p = 0.007$; 61st day: $F_{2, 34} = 3.43$, $p = 0.04$; (T > C) > B, Tukey post hoc for $p < 0.05$). We also observed that the glands of group B birds shrank significantly, compared to the controls, during the course of the Treatment phase ($F_{2, 32} = 3.51$, $p = 0.04$).

We found no significant overall effect of EB treatment on plasma carotenoids ($F_{2, 33} = 1.51$, $p = 0.23$). However, on treatment, group B females (test) had significantly elevated plasma carotenoids levels than control females in other groups [Fig. 4; $F_{2, 17} = 7.38$, $p = 0.0059$; B > (C > T), Tukey post hoc for $p < 0.05$], after 30 days of treatment. Males between the Treatment groups failed to show any significant differences, with respect to their plasma carotenoid levels ($F_{2, 15} = 0.34$, $p = 0.71$).

We also found that group B birds did not respond significantly differently to PHA, compared to their control counterparts ($F_{2,34} = 2.03$, $p = 0.14$).

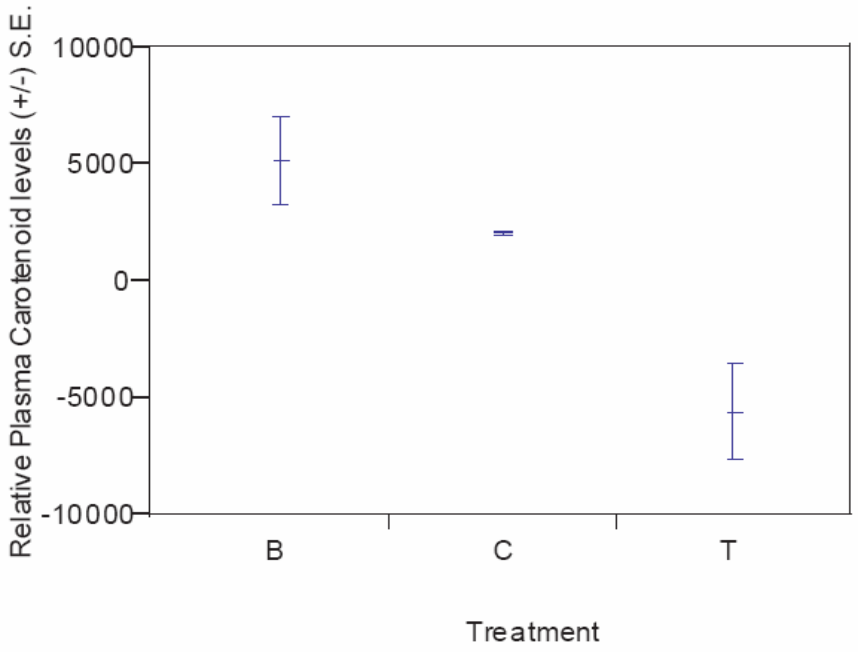

Figure 4: Relative plasma carotenoids levels in females of different Treatment groups.

Discussion:

This study shows that EB treatments of birds, leading to changes in their bacterial loads, can modify certain morphological traits such as the uropygial gland volume and the intensity of bill color in males. Our results demonstrate for the first time that an enhanced load of ecto-bacteria, which comprise the hosts' normal flora, alters the expression of condition-dependent traits. Experimental manipulation of ecto-bacterial loads neither altered the body mass, nor evoked a cellular immune response in hosts, implying that such bacteria and their loads did not cause disease in zebra finches.

We found that the bacterial treatments had an effect on the males' bill hue, wherein males with higher bacterial load had higher bill hues. This observed effect of

treatments on bill hue disappeared, after treatments were stopped and the bacterial loads of both the test and the control birds became similar. Bill coloration in zebra finches is a sexually selected trait and is sexually dimorphic, with males having bills redder than females, caused by deposition of red carotenoids (Burley *et al.* 1992; Birkhead *et al.* 1998; Blount *et al.* 2003). Given that hue is the inverse of intensity of redness (Bright *et al.* 2004), we infer from our results that bacterial loads act as a constraint in the expression of a sexually selected trait in male zebra finches. We also observe that males respond to the presence of females and hence mating opportunities, by decreasing their bill hues. Such a response is demonstrated to be dependent on their ecto-bacterial loads, as males with unaltered bacterial loads effect a significantly greater decrease in bill hues in response to mating opportunities than males with elevated bacterial loads.

Bill redness depends on the amounts of red carotenoids stored in bills. Although red carotenoids do not play a direct role in affording a defense response to pathogens, they help in boosting the immune system (McGraw & Ardia 2003). A decrease in bill redness, with an increase in bacterial load on EB treatment, implies the depletion of bill carotenoids due to their utilization in response to elevated bacterial loads. The mechanism of such a response is unknown, as decreased levels of circulating carotenoids failed to change the cellular immune response of birds. Results suggest that in response to increased bacterial presence, males use carotenoids from their stored depots in bills, leaving their levels of plasma carotenoids unchanged. Female zebra finches, on the other hand, do not store red carotenoids in their bills in the same amounts as males and resort to using circulating plasma carotenoids to respond to increased bacterial loads. This leads to a significant difference in plasma carotenoids levels in females of different groups. Recent data documents a sex-based difference in carotenoid status and immune function in zebra finches, wherein dietary carotenoids aid the immune function of both the sexes differently (McGraw & Ardia 2005). Our results suggest that zebra finches use carotenoids in response to microbial symbionts and that there is a sex-based difference in the source of carotenoids used to afford such a response, wherein males use carotenoids stored in their bills and females use their circulating plasma carotenoids.

Birds treated with EB, having elevated bacterial loads, had significantly lower uropygial gland volumes when compared to control birds. After treatment with EB community, the uropygial gland volume in birds with elevated bacterial load shrank significantly from its original volume, as compared to birds with un-altered bacterial load. The uropygial gland and its secretions are condition-dependent traits (Sandilands *et al.* 2004a; Sandilands *et al.* 2004b), with some preen oil fractions having anti-microbial activity (Shawkey *et al.* 2003). Preen oil maintains the plumage quality, a sexually selected trait in zebra finches (Clayton 1990) and a cessation of preen oil makes the feathers brittle (Moyer *et al.* 2003). Preen oil also gives the avian plumage a characteristic social odor (Sandilands *et al.* 2004a). Putting our results in context of previous work, we suggest that the shrinkage in gland volumes is due to an increased secretion of the preen oil. We speculate that such an increase in the secretion of preen oil is a defense response to the elevated bacterial load. In addition, the shrinkage in gland volume effected by EB loads and the resulting reduction or cessation of preen oil could alter attractiveness and odor of the birds.

The mechanism by which birds are able to detect and respond to the elevated loads of microbial symbionts cannot be revealed by our experiment, as the EB treatments do not change the body mass or cellular immune state of zebra finches. This requires further investigation, though we hypothesize that such a response could be mediated by either the feather-degrading capability of the EB community, causing degradation of plumage and local irritation of the uropygial gland and epidermis; or by the EB getting ingested during preening and being recognized by intestinal immune tissues (Klipper *et al.* 2000).

This study demonstrates that the ecto-microbial load of birds modulates the expression of certain condition-dependent traits, some of which play a significant role in their mate choice. In addition, by altering the expression of such traits, we suggest that the birds are able to respond to increased symbiont load. We also show

that the mechanisms of such responses are sex-biased, with both sexes using sources of body carotenoids to respond to an increase in microbial loads.

Further work should focus on identifying the mechanisms behind the hosts' detection and response to EB. It should also be investigated whether such elevated bacterial loads lead to a change in the composition of the preen gland oil, altering their anti-microbial fraction. Defining an ecological role for the interactions between birds and their EB symbionts requires further work. Though based on present work, we hypothesize that EB are opportunistic parasites of birds, as they depend on their loads, to diminish hosts' expression of condition-dependent traits and evoke a hosts' response to their increased loads.

Summary:

Birds harbor diverse bacteria in varied loads on their plumage, some of which are capable of degrading feathers. By experimentally enhancing the load of feather-degrading bacteria on zebra finches (*Taeniopygia guttata*), we show the effects of ecto-bacteria on some of their condition-dependent morphological traits. We demonstrate that such bacteria reduce the expression of male bill color, a sexually selected trait in zebra finches, without changing the hosts' body mass. Our experiment also demonstrates that an experimental increase in ecto-bacterial load of hosts leads to a reduction in plasma carotenoids levels in females and a reduction in uropygial gland volumes of zebra finches.

Acknowledgements:

We thank the *Swiss National Research Foundation* (project No 26072464) and a professorial fellowship from the *Fondation de Famille Sandoz* (both to P. Heeb). We thank Dr. Hans Weber for his help with the HPLC analysis. We also wish to thank Dr. Rob Hammond and Tanja Schwander for their inputs. This work was conducted in accordance with Swiss laws on animal research.

Chapter 3

Microbial loads and male size govern male mate choice in zebra finches (*Taeniopygia guttata*)

The work presented here is in preparation for submission to *Behavioral Ecology and Sociobiology*.

Introduction:

Sexual selection generally operates by a higher preference for mates with an enhanced expression of costly morphological traits. Reproductive success has been hypothesized to be correlated with the expression of such morphological traits, also known as mate choice traits, which are honest signals of mate condition (Zahavi 1975). The intensity of sexual selection, operating through mate choice, depends on the difference in the reproductive and parental investment between both sexes, the operational sex ratio, and the costs of mate search and courtship (Krebs & Davis 1993; Andersson 1994; Jennions & Petrie 1997; Widemo & Saether 1999). Sexual selection in many species is more intense among males, as they vary more in reproductive success than females (Wade & Arnold 1980), suggesting that female mate choice is more intense, than male mate choice. As choosiness varies with reproductive investment, female mate choice is usually more significant because females invest significantly more in reproduction and parental care. Female mate choice of males has been well documented partly because it is based on observable and exaggerated males' sexually selected traits (Peters *et al.* 2004) and as females are usually the choosier sex (Andersson 1994).

Male mate choice, the choice exerted by males on females, is significant when mating is costly for males (Cunningham & Birkhead 1998; Bergstrom & Real 2000; Kokko & Johnstone 2002). This can occur due to elevated levels of male parental investment, reduced male mating opportunities, or reduced male bias in the operational sex ratio (Bonduriansky 2001; Kokko & Johnstone 2002). In zebra finches (*Taeniopygia guttata*), a monogamous species, there is evidence of mutual mate choice as male zebra finches are also known to choose females (Zann 1996). Male mate choice operates by discriminating on the basis of traits indicative of female quality and condition, such as bigger body size (Jones *et al.* 2001) or structural coloration of plumage (Siefferman & Hill 2005).

The average parasite loads of hosts are negatively correlated with hosts' body condition and the expression of their sexually selected traits (Hamilton & Poulin 1999). Parasite loads can alter mate choice as mate choice favors the avoidance of mates with higher parasite loads (Mazzi 2004). In house finches (*Carpodacus mexicanus*), males show reduced expression of their sexually selected traits on infection with ecto- and endo-parasites (Thompson *et al.* 1997). In some species, males choose to avoid parasitized and infected mates, as parasite loads are inversely linked to female fecundity (Rosenqvist & Johansson 1995). This experiment investigates the effect of microbial symbionts on the male mate choice of zebra finches (*Taeniopygia guttata*). We hypothesize that the microbial symbiont load of females could bias male mate choice and males would prefer females with lower symbiont loads.

In our study, we experimentally manipulate the load of keratinolytic bacteria (called EB: Ecto-Bacteria) on the plumage of zebra finches (*Taeniopygia guttata*) to create groups of individuals with significant differences in their ecto-bacterial loads. By investigating the male mate choice for females with higher and lower ecto-bacterial load, we expect males to avoid females with higher microbial loads and therefore prefer females with lower microbial loads. In zebra finches, male mate choice depends on female's sexually selected traits, which are orange bill color, tail length (Zann 1996), and fecundity (Jones *et al.* 2001). We predict that an increase in hosts' microbial load would reduce the expression of female's sexually selected traits and hence, skew the male mate choice towards lower bacterial load females with a better expression of sexually selected traits.

In addition, male mate choice can vary with male quality (Amundsen & Forsgren 2003; Wong & Jennions 2003). Low quality males are theoretically expected to be less choosy (Parker 1983) and higher quality males can be expected to be more choosy. Male size is an indicator of male quality in zebra finches (Zann 1996) and hence, male mate choice in zebra finches could also vary with male size. It would be interesting to investigate the effect of male size on the male mate choice of zebra finches. Generally, it is thought that hosts' parasite load and male size governs their

male mate choice, hence this experiment would also test the interaction between male size, male mate choice, and the microbial loads of birds.

Methods

Experimental setup:

Thirty-six birds (18 males and 18 females) were used for this experiment. The birds were previously color ringed, which since would interfere with mate choice of zebra finches were changed to numbered metallic rings (Hunt *et al.* 1997). Birds were housed in cages of same sex pairs for 5 months before the experiment, with food, water, and sand available *ad libitum*. The temperature and relative humidity in the cages were maintained at 27º C and 60% (± S.D.). Light and dark periods were 18 and 6 hours, respectively. Tarsal lengths of all birds were measured before the start of the experiment using vernier calipers, whereas their weights were measured before and during the experiment using an electronic balance (Mettler). The experiment consisted of a Treatment phase, followed by the Mate Choice experiment.

The Treatment phase:

In the Treatment phase, the birds were divided into three Treatment groups, one 'test' group: B (n = 16) and two 'control' groups: S (n = 14) and C (n = 6). All the groups had an equal number of both sexes and there was no significant difference in size or body mass (for statistical analyses, see Chapter 2, *Methods*) between the groups. Each group B bird was treated on their wing, breast, and tail feathers with 1 ml of a bacterial suspension [cell density: 10^5 cells/ml, estimated by Direct Microscopic Count (Atlas 1995)] using a sterile paintbrush. The bacterial suspension was composed of a community of ten different feather-degrading bacteria [called EB: Ecto-Bacteria; (Lucas *et al.* 2003)] present in similar cell densities. In the control groups, group S birds had their wing, breast, and tail feathers treated with 1 ml of sterile PBS using a sterile brush and group C birds had similar feathers ruffled using a dry paintbrush. The birds were treated once every seven days, for 30 days. The

treatment was aimed at increasing the bacterial loads of birds within a range that is observed in the wild. Therefore, the cell density of the EB suspension was made similar to that of pond water (< 10^6 cells/ml) frequented by wild birds (Diler *et al.* 2000; Al-Harbi 2003). One female from control group S died during the Treatment phase, but her death appeared to be unconnected with the treatment regimes.

Bacterial sampling:

To test that EB treatments of group B birds successfully increased their microbial loads, the bacterial load on the plumage of all birds was determined on the 30^{th} day of the Treatment phase. We developed a sampling method in which a print of two adjacent wing and two adjacent tail feathers of every bird was impressed on separate sterile agarose slabs. An area of 64 mm^2 of the agarose imprint of every feather (four samples per bird) was removed and aseptically transferred into a micro-centrifuge tube, containing 200 µl of sterile PBS. After 10 seconds of vigorous shaking, each tube was plated out on Casein Starch Agar (CSA), in two dilutions (Atlas 1995). The CSA plates were incubated at 25° C for 24 hours, following which the numbers of bacterial colonies on all plates were enumerated (Atlas 1995). There was a strong correlation between the number of colonies from feather sub-samples (for statistical analysis; see Chapter 2, *Materials and Methods, Bacterial sampling*), showing that this method was highly repeatable.

Treatment of group B (test) birds with EB suspension had a significant effect on their ecto-bacterial loads, with group B birds having significantly higher bacterial loads than the control groups C and S (Fig. 1; See *Results*). Control individuals between groups C and S had no significant differences in their bacterial loads and were consolidated in a single control group (T) for all further analyses. Effectively, test group (B) contained birds with enhanced EB loads compared to control group (T) birds that had lower bacterial loads.

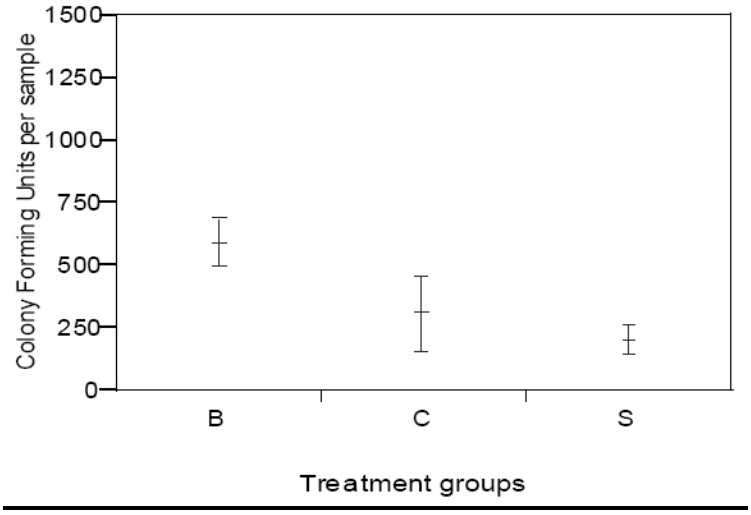

Figure 1: Ecto-bacterial loads for all birds in the Treatment groups B, C, and S.

Measure of females' sexually selected traits:

After 30 days of treatments, the females' traits implicated in male mate choice in zebra finches were measured. These traits were intensity of bill color, tail length of females and the number and weight of unfertilized eggs laid by females per cage (we assume that the number of unfertilized eggs laid by females are an indicator of their fecundity). Bills of all birds were digitally photographed at a fixed distance from a neutral light and their hues were calculated as the mean of five RGB (Red, Green and Blue) hue values taken at five random locations on the bill. Hue for each bill was calculated using Adobe Photoshop 5.0 (Fitze & Richner 2002). Bill hues were positively correlated with the orangeness of the bills (Bright *et al.* 2004).

Mate choice set-up and experiment:

The mate choice experiment was performed in the setup shown in Fig. 2. The central cage housed the male and each of the adjacent two cages housed a single female. Food and water was available in all cages *ad libitum*. The setup was lit with two fluorescent tubes of 60 Hz and 17 W. Cardboard blinds were arranged such that both females could see the male, but not each other (Fig. 2).

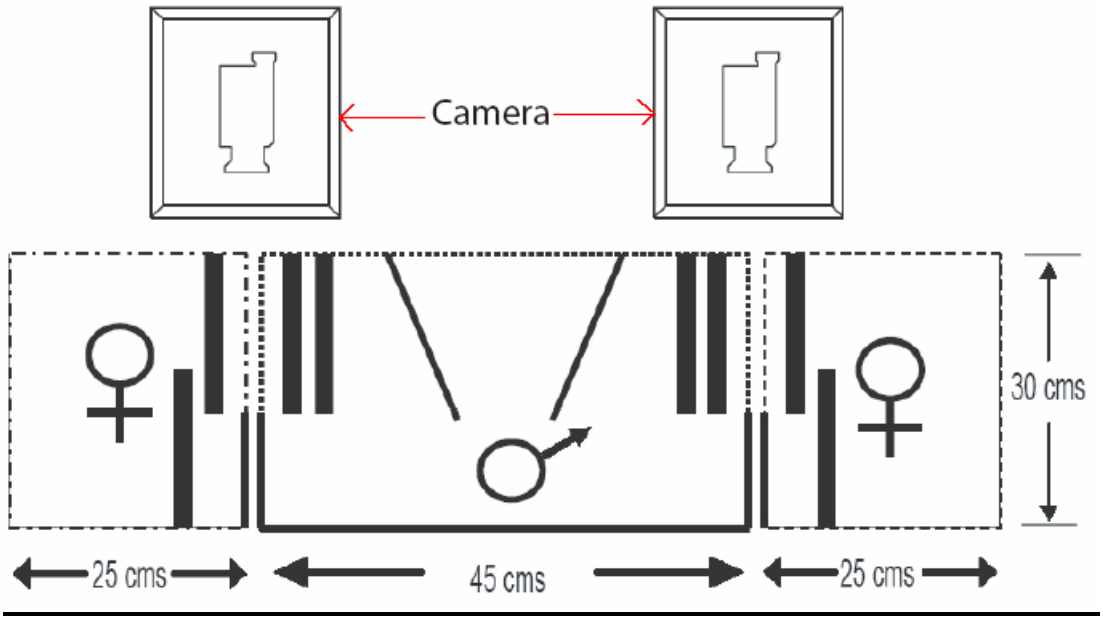

Figure 2: Cage for mate choice experiment. Complete lines indicate permanently blinded sides, serrated lines indicate two-way visibility sides, and the thick lines indicate perches. Area between the angled lines indicates the opaque male release chamber.

To avoid any bias of placing the male towards any side of the cage, males were first released into a closed opaque box in the middle of the male cage. The pair of females was then released into adjacent cages and each male was tested using a pair of females, containing one from test group (B) and another from control group (T). Females were paired based on similarity of their tarsal lengths and hence size [For pairs: (Size Female 1) = 2.79 + 0.81 X (Size Female 2); $r^2 = 0.45$, n = 17, p = 0.003] and each pair was used to test two males. The mate choice experiment commenced 30 minutes later, by opening a trap door of the opaque box, releasing the male into the main cage enabling it to see and court females. The time spent by the male on both sides of the male cage was video-recorded for 45 minutes in total using two hidden cameras, placed on either side of the male cage, recording simultaneously (Fig. 2). The experiment and recordings were stopped after 45 minutes and the cages were blinded, blocking visibility between the cages of males and females. To control for the effect of side preference, females were swapped between the two cages. Following a 15-minute habituation period, during which the male was placed again in the central opaque box, we removed the cage blinds, released the male into the

main cage, and re-commenced our experiment and recordings for another 45 minutes. By performing such a swap of females between cages, we tested the male's choice between the same pair of females in different cages for a total of 90 minutes.

Male mate choice:

Male choice for a female was calculated as the percentage of time spent by the male on the perch near to a female, out of the total time spent by the male on perches (Jones *et al.* 2001). Courting time was calculated as the total time male spent on perches in front of both the females. We assumed that a male had chosen a female when he spent more than 50% of his perch time on the perch near to her. One male belonging to the control group (T) did not spend any time on any perch and hence was removed from the analyses. Statistical analyses were done using the software JMP 5.0.1a

Results:

Effect of EB treatments on bacterial loads of birds

Treatment of group B (test) birds with EB suspension had a significant effect on their plumage bacterial loads. Bacterial sampling of the wing and tail feathers revealed that test birds (group B) had significantly higher feather bacterial loads than control (groups C and S) birds (Fig. 1; mean group T: 396.4, mean group B: 562.0, $F_{2, 33} = 4.10$, $p = 0.02$). The data was tested for homogeneity of variance across groups using Bartlett's test and was found to be equal across all the three Treatment groups (F ratio = 1.8, $df = 2$, $p = 0.16$), satisfying the criteria for One-Way ANOVA. All control individuals were consolidated in a single control group (T) for all further analyses. Effectively, group B contained birds with enhanced EB loads compared to group T birds that had lower bacterial loads. Bacterial loads for all feathers, were found to exist within the observed bacterial loads of wild birds (Burtt & Ichida 1999).

Effect of females' bacterial loads on male mate choice and females' sexually selected traits:

We found no significant effect of the females' bacterial loads on the male mate choice (t test: $t = 0.87$, $n = 16$, $p = 0.39$). We also found no significant effect of bacterial loads on the measured sexually selected traits of females (One-Way ANOVA: Bill hue: $F_{1, 17} = 0.03$, $p = 0.96$; Tail length: $F_{1, 17} = 0.37$, $p = 0.69$; Number of eggs laid: $F_{1, 8} = 0.39$, $p = 0.68$, Weight of eggs laid: $F_{1, 8} = 0.27$, $p = 0.77$). Body mass of birds of both sexes was not affected by their EB treatments (Males: $F_{1, 17} = 0.73$, $p = 0.49$; Females: $F_{1, 16} = 2.36$, $p = 0.13$). Also, the bacterial loads of EB community did not cause any change in the immune status of their hosts (see Chapter 2: *Materials and Methods, Results*)

Effect of males' bacterial load on male mate choice:

Males' preference and their courting time could be governed by males' microbial load. We found no significant effect of ecto-bacterial loads of males on the time they spent with females of either group ($F_{1, 16} = 0.46$, $p = 0.51$). We also found that the treatment of males did not significantly alter the courting time of males ($F_{1, 16} = 0.006$, $p = 0.94$).

Effect of absolute male size on male mate choice:

Males' preference and their courting time could be a function of absolute male size (Amundsen & Forsgren 2003). We found that male mate choice with respect to females' group was significantly correlated to the tarsal length of the choosing male (Logistic regression: $r^2 = 0.52$, $n = 17$, $\chi^2 = 12.12$, $p = 0.0005$; Wilcoxon Rank Test: $\chi^2 = 8.63$, $p = 0.003$; Fig 3). Males with longer tarsi (larger males) were found to prefer females with enhanced bacterial loads, whereas males with shorter tarsi (smaller males) preferred females with un-altered (lower) bacterial loads (the mean +/- S.E. of the percentile time spent by males with preferred females was found to be 76.84 +/- 4.05). Although the biomass of males is significantly correlated with their body size ($r^2 = 0.33$, $n = 17$, $p = 0.015$), there was only a marginal significance for the correlation observed between the choice of males and their body mass ($r^2 = 0.16$, $n = 17$, $\chi^2 = 3.75$, $p = 0.052$). Courting time of males was also found to be

significantly affected by male size ($r^2 = 0.35$, n = 17, p = 0.012), with larger males investing more time in courting females, compared to smaller males.

Effect of relative male size on male mate choice:

Male preference could also be a function of male size relative to the size of the females in the experiment (Foote 1988). Relative male size was computed as the difference between the absolute male tarsal length and the mean tarsal lengths of the females in one test (Amundsen & Forsgren 2003). We found no significant effect of the relative male size on male preference ($r^2 = 0.09$, n = 17, $\chi^2 = 2.27$, p = 0.13) or on their courting time ($r^2 = 0.005$, n = 17, p = 0.77).

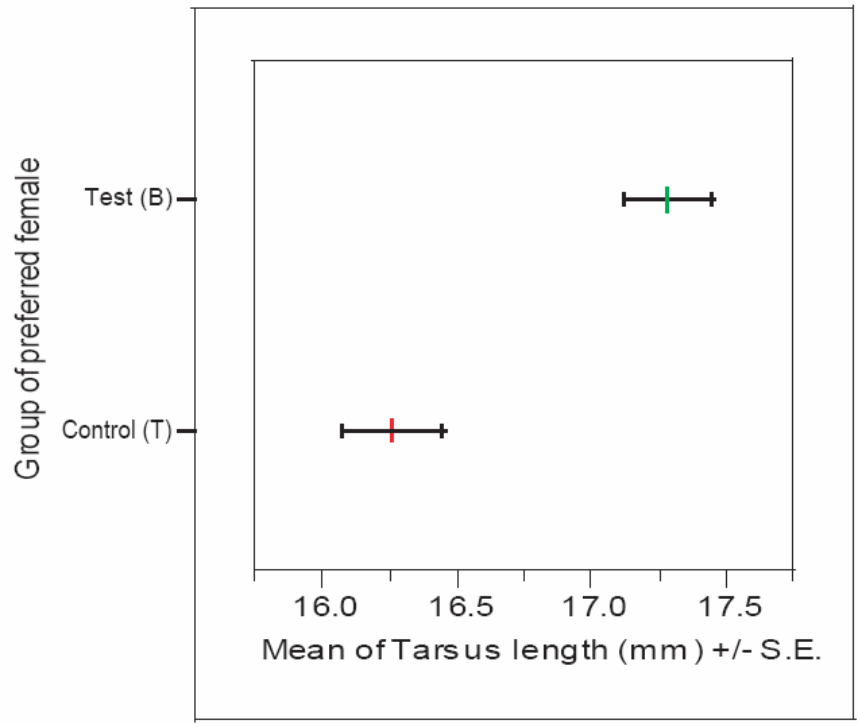

Figure 3: Male association with preferred females in relation to male tarsal length and Treatment group of female.

Discussion:

Our results suggest an interaction between the body size of males and their mate choice with reference to the bacterial load of females. As male size in zebra finches is an indicator of male quality (Zann 1996), we suggest that a variation in male quality leads to a change in the male mate choice. We demonstrate that the observed variation in male mate choice was linked to the absolute size of males and not their size relative to females under choice. Our results also show that the courting time of males, measured by their total perch time, significantly increased with male size. This suggests that larger males invested more time to exercise their choice, compared to smaller males. However, we find that our results do not support our main hypothesis, as the enhanced bacterial loads of birds did not skew the overall male mate choice of zebra finches towards females with lower bacterial load. In addition, our results demonstrate that the interaction between the body size of males and their male mate choice of females with higher and lower bacterial loads occurred independent of the effect of bacterial loads on the measure of sexually selected traits of females.

We find that larger males preferred females with enhanced bacterial loads whereas smaller males preferred those with un-altered bacterial loads. These findings suggest that males are able to distinguish between females of different treatments having different bacterial loads. Since the treatments had no measurable effect on the female body mass or other observed morphological traits, we suggest that the detection of increased bacterial load is independent of their effect on the measured traits.

Mating opportunities vary with male size in the wild, with bigger males getting mating opportunities with more and particularly better females (Reid & Roitberg 1995; Amundsen & Forsgren 2003). Smaller males, often excluded from mating in the wild, are less choosy in accepting mating opportunities (Parker 1983). We speculate that zebra finch males use different mate choice strategies depending on their size. We suggest that in wild birds, larger males infer females with an

asymptomatic increase in bacterial loads to have better tolerance to microbes, as an increase in their microbial load does not reduce their body condition. We suggest that in our experiment, larger males perceived females with an elevated bacterial load but an un-altered body condition to possess better tolerance to microbial loads. Better tolerance to microbes could be an indicator of mates' body condition, immune response, and hence, fitness. Therefore, we suggest that larger males choose group B females, as they perceive them to possess better body condition, in view of their perceived tolerance to EB loads.

Mating opportunities for small males are limited for two reasons: (1) due to a higher rejection rate by females and / or (2) by being competitively inferior to larger males (Amundsen & Forsgren 2003). Small males, if infected with higher microbial loads would experience a further reduction in their body condition and mating opportunities. In our experiments, we observe that smaller males prefer females with lower bacterial loads. We speculate that such a strategy, could aid the small males avoid contracting parasites and help them sustain their body condition and long-term mating opportunities. In addition, females with un-altered body condition and lower microbial loads can be perceived to have better microbe clearance rates. Together, this suggests, that given a choice, smaller males would court and mate with females having lower microbial loads to avoid getting infected and to select for better 'parasite clearance rate' traits.

The mechanism(s) by which males are able to detect the differences in bacterial loads of females, cannot be revealed by our experiment, as the enhanced bacterial load did not change any of the measured morphological traits. This requires further investigation, although we suspect that such a change in the ecto-bacterial load of females could lead to subtle, yet detectable changes in certain behavioral and morphological traits, which could not be measured in this experiment.

EB community isolates occur on wild birds (Burtt & Ichida 1999) and their loads in our experiment were found to in a range found in the wild. As male mate choice was found to be influenced by these microbial loads in captivity, we suggest that such

ecto-microbial loads could also alter male mate choice in wild. The present study demonstrates that variable loads of normal flora of birds can alter their male mate choice. Male mate choice in such cases depends on the size of the males and the microbial load of females. Further work should be done by using males, grouped based on their size, to mate with females of enhanced and un-altered bacterial loads, to understand fitness consequences of their choice.

Summary:

The parasite loads of individuals alter their mate choice. Socially monogamous species have mutual mate choice, wherein male mate choice gains significance with a decrease in mating opportunities. In this experiment, by experimentally enhancing the ecto-bacterial loads of zebra finches (*Taeniopygia guttata*), we test the hypothesis that male mate choice will be altered by the ecto-bacterial loads of females. We found that the size of males and the microbial load of females together governed the male mate choice, without the enhanced microbial load changing the expression of the females' sexually selected traits. We thus establish a new interaction between microbial loads and male mate choice in zebra finches.

Acknowledgements:

We thank the *Swiss National Research Foundation* (project No 26072464) and a professorial fellowship from the *Fondation de Famille Sandoz* (both to P. Heeb). We wish to thank Sabrina, Jessica, Dr. Robert Hammond, Dr. John Wang, Dr. Pierre Bize, Dr. Lori Handley, Daniel Croll, and Tanja Schwander for their inputs. This work was conducted in accordance with Swiss laws on animal research.

Chapter 4

Differential degradation of black and white feathers by a community of feather-degrading bacteria

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The study presented in this chapter is in preparation for submission to *Auk*.

Introduction:

Feathers contain more than 90% α-keratin and their chemical composition is conserved among bird species (Odonnell & Inglis 1974; Brush 1976). Feathers show large structural variation between species which lead to diverse morphologies and pigmentations (Prum *et al.* 1999; Shawkey & Hill 2004). Structural diversity in feathers could also create potential micro-niches for microbial growth (Shawkey & Hill 2004). Feathers are prone to bacterial degradation. Although a high diversity of feather-degrading bacteria have been isolated and characterized (Lucas *et al.* 2003; Shawkey *et al.* 2005), their precise role in evolution of feather traits is debatable (Grande *et al.* 2004).

Melanin could play a role in reinforcing feather structure, as an increase in melanin is associated with a reduction in feather abrasion (Bonser 1996; Kose & Moller 1999). Alternatively, some authors suggest that the susceptibility to abrasion of white feathers arises from feather structure, rather than the presence or absence of melanin (Butler & Johnson 2004). Recent data indicates that both melanin-based and structure-based plumage ornamentations appear to be honest signals of mate quality, assessed either by competitors or by potential mates (Siefferman & Hill 2003). Feather ornamentation has been hypothesized to follow Gloger's rule, wherein feathers tend to be darkly colored in habitats with high relative humidity and pale in low relative humidity (Burtt & Ichida 2004). Recent studies suggest that Gloger's rule is linked to parasite loads, wherein high and low humidity areas correspond with high and low parasite loads, respectively (Burtt & Ichida 2004). This indicates that bacterial activity could be linked to feather ornamentation and the evolution of feather melanin polymorphism.

The aim of our study was to find out whether bacterial feather degradation is a function of feather melanin dimorphism. A recent study (Goldstein *et al.* 2004) observed that *Bacillus licheniformis*, a keratinolytic bacterium, degraded non-melanized white feathers significantly more than the melanized black feathers, when incubated in broth. This study, though did not observe bacterial growth associated

with feather degradation. Such a deficiency makes it difficult to conclude whether the observed difference in feather degradation was due to bacterial activity. In our studies, we further explored the prediction that melanized feathers should undergo less microbial degradation, as compared to non-melanized feathers. We tested our prediction in three different experimental setups, namely: feather degradation in broth, in air without broth (involving minimum exposure of feathers to water), and bacterial growth on feathers embedded in Petri plates. In our setup, we also tested whether feather degradation was correlated with bacterial growth. A community of bacteria is found on feathers of wild birds, which could effect feather degradation (Lucas *et al.* 2003). Therefore, we used a community of feather-degrading bacteria (called EB: Ecto-Bacteria) in our studies, rather than focusing on the dynamics of a single bacterium in culture.

Methods:

EB growth on feathers in Agar:

Seventeen Tail feathers taken from separate zebra finches (*Taeniopygia guttata*), having alternate white and black bands, were UV sterilized (30 minutes) and fixed in Petri plates on sterile Water Agar. The plates were divided into two groups: a treatment group B, with 14 plates overlaid with 10 ml warm agar solution containing 10^5 cells/ml of EB [a community of feather-degrading bacteria; (Lucas *et al.* 2003); cell number estimated by Direct Microscopic Counts] and a control group C, containing 3 plates overlaid with 10 ml sterile warm agar solution (Atlas 1995). All plates were incubated at 25°C for 10 days and the numbers of colonies on the black and white part of the feathers were counted under microscope, at 45X magnification (Fig. 1). Every black and white feather area was counted in three microscopic fields. The mean number of colonies counted, on the black and white part of each feather, was used for statistical analysis.

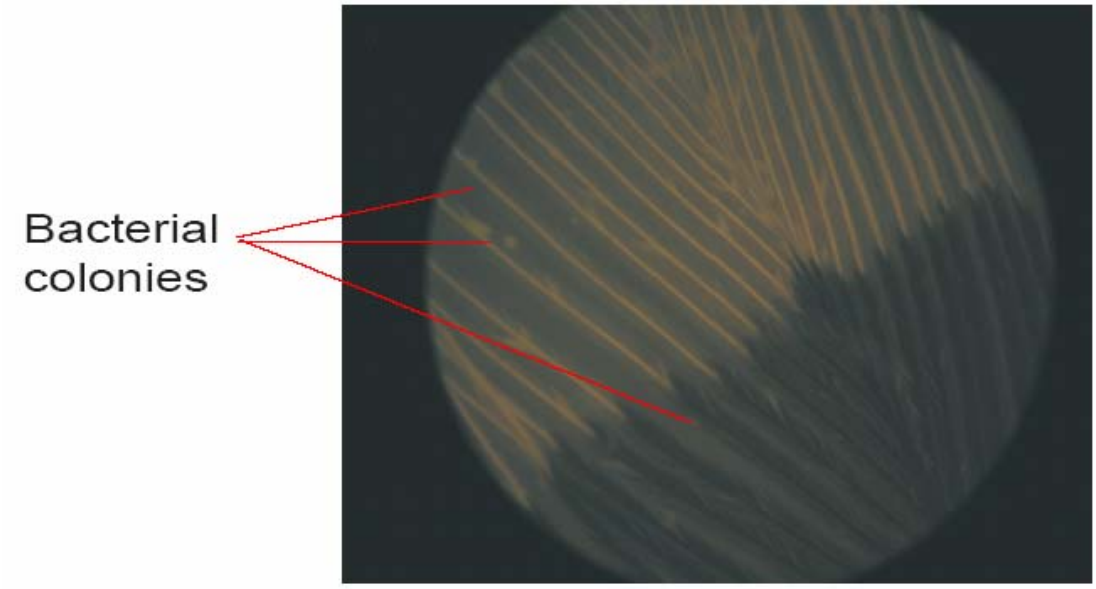

Figure 1: Image of bacterial colonies under magnification (45X) on black and white feathers of a zebra finch tail feather.

Feather degradation by EB without broth:

Twelve tail feathers, taken from separate zebra finches and containing alternating black and white bands, were UV sterilized (30 minutes) and kept aseptically in separate sterile 1.5 ml micro-centrifuge tubes. The tubes were divided equally into groups B and C. The six feathers in the group B tubes were soaked in 10^5 cells/ml of EB suspension [cell density estimated by Direct Microscopic Counts (Atlas 1995)], whereas the other six feathers (group C) were soaked in sterile distilled water. All tubes were then sealed with wax tapes and incubated at 25°C. The numbers of black and white broken ends (called barbs) on all feathers were counted at 10 X magnification, on the 30^{th} day of their incubation. Every black and white feather was counted in three separate low power fields and their mean numbers of black and white barbs were computed and used for the statistical analysis. Thus, the setup aimed at observing the disparity in feather degradation between melanized and non-melanized feathers, involving minimum exposure of feathers to water.

Feather degradation by EB in broth:

Black and white feathers from fowl were UV sterilized (30 minutes). 100 µg black and white feathers were added to 20 tubes (ten for either feather type), containing 10 ml sterile PBS. 100 µl of the EB suspension [10^5 cells/ml of EB suspension: cell density estimated by Direct Microscopic Counts (Atlas 1995)] was added to 18 test tubes (nine of either feather color) and the remaining two tubes, were kept as controls. All 20 tubes were incubated at 25°C for 10 days. Following incubation, a 100 µl sample from all test and control tubes was plated out on sterile nutrient agar plates in two dilutions (Atlas 1995). The plates were incubated at 25°C overnight and their numbers of colonies were counted following the incubation (Atlas 1995). Immediately after sampling, the tubes were centrifuged at 5000 rpm for 5 minutes. The total protein present in 1 ml of the supernatants of all feather broths was quantified, using the Biorad Protein Assay kit. The total protein present in the supernatant of a sample was assumed to be an indicator of the feather degradation observed in that sample. All statistical analysis were done using JMP 5.0.1.

Results:

EB growth on feathers in Agar:

The white bands of the feathers were found to have significantly higher numbers of colonies, than the black bands. (Fig. 2, t test; $t = 2.38$, $n = 14$, $p = 0.003$). We also found that the difference between the number of colonies on white and black bands, increases significantly with an increase in the total number of colonies (Fig. 3, $r^2 = 0.69$, $n = 17$, $p < 0.001$).

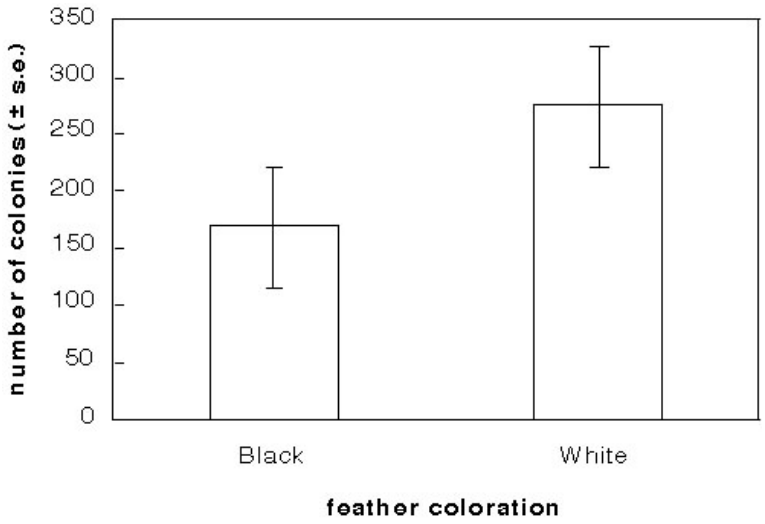

Figure 2: Number of colonies (mean ± SD) growing on white and black feathers.

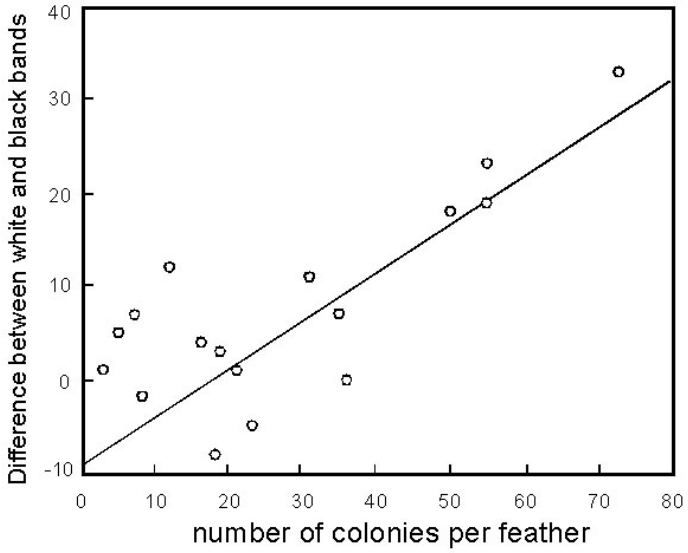

Figure 3: Difference in the number of colonies growing on white and on black bands in relation to the total number of colonies growing on the feathers.

Feather degradation by EB without broth:

We found that on EB treatment, feathers had significantly more white barbs breaks, than black barb breaks (Fig.4, t test; t = 5.39, n = 6, p = 0.003). We found that feathers treated with EB developed more breaks, than control feathers (Analysis of Variance, $F_{1, 11} = 41.67$, $p < 0.001$). We also found that the difference in the number of breaks between white and black feather barbs, increases significantly with an increase in the total number of feather breaks ($r^2 = 0.73$, n = 6, p = 0.02).

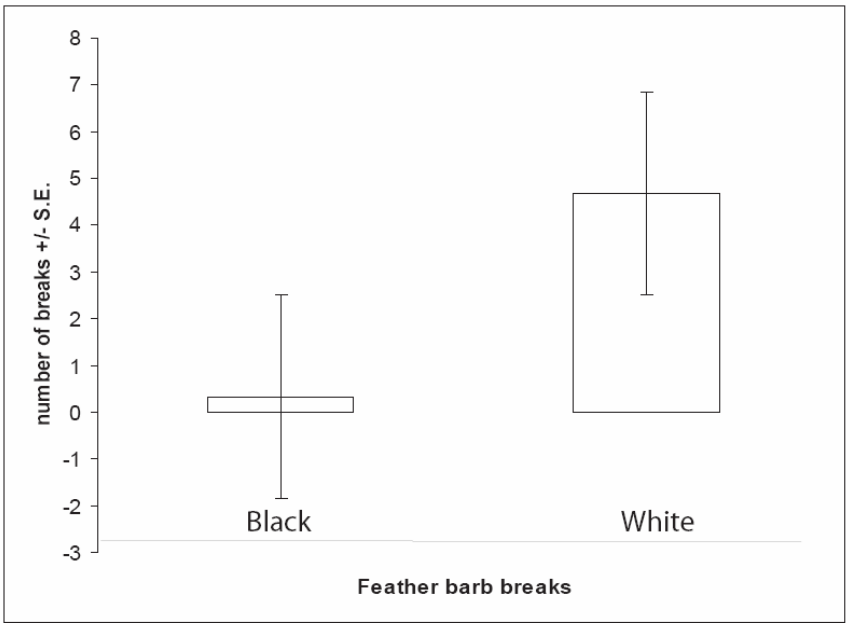

Figure 4: Number of breaks on barbs of white and black feather

Feather degradation by EB in broth:

We found that on incubation in broth with EB, white feather broths had significantly more protein in supernatant, as compared to black feathers broths (Oneway ANOVA, $F_{1, 17} = 10.67$, $p = 0.005$). The bacterial density was also found to be significantly higher in white feather broths, than black feather broths (Fig.5, $F_{1, 17} = 28.66$, $p < 0.0001$). We found the quantity of protein in supernatants of broth cultures to be positively correlated with their bacterial densities (Black feather broths: $r^2 = 0.72$, n = 9, p = 0.003; White feather broths: $r^2 = 0.73$, n = 9, p = 0.003).

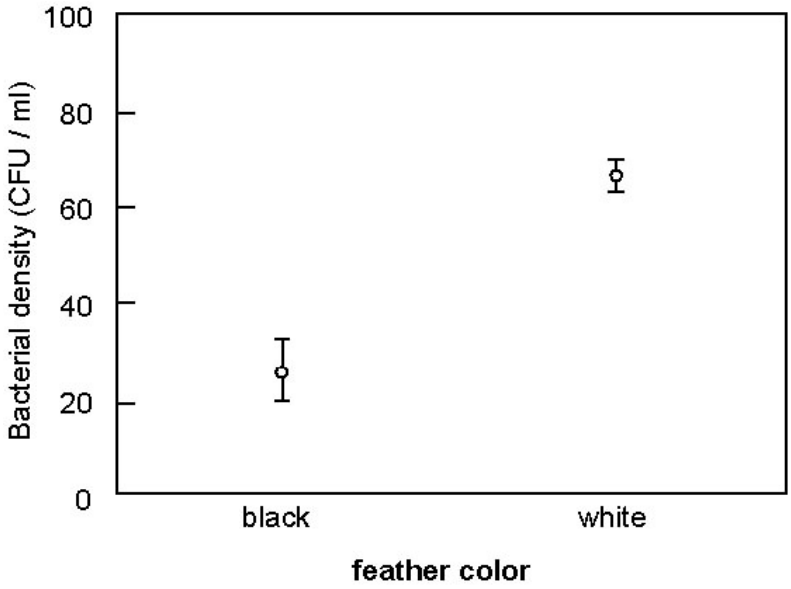

Figure 5: Bacterial densities in broth containing black or white feather fragments

Discussion:

Our experiments provide new data supporting the hypothesis that melanized feathers undergo less microbial degradation, compared to non-melanized feathers. Goldstein *et al* (2004) used a single bacterial strain and provided some evidence for this hypothesis in an earlier study, though they neither showed that the observed feather degradation was a result of bacterial activity nor commented on the bacterial growth and activity in the presence and absence of melanin. Through our experiments, we demonstrate that the presence of melanin reduces both the degradation activity and the growth of feather-degrading bacteria (EB). By testing the hypothesis in separate experiments, we find that the EB community achieves significantly higher loads on white feathers, than on black feathers. We also observe that such differential degradation of black and white feathers occurs in humid air, outside broth cultures.

We found that on incubation with EB, white feather broths witnessed significantly more feather degradation, than black feather broths. White feather broths also had significantly higher bacterial loads, than black feather broths, which significantly correlated with their observed feather degradation. Therefore, we conclude that in

broth cultures, EB degrades white feathers faster than black feathers. This observed feather degradation correlates positively with bacterial growth and density. Our results also imply that at higher bacterial loads, the difference in feather degradation between black and white feathers is significantly enhanced. We infer that the presence of melanin retards the rate of feather degradation by bacteria. We suggest that this inference be further tested in a separate experimental set-up, using increasing EB loads to degrade melanized and non-melanized feathers, to quantify the rate of feather degradation. Feathers from zebra finches and fowl followed similar pattern of differential degradation, which leads us to infer that the effects found in this experimental set-up would hold true across bird species.

According to Gloger's rule, relative humidity of an environment dictates the amount of melanin pigmentation observed in organisms habituating it (Burtt & Ichida 2004). Previous data also suggests that relative humidity is positively correlated with parasite load (Burtt & Ichida 2004). Our experiments present evidence on the inhibitory effect of melanin on the growth and activity of bacteria. This supports the hypothesis that Gloger's rule operates through hosts' bacterial loads, wherein increased humidity leads to increased bacterial loads, driving the hosts' evolution of increased melanin production as a protection against feather degradation by bacteria. In nature, feather-degrading bacteria rarely occur in pure cultures (Lucas *et al.* 2005) and hence our results showing differential feather degradation by a community of bacteria could constitute a more suitable set-up to understand the effect of melanin on microbial dynamics, as compared to those of Goldstein *et al* (2004).

Summary:

Feathers are prone to abrasion and degradation by biotic and abiotic factors. Melanin is a pigment responsible for brown and black coloration of feathers and helps increase their resistance to abrasion. Recent studies have described that a number of microbes have the ability to degrade feathers individually. However, in nature, bacteria are always found in communities, instead of single isolates, leading to

potentially different results. In this study, we applied a community of feather-degrading bacteria on black and white feathers, to test whether feather coloration altered the ability of micro-organisms to degrade feathers. We found, in three separate tests, that the presence of melanin significantly reduced the growth of a bacterial community and its' associated feather degradation. Feather degradation was found to be associated with bacterial growth and the amount of free protein produced in solution. Our results confirm earlier results found for only one bacterial strain.

Acknowledgements:

The authors acknowledge the Swiss National Science Foundation and the Fondation de Famille Sandoz (both grants to P. Heeb) for financing this research.

Appendix to Chapter 4

Niche specialization of the cultivated community of feather-degrading bacteria on feathers of live birds.

Introduction:

The total microbial load in a ton of soil is speculated to be more than 10^{16} cells (Curtis & Stoan 2005). There are estimated to be atleast 10^{6} diverse bacteria in a gram of soil (Gans *et al.* 2005) of which only a fraction have been cultivated (Watve *et al.* 2000). Bacteria in different environments have been shown to exist either attached or unattached to stratum. Bacterial populations in environments as diverse as coastal lagoons (LaMontagne & Holden 2003), peat (Barkovskii & Fukui 2004), and human buccal cavity (Zhang *et al.* 2005) were found to contain co-existing communities of attached and un-attached bacteria.

Many bacteria living attached to stratum, do so by either producing adhesins (Patti & Hook 1994), pili (Mouricout & Julien 1987), lipoteichoic acids (Sugarman 1980), or simply by producing amorphous layers of extra-cellular polysaccharides (Roberts 1996). Attachment of micro-organisms to host tissues represents a critical phase in the development of many types of infections (Zhang *et al.* 2005). On the other hand, un-attached bacteria by definition are bacteria that do not attach themselves to the stratum. Recent data suggests that the un-attached and attached communities of bacteria sharing a niche do not differ functionally with respect to primary ecological roles (Worm *et al.* 2001), but that they differ metabolically in their *in situ* activities (Bonin *et al.* 2001).

The avian plumage is one such ecological niche harbouring a diverse bacterial population (Muza *et al.* 2000), some of which have the capacity to metabolize feather keratin as their primary carbon and energy source (Burtt & Ichida 1999). The avian plumage is co-habited by both attached and un-attached bacterial communities, wherein the self-cleaning mechanisms of avian hosts decrease the loads of un-attached, but not of bacteria living attached to the feathers (Lucas *et al.* 2005). Lucas *et al.* (2005) also documented that such different communities could be separated by differential sonication of feathers in broth. Soil, the major source of avian ecto-bacteria, contains bacteria known to be capable of feather degradation *in vitro* (Lucas

et al. 2003), though there is yet no indication on their feather degradation capability on intact avian plumage.

Our previous data, using feather-degrading bacteria cultivated from soil, suggests that such bacteria are active and are able to degrade feathers *in vitro* with minimal water (Chapter 4). In this experimental set-up, we wanted to test the niche specialization of our bacterial isolates. Here, we investigate whether and which of our feather-degrading bacterial isolates (called EB: Ecto-Bacteria), are present on avian plumage as un-attached bacteria, or whether they attach themselves to feather stratum.

Materials and Methods:

Sixteen zebra finches (*Taeniopygia guttata*) were divided equally into two groups: T (Treatment) and C (Control). Both groups contained equal number of males and females and all individuals were caged individually. One hour prior to the treatment, the tail feathers of all the 16 birds were cleaned with 30% alcohol solution and dabbed dry with sterile filter paper.

A bacterial suspension composed of a known feather-degrading bacterial community (EB; Table. 1) was prepared with a cell density of 10^6 cells/ml [cell density estimated by Direct Microscopic Counts (Atlas 1995)]. Tail feathers of each of the group T birds were treated with 1 ml of the bacterial suspension, applied using a sterile brush. Tail feathers of all group C birds were treated similarly, with one ml sterile PBS and both the groups were sampled 30 hours after the treatment. For sampling, four tail feathers of all the 16 birds were removed with sterile forceps and feathers from each bird were stored in separate sterile Petri plates.

Following the sampling, three feathers from each individual were immersed in 10 ml sterile PBS, labelled (1) and sonicated for 10 seconds. The feathers were then removed and immersed in another tube containing 10 ml PBS, labelled (2) and

sonicated for 5 minutes. We assumed that bacteria that lived un-attached on feathers would require a smaller force (less sonication) to dislodge them from feathers, compared to bacteria living attached to feathers (which would require greater force and hence more sonication). The sonicated feathers were then discarded and all the (1) and (2) tubes were centrifuged at 5000 rpm for 20 minutes. The resulting pellet was re-suspended in 500 µl sterile PBS, 100 µl of which, was used to inoculate 10 ml sterile Luria Broth (LB). On incubation at 25° C for 16 hours, the LB culture tubes were centrifuged at 5000 rpm for 15 minutes and the resulting pellet was used for DNA isolation using Qiagen DNA Isolation kit. The fourth tail feather from all individuals was incubated at 25° C for 5 days, in sterile falcons labelled as (G), containing sterile PBS. Following incubation, the tubes were sonicated for 5 minutes and after discarding the feathers, were centrifuged at 5000 rpm for 20 minutes. The resulting pellet was used for DNA isolation using Qiagen DNA Isolation kit.

Isolate	**Bacteria**
N	*Janthinobacterium lividum*
BL	*Bacillus licheniformis* PWD1
19S	*Pseudomonas fragi*
7	CFB group bacterium
E	*Bacillus sp.*
1B	*Stenotrophomonas maltophilia*
5	Previous uncultured soil bacterium
20	*Arthrobacter sp*
J	*Bacillus sp.*
2Bf	Actinomycetal soil bacterium

Table 1: Composition of the EB community isolated from soil and used for this experiment.

DNA isolated from all the tubes, along with the genomic DNA of pure cultures of all the 10 bacterial strains, was subject to amplification and endonuclease treatment

(Lucas *et al.* 2003). The reaction products were then run on a 2% agarose gel with 100 bp. DNA marker along with the products of restriction and amplification of DNA from pure cultures, to identify and mark the presence or absence of known bacteria in all the test profiles.

Bacteria that were detected only in (1) and in both (1) and (2) samples of the same individuals together were assigned to the group U. Bacteria detected only in (2) samples of individuals were assigned to group A. Bacteria that were absent in both (1) and (2) samples, were scored to be found missing and not assigned to any group. Bacteria found present in (G) samples were scored as positive.

We counted the number of times every isolate occurred in groups U and A and we analysed the data using Student's t test and Tukey-Kramer's post hoc test. We assume that bacteria found in group U required a smaller force to be dislodged from feathers, as compared to those found in group A. Bacterial isolates found to be occurring in significant numbers in groups U and A were inferred to be living un-attached and attached, respectively. The data was analysed for statistical significance using JMP 5.0.1.

Results and Discussion:

We found that EB community is divided between bacteria that live attached to the feathers and those that live un-attached (Table 2). Bacterial isolates 19S (*Pseudomonas fragi*), 7 (CFB group bacterium), E (*Bacillus sp.*), and 5 (previously uncultured bacterium) were found to be present only in group U in all samples. This denotes that these isolates live un-attached on feathers, having little attachment with the substratum. Isolates N (*Janthinobacterium lividum*), BL (*Bacillus licheniformis* PWD1), and 1B (*Stenotrophomonas maltophilia*) were found to be present significantly in group U, although in some samples they were also found to be in group A. This implies that these isolates live predominantly as un-attached, but can attach themselves to feathers.

On the other hand, isolate J (*Bacillus sp.*) was found to be present only in group A, denoting that this isolate attaches itself strongly to feathers and requires a strong force to break its attachment. Isolates such as 20 (*Arthrobacter sp.*) and 2Bf (Actinomycetal soil bacterium) also were scored as bacteria living attached to feathers, though found to be present in group F of some samples. We suggest that isolates 20 and 2Bf are capable of attaching to feathers, though more than isolates N, BL, and 1B.

The bacteria in the EB community were taken from the same source and share the function of feather degradation *in vitro*, but they behave differently with respect to inhabiting their niche in feathers, wherein some will attach to the feathers and others will exist un-attached (Table 2). All the EB isolates that attached themselves to feathers were gram positive. Previous studies document the ability of other gram positive bacteria to attach and adhere to keratin from different cell types (Cole & Silverberg 1986; Tamura & Nittayajarn 2000), while there is no evidence for the adherence and attachment of gram negative bacteria to keratin and keratinous substrates. Therefore, it needs to be investigated whether the property of keratin attachment is related to the gram character of the bacterium and whether this is aided by production of specific structures such as adherins.

Attachment to a substrate involves overcoming the free energy of its surface (Bakker *et al.* 2004), which if done by production of specific receptors or other organelles and compounds would be an energy dependent task. In such a scenario, the bacteria would have to be active on the surface. Our previous work shows that members of EB community are active and functional *in vitro* on feathers, with minimal access to water. Combining it with the observation that bacteria can attach to feathers *in situ*, we suggest that such bacteria are active on the feathers using either, the feather degradation, or degradation of other compounds on feathers, for their carbon and energy needs. Further work, should involve observing the gene expression of bacteria found to be living attached to feathers, to test the hypothesis that bacteria living attached to avian plumage are active, when present on live birds.

Isolate	**Bacteria**	**Tukey - Kramer $p < 0.05$**	**p**	**Exists as**	**Growth *in vitro***
N	*Janthinobacterium lividum*	U > A	0.0002	Un-attached	+
BL	*Bacillus licheniformis* PWD1	U > A	0.0002	Un-attached	+
19S	*Pseudomonas fragi*	U > A	< 0.0001	Un-attached	+
7	CFB group bacterium	U > A	< 0.0001	Un-attached	+
E	*Bacillus sp.*	U > A	< 0.0001	Un-attached	+
1B	*Stenotrophomonas maltophilia*	U > A	0.0025	Un-attached	+
5	Previously uncultured soil bacterium	U > A	< 0.0001	Un-attached	+
20	*Arthrobacter sp*	A > U	0.0002	Attached	+
J	*Bacillus sp.*	A > U	< 0.0001	Attached	+
2Bf	Actinomycetal soil bacterium	A > U	0.0486	Attached	+

Table 2: Niche preference of all the isolates of EB community.

Acknowledgements:

The authors acknowledge the Swiss National Science Foundation and the Fondation de Famille Sandoz (both grants to P. Heeb) for financing this research.

Chapter 5

Social and asymmetric sexual transmission of bacteria in birds

The study presented in this chapter has been submitted and is in review in *Oecologia*.

Introduction:

In the study of emerging diseases and zoonotic infections, little is known about the factors and mechanisms that promote the transmission of pathogens in wild and domestic animal populations (Daszak *et al.* 2000; Harvell 2004; Morens *et al.* 2004). Animals often carry various pathogens that are responsible for high economic losses in domestic animals, pose conservation issues in wild populations. Furthermore, three -quarters of emerging human diseases are of zoonotic origin (Woolhouse 2002). Observational evidence in wild bird populations suggests that contact among individuals increases bacterial transmission (Faustino *et al.* 2004).

Epidemiological models have been developed in order to understand the dynamics of zoonotic infection and transmission rates (Ludwig *et al.* 2003). These models have shown that an understanding of pathogen dynamics also requires an understanding of the physiology and social and sexual behaviors, of vertebrate hosts (Moutou & Artois 2001; Dhondt *et al.* 2005). Recent studies in captive mammals suggest that grooming behaviors in animals could promote pathogen transmission (Weiss 2003; McGee *et al.* 2004). Most epidemiological studies of emerging diseases focus on the population patterns of diseases that rely on non-quantified behavioral mechanisms to explain the observed population patterns (Hutchings *et al.* 2003). Currently, there is a need for developing a better understanding of the routes and frequency of horizontal transmission and infection of animal pathogens.

Birds are important animal reservoirs of pathogens such as *Leshmania*, West Nile Virus, H5N1 Avian Influenza virus and various bacteria, which can lead to zoonotic infections (Webby & Webster 2003). In nature, birds carry significant microbial loads on their feathers (Lucas *et al.* 2005), in their cloaca (Lamberski 2003) and in their semen (Westneat & Rambo 2000), some of which can be potential pathogens.

Given the essential function of feathers for efficient flight, insulation and signalling, birds spend a significant proportion of their time preening their feathers (self-preening) with their beaks, to remove bacteria and other parasites (Moller 1991).

This behavior results in the maintenance of plumage condition, ensuring better functionality of feathers (Zampiga *et al.* 2004). Furthermore, birds are also known to preen the plumage of co-specifics [allo-preening; (Adkins-Regan & Robinson 1993; Pozis-Francois *et al.* 2004)] and this behavior could promote the horizontal transfer of bacteria between individuals.

Avian copulation involves cloacal contact where sperm is transferred from males to females. The avian cloaca has the dual function of being the chamber for gamete transfer, as well as for excretion (Lombardo 1998) and has been documented to host bacterial communities (Stewart & Rambo 2000). The risks of bacterial transmission during copulations in birds are expected to be high. It has been proposed that the cost of multiple copulations in birds is the risk of exposure to pathogenic cloacal microbes, as this can decrease reproductive success and /or survival (Lombardo & Thorpe 2000).

Using a captive population of zebra finches (*Taeniopygia guttata*) sharing the same environment and a traceable non-pathogenic soil bacterium *Bacillus licheniformis* PWD1 (Lin *et al.* 1995) as an experimental model system, we studied the frequency of self-infection and transmission of ecto-bacteria (EB) from avian plumage, to their gastro-intestinal tracts. We also examined whether the frequency of sexual transmission of bacteria in gender biased. We expected that, since males are sperm donors, copulations from infected males should have higher transmission rates of bacteria, than copulations from infected females.

Methods:

Birds

Forty-eight zebra finches (*Taeniopygia guttata*) of the same sex were kept in cages (50 X 40 X 35 cm) for more than two months, prior to the experiments. Food and water were given to the birds *ad libitum*. The light and dark cycle was fixed to 16:8

hours and temperature and relative humidity was controlled to 25˚C and 60% (± S.D.), respectively.

Microbiological techniques

Bacillus licheniformis PWD1 (BL), a non-pathogenic soil bacteria, was procured from ATCC (American Type Culture Collection). Primers were designed specific to the kerA gene of the bacteria . The primers were designated as **kerAfwd** (5' – AAC GGG TGT ATT AGG CGT TG – 3') and **kerArev** (5' – TTG TGA AGC TGA AAG GTT CG – 3'), which gave an amplification of 482 bp with the DNA of *Bacillus licheniformis* PWD1 (positive control). PCR was done with kerAfwd and kerArev primers at annealing temperature of 49˚C, along with a positive and negative control (genome of *Bacillus licheniformis* PWD1; PCR water and sterile buffer used for sampling, respectively). The PCR products were analyzed on a 2% agarose gel, alongside a 100 bp DNA marker, where the positive control gave an amplification of approximately 500 bp and the negative control did not respond. Hence, the presence of *Bacillus licheniformis* PWD1 in a sample was scored as a positive result by the presence of an amplified fragment of 500 bp, whereas the absence of BL was scored as a negative result, by the absence of amplified product. The numbers of positive and negative results were then counted and assigned to respective groups.

The primers were tested with other bacteria in our collection (Lucas *et al.* 2003) and did not give any amplification. The primers were also tested with bacteria on skin, plumage and cloacal bacterial samples of the birds prior to the experiment and they did not give any detectable amplification.

Ingestion and horizontal transmission

All birds were tested to be BL free before the start of the experiments. Six birds were kept as controls and were housed in three separate cages. On nine birds in the Treated group, we applied once, a BL suspension of 10^5 cells/ml [cell density estimated using Direct Microscopic Counts (Atlas 1995)] on the plumage of the neck

and wings, with a sterile brush. Care was taken not to touch the beak of the birds with the brush. Nine Untreated birds were co-housed in pairs with the Treated birds. Treated and Untreated birds were handled in a similar way, using new and sterile rubber gloves for every bird.

Cloacal bacterial sampling was done immediately before the treatments and 24, 48, 96 hours afterwards. A sterile pipette tip was rubbed on the surrounding skin of the cloaca and was later rinsed in 100 µl sterile PBS for the cloacal skin sampling. A 100 µl sterile tip was inserted in the cloacae and 100 µl of sterile PBS was used to flush sample the cloacae. The skin surrounding the cloacae was cleaned with 10% ethanol after the cloacal skin sampling, but prior to cloacal sampling. All the samples were then incubated in 10 ml sterile nutrient broth at 37˚C overnight. Cells were then harvested and their DNA isolated using Qiagen DNeasy tissue kit. This DNA was then used for PCR with the primers specific for kerA gene. The skin surrounding cloacae of all the birds (n = 48) and cloacal samples from all the control birds (n = 12), were tested to be free of BL.

Sexual transmission

Males and females were randomly assigned to the "recipient" (12 males and females) and the "donor" group (12 males and females). Before the experiment, birds were tested for the absence of *Bacillus licheniformis* PWD1 (BL), by sampling their cloaca with 25 µl of sterile PBS buffer, using a sterile micropipette. Samples, from wings, neck and the soil of their cages, were adsorbed on sterile agarose slabs. After culturing these samples overnight at 37° C, in nutrient media and harvesting their DNA, we tested them for presence of kerA gene. All the samples showed no amplification for the BL specific gene primers and hence BL was known to be absent in all the samples before the experiments were carried out.

For the experiment, we injected 25 µl of 10^5 cells/ml of BL as a tracer [estimated by Direct Microscopic Counts (Atlas 1995)], with a micropipette in the cloacae of the "donor" bird (of both sexes). We placed the "donor" bird immediately in a cage, where the "recipient" bird (of the opposite sex) had been placed 5 minutes before introducing the "donor" bird. The mating was video-recorded and the two birds where allowed to mate only once. The time interval between the release of the bird to the copulation was noted. The pairs were separated immediately after the first mating (mating time interval range: 31 – 346 seconds) and the cloacae, beaks and wing feathers of recipients and donors were sampled and tested for the presence of BL. A positive sexual transmission of bacteria was determined by the presence of BL in cloacae (and only the cloacae) of the recipient after a single copulation. The data was analyzed using the software JMP 5.1.

Results and Discussion

Ingestion and horizontal transmission of bacteria:

After applying BL on feathers of zebra finches, we found that this ecto-bacterium is ingested by the birds and infests their digestive tract (Fig 1 a). The oral route of infection also led to the contamination of the cloacae (and hence the gut) of the untreated individual sharing the same cage (Fig 1 a), possibly through allo-preening (Adkins-Regan & Robinson 1993; Pozis-Francois *et al.* 2004). In contrast to controls, a significantly high proportion of zebra finches had their guts infested with BL, contracted from their own plumage (self-infected), or from the plumage of another co-habited (allo-infected) individual (Fisher test: Self Infection: n = 9, p = 0.0004; Allo-Infection: n = 9, p = 0.002). After 48 hours of treatment, none of the control individuals were contaminated by BL, whereas we still detected BL in the cloaca of a few birds from the treated and untreated individuals. In all the individuals, BL was found to be absent from all samples 96 hours after the treatment (Fig. 1 a), revealing that, at the concentrations used in this experiment, BL led to transient gut infestations.

Here, we show that bacteria present on the plumage will be ingested and infest the gut of the host (Fig. 1 a). We propose that since bacteria were neither applied, nor found outside the cloaca of the birds, the ingestion of BL was probably mediated by the hosts' self-preening activities. Our results suggest that self-preening of the plumage will lead to risks of contamination by ecto-bacteria present on feathers.

Our study also shows that ecto-bacteria on the plumage of one bird will be rapidly transmitted to another bird sharing the same spatial location (Fig. 1 a). Birds in close contact commonly engage in social behaviors like allo-preening (Adkins-Regan & Robinson 1993). Hence, in our study allo-preening could have led to the transmission of bacteria among birds sharing the same environment. It has been proposed that close contact and social interactions, among individuals living in the same group could lead to horizontal transmission of bacteria (Faustino *et al.* 2004). Our study provides evidence for fast horizontal transmission and gut contamination by bacteria. This effect is likely to be important in wild bird populations, which harbour a variety of bacteria are found on their plumage (Lucas *et al.* 2005).

Sexual transmission of bacteria:

After infecting the cloaca of males and females with BL, we found that after a single copulation the sexual transmission of bacteria between sexes was significantly more frequent when infected males copulated with uninfected females, than vice versa (Fig 1 b; Fisher exact-test, $n = 24$, $p = 0.0006$). This asymmetry in frequency of sexual transmission of BL between sexes was expected as the males are transferring sperm to the females (Westneat & Rambo 2000). In addition, we found that females can also transmit bacteria at a certain rate to males, suggesting that sexual transmission of pathogens can also take place through physical contact of the two cloaca. As proposed, sexual transmission of pathogens has thus the potential to affect the evolution of mating patterns and sexual behaviors (Sheldon 1993).

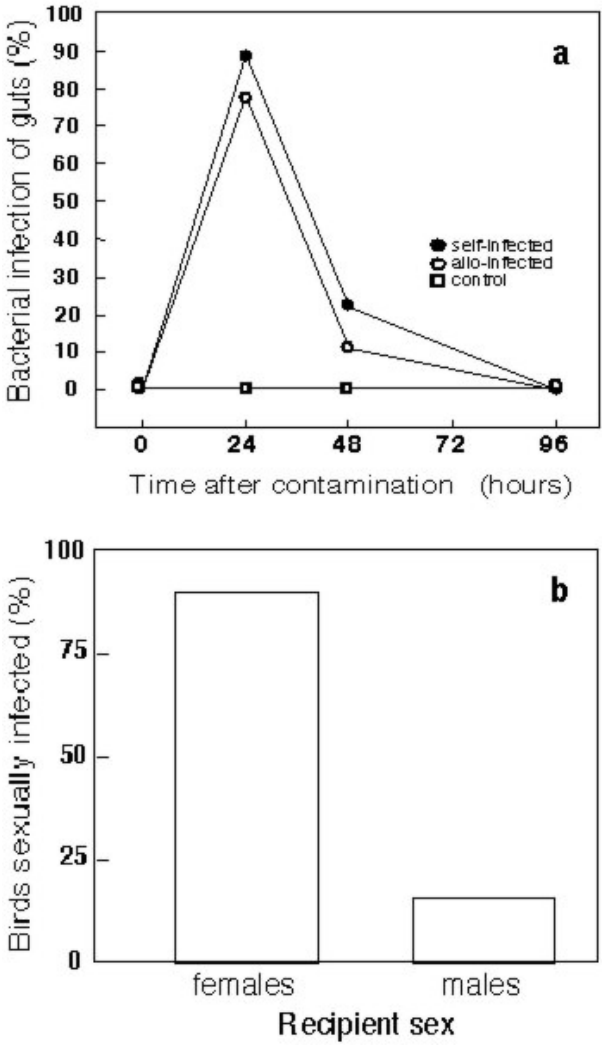

Figure 1: **Horizontal and sexual transmission of *Bacillus licheniformis* PWD1 (BL) in zebra finches**

a. Proportions of zebra finches with BL gut infestation contracted from BL placed on their feathers (self-infected) or on the feather of another individual (allo-infected). BL was not applied on the feathers of control birds.

b. Proportions of zebra finches contracting BL after a single mating with birds from the opposite sex that had been experimentally infected.

Results from our two experiments suggest the existence of a potential transmission link between the oral, faecal and genital routes of pathogens in birds. In this route, infectious micro-organisms present on the feathers can gain access to avian guts through preening and can get transmitted between individuals by allo-preening and sexual contact. Many wild birds live in large social groups, where horizontal bacterial transmission has been proposed to increase with group size (Spilling *et al.* 1999). Our study suggests that external contamination in association with social contacts and copulations can promote rapid dispersal and horizontal transmission of pathogens among host population.

Prevalence of avian infectious diseases is known to have seasonal and yearly dynamics and social and other behaviors have been hypothesized to be a factor in these dynamics (Altizer *et al.* 2004). Our data suggests that the evolution of self and allo-preening rates and mating frequencies are likely to be shaped in response to bacterial loads present on feathers and the risks of horizontal transmission of pathogens. In absence of experimental epidemiological data on wild vertebrates, such experimental models of sexual transmission could be used to quantify the behavioral factors shaping the dynamics of emerging diseases.

Summary:

Understanding the behavioral mechanisms that mediate pathogen transmission in social hosts like birds could provide the empirical bases for explaining the epidemiological dynamics of zoonotic infections. By experimentally infecting the feathers and cloaca of captive zebra finches (*Taeniopygia guttata*), with the bacterium *Bacillus licheniformis* PWD1 (BL), we examined the self contamination and horizontal transmission of birds sharing the same environment. We also examined whether sexual transmission of bacteria is gender biased. Our results show that bacteria placed on the plumage of the birds lead to self and allo-infections, possibly through preening behaviors. Furthermore, we found that sexual transmission of the bacteria was asymmetrical, being higher when males are the transmitting sex.

Our results suggest the existence of an oral – faecal – genital route of transmission for avian hosts, wherein bacteria on feathers get ingested through self and allo-preening. Gut bacteria can then be transmitted sexually with transmission rate being higher when males are the infected sex.

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Chapter 6

Conclusion

This thesis documents the interactions between the load and activity of ecto-bacteria and their hosts' morphology, physiology, and behavior. It also demonstrates the importance of social and sexual behaviors of birds in the transmission of potential pathogens within a population.

Chapter 2 supports the hypothesis, that enhanced loads of a community of feather-degrading ecto-bacteria (EB: Ecto-Bacteria), which comprise the normal flora of birds, can significantly alter the hosts' sexually selected traits. Results demonstrate that an increase in the EB load of zebra finches significantly reduces the intensity of males' bill color, a sexually selected trait (Zann 1996). They also significantly reduce the ability of males to enhance their bill color, in response to mating opportunities. Although the EB treatments do not elicit a cellular immune response from hosts, results indicate the presence of a carotenoid based response afforded against these symbionts. The source of carotenoids to mount such a response was found to be different between the sexes, with females recruiting circulating plasma carotenoids and males using carotenoids stored in their bills, effecting a change in intensity of males' bill color. On EB treatments, the enhanced bacterial load was accompanied with a decrease in the uropygial gland volume. The uropygial gland secretions (preen oil) possess anti-microbial properties (Shawkey *et al.* 2003). This suggests that an increased secretion of preen oil, as a defense response to EB, leads to a decrease in uropygial gland volume over time. Such a reduction in uropygial volume can alter preen oil contents and reduce preen oil secretion over time, changing the plumage condition and attractiveness of birds. Altogether, these results indicate that the observed reduction in condition-dependent morphological traits, is effected by a trade-off between investment in morphological displays (such as beak color intensity) and in defense response to EB (carotenoid and preen oil based response). Hypothetically, the ingestion of EB while preening and their subsequent detection by intestinal immune tissues can trigger such a trade-off. Alternatively, EB activity on the feathers and skin of birds could mediate their detection and trigger a carotenoid response from their hosts.

Chapter 3 studies the effect of EB community on male mate choice in birds, following Chapter 2 results, where enhanced bacterial loads did not alter mate choice traits (body mass and intensity of bill color) in females. Chapter 3 results show that male mate choice varies with male size. They indicate that the bacterial load of females also affected the male mate choice, wherein larger males chose females with enhanced bacterial load and smaller males preferred females with lower bacterial load. This suggests that males of different sizes use different male mate choice strategies, while choosing between females with differing bacterial loads but similar morphological displays. I speculate that larger males choose for females with better tolerance to EB, implying preference for females with un-altered morphological displays despite enhanced bacterial loads. On the other hand, smaller males, with limited mating possibilities, would further reduce their mating opportunities on infection with microbes. Hence, given a choice, smaller males would avoid mating with infected females, thus avoiding infection to sustain their body condition and long-term mating opportunities. Results support this speculation, as they demonstrate smaller males avoiding proximity to females with higher bacterial load. Males can also perceive females with un-altered morphological displays and lower bacterial load, to have better microbial clearance rates. Together, this implies that the smaller male's strategy is to avoid females with higher bacterial load and choose for females with a better microbial clearance rate, supporting the case for their choice of females with un-altered bacterial loads. Results also suggest that males detect a difference in the bacterial load of females, without the EB altering females' condition-dependent morphological traits measured in this experiment. Identifying the mechanism for such detection requires further work. Hypothetically, the males could detect an effect of EB on some behavioral traits (such as song, courtship displays) of females. Such a change could mediate the detection of females' bacterial loads.

Taken together, Chapters 2 and 3 document the effect of EB on the behavior and morphology of birds. Chapter 4, on the other hand, documents the effect of avian morphology on the activity and growth of EB. The experiments tested the effect of feather melanin on the activity and growth of EB community in different conditions *in vitro* and found that the presence of melanin lowers bacterial growth and activity.

They also demonstrate the ability of EB community to degrade feathers outside broth. These results also help support speculations for a role for keratinolytic bacteria in the evolution of melanin based feather coloration. Additional experiments document that some EB isolates attach themselves to feathers on live birds, whereas others live freely on them. Activity of EB on feathers in minimal water conditions and their *in situ* attachment to feathers leads to the speculation of EB isolates being active on live birds. Such active EB can effect feather degradation and cause deterioration in plumage quality, a sexually selected trait in hosts. This could in turn, mediate the recognition of females with enhanced EB presence, as reported in Chapter 2. In addition, all the EB isolates found attached to feathers were found to be gram positive. Hence, it should be tested whether attachment to feather keratin is a trait linked to the gram character of a bacterium.

The effect of symbionts on host body condition and mate choice, has been largely studied using pathogens and obligate parasites (Blanco *et al.* 1999). Contrasting with the stark differences caused by the presence of parasitic symbionts and pathogens (Latta 2003; Navarro *et al.* 2003; Sol *et al.* 2003), experimental manipulation of EB loads caused alterations in condition-dependent morphological traits and physiology of hosts without causing a change in body mass or cellular immune response of the hosts. The EB community used to treat hosts in Chapters 2 and 3 had a cell density similar to that of pond water frequented by birds (Al-Harbi 2003). Both the elevated and the un-altered EB load of birds were found to occur in wild (Burtt & Ichida 1999; Lucas *et al.* 2005). This implies that the observed alterations in hosts' morphology, physiology, and behavior, mediated by EB, could also occur in wild. Taking together Chapters 2, 3, and 4, we speculate that hosts' body condition is dependent on symbiont load and that the symbionts' activity and load depend on host morphology. In addition, we observe that the hosts' response to symbionts depends on hosts' physiology, which in turn depends on symbiont load. This implies that the hosts body condition and symbiont load and activity could be linked in a loop, wherein hypothetically, there should be no conflict of interest.

Finally, Chapter 5 suggests that preening (self-cleaning) behavior of birds aids EB access the avian gut; whereas allo-preening, a social interaction, is implicated in the horizontal transmission of EB. Since pathogens are often present on the plumage of birds (Hubalek *et al.* 1995; Nuttal 1997), such self-cleaning and social interactions can act as the route of infection and horizontal transmission of pathogens. These results also support the prediction from Chapter 2, that preening aids the EB access avian gut. Possibly, such ingestion of bacteria could also help hosts detect and respond to some symbionts, using intestinal immune tissues, apart from actively reducing ecto-bacterial load. Experiments also suggest that symbionts ingested during preening can be sexually transmitted, with the cloacae being the common chamber for faecal and genital exudates, implying a linked oral – faecal – genital route of transmission. Results demonstrate that sexual transmission is asymmetric between the sexes, with males sexually transmitting bacterial symbionts significantly more frequently than females. The risk of sexually transmitted pathogens has been suggested to drive the evolution of mating behaviors (Boots & Knell 2002). Together with Chapter 5, they suggest that bacterial loads are likely to shape the evolution of self-maintenance, social interactions, and mating behaviors and frequencies, due to the risks of horizontal transmission of pathogens. This result is significant to epidemiology of wild and zoonotic diseases, as it implicates important self-maintenance, social, and sexual (intra-specific) interactions of birds in the dispersal of pathogens. In addition, this study proposes the use of similar animal models in investigating the social and behavioral factors shaping the epidemiological patterns of emerging diseases.

In review, I conclude that this thesis has helped understand the effects of keratinolytic bacteria on their hosts. It has done so by contributing to establish a link between the load and activity of these symbionts and some morphological, physiological, and behavioral traits of their hosts. This work suggests that the hosts' sex determines the mechanism of its defense response to symbionts. The thesis also sheds light on the effect of these symbionts on mate choice of birds. Although these experimental results do not clearly indicate the effect of such symbionts on the fitness of their hosts, we predict that these bacteria are opportunistically parasitic

symbionts of birds, as they reduce condition-dependent morphological and physiological traits and evoke hosts' response to their increased presence. This thesis implicates social and sexual behaviors of birds in the transmission of bacterial symbionts, suggesting that hosts' behavior negotiates pathogen dispersal. It also suggests a linkage between the faecal – oral and genital routes of pathogen transmission, in birds. Effectively, it provides us with an experimental system, which could test existing epidemiological models of transmission of avian pathogens.

Abstract/ Résumé

Birds harbor a variety of bacteria on their plumage, some of which can degrade feathers *in vitro*. Whether these keratinolytic bacteria are active on live birds and can effect feather degradation on birds is debatable. The effect of such bacteria on the body condition and behavior of birds, is unknown. Using a community of feather-degrading bacteria (EB), we investigate the interaction between the activity and load of such bacteria, on the morphology, body condition, and behavior of zebra finches (*Taeniopygia guttata*).

In Chapter 2, we find that the elevated loads of such microbes lead to a reduction in the expression of morphological traits, such as male bill color (a sexually selected trait) and uropygial gland volume, without reducing body mass, or evoking a cellular immune response. We also suggest the presence of a carotenoid based defense response in hosts, to such elevated loads of microbes and document a sex-based difference in the source of carotenoids used for such a response. In Chapter 3, we investigated the effect of EB loads on male mate choice of zebra finches, wherein male choice of females with elevated and un-altered bacterial loads, varied with male size. We found that larger males preferred females with higher bacterial load and smaller males preferred females with lower bacterial load. Chapter 4 demonstrates that the presence of melanin in feathers reduces the growth and activity of the community of feather-degrading bacteria (EB) and that the EB community can effect feather degradation in humid conditions, without broth. Additional results also demonstrate that the EB community consists of bacteria that can attach themselves to feathers on live birds and those that can live freely on avian plumage. Finally, chapter 5 demonstrates that the self-maintenance, social and sexual behaviors of birds are implicated in the infection and horizontal transmission of bacteria. It also suggests a linked oral – faecal – genital mode of transmission of pathogens in birds.

These results demonstrate that differential loads of normal flora of vertebrate hosts can effect changes in their morphology and behavior. They also shed light on the role of feather-degrading bacteria in the evolution of melanin polymorphism in birds and suggest that bacteria can be active on live birds. This thesis also highlights the importance of social and, sexual behaviors of birds, in epidemiology.

Les Oiseaux ont dans leur plumage diverses bactéries dont certaines dégradent les plumes *in vitro*, néanmoins. Il n'est pas clair, au vu de précédentes études, si ces bactéries kératinolytiques sont actives sur des oiseaux vivants, et si celles-ci dégradent effectivement le plumage de leur hôte. L'effet de ces bactéries sur la condition corporelle ainsi que le comportement des oiseaux n'est pas connu. A l'aide d'une communauté de bactéries dégradant les plumes (EB), non pathogènes, nous examinons les interactions entre l'activité et la charge bactérienne sur la morphologie, la condition corporelle et le comportement du diamant mandarins (*Taeniopygia guttata*).

Dans le chapitre 2, nous montrons qu'une charge élevée de ces microbes mène à une réduction de l'expression de certains traits morphologiques, tels que la couleur du bec chez le mâle (un trait soumis à sélection sexuelle), ainsi que le volume de la glande uropygienne, sans qu'il y ait une réduction de la masse corporelle, ni déclenchement d'une réponse immune cellulaire. Nos données suggèrent la présence d'une défense chez l'hôte à des charges élevées de bactéries basée sur la présence de caroténoïdes. Nous montrons, de plus une différence liée au sexe dans la source des caroténoïdes utilisé pour cette réponse. Dans le chapitre 3 nous examinons l'influence de la charge bactérienne EB sur le choix des mâles chez le diamant mandarins. Des femelles avec une charge bactérienne normale et augmentée sont choisies par les mâles et ce choix varie avec la taille des mâles. Nous avons mis en évidence que les grands mâles préfèrent les femelles avec une charge bactérienne plus élevée. Les petits mâles préfèrent les femelles avec une charge bactérienne réduite. Le chapitre 4 démontre que la présence de mélanine dans les plumes réduit la croissance et l'activité de la communauté de bactéries dégradant le plumage (EB), et que cette communauté EB peut dégrader les plumes dans des conditions humides, sans milieu de culture liquide. De plus nous montrons que cette communauté consiste en des bactéries qui peuvent s'attacher sur les plumes d'oiseaux vivants ainsi que des bactéries libres. Pour finir nous montrons dans le chapitre 5 que la maintenance corporelle, l'interaction sociale et le comportement sexuel de ces oiseaux sont impliqués dans l'infection et la transmission horizontale de ces bactéries. Nos données suggèrent une transmission orale-fécale-génitale des pathogènes chez les oiseaux.

Ces résultats montrent que des charges différentes de la flore bactérienne habituelle et non pathogène de vertébrés peuvent affecter leur morphologie et leur comportement. Ils éclaircissent également le rôle des bactéries dégradant les plumes dans l'évolution du polymorphisme mélanique chez les oiseaux et suggèrent que ces bactéries peuvent être actives sur des oiseaux vivants. Cette thèse souligne également l'importance du comportement social et sexuel des oiseaux dans l'épidémiologie.

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