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Haider Al-Murayati

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Par

Haider Yousif Ahmed AL-MURAYATI

**Diversity of the bacterial community and secondary sexual
characters in the peacock**

Thèse présentée et soutenue à Orsay, le 28 Avril 2017 :

Composition du Jury :

Dr. Puri Lopez	ESE, Université Paris-Saclay, FRANCE	Président
Pr. Marion Petrie	Université Newcastle, ROYAUME-UNI	Examineur
Dr. Julien Gasparini	IEES, Université Pierre et Marie Curie, FRANCE	Rapporteur
Pr. Manuel Martín-Vivaldi	Université Grenade, ESPAGNE	Rapporteur
Dr. Anders Pape Møller	ESE, Université Paris-Saclay, FRANCE	Directeur de thèse

TITLE

Diversity of the bacterial community and secondary sexual characters in the peacock

ABSTRACT

Bird feathers harbour numerous microorganisms that could be acquired from the surrounding environment, these microorganisms may exert intense selection on their hosts by reducing fecundity and survivorship. Several bacterial taxa that live on feathers have the ability to degrade feather keratin and cause damage to feather structure and may alter the feather colouration. Birds use visual signals such as bright colours or exaggerated ornamentation for socio-sexual communication as well as species recognition. Only healthy individuals are able to produce exaggerated secondary sexual characters and still remain resistant to debilitating parasites. Peacocks (*Pavo cristatus*) is a polygamous species that have different exaggerated ornamentation, the most notable secondary sexual characters of the peacock are their long-decorated trains that comprise the magnificent ocelli which contain three different iridescent colours.

Through a culture based technique we isolate feather bacterial community from differently coloured parts of the ocelli of the peacock's train. The study reveal that there was a heterogeneous distribution of bacteria among the differently coloured parts of ocelli. The abundance and prevalence of specific bacterial taxa was related to the degree of feather degradation, expression of different secondary sexual character, changes in ocelli colouration and daily growth increment. Furthermore, we found a small effect of the expression of secondary sexual characters on biasing of brood sex ratio towards production of more sons than daughters.

The work presented in this thesis provide evidence that feather ocelli may consider as a reliable signal of the diversity and the abundance of bacteria in peacock and in consequence indication for the individual quality and that allowing the choosy females to pick males with a specific bacterial community.

KEY WORDS: Feather bacteria; barb breakage; daily growth increments; feather colouration; feather degradation; feather; moult; ocelli; peacock train; sex ratio; spur length; train length.

TITRE

Diversité de la communauté bactérienne et caractères sexuels secondaires chez le paon

SYNTHÈSE EN FRANÇAIS

Les plumes d'oiseaux abritent de nombreux microorganismes qui pourraient être acquis dans l'environnement, ces microorganismes pouvant exercer une sélection intense sur leurs hôtes en réduisant leur fécondité et leur survie.

Plusieurs taxons bactériens qui vivent sur des plumes ont la capacité de dégrader la kératine des plumes et causent des dommages à leur structure et peuvent modifier aussi leur coloration. Les oiseaux utilisent des signaux visuels tels que des couleurs vives ou des ornements exagérés pour la communication socio-sexuelle ainsi que la reconnaissance des espèces. Seuls les individus en bonne santé sont capables de produire des caractères sexuels secondaires exagérés et restent résistants aux parasites. Le paon (*Pavo cristatus*) est une espèce polygame qui a plusieurs décorations exagérées, les caractères sexuels secondaires les plus remarquables du paon sont leur traîne décorée avec des ocelles magnifiques qui contiennent trois couleurs irisées différentes.

Grâce à une technique basée sur la culture, j'ai isolé des bactéries à partir des plumes de différentes parties colorées des ocelles de la traîne du paon. Cette thèse traite des cinq questions suivantes concernant l'association entre la communauté bactérienne et l'expression des plumes ocelles de la traîne du paon.

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Chapitre un :

Le problème de gradation de Darwin chez paon ocelles :

Dégradation des ocelles par les microorganismes

Darwin (1871) a noté que les paons *Pavo cristatus* ont des ocelles qui sont entourés par des anneaux partiellement transparents provoqués par une absence de barbules faisant apparaître les ocelles comme isolés de la traîne. Ces zones translucides peuvent rendre les plumes susceptibles de se briser soit mécaniquement, soit par suite de l'action des microorganismes. Nous avons testé s'il y avait une différence dans l'abondance et la diversité des bactéries dans les trois parties de couleurs différentes des ocelles, si le degré de dégradation des plumes diffère entre les parties de couleurs différentes, et si le degré de perte de barbule était lié à la force requise pour les rompre. Nous avons mis en évidence une répartition hétérogène des bactéries parmi les différentes parties colorées des ocelles, que le degré de dégradation des plumes dans des parties colorées différentes des ocelles dépendait de l'abondance et de la diversité des bactéries, que la force nécessaire pour briser les barbules était liée à la diversité des bactéries dans les différentes parties colorées des ocelles, et que les paons avec de grandes ocelles ont perdu relativement peu de barbules. Ces résultats sont compatibles avec l'hypothèse selon laquelle les bactéries peuvent jouer un rôle important dans les dommages et la dégradation des parties colorées différentes des ocelles des paons et que le phénotype des ocelles peut révéler des informations fiables sur l'infestation par des microorganismes chez les femelles et les mâles concurrents.

Mots-clés : Diversité bactérienne ; Barbules ; Barbes ; Dégradation des plumes ; Ocelles ; Paon.

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Chapitre deux :

Pourquoi les paons ont-ils tant de caractères sexuels différents ?

L'évolution de caractères sexuels exagérés dans un seul sexe, habituellement masculin, reste une énigme car les facteurs sous-jacents limitant leur expression sont mal compris. Nous suggérons ici que la diversité et l'abondance de bactéries peuvent réduire la quantité de ressources allouées à la production de caractères décoratifs extravagants et constituent donc un facteur négligé. Nous avons étudié la relation entre la prévalence et l'abondance de la communauté bactérienne dans des parties colorées différentes des ocelles de la traîne du paon *Pavo cristatus* et leur relation avec l'expression de caractères sexuels secondaires (nombre d'ocelles, longueur de la traîne, croissance de la traîne, longueur d'éperon et croissance de l'éperon). La communauté bactérienne dans les parties vertes, bleues et brunes de la traîne du paon variait parmi les trois zones des ocelles. Le nombre d'ocelles semble négativement lié à l'abondance et à la diversité des bactéries. De même, le nombre de barbules perdues de la partie inférieure des ocelles apparaît positivement lié à l'abondance et à la diversité des bactéries. La présence de quelques taxons bactériens tels que *Paenibacillus* sp. et *Solibacillus silvestris* semble lié à la longueur de la traîne et aux éperons. Ces résultats sont compatibles avec l'hypothèse selon laquelle différents traits sexuels secondaires fournissent une image partielle de l'état général des paons mâles.

Mots-clés : Diversité bactérienne ; Dégradation des plumes; Paon ; Barbes ; Ocelles ; Éperon ; Traîne.

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Chapitre trois :

Le sex ratio chez le paon est-il lié à l'expression de caractères sexuels secondaires?

On prétend souvent que les femmes qui s'accouplent avec des camarades attrayants devraient produire plus de fils parce que ces fils hériteront des traits d'attractivité de leur père et, par conséquent, augmenteront leur succès reproductif à travers les accouplements de leurs fils. La manipulation du sex ratio adaptatif par les femelles chez les oiseaux nicheurs est devenue une priorité majeure en biologie évolutive ces dernières années et plusieurs études empiriques et théoriques ont abordé cette hypothèse, avec des résultats incohérents qui ont entraîné une confusion considérable.

L'incohérence des résultats dans ce domaine est principalement attribuée à le biais d'échantillonnage. Dans la présente étude, et en utilisant un grand ensemble de données pour éviter les problèmes de biais d'échantillonnage, nous avons utilisé les traits sexuels secondaires multiples du paon *Pavo cristatus* qui sont supposés être impliqués dans le choix des femelles et qui jouent un rôle important dans la sélection sexuelle ; afin de voir si l'expression de ces traits est corrélée avec le sex ratio dans la couvée du paon. Nous avons constaté une faible corrélation positive au sein de l'expression de caractères sexuels secondaires qui tend à biaiser légèrement le sex ratio en faveur des mâles. Le sex ratio observé était significativement plus petit que celui rapporté dans la méta-analyse par Ewen et al. (2004), ce qui implique qu'il est possible de démontrer des effets de taille, modestes mais significatifs.

Mots-clés : Barbes ; Ocelles ; Paon ; Sex ratio ; Longueur d'éperon ; Longueur de la traine.

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Chapitre quatre :

Les signaux visuels des paons révèlent-ils l'abondance et la diversité des microorganismes ?

Les oiseaux utilisent des signaux visuels tels que des couleurs vives ou des ornements exagérés pour la communication socio-sexuelle ainsi que la reconnaissance des espèces. Les plumes d'oiseaux abritent de nombreux microorganismes, dont certains sont en mesure de dégrader la kératine des plumes, comme certaines bactéries, qui peuvent affecter l'intégrité des plumes et altérer leur coloration. Les ocelles de la traine du paon (*Pavo cristatus*) contiennent trois couleurs irisées distinctes. De tels ornements sont considérés comme des signaux de qualité «honnête» parce qu'ils sont coûteux à produire et reflètent la condition physique de l'individu. Nous avons émis l'hypothèse que les différentes parties d'ocelles sont sensibles à la dégradation par des microorganismes dans une mesure différente. Nous avons étudié s'il y avait une relation entre l'abondance et la diversité de la communauté bactérienne dans les ocelles de la traine du paon et la coloration des taches brunes, vertes et bleues des ocelles. Nous avons montré que la communauté bactérienne dans les ocelles était liée à des changements dans la coloration des trois parties de couleurs différentes, les principaux changements étant trouvés pour la zone brune des ocelles. Ces résultats soulignent l'importance de la zone brune dans la sélection sexuelle chez le paon.

Mots-clés : Diversité bactérienne ; Dégradation des plumes ; Coloration des plumes ; Ocelles ; Paon.

Traduit par Google

Chapitre cinq :

Les bactéries des plumes peuvent influencer la croissance quotidienne des ocelles des plumes de paon

Les animaux peuvent voir l'intégrité de leur tégument affectée via la présence de microorganismes acquis dans leur environnement. Les agents pathogènes tels que les microorganismes peuvent exercer une sélection intense sur leurs hôtes en réduisant la fécondité et la survie. Les bactéries sont fréquentes dans le corps de tous les organismes vivants, mais aussi sur la peau, les écailles, les cheveux et les plumes. Plusieurs taxons bactériens qui vivent sur des plumes ont la capacité de dégrader la kératine de plumes et de causer des dommages à leur structure. Le développement et l'entretien des écailles, des plumes et des cheveux demandent de l'énergie et du temps, car ils sont essentiels pour les fonctions fondamentales de ces structures. Les taux d'incrément de croissance quotidienne des plumes peuvent être facilement quantifiés à partir des bandes claires et foncées alternées sur les plumes. Nous avons étudié la relation entre la prévalence et l'abondance de la communauté bactérienne dans les différentes parties colorées des ocelles de la traine du paon et le taux d'incrément de croissance quotidienne. Nous avons également étudié la relation entre trois variables de couleurs différentes (θ , ϕ et r) obtenues pour les différentes taches des ocelles et les incréments quotidiens de croissance des plumes. La communauté bactérienne dans les différentes parties colorées des ocelles différait considérablement. L'abondance de *Bacillus licheniformis* et *Paenibacillus* sp. était positivement associé à des incréments de croissance quotidiens plus élevés, alors que l'abondance de *Micromonospora* sp. Et *Bacillus pumilus* a été associée à des augmentations de croissance quotidienne réduites. Les

trois variables de couleur ont montré des variations considérables chez les individus, même si seulement la couleur du patch bleu était négativement liée à la largeur des incréments de croissance des plumes. Ces résultats sont compatibles avec l'hypothèse selon laquelle différentes parties d'ocelles abritent différents types de bactéries qui ont un impact différent sur leurs hôtes. Ces différences impliquent que les parties colorées différentes des ocelles révèlent des informations sur la croissance des plumes et le microbiome des paons mâles.

Mots-clés : Diversité bactérienne; Dégradation des plumes ;
Augmentations quotidiennes de la croissance ; Mue; Ocelles; Paons.

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INTRODUCTION

Microorganisms are microscopic organism, commonly known as microbes. Before microorganisms were first observed some scientists postulated that diseases may be caused by "living invisible creatures" (Kumar 2015). However, these "invisible creatures" were not well described until late in the sixteenth century by the Dutch scientist Antonie van Leeuwenhoek with aid of his simple microscope. "Little animals" is the term that he used to describe protozoa and bacteria (Dobell 1958). Later and until the end of the eighteenth century, Robert Koch revealed the importance of these "little animals" as the cause of disease when he discovered and described that *Mycobacterium bacilli* was the cause of tuberculosis (Kumar 2015).

Microorganisms are found almost everywhere and they constitute the major part of the earth's biomass (Krasner 2010). The term microorganism includes a massive range of organisms including bacteria, fungi, viruses, algae, archaea and protozoa (Pepper *et al.* 2014).

Birds occupy most existing environments on the planet and there are a huge amount of data and literature on this class. Thus, birds may be considered an appropriate group of organisms for testing different hypotheses and theories in ecology, behaviour and evolution (Bennett and Owens 2002). In addition, their phylogeny is particularly well studied compared to other groups, facilitating the use of appropriate methodologies for comparative studies. Therefore, they have been used as a model to explore the evolution of life history traits (Starck and Ricklefs 1998).

Bird - parasite interactions in nature

Most birds share their environments with a diverse fauna of parasites. Parasites are defined as organisms that live in, with, or on another living being to gain access to necessary resources for their life cycle. Parasites are generally costly for their hosts due to their use of limiting resources or due to their damage imposed on hosts (Price 1980). Birds are occupied by various types of parasites that fall into two main groups, macro-parasites and micro-parasites (Anderson and May 1979). The first group comprises helminths and arthropods while the chewing lice (Insecta: *Phthiraptera*) and feather mites (Acari: *Astigmata*) represent the most diverse and abundant group in birds (Johnson and Clayton 2003; Proctor 2003). Micro-parasites include virus, bacteria and fungi. Both of these groups include ecto- and endoparasites (Campbell and Lack 2013). The whole pool of microorganisms is generally referred to as the “microbiome” (Morgan *et al.* 2013).

Ectoparasites and endoparasites impose great fitness costs on their hosts partially shaping the evolution of life history traits of the hosts (Poulin 2010). Hosts invest their resources in different life history traits like growth, reproduction and survival, with increasing investment in one component leading to a decrease in available resource for other life history traits (Stearns 1992).

Host interactions with the environment can alter their resource allocation, and parasites are one of these environments factor that reduce the availability of overall resources (Pagán *et al.* 2008). Parasites impose different types of fitness costs their hosts, directly through loss of blood and nutrients and energy allocation to defence mechanisms.

Bacteria and birds

Among microorganisms inhabiting birds, bacteria represent the vast majority. Bacteria that can cause infection in birds are phylogenetically very diverse and belong to different bacterial groups (Nuttall 1997). Birds are susceptible to pathogenic bacterial infection at all stages of their life cycle even before and after hatching, bacteria have the ability to break through the eggshell and thus infect the egg contents and eventually kill the developing embryo (Cook *et al.*, 2003; Soler *et al.* 2012). After hatching, nestlings are fully exposed to different microorganisms through food provided by parents which is usually mixed with their saliva, but chicks may also be in direct contact with nest material that already contains different microorganisms (Berger *et al.* 2003; Kyle and Kyle 1993; Mills *et al.* 1999; Singleton and Harper 1998). During the breeding season the cloacal passage (channel for both gamete transfer and faeces excretion) can be a route for transmission of sexually transmitted diseases in case of presence of pathogenic bacteria in the gut (Reiber *et al.* 1995; Sheldon 1993).

Bacteria and bird feathers

Feathers are important for birds by being related to locomotion (Rayner 1988), thermoregulation (Stettenheim 2000) and communication (Andersson 1994; Shuster and Wade 2003). Birds in order to maintain the essential functions of feathers might be affected by the presence of parasites. Birds tend to moult their feathers regularly or replace damaged feathers or feathers that are accidentally lost to restore their plumage function. Feather growth is energetically highly demanding but also time-consuming (Dietz *et al.* 1992; Murphy *et al.* 1992; Lindström *et al.* 1993; Klaassen 1995; Murphy and Taruscio 1995; Bonier *et al.* 2007; Cyr *et al.* 2008). Bird feathers have

surface structures that reflect diurnal patterns of growth with alternating light and dark bands that reveal daily growth increments, and they may be considered to reflect the quality of the individual (Grubb 1995; 2006; Clarkson 2011; Saino *et al.* 2012, 2013). Each set of dark and light bands show the growth during a twenty-four-hour cycle. The width of these growth bands represents the rate of feather growth and a wider daily growth increments mean a faster feather growth. Feather growth is related to the nutritional status of the individual at the time of feather production, and thus it can be used as an estimator for overall condition, but also as assessment for ecological stress (Grubb 1995). Several studies have shown a positive link between width of growth increments and body condition, implying that such growth increments can serve as useful tools in the study of physiological trade-offs (e.g. the better nutritional status of the individual, the wider the daily growth increments (Riddle 1908; Wood 1950; Grubb 1991; Grubb *et al.* 1998; Saino *et al.* 2014).

The complex community of feather ectoparasites can be acquired through contact with soil, which is considered a prime source of feather bacteria and other microorganism (Lucas *et al.* 2003; Burt and Ichida 1999; Shawkey *et al.* 2005), the other way of microorganism acquisition is through contact with vegetation, unrelated and related birds in the community (horizontal transmission) and from parents to offspring (vertical transmission) (Darolova *et al.* 2001).

The prevalence and the abundance of the bacterial community in bird feathers are influenced by different extrinsic and intrinsic factors. Extrinsic factors are the ecological environment of the host, through access to food (Maul *et al.* 2005; Blanco *et al.* 2006), habitat, climate and soil characteristics (Lucas *et al.* 2003; Bisson *et al.* 2009), and social behavior

(Waldenström *et al.* 2002; Bisson *et al.* 2009; Benskin *et al.* 2009). Intrinsic factors are related to overall condition, an ability to cope with infection may suggest the maintenance of an efficient immune system (Sandland and Minchella 2003; Schmid-Hempel 2003; Hawley and Altizer 2011).

A large diversity of potential bacteria can be carried on feathers, but some can gain access to host tissue and act as opportunistic pathogens (Otto 2009). Feathers of most wild birds are colonized by feather-degrading bacteria (FDB), a polyphyletic group of bacteria (Burt and Ichida 1999; Lucas *et al.* 2003; Whitaker *et al.* 2005; Shawkey *et al.* 2007). Much experimental evidence suggests these bacteria are active on the feather and capable of degrading keratin, a protein that constitutes the major bulk of feathers (Burt and Ichida 1999; Sangali and Brandelli 2000; Shawkey *et al.* 2007). Feather degradation might not be lethal to birds, but can still have important consequences like a decrease in thermoregulatory ability (Brush 1965), aerodynamic efficiency of feathers (Swaddle *et al.* 1996) and reducing protection from other bacterial infections (Muza *et al.* 2000). An excessive bacterial load may reduce reproductive success of the host either via changes in parental condition through a trade-off between reproductive effort and preening (Leclaire *et al.* 2014), or because of reduced social communication based on feather coloration that will affect reproductive success through social dominance and mate choice (Gunderson *et al.* 2009; Shawkey *et al.* 2009a; Ruiz-de-Castañeda *et al.* 2012). On the other hand, some bacteria might be beneficial for the host. Many bacteria isolated from feathers have the ability to produce antimicrobial substances that will help the bird protect its eggs from pathogenic bacteria (Riley and Wertz 2002; Peralta-Sanchez *et al.* 2010; Soler *et al.* 2010). Furthermore, such bacteria can maintain the stability of the microbial community through competition

and cooperation thus preventing colonization by environmental pathogens (Dillon *et al.* 2005; Faust *et al.* 2012).

Birds have evolved a wide range of defence mechanisms to prevent or minimized parasite colonization in order to maintain the integrity and functions of their feathers (Clayton and Moore 1997). The first line of defence against feather degrading microorganisms is the chemical structure of the feather which is mainly composed of β -keratin that constitutes more than 90% of feather mass (Onifade *et al.* 1998). β -keratins are extensively cross-linked within and between polypeptides through hydrogen and disulfide bonds, and with the high prevalence of cysteine residues all that make the keratin a highly rigid structure and cause it to be very difficult to break down by most proteolytic enzymes to be taken into the cell. Therefore, microorganisms must rely on extracellular enzymes to accomplish feather degradation (Kornilowicz-Kowalska 1999). Furthermore, birds evolved a complex system of behavioural and physiological defences. Preening is the prime behavioural defence against feather bacteria by use of secretions of the uropygial glands. These produce a secretion that acts either by forming a physical barrier between bacteria and the feather surface or through the antibacterial properties of uropygial secretion compounds (Jacob *et al.* 1997; Shawkey *et al.* 2003; Martín-Platero *et al.* 2006; Soler *et al.* 2008; Ruiz-Rodríguez *et al.* 2009). Other behavioural defences include sun bathing, dust bathing, anting or the use of other objects that contain antimicrobial compounds such as snails, fruit and fresh green vegetation with highly volatile compounds for lining their nests to prevent bacterial growth (Saranathan and Burt 2007; Ehrlich *et al.* 1986; VanderWerf 2005; Clayton and Vernon 1993; Clark and Mason 1985; Petit *et al.* 2002).

These behavioural and physiological defences are a significant investment in terms of time because birds devote on average 9% of their daily time budget on maintenance behaviour (Cotgreave and Clayton 1994). Not surprisingly these defence mechanisms are energetically costly, and hence the benefits from such defences are traded against the costs (Croll and McLaren 1993; Carey 1996; Goldstein 1988; Hasselquist and Nilsson 2012; King and Swanson 2013).

Bacteria and feather coloration

Feather coloration is the outcome of two main mechanisms, pigmentary colours which are the result from pigments that are incorporated in the feather keratin matrix where absorption and reflection of the incident light is influenced by pigments concentration (D'Alba *et al.* 2012). Different classes of pigments have been found in bird feathers, the most common pigments are melanins and carotenoids. Melanin exists in two main forms, eumelanin that gives black and grey colours whereas the earth-toned colours are the result from the presence of pheomelanin pigments (Haase *et al.* 1992; Fox and Vevers 1960). Carotenoid pigments that are acquired by birds through their diet can be classified according to their molecular structure as different carotenes and xanthophylls. Each can produce different colours (bright yellows and brilliant orange yellow), but carotenoids are also known to interact with melanin to produce a different colour like olive-green (Goodwin 1984). Porphyrins are another type of pigments that are attributed to the presence of modifying amino acids. These types of pigments produce different arrays of colour such as pink, browns, reds, and greens, but also brilliant greens and reds (Proctor and Lynch 1993). Furthermore, all the

previous pigments may interact to produce several other hues (McGraw 2006).

A second mechanism of colour production is known as structural coloration. Coloration of this type rely on the interaction between incident light and the nanostructures of feather barbules. Structural coloration produces an amazing range of green, blue, violet and ultraviolet colours (Dyck 1976; Kinoshita *et al.* 2008). Keratin, melanin (as a granule, called melanosomes) and air are the main components in structurally coloured feathers (Prum 2006). Structural colorations can be classified in two subgroups: iridescent and non-iridescent (Shawkey *et al.* 2009b). Within the barbule cortex of the iridescent feathers melanin rods are arranged in manner to produce two-dimensional (2D) photonic crystal-like structures at the sub-micron scale (Zi *et al.* 2003; Li *et al.* 2005; Yoshioka and Kinoshita 2002). Thus, the colour is an outcome of the thin-film interference phenomena where the refraction acts like a prism that splits the light into rich component colours where the colour could be changed with the change of the viewing angle to produce several shimmering, glowing iridescent colours. In contrast to iridescent feathers, non-iridescent feather colours are produced by 3D spatial periodicity in feather barbs (the tiny air pockets in the barbs of feathers can scatter incoming light), and the change in the observer angle does not lead to a change in colours (Proctor and Lynch 1993).

In addition to the functions of feather coloration in visual signalling, feather colour is also thought to play a mechanical role like abrasion resistance (Burt 1986) and metal binding (McGraw 2003). For instance, an increase in melanisation in feathers is associated with a reduction in feather wear due to abrasion (Butler and Johnson 2004). Thus, melanin is expected to be observed in feathers that are more exposed to wear, such as tips of

wing feathers (Gill 1995). Well-supported studies suggest that feather coloration may affect the bacterial community, and increased melanization of the plumage may minimize microbial damage (Burt and Ichida 2004; Peele *et al.* 2009). Selection of melanin for its resistance to bacterial degradation raises the possibility that other feather pigments may serve a similar protective function. Burt *et al.* (2011) found that parrot coloured feathers that contained the red psittacofulvin pigments are degraded by *Bacillus licheniformis* at a similar rate as melanized feathers and more slowly than white feathers (lack pigments). Furthermore, blue feathers, in which colour is based on the microstructural arrangement of keratin, air and melanin granules, and green feathers, which combine structural blue with yellow psittacofulvins, degrade at a rate similar to that of red and black feathers.

Many studies have suggested that feather-degrading bacteria could alter feather-based communication by affecting feather colouration. Gunderson *et al.* (2009) and Shawkey *et al.* (2007) found that the occurrence of feather-degrading bacilli in the plumage of eastern bluebirds (*Sialia sialis*) had potential consequences, because they altered the non-iridescent colour of feathers accompanied with a reduction in body condition and lower reproductive success. Similarly, Leclaire *et al.* (2014) showed in their experimental study by manipulation feather bacterial load in captive feral pigeons (*Columba livia*) that individuals (both sexes) with lower bacteria load on their feathers had more brightly iridescent neck feathers. Meanwhile, Jacob *et al.* (2014) found no significant relationship between increased bacterial load and a reduction in feather colour in their experimental study on great tits (*Parus major*).

Sexual selection

Sexual selection was first presented by Charles Darwin in *The Origin of Species* (1859) and he developed this idea in *The Descent of Man and Selection in Relation to Sex* (1871), as he believed that natural selection alone was unable to explain certain types of characters such as the train of the peacock (*Pavo cristatus*), and bright plumage and naked patches of skin in many birds. They could clearly not improve survivorship for their bearers. Thus, he suggests that such traits may have become exaggerated because they provided individuals with the most exaggerated traits with an advantage in terms of mating success and he termed them secondary sexual characters. Therefore, sexual selection can be considered part of natural selection where members of one sex (usually females) compete for choosing mates (intersexual selection), while members of the same sex (usually males) fight over access to members of the opposite sex (intrasexual selection) (Andersson 1994). In few words, sexual selection implies that some individuals have higher reproductive success than others by being more attractive through their display of exaggerated secondary sexual characters.

Multiple ornamentation

For communication, animals have evolved an amazing array of different signals. Signals fall into three main groups: vocal, olfactory or chemical, and visual signals. Furthermore, some fish species produce electric signals released in the form of pulses (Andersson 1994). Signals are traits that have evolved to transmit information for signallers in an effort to manipulate the behaviour of others to their own advantage. Signals between conspecifics are used in different social situations (attraction of mates, defence of the territory against competitors, or warning of conspecifics in the case of the

presence of a predator). Interspecific signals are less common. The types of signals that have evolved rely on the type of messages that are transferred, the environment, and the sender of the signal (Drickamer *et al.* 2002).

Most males and even some females have multiple sexual ornaments which are mainly used to advertise their quality to compete for and attract mates. These multiple sexual ornaments are often used simultaneously rather than relying on one type of signal at a time. Thus, animals may display a number of different visual, auditory and olfactory components (Zuk *et al.* 1990, 1992; Redondo and Castro 1992; Edmunds 1974).

To explain the existence of such multiple ornaments, several hypotheses have been proposed. Three explanations for this diversity of signals were presented by Møller and Pomiankowski (1993):

1-The multiple message hypothesis suggests that each ornament signals a particular feature of individual condition, and that in turn each signal delivers different messages about the overall condition of the individual. Alternatively, these multiple signals may reveal individual condition throughout their life, where some traits suffer from changes over time, as ornamented feathers of the birds or antlers of a deer that grow each year. In contrast, inflatable bare skin patches of grouse or colourful patches in primates are more likely to reflect current body condition. In brief, this hypothesis assumes that different signals act as indicators of different aspects of individual quality.

2- The redundant signal hypothesis (also known as the back-up signal hypothesis) proposed that each secondary sexual trait provides a partial picture about overall male condition (Møller and Pomiankowski 1993). Thus, females can make a better assessment of overall condition of males by inspecting different traits that each is correlated with individual condition.

3- The unreliable signal hypothesis suggests that different multiple sexual traits actually may not offer a good indication of current male condition (Møller and Pomiankowski 1993). These traits could have evolved by exploiting pre-existing female preferences (Ryan 1990), and over time they lost the correlation with individual condition.

Another hypothesis presented by Andersson *et al.* (2002) is **the multiple receiver hypothesis**, which suggests that the presence and the development of costly multiple traits may be due to inter- and intrasexual selection, in which different signals are selected by separate receivers (males and females relying on the use of different signals).

Mate choice and feather parasite load

Many observational and experimental studies refer to the important role of bird feather ornamentation in female mate choice, but in contrast it has less value in male-male competition (Andersson 1994; Møller 1994). Female choice is therefore expected to account for the maintenance of extravagant plumage ornamentation in birds. A series of handicap-models of sexual selection were developed to elucidate the benefits that may derive from the female choice (Zahavi 1975; Grafen 1990a, b). These models suggest that male ornamentation is costly to produce and maintain (Dale 2006), and consequently only high-quality males should be able to withstand such costs. Therefore, these displays can be considered reliable signals of male quality (Zahavi 1975; Hill 1991; Andersson 1994). Thus, if male quality and ornament size are positively correlated, and heritable, females should gain from their mate choice by increasing the survival prospects of their offspring. Female choice based on signals of individual quality could also lead to adjustment in the sex ratio of their offspring (the proportion of males

to females in a population of any sexually reproducing species (Allaby 2003)) in response to the degree of attractiveness or quality of their mate (Weatherhead and Robertson 1979). This assumption is similar to the classic Trivers and Willard (1973) argument where the only difference being that it is mate quality rather than maternal condition that influences offspring fitness. Another hypothesis (the differential allocation hypothesis) suggests that females may manipulate reproductive efforts for their offspring on the basis of attractiveness of their mates (Burley 1988; Sheldon 2000). Thus, if male attractiveness contributes to offspring fitness (either by providing maximum paternal care or high genetic quality), females that choose to mate with highly attractive males may produce more offspring or offspring of superior quality than those that mate with less attractive males. On this basis, the attractiveness hypothesis proposes that females that gain mating with the mostly attractive males should produce more sons that inherit the sexually attractive traits of their sires (Burley 1986; Cockburn *et al.* 2002). Thus, females can enhance their fitness by producing chicks with male-biased sex ratios if they mate with attractive males.

Further development of the handicap-model by Hamilton and Zuk (1982) suggested that the ability to resist predominant parasites is a reflection of quality that is revealed by male ornaments. This hypothesis predicts that within species there should be a negative relation between ornament expression and parasite load.

Many studies suggest that females prefer to mate with males that have few parasites to avoid parasite transmission to the next generation (Freeland 1983; Borgia 1986; Hillgarth 1996), to gain more paternal care (Hamilton 1990; Milinski and Bakker 1990; Møller 1990) or to pass on genes for parasite resistance to the offspring (Hamilton and Zuk 1982).

In many lekking species and prior to mate choice, females regularly visit several males before choosing one (Lill 1974, 1976; Gronell 1989; Trail and Adams 1989; Dale *et al.*, 1990; Petrie *et al.* 1991; Bensch and Hasselquist 1992; Byers *et al.* 1994; Fiske and Kålås 1995). Darwin (1871) proposed that females prefer to mate with certain males depending on different cues that may signal their quality. Many studies have indicated that the decision to mate can be taken according to morphological (males with bright feathers or long tails) or behavioural traits (males able to defend their territories or other sources that can be essential for reproduction) (Burley 1981; Johnstone 1996; Lozano 2009; Dolnik and Hoi 2010; Hoi and Griggio 2012).

Blue peafowl (*Pavo cristatus*)

Peacocks are resident polygynous birds in South-East Asia, where they prefer deciduous open forest, but they can also be found in captivity and have the ability to adapt easily to colder climates if provided with a simple shelter (Jackson 2006). The adult male vary in size from the bill to the end of the tail from 100 to 115 cm, and the train can reach as much as 195 to 225 cm (Whistler and Kinnear 1949). The Peacocks weigh about 4-6 kg, while females (peahens) tend to be smaller and lighter.

The peacock possesses different visual and vocal secondary sexual characters. The head of the peacock is characterised by its short and curled feathers that are metallic blue in the top and iridescent greenish blue feathers on the side of the face, at the top of the head a fan-shaped crest made of feathers with bare black shafts and tipped with bluish-green webbing (Fig.

1). The eyes are surrounded by a bare white skin (an upper white stripe and a crescent shaped patch below the eye) (Whistler and Kinnear 1949).

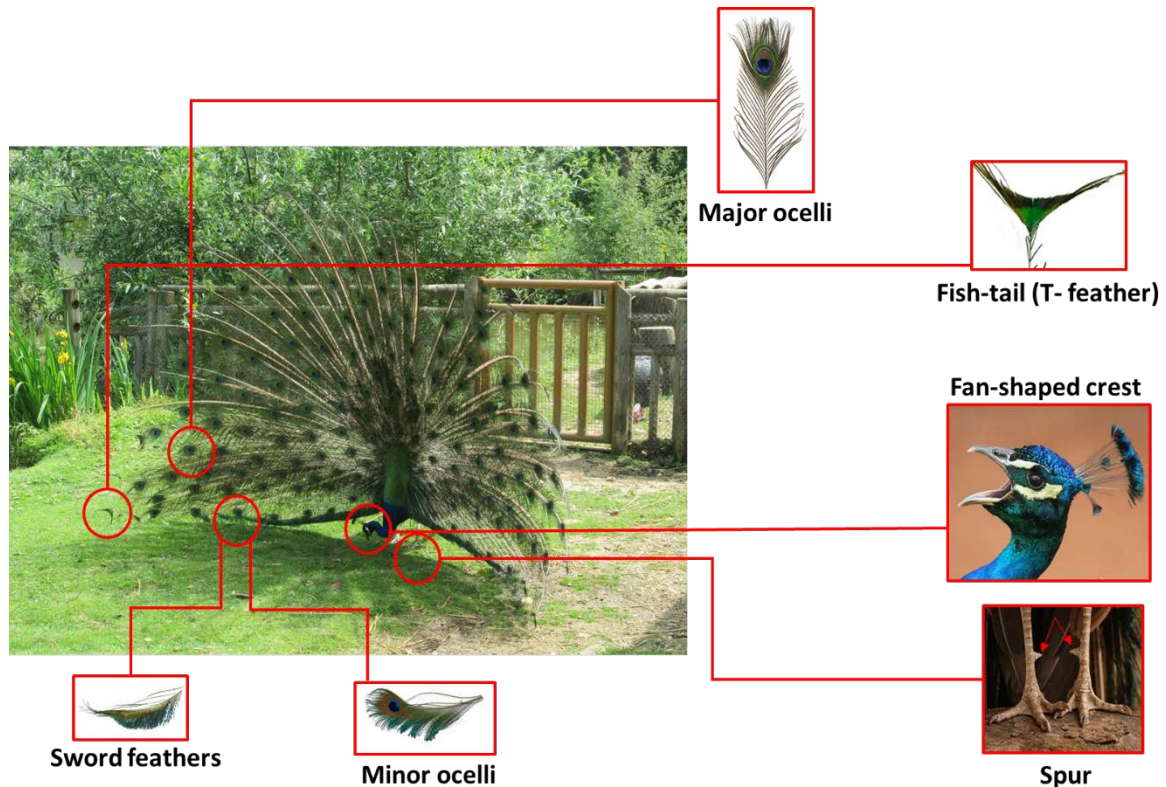


Fig. 1. Peacock with fully erected train, with red boxes showing the positions of the four-different types of the train feathers, crest and spur (white arrows).

Among the most notable features of adult peacocks are their magnificent long train, four specialized feather types can be recognised in the erected train, the outermost edge of the train is lined by the longest “fish-tail” (also referred to as the T feathers), meanwhile the bilaterally symmetrical major ocelli feathers are distributed throughout the main part of the train and form the majority of its feathers. In the lower edges, the erected train is bordered by both curved asymmetrical minor eyespot feathers and curved asymmetrical sword feathers (Sharma 1974; Manning 1989; Fig. 1). The number of ocelli feathers vary among studies and range between 105

and 177 (Dakin and Montgomerie 2011). Peacocks moult all four feather types annually following the breeding season in autumn (over an 8-week period), and they are regrown before the breeding season in spring (over an 8-12-wk period) (Sharma 1974).

Outside the breeding season peacocks live in flocks, while during the breeding season they aggregate at open communal display grounds so-called leks. There they maintain their territories calling to attract females from a distance, displaying their erect trains to females when they arrive on the lek. Peahens follow the sampling behaviour of many lekking species during the breeding season. Males form leks (on small display territories), where they advertise their extravagant traits in an attempt to attract a potential mate. Females arrive at these display grounds for several days before deciding to copulate with the most suitable mate (Rands *et al.* 1984; Harikrishnan *et al.* 2010). Subsequently, peahens construct a nest on the ground away from these display sites, where they lay buff coloured eggs which they then incubate for 28-30 days. Peacocks play no part in post-mating reproduction and never interact with the offspring.

The peacock was a particularly difficult enigma for Darwin, and the complexity of the elongated decorated trains caused him to be almost sick with worry of this exaggerated display, as he expressed it in a letter to Asa Gray on 3 April, 1860: “The sight of a feather in a peacock’s tail, whenever I gaze at it, makes me sick!” (Burkhardt *et al.* 1994). In particular, he was completely aware that the evolution of the train was at the expense of the survival of individuals, and that an increase in train size would make males easier prey for any predator, but also increase the mating success of such males (Gadagkar 2003). Certainly, peacocks with longer trains, in a free

ranging UK population, were better able to survive predation attempts by foxes *Vulpes vulpes* than males with short trains, but they also experienced higher mating success (Petrie 1992). Thus, Darwin proposed that the evolution of the extravagant plumage in polygynous birds in general was the outcome of sexual selection and most likely by female choice (Darwin 1871). Ocelli feathers comprise a centric iridescent deep blue part with indentation in the lower section along the line of the shaft of the feather, a blue part enclosed by a rich green section, and this in turn by a wide bronze-brown area. Darwin noticed that the base of the free barbs at the top of the ocelli is derived from the missing barbules that make two clear translucent zones surrounding the ocelli thereby making it clearly separate from the rest of the feather (illustrated in Fig. 2). Darwin speculated that these clear zones may be related to the development of the ocelli (Darwin 1871). However, he did not notice that these translucent zones also made the feathers particularly susceptible to breakage. Such breakage could affect the appearance of the ocelli and thereby reveal the causes of such breakage, either mechanical or caused by microorganisms.

Darwin meditated about the question why not all the peacock develops such most exaggerated secondary sexual characters, and he suggested that the train was an engaging trait rather than being of any utility. Peacocks do not provide their mates with any material benefits, and hence the peacocks have been hypothesized to be a source of good genes for choosy females (Davies 1978; Payne 1984). If the most ornamented males are in better condition before, but also after development of their secondary

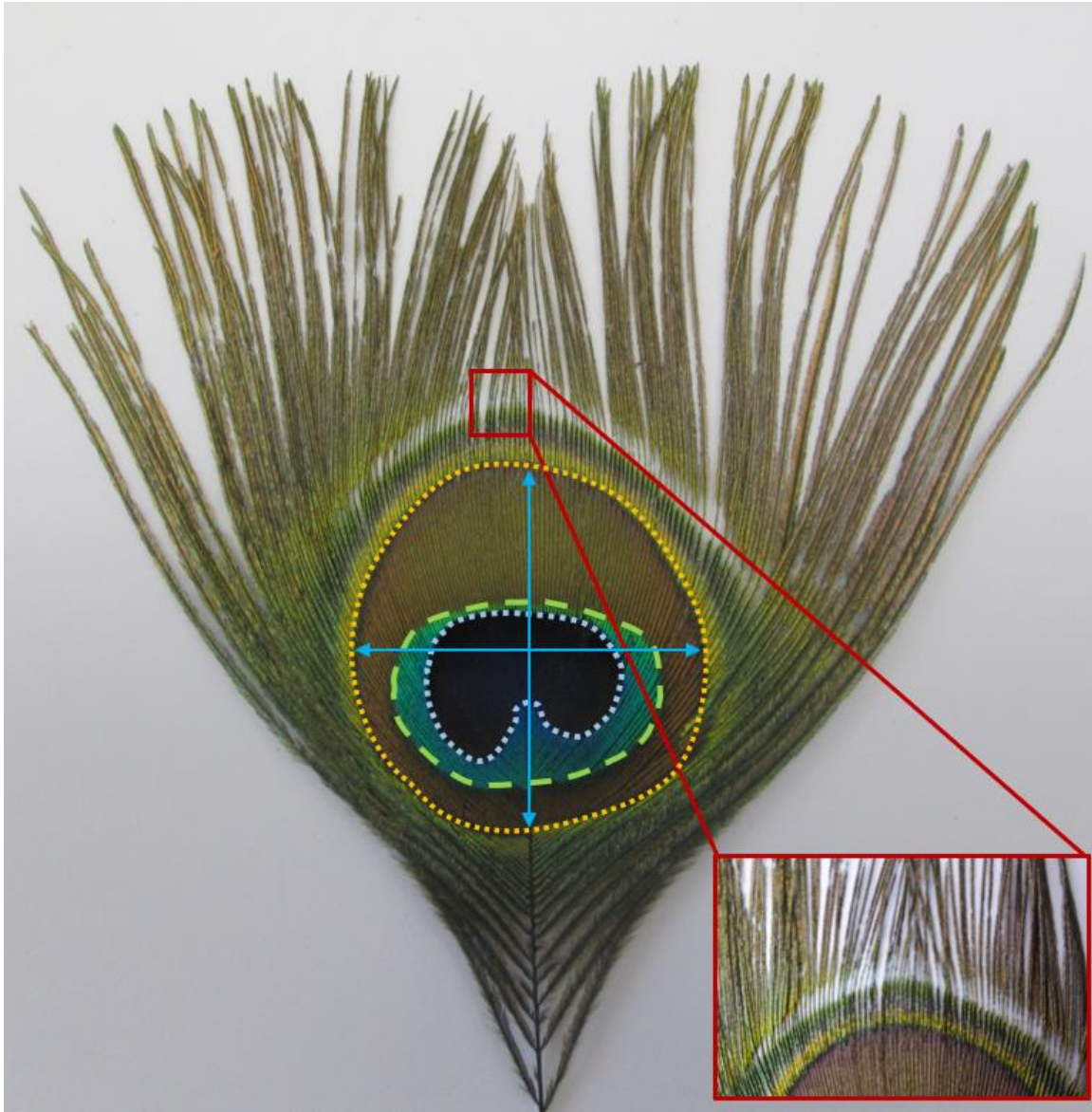


Fig. 2. Ocellus of a peacock showing the brown, green and blue parts. Orange dotted line indicates the area where HAM measured the size of ocelli (mm), while the width and height were measured at the widest points of the ocelli (light blue arrow). The red box shows a higher magnification of the clear translucent zones that surround the upper part of ocelli.

sexual characters, such males might signal their superior quality (Zahavi 1975). Subsequent analyses have shown that male peacocks may reliably signal their condition (Møller and Petrie 2002), quality (Loyau *et al.* 2005b) or disease resistance (Møller and Petrie 2002; Hale *et al.* 2009). Indeed, peahens gain indirect genetic benefits for their offspring since peacocks with elaborate trains leave more surviving offspring (Petrie 1994). On this basis, Darwin (1871) suggested that females prefer to mate with certain males depending on different cues that may signal their quality and thus the suitable male for the choosy female usually has a highly ornamented train as confirmed by observations (Petrie *et al.* 1991; Loyau *et al.* 2005a, b) and experiments (Petrie and Halliday 1994; Dakin and Montgomerie 2011; Dakin and Montgomerie 2013). Furthermore, the structure and size of ocelli (Møller and Petrie 2002; Dakin and Montgomerie 2013), the length of feathers in the train (Manning and Hartley 1991; Yasmin and Yahya 1996; Loyau *et al.* 2005b), the crest (Dakin 2011), the direction of the expanded train relative to the sun (Dakin and Montgomerie 2009), calls (Yasmin and Yahya 1996; Dakin and Montgomerie 2014; Yorzinski and Anoop 2013) and the shaking of the train feathers that produces two faintly distinct types of infrasonic mating calls (Freeman 2012) all have been reported to contribute to male mating success. However, there is considerable variation in these preferences among populations (Dakin and Montgomerie 2011). Feather growth bars linked to overall individual condition are influenced by nutritional status during moult, and they have been proven to be a potentially honest signal of individual quality and hence for total annual reproductive success (Takaki *et al.*, 2001). Thus, growth bars might be a reliable clue to peahens when selecting their mates. Furthermore, Petrie (1994) showed that the degree of train elaboration is a heritable trait, and that it is positively

related to offspring survival, with a stronger effect in sons than in daughters. Thus, females tend to change the sex ratio of their offspring by producing significantly more daughters when mated with less attractive males, suggesting that peahens have the ability to control the sex of their offspring (Pike and Petrie 2005).

OBJECTIVES OF THE THESIS

During the daily activities of the peacock like foraging, roosting and interaction with the other members of a flock, males may expose their elaborate train feathers to different kinds of microorganisms that originated from the surrounding environment especially from the ground that contains a vast majority of feather degrading microorganisms (Clayton 1999; Shawkey *et al.* 2003; Sangali and Brandelli 2000; Lucas *et al.* 2003; Riffel *et al.* 2003). One important group among these microorganisms is feather-degrading bacteria (FDB), a polyphyletic assemblage of bacteria (Burt and Ichida 1999; Whitaker *et al.* 2005; Shawkey *et al.* 2007; Gunderson *et al.* 2009; Shawkey *et al.* 2009) that has the ability to degrade feather keratin (Muza *et al.* 2000; Sangali and Brandelli 2000; Lucas *et al.* 2003; Gunderson *et al.* 2009; Shawkey *et al.* 2009). Thus, bacteria may lead to deterioration of feather structure and consequently reduce the fitness of their hosts by reducing thermoregulatory efficiency, flight performance and socio-sexual communication (Swaddle 1996; Clayton 1999; Shawkey *et al.* 2007).

This thesis addresses the following five questions about the association between the bacterial community and the expression of the ocelli feathers of the peacock train.

1. Darwin's problem of gradation in peacock ocelli: Degradation of ocelli by microorganisms

The objectives of this chapter were to assess the extent to which the structure of the ocelli of peacocks reflects the abundance and the diversity of microorganisms. Specifically, we tested whether male peacocks with larger ocelli have a lower abundance and diversity of microorganisms, in particular pathogenic microorganisms, and whether the size of ocelli reveals susceptibility to feather degradation as judged from missing barbs. We isolated bacteria from the different coloured part of ocelli in order to determine their identity, diversity and abundance, and we subsequently analysing the relationship between diversity and abundance of bacteria and size of ocelli, feather degradation and force required to break barbs of the feathers with ocelli.

2. Why do peacocks have so many different signals?

Most secondary sexual characters are costly to produce and maintain and the prevalence of different bacterial taxa may reduce the ability to produce and maintain extravagant ornamental characters. In this chapter, we suggest that the abundance and the diversity of bacteria in the different coloured parts of the ocelli of the train play important roles in the expression of secondary sexual characters like the number of ocelli in the train, the size of different parts of these ocelli, spur length and train length. Thus, different secondary sexual characters may signal different properties of individual quality, which in turn may allow females to assess male quality and hence decide the quality of their future partner.

3. Is sex ratio in the peacock related to the expression of secondary sexual characters?

Females may assess male quality from their degree of ornamentation which represents a particularly reliable indicator of parasite load of the bearer, if only healthy individuals are able to produce exaggerated secondary sexual characters and still remain resistant to debilitating parasites. If sons are able to inherit the attractiveness of their fathers, consequently sons of the attractive males might be of higher reproductive value than daughters of such males. Thus, females can enhance their fitness by producing chicks with male-biased sex ratios if they mate with attractive males. In this chapter, we explore if there was a relationship between primary sex ratio and expression of peacock secondary sexual characters such as train and spur length, number of ocelli, and degree of feather degradation as caused by feather degrading microorganisms and offspring sex ratio.

4. Do peacock signals reveal abundance and diversity of microorganisms?

Peacocks have coloured feathers that are degraded to a different degree in different parts of the ocelli, suggesting that different parts of ocelli are susceptible to degradation by microorganisms to a different extent. The aim of this chapter was to explore if there is a relationship between the abundance and the diversity of the bacterial community in the ocelli of the train of the peacock and the characteristics of the colouration of the brown, green and blue parts of the ocelli.

5. Feather bacteria may influence daily growth increments of peacock ocelli feathers

Bird feathers have a set of dark and light bands which reflect the diurnal patterns of feather development, the width of these growth bands representing the rate of feather growth. The objectives of this chapter were to investigate (1) the relationship between the abundance and the diversity of the bacterial community in peacock ornamental tail feathers and the daily rate of feather growth during the annual moult; and (2) the relationship between characteristics of the colouration of the brown, green and blue parts of the ocelli and daily growth increments. We did so by investigating a sample of feathers collected from a population of peafowl kept at a commercial farm as part of a long-term experiment to investigate the functional significance of the exaggerated train of peacocks.

MATERIALS AND METHODS

Captive breeding experiment

We conducted a captive breeding experiment in which a total of 46 adult males (fathers) each were randomly mated with four females, in total 184 adult females (mothers). Each mother was allocated to a single male for the entire breeding season, so each mother was mated with only one male. Eggs were collected daily, numbered and weighed on a digital top pan balance to the nearest 0.1 g. Any mother that died during the experiment was replaced and mothers and fathers were allocated to pens at random at the start of the breeding season. During the experiment, only four females were replaced (2% of total females) and all replacements occurred during the first four weeks of the 20-week breeding season. Blood samples were taken from each

individual for MHC and microsatellite genotyping, and parentage and sex of offspring were determined genetically.

At the start of the breeding season, on the day adults were allocated to pens, body weight (g) was recorded and spur length (mm) and tarsus length (mm) were measured with digital calipers for all adults, and train length (length of the longest feather in mm), number of ocelli was the number of ocelli counted from photographs taken of each male. Because the count was taken from photographs, and it is difficult to get a photograph where the train is completely visible, this measure is an estimate of number of ocelli rather than an accurate count. The peafowl were provided with water and fed a poultry layers' pellet that did not contain any antibiotics. The breeding experiment was conducted over two years (in 1998 and 1999) in a commercial peacock farm in Norfolk, UK (see Hale *et al.* 2009 for further details), with mothers and fathers randomly reassigned to mates between the two years. The random allocation of four mothers to each father was designed to reduce the impact of any maternal effects in the analyses of reproductive output, including any impact of previous mate history.

The 4,977 collected eggs over the two years were either incubated to term (28 day) in separate compartments or incubated to day 10. Approximately half the eggs laid were incubated to day 10 and half to full term. For those eggs incubated to term any unhatched eggs were dissected and the stage of development recorded. Blood and/or tissue samples were taken from any fertilized and unhatched eggs. Blood was taken from all hatched chicks. All eggs incubated for 10 days were dissected and the state of development recorded and blood and tissue samples were taken.

Outside of the breeding season all individuals were sheltered in large outdoor aviaries, and allowed free access to invertebrates in the soil and to

green vegetation. Therefore, all individuals were supposed to have similar contamination levels with microorganisms, since all were housed in the same farm and cared for by the same persons, suggesting that the difference in infection level is due to resistance and anti-microbial defences of each individual.

The study was approved by the UK home office and there were no signs of negative effects of any of the procedures adopted throughout the study that may adversely have affected the peacocks.

Ocelli feather collection

At the beginning of the breeding season (spring 1999), 10 ocelli feathers were removed aseptically from each of the 46 peacocks by using a pair of sterile examination gloves and sharp scissors, and the removed feathers were placed in dry clean plastic bags. All samples were transported in a cool box to the laboratory and stored under the same conditions until processed.

A single ocellus feather was chosen randomly from the 10 feather samples that were collected from each individual (in total 46 individuals) to estimating the ocelli feather degradation, estimating the force required to break feather barbs, bacterial isolation, colour reflectance measurements and measuring the daily growth increments. All measurements were made blindly with respect to identity and phenotype of individuals.

Ocelli measurements and estimation of ocelli feather degradation

Feathers were photographed with a digital camera, and all ocelli measurements were taken from the pictures by using the 'ruler' tool in Adobe Photoshop CC software. All measurements were done by HAM to avoid inter-observer variation, and the size of ocelli (measured in mm) was

calculated as follows: the product of the width at the widest point multiplied by the height at the greatest vertical distance (Fig. 2). All measurements were done blindly without prior knowledge about individual identity and phenotype.

To estimate degradation of ocelli of the train, HAM adopted three different methods: First, HAM counted the number of barbs that were missing from the lower part of ocelli feathers as revealed by remaining stubs of barbs. Secondly, the degree of barb loss from the upper part of the ocelli was estimated according to the following scale: 0 = no loss due to degradation; 1 = no loss with degradation; 2 = one third of barbs from the upper part were lost or degraded; 3 = two thirds of barbs from the upper part were lost or degraded; and 4 = more than two thirds of barbs from the upper part were lost or degraded (Fig. 3).

The third scale was the estimation of degradation level in different parts of the ocelli (brown, green and blue), with degradation ranked on a four-point scale: 0 = no degradation; 1 = slight degradation (less than 1-3 small spots of degradation); 2 = medium degradation (large area of degradation); and 3 = high degree of degradation (more than half of the area degraded) (Fig. 4).

To estimate the precision of our measurements and scoring of the ocelli characters, repeatability (R) (Becker 1984; Falconer and Mackay 1996) was calculated from 30 individuals for which measurements were repeated twice on different days without prior knowledge of the first set of measurements and scoring. HAM found that the repeatability was high in all cases (Table S1).

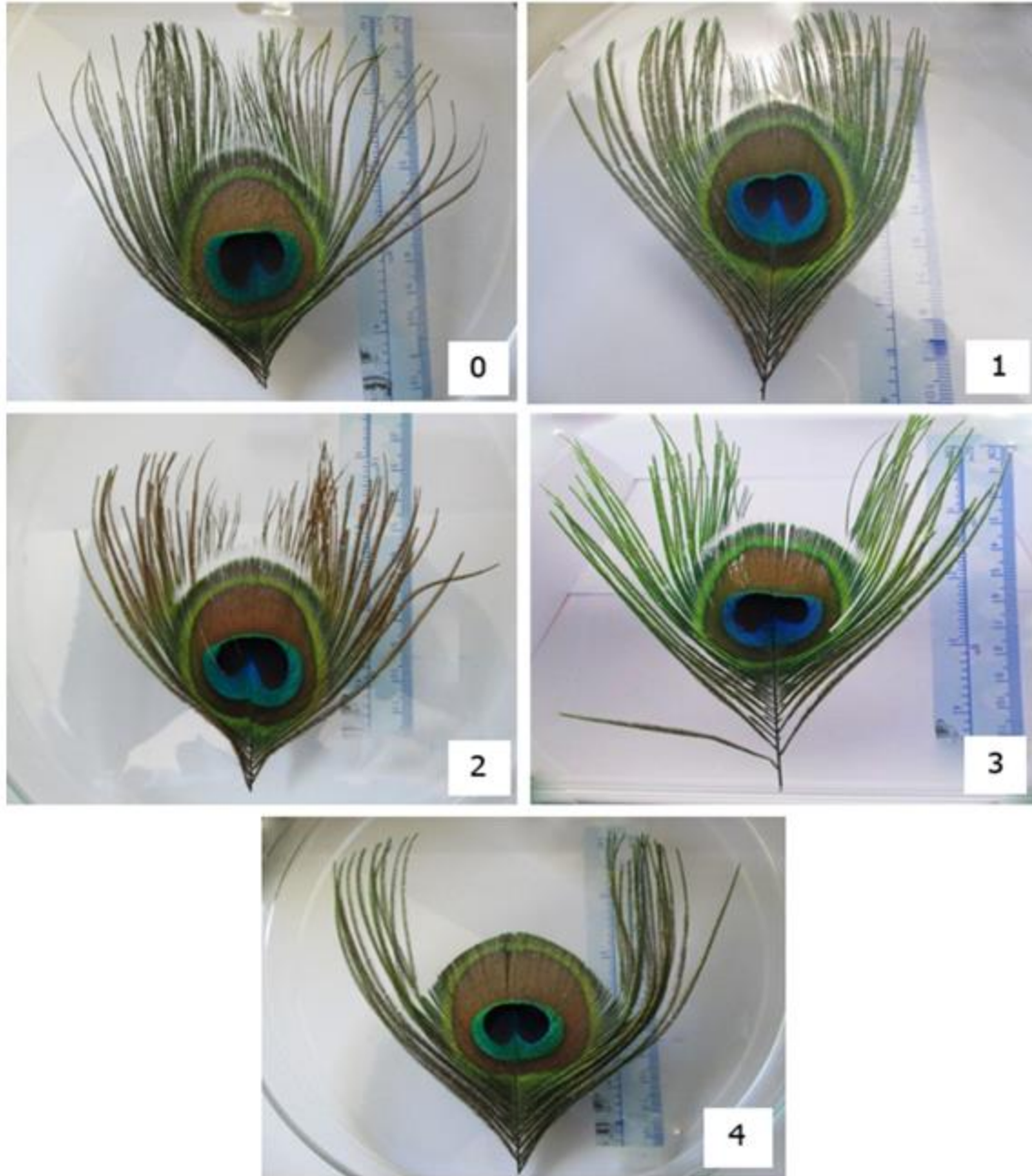


Fig. 3. Degree of loss of barbs from the upper part of ocelli: 0 = No loss due to degradation, 1 = no loss with degradation, 2 = one third of barbs from the upper part was lost or degraded, 3 = two thirds of barbs from the upper part were lost or degraded, and 4 = more than two thirds of barbs from the upper part were lost or degraded.

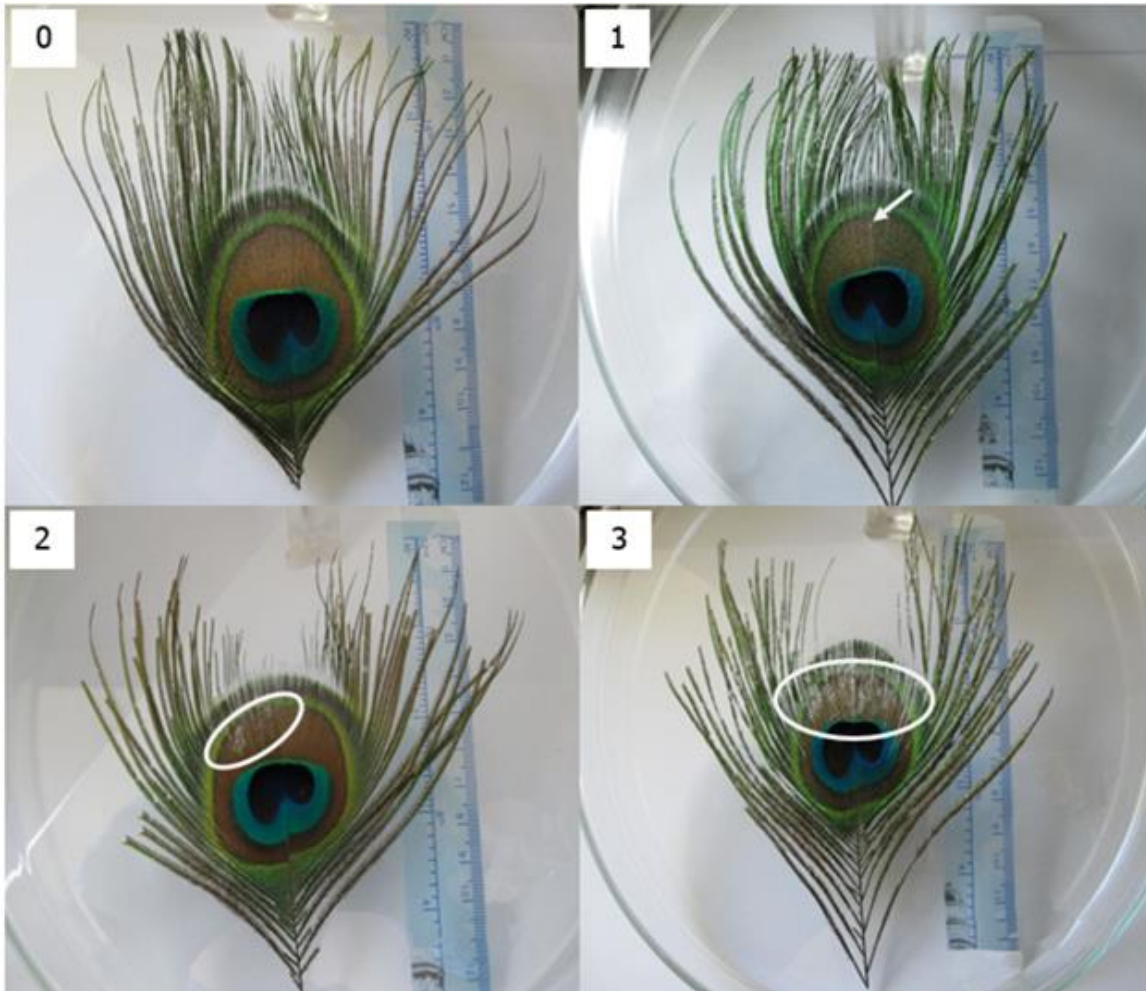


Fig. 4. Levels of feather degradation:0 = No degradation, 1 = slight level degradation (less than 1-3 small spots of degradation), 2 = medium level degradation (large area of degradation), and 3 = high degree of degradation (with more than half of the area degraded).

Estimating the force required to break feather barbs

We estimated the force required to break a feather barb that surrounds the ocelli by adopting a previously used method (Møller *et al.* 2006). First, Johannes Erritzøe (JE) fastened the end of the barb to a clamp. Subsequently, the barb was attached to a Pesola spring balance, which was pulled slowly until the barb broke, carefully reading the number on the spring balance when the barb broke. We repeated this exercise three times for a feather to allow for estimation of repeatability and for calculating more precisely the force required (the repeatability (R) for force required to break a feather barb is reported in Table S2). It is well-known that traits measured with error can be measured more precisely by measuring the same trait repeatedly. Finally, we calculated the mean of the three estimates as a best estimate of the required force for inducing breakage. JE was unaware of the purpose of the study, and he had no prior knowledge of any of the other variables when estimating the forces. Therefore, the measurements were made blindly with respect to the objectives of the study.

Bacterial isolation

For bacterial isolation HAM choose feather samples randomly from 30 individuals of overall 46 individuals that include in this study (the remaining 16 males were not included for logistic reasons), in addition to a second sample from 10 randomly chosen individuals to estimate repeatability of total number of bacterial and the number of bacterial species (Becker 1984; Falconer and Mackay 1996). The degree of repeatability was high in all cases except for number of species recovered from TSA plates of the brown and blue parts of the ocelli (repeatability of total number of bacteria and the number of bacterial species on TSA and FMA media from differently

coloured parts of the ocelli are reported in Tables S3A and B). Each coloured part of peacock feathers was cut by using a sterilized scalpel and with the help of forceps in the laboratory. HAM used a pre-weighed 2 ml eppendorf tube which after receiving the feather sample was weighed again to calculate the mass of the feather and to determine the volume of sterile phosphate buffer saline (pH 7.2) to be added to the tube (each mg feather sample equalled 100 μ l PBS). This was followed by 3 vortex periods 1 min each. Free-living bacteria were washed out from the feathers and collected in PBS solution (Saag *et al.* 2011a). To quantify the cultivable and feather-degrading bacteria, duplicates were made by spreading 100 μ l of the resulting PBS with a sterile spreader loop on two different growth media: (1) Tryptic soy agar (TSA), which is a rich medium on which heterotrophic bacteria can grow, thus enabling us to assess total cultivable microorganism load of feathers. (2) Feather meal agar (FMA), which is a medium highly selective for keratinolytic bacteria with the unique source of carbon and nitrogen being keratin. Hence, only bacteria that are able to digest it can proliferate, allowing for quantification of the feather-degrading bacterial load. The FMA contains 15 g L⁻¹ feather meal, 0.5 g L⁻¹ NaCl, 0.30 g L⁻¹ K₂HPO₄, 0.40 g L⁻¹ KH₂PO₄, and 15 g L⁻¹ agar (Williams *et al.* 1990; Sangali and Brandelli 2000; Shawkey *et al.* 2003, 2007, 2009). (3) Control plate inoculated with the same volume (100 μ l) of sterilized distilled water in order to detect any contamination of media (negative controls).

Fungal growth was inhibited by adding cycloheximide to TSA and FMA media (Smit *et al.* 2001). Plates were incubated at 28°C for 3 days in the case of TSA, and for 14 days in the case of FMA. After incubation, HAM counted the number of colony-forming units (CFU) of each morphotype per plate by using dissecting microscope, and distinguished the morphotypes on

the basis of colony colour, shape, size, and presence or absence of glutinous aspects.

Isolate preservation

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

DNA extraction

Genomic DNA was extracted from each isolate by using two different protocols: (A) Freeze-thaw protocol. (1) With a wooden toothpick, HAM transferred and squashed a part of a bacterial colony in a 0.5 ml eppendorf tube containing 50 µl Tris (10 µM, pH 8.0). (2) Start of freezing and thawing by putting the eppendorf tube in liquid nitrogen for one to two minutes until completely frozen, then quickly placing the tube in a hot water bath until completely thawed. This process is known as a freeze-thaw cycle, and HAM repeated the same process for a total of three cycles. HAM made sure that he mixed the sample tube between each cycle. (3) To ensure complete cell lysis HAM put the tubes in a microwave at 270W for 5 to 6 s followed by 10 s of waiting, repeated these three times. (4) HAM centrifuged the sample tube for five minutes at 12,000 x g in a microcentrifuge. Using a micropipette, he transferred the supernatant that contains the DNA into a new clean, sterile microcentrifuge tube and discarded the pellet that contains cellular debris. HAM stored the tubes in -20 °C for the Polymerase Chain Reaction (PCR). (B) DNA Extraction kit: HAM used the Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA) to extract DNA according to the protocol that is supplied with the kit.

PCR amplification of bacterial 16SrRNA gene

DNA isolated from samples was used as a template for PCR to amplify the bacterial 16S rRNA gene by using the forward primers: 16S rDNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 16S rDNA-63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 25 µl containing 16 µl ultrapure water, 4 µl 5x buffer, 0.4 µl dNTPs 10nM, 1 µl 10 µM 27F, 1 µl 10 µM 1492R, 0.2 µl Go Taq DNA polymerase and 3-5 µl genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s, annealing for 30 sec. at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Agarose electrophoresis

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

DNA sequencing

PCR products were sent for DNA sequencing in to Beckman Coulter Genomics (Takeley, Essex CM22 6TA, United Kingdom). The sequence results were processed by using the web-based blasting program, basic local

alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Gene bank database.

Offspring sexing

Offspring were sexed by M. Hale via PCR amplification of the CHD genes using primers P2 and P8 (Griffiths *et al.* 1998) in 10µl reactions containing 1 x *Taq* buffer, 2mM MgCl₂, 200µM each dNTP, 1.0µM each primer, 0.3U *Taq* and 0.5µl template DNA. The reaction cycle was 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, 50°C for 20 seconds and 72°C for 25 seconds. PCR products were detected on 8% denaturing polyacrylamide gels. Females produced three bands, males two bands.

Colour reflectance measurements

To measure reflectance spectra of differently coloured patches of the ocelli (brown, green, and blue) under different incident light angles (60° and 30°) (Fig. 5). Alessandra Costanzo used an Avantes dh 2000 spectrophotometer with a deuterium-halogen light to measure coloration of ocelli (<http://www.avantes.com/products/light-sources/item/237-avalight-d-h-s-deuterium-halogen-light-sources>). Collimating lenses (<http://www.avantes.com/products/fiber-optics/item/256-collimating-lens>) were mounted onto the ends of the reflection probes (<http://www.avantes.com/products/fiberoptics/item/245-reflection-probe-standard>) for both illumination (3 cm from the feather surface) and measurement (6 cm from the feather surface for 60° measurement and 3 cm from the feather surface for 30° measurement) of a spot of feather from the ocelli about 2 mm in diameter. The measurement was performed in a dark

room. We took on average 5 scans at 800 ms integration time for the 60° measurements, and on average 5 scans at 150 ms integration time for the 30° measurements. The difference in the integration time did not affect the result of measurements. Recalibration of the dark and white standards was done every 15 minutes. Each sample was measured twice, remounting it in the apparatus between measurements. We measured again those samples that deviated by more than 3 SD from the mean value. Reflectance spectra (Fig. 6) were then processed according to the Tetrahedral colour space model (Stoddard and Prum 2008) to adopt the spectral sensitivity of the peacock. Feather colour was thus described by three colour variables: θ , which represents the visible light, ϕ , which accounts for the ultraviolet component and achieved r , which is a measure of colour saturation.

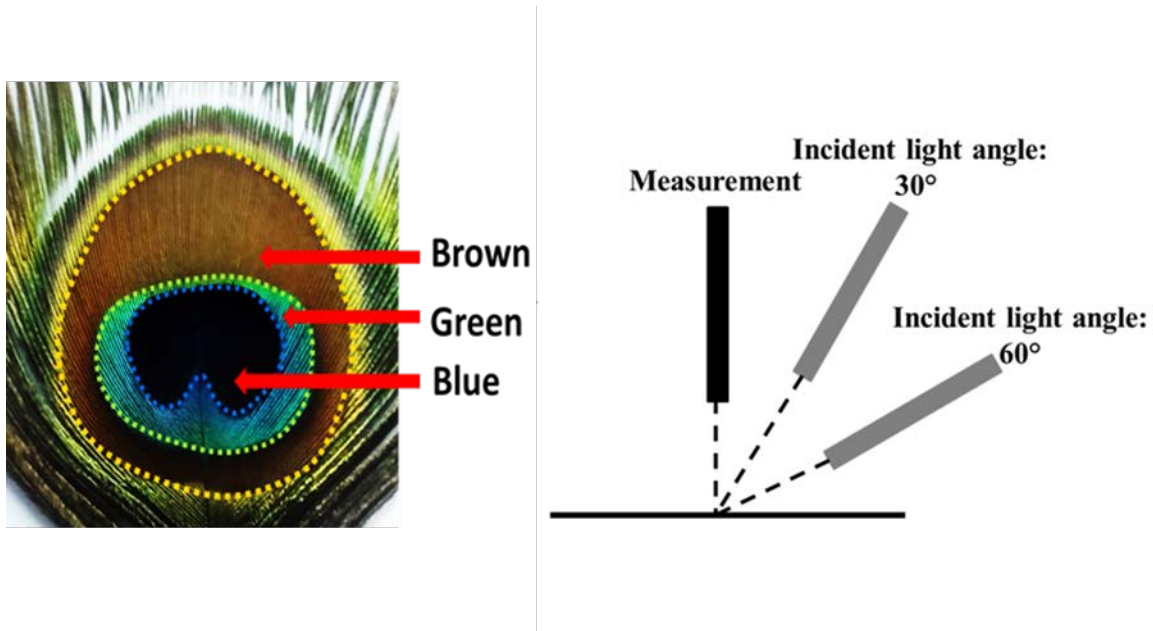


Fig. 5. On the left, the three parts of the ocelli measured (brown, blue and green), marked with a white dot. The measurements were always performed on the left side of the feather when viewed from the front. On the right, the reciprocal position (30° and 60°) of measurement and light source probes (modified from Dakin and Montgomerie, 2013).

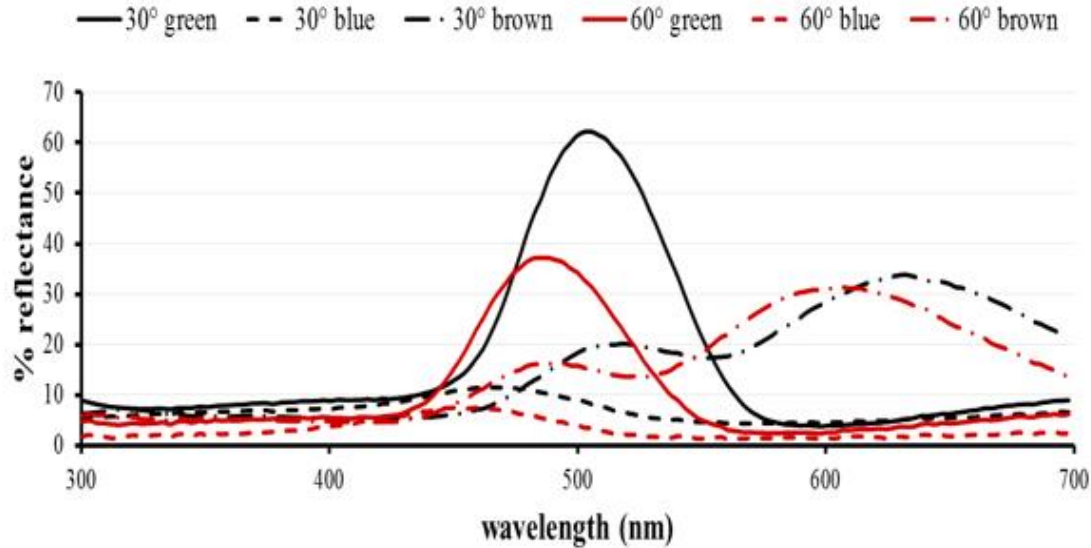


Fig. 6. Reflectance spectra of the brown, blue and green parts of the ocelli, measured at illumination angles of 30° and 60° (black and red curves, respectively) from the female’s typical viewing position in front of the male during courtship. Curves represent the average of the measurements taken from a single feather from each of the 46 individuals’ ocelli.

Growth increment measurement

The width of daily growth increments was measured according to Takaki *et al.* (2001). HAM used the back side of the feathers (with the aid of a faint light) to measure the width of bands (Fig. 7). The feather was placed on white plain paper fixed to a polystyrene board. HAM stuck insect pins through the paper at the distal edge of each growth band on the ocelli. After removal of the feather, HAM measured the distances between the first and last pin marks with a digital calliper to the nearest 0.01 mm (Mitutoyo CD-6” BS). The mean width of each band was estimated by dividing the measured distance by the number of bands measured. All measurements were made blindly with respect to ornamentation or other phenotypic characters.

The measurements of the width of daily feather growth for 46 individuals were repeated twice on different days without any recent prior knowledge of the first set of measurements. All bands were measured by HAM to eliminate any variation in measurements due to among-observer variability. The repeatability of measurements of daily growth increments in train feathers of 46 individual peacocks was high at R (SE) 0.88 (0.033), $F = 15.78$, $df = 45, 46$, $P < 0.0001$.



Fig. 7. The back side of peacock train feathers showing daily growth increments (indicated by white arrows).

Statistical analyses

We analysed repeatability of measurements and scores for randomly chosen subsamples of individuals that were measured twice, using equations in Becker (1984) and Falconer and Mackay (1996) for estimating repeatabilities and their SE.

Simpson's index (a measure of diversity independent of sample size) was calculated to determine the diversity of microorganisms in the differently coloured parts of the ocelli, which takes into account the number of species present, as species richness and evenness increase, so does diversity (Magurran 2004).

We used multivariate analysis of variance (MANOVA) with a repeated measures design when analysing the relationship between the degree of degradation of green, blue and brown parts of ocelli and abundance of bacteria. MANOVA is typically used when two or more response variables are related to one or more predictor variables.

The relationship between the area of ocelli and the degree of feather degradation in differently coloured parts of the ocelli was made using standard least squares regression.

The relationship between the different bacterial taxa from differently coloured parts of the ocelli with different secondary sexual characters was made using standard least squares regression.

Sex ratio of the offspring for each male was expressed as the proportion of males to females produced by a female. Generalized linear models with binomial error distribution and a logit link was used to express the strength of association between the sex ratio and the expression of secondary sexual characters by calculating the effect sizes (r) from the value of chi square according to the following equation (Rosenthal 1994):

$$r = \sqrt{\frac{\chi^2 (1)}{N}}$$

The sample correlation r was subsequently transformed for analysis using Fisher's z -transformation (Fisher 1915)

$$z = 0.5 \times \ln \left(\frac{1 + r}{1 - r} \right)$$

The measures of effect size were weighted by sample size to account for differences in sampling effort among males.

To express the strength of the association between the colour variables (θ , ϕ , and achieved) at different incident light angles (60° and 30°) and the abundance of bacteria and the different bacterial species, Pearson's product-moment correlation coefficients (r) were calculated from the (t) ratio of the partial effect for each variable according to the following equation (Rosenthal 1994):

$$r = \sqrt{\frac{t^2}{t^2 + df}}$$

The sample correlation (r) was subsequently transformed for analysis by Fisher's z -transformation (Fisher 1915):

$$z = 0.5 \times \ln \left(\frac{1 + r}{1 - r} \right)$$

The relationship between the three-colour variables at different incident light angles for all coloured patches of the ocelli and the abundance of bacteria and the taxa diversity was made using standard least squares analyses.

The relationship between the width of growth increments and the abundance of different bacterial taxa in differently coloured parts of the ocelli including the three colour variables was made using standard least squares regression. All statistical analyses were made using JMP (SAS 2012).

GENERAL RESULTS

Bacterial community from ocelli

We found a heterogeneous abundance of bacteria in different parts of the ocelli (Tables S4A and B). In addition, there were variable numbers of bacterial taxa in differently coloured parts of the ocelli. From the eight bacterial isolates that were recovered from TSA medium, HP-F isolates (Unidentified bacterium) form the largest percentage in brown and green parts of the ocelli (19% and 20%, respectively), while the largest group for the blue area was 19% for *Paenibacillus* sp. (HP-D). As for FMA medium HAM contained seven bacterial isolates for which *Streptomyces marokkonensis* (HP-AF) was the most dominant bacterial isolate in brown, green and blue areas of the ocelli (29, 35 and 27%, respectively) (Tables S5A and B). The different bacterial taxa were widely distributed across the phylogenetic tree of bacteria (Fig. S1 phylogenetic tree).

Summary statistics for secondary sexual characters

The different secondary sexual characters varied considerably among peacocks. Coefficients of variation were generally high thus providing males and females with considerable variation for evaluation of potential competitors and mates. The largest coefficients of variation were in train growth and barb loss (Tables 1 A, B, C).

Table 1. (A) Mean, SE, range and coefficient of variation (CV) for secondary sexual characters in peacocks.

Character	Mean	SE	Min.	Max.	CV (%)	N
Number of ocelli	125	2.37	58	147	12.72	45
Ocelli area - second year (cm²)	17.58	0.36	12.74	22.46	13.87	46
Force required to break barbs (g)	54.60	2.37	25.67	91	29.45	46
Lower barb loss - second year	5.24	0.40	0	12	51.77	46
Feather daily growth increments (mm)	0.42	0.01	0.28	0.66	19.95	46
First year train length (mm)	1327	13.98	1060	1510	7.14	46
Second year train length (mm)	1488	11.71	1320	1690	5.34	46
Train growth	162	15.00	- 80	300	62.80	46
First year spur length (mm)	17.97	0.39	11.98	26.22	14.76	46
Second year spur length (mm)	32.98	0.40	27.1	40.6	8.23	46
Spur growth (mm)	15.01	0.26	9.91	18.8	11.75	46

(B) Frequency and percentage of degree of loss of barbs from the upper part of ocelli from 46 peacocks.

Degree of loss	Frequency	%
0	6	13
1	4	8
2	22	48
3	12	26

(C) Frequency and percentage of degree of degradation of differently coloured parts of ocelli from 46 peacocks.

Degree of degradation	Brown		Blue		Green	
	frequency	%	frequency	%	frequency	%
0	9	20	20	43	40	87
1	20	43	18	39	3	7
2	12	26	5	11	3	6
3	5	11	3	7	0	0

1. Darwin's problem of gradation in peacock ocelli: Degradation of ocelli by microorganisms

Bacterial abundance and Simpson diversity index in relation to the area of ocelli.

The area of ocelli ranged from 12.74 to 22.46 cm² with a mean \pm SE of 17.58 \pm 0.36 cm², $n = 46$ males, almost a two-fold difference. The total number of colonies and the Simpson diversity index based on microorganisms from TSA medium for the green and the blue parts, respectively, were negatively related to the area of ocelli. The mean number of colonies based on FMA medium was positively correlated with the area of the blue part of the ocelli ($F = 7.27$, $r^2 = 0.46$, $df = 3,29$, $P = 0.001$). The abundance of different bacterial taxa was related to the area of ocelli ($F = 6.98$, $r^2 = 0.68$, $df = 7,29$, $P = 0.0002$). Four taxa showed a significant negative relationship with the area of ocelli, while three showed a significant positive relationship (Table S6).

Bacterial abundance and in relation to the force required to break barbs

The force required to break a barb from train feathers of 46 peacocks ranged from 25.7 to 91.0 g with a mean \pm SE: 54.6 \pm 2.37 g. The mean force required to break a barb was related to the abundance of three bacterial taxa from FMA medium, two specific for the brown area and one for the blue area ($F = 8.40$, $r^2 = 0.49$, $df = 3,29$, $P = 0.0005$). The abundance of two taxa was positively related to the mean force while one was negatively related (Table S7). A second series of analyses including both bacteria from TSA and FMA media showed qualitatively similar results (results not shown).

Loss of barbs from ocelli and its predictors

Peacocks varied in loss of barbs from ocelli with most individuals having a score of 2 from the upper part of ocelli (Table S8). The number of barbs lost from the lower part of ocelli ranged from 0 to 12 with a mean \pm SE: 5.24 ± 0.40 , $n = 46$ males.

The degree of barb loss differed between the upper and the lower part of the ocelli, greater barb loss being associated with a stronger force required to break a barb, while the interaction between upper and lower part of the ocelli and the force required to break a barb was not statistically significant (Table 2). Finally, we found that loss of barbs from the lower part of the ocelli was negatively related to the area of ocelli controlled statistically for the degree of loss from the upper part of the ocelli (Table 3). This implies that peacocks with large ocelli lost relatively fewer barbs.

Table 2. Degree of barb loss in relation to mean force required to break a barb, whether barb loss was from the lower or the upper part of the ocelli, and the interaction between these two predictors

Character	<i>F</i>	Estimate \pm SE	<i>P</i>
Mean force	5.20	0.03 \pm 0.01	0.026
Upper/Lower	61.00	1.62 \pm 0.21	< 0.0001
Mean force*Upper/Lower	3.57	0.02 \pm 0.01	0.062

Table 3. Relationship between loss of barbs from the lower part of ocelli and the area of ocelli and the degree of loss of barbs from the upper part of ocelli in 46 male peacocks

Character	<i>F</i>	Estimate ± SE	<i>P</i>
Area of ocelli	4.57	− 0.33 ± 0.16	0.038
Degree of barb loss	4.98	0.82 ± 0.37	0.032

Feather degradation and its predictors

Degradation levels of the brown area of ocelli was the largest, while the green area had the lowest level of degradation, with the degradation score being intermediate for the blue part of the ocelli (Table S9).

We found a significant negative relationship between the area of ocelli (cm²) and the degree of degradation for the brown patch ($F = 6.97$, $r^2 = 0.14$, $df = 1,45$, $P = 0.011$, estimate ± SE: 0.99 ± 0.38 ; Fig. 8).

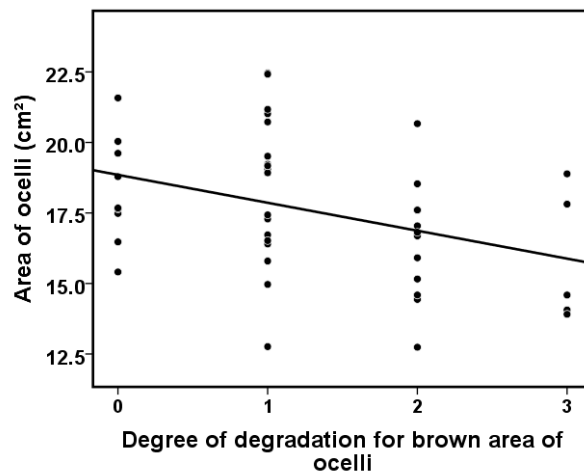


Fig. 8. Area of ocelli (cm²) in relation to degree of degradation of the brown part of ocelli for 46 peacocks. The line is the linear regression line.

We used MANOVA to test for differences in degradation among brown, green and blue parts of ocelli in relation to area of ocelli (Table 4). For between-subjects there was no significant effect of area of ocelli. However, for within-subject effects there was a significant difference among colours, implying that the relationship was different for brown, green and blue parts of ocelli. This effect differed among areas of ocelli as shown by the significant interaction between coloration and area (Table 4). These results show that the brown area is more affected by degradation than the blue and green areas.

Table 4. Multivariate analysis of variance (MANOVA) of the relationship between degree of degradation of differently coloured parts of ocelli feathers of peacocks by area of ocelli

Between subjects	Term	<i>F</i>	df	<i>P</i>
	Area of ocelli	2.25	1, 44	0.14
Within subjects				
	Feather colour	9.46	1.94, 85.43	0.0002
	Feather colour*Area of ocelli	4.75	1.94, 85.43	0.0118

Values in bold are statistically significant ($P < 0.05$).

There was a significant positive relationship between the loss of barbs from the lower part of the ocelli and degree of degradation of the brown patch ($F = 12.71$, $r^2 = 0.22$, $df = 1,45$, $P = 0.001$, estimate \pm SE: 1.42 ± 0.40 ; Fig. 9). In contrast, there was no such relationship between the loss of barbs from the lower part of the ocelli and degradation of the blue and the green patch ($F = 5.49$, $r^2 = 0.11$, $df = 1,45$, $P = 0.02$, estimate \pm SE; 1.03 ± 0.44); $F = 1.44$, $r^2 = 0.03$, $df = 1,45$, $P = 0.24$, estimate \pm SE = 0.89 ± 0.74).

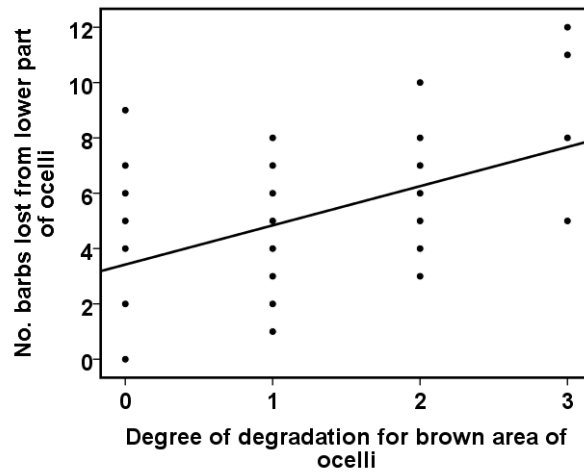


Fig. 9. Loss of barbs from the lower part of ocelli in relation to degree of degradation of the brown part of ocelli in 46 peacocks. The line is the linear regression line.

Mean number of bacteria based on TSA medium were positively correlated with degree of degradation in the brown and blue patches of ocelli (brown patch: $F = 4.67$, $r^2 = 0.14$, $df = 1,29$, $P = 0.039$, estimate \pm SE: 1.03 ± 0.48 ; blue patch: $F = 0.5.36$, $r^2 = 0.16$, $1,29$, $P = 0.028$, estimate \pm SE = 0.99 ± 0.43). In contrast, the degree of degradation in the green area of ocelli was positively correlated with the total number of colonies based on FMA medium ($F = 6.74$, $r^2 = 0.20$, $df = 1,29$, $P = 0.015$, estimate \pm SE: 0.52 ± 0.20).

A MANOVA revealed that the abundance of different kinds of bacteria was related to the degree of degradation of differently coloured parts of the ocelli (Table 5). There were statistically significant differences among differently coloured parts of ocelli between individuals, there was a significant within individual effect among colours, and the interaction between colour and

abundance of bacteria was significant for four out of 11 bacterial taxa (Table 5).

Table 5. Multivariate analysis of variance (MANOVA) of the relationship between degradation of differently coloured parts of ocelli feathers of peacocks by different microorganisms

Between subjects	Term	<i>F</i>	df	<i>P</i>
	<i>Bacillus mycoides</i> (HP-E)	0.32	1, 18	0.58
	<i>Bacillus megaterium</i> (HP-B/C)	0.51	1, 18	0.49
	<i>Bacillus pumilus</i> (HP-G1/G2)	0.44	1, 18	0.52
	<i>Bacillus licheniformis</i> (HP-A1)	0.04	1, 18	0.85
	<i>Bacillus subtilis</i> (HP-DF)	5.62	1, 18	0.029
	<i>Bacillus licheniformis</i> (HP-CF)	0.05	1, 18	0.82
	<i>Bacillus licheniformis</i> (HP-CF)	0.86	1, 18	0.37
	<i>Bacillus megaterium</i> (HP-BF)	1.05	1, 18	0.32
	<i>Streptomyces thermocarboxydus</i> (HP-EF)	0.05	1, 18	0.83
	<i>Streptomyces marokkensis</i> (HP-AF)	8.40	1, 18	0.0096
	<i>Streptomyces thermocarboxydus</i> (HP-EF)	1.88	1, 18	0.19
Within subjects				
	Feather color	13.38	2, 36	< 0.0001
	Feather color * <i>Bacillus mycoides</i> (HP-E)	0.64	2, 36	0.53
	Feather color * <i>Bacillus megaterium</i> (HP-B/C)	10.18	2, 36	0.0003
	Feather color * <i>Bacillus pumilus</i> (HP-G1/G2)	11.73	2, 36	0.0001
	Feather color * <i>Bacillus licheniformis</i> (HP-A1)	0.04	2, 36	0.96
	Feather color * <i>Bacillus subtilis</i> (HP-DF)	0.10	2, 36	0.90
	Feather color * <i>Bacillus licheniformis</i> (HP-CF)	1.56	2, 36	0.22
	Feather color * <i>Bacillus licheniformis</i> (HP-CF)	1.20	2, 36	0.31
	Feather color * <i>Bacillus megaterium</i> (HP-BF)	7.96	2, 36	0.0014
	Feather color * <i>Streptomyces thermocarboxydus</i> (HP-EF)	2.58	2, 36	0.090
	Feather color * <i>Streptomyces marokkensis</i> (HP-AF)	5.88	2, 36	0.0062
	Feather color * <i>Streptomyces thermocarboxydus</i> (HP-EF)	2.02	2, 36	0.15

Values in bold are statistically significant ($P < 0.05$).

2. Why do peacocks have so many different signals?

Bacterial abundance and diversity in relation to ocelli number and degree of degradation

The mean number of bacterial colonies in the brown area (recovered from TSA medium) and blue area (recovered from FMA medium) were negatively related to the number of ocelli in the train ($F = 8.11$, $df = 1, 28$, $P = 0.009$, estimate (SE) = -18.97 (6.66); $F = 8.00$, $df = 1, 28$, $P = 0.0023$, estimate (SE) = -31.60 (11.17)). In addition, the diversity index based on microorganisms from FMA medium for the brown area was positively correlated with the number of ocelli in the train ($F = 11.47$, $df = 1, 28$, $P = 0.009$, estimate (SE) = 96.66 (28.54); whole model statistics: $F = 6.34$, $df = 3, 28$, $r^2 = 0.43$, $P = 0.002$). Furthermore, among the fifteen bacterial isolates in this study, *Paenibacillus* sp. (HP-D) was the only species from the blue area that was significantly negatively related to the number of ocelli of the train ($F = 7.08$, $df = 1, 28$, $r^2 = 0.21$, $P = 0.013$, estimate (SE) = -11.44 (4.3); Fig. 10).

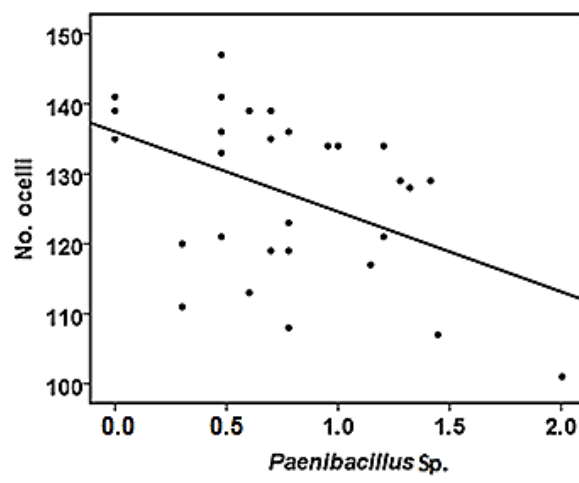


Fig. 10. Number of ocelli in relation to the abundance of *Paenibacillus* sp. in the blue part of ocelli for 30 peacocks. The line is the linear regression line.

The degree of barb loss from the lower part of the ocelli was positively associated with the number of ocelli in the peacock's train ($F = 6.82$, $df = 1, 44$, $r^2 = 0.14$, $P = 0.0123$, estimate (SE) = 0.06 (0.02); Fig. 11), while the relationships between the degree of barb loss from the upper part of the ocelli and the number of ocelli was not statistically significant (results are not shown). This indicates that the number of barbs lost from the lower part of ocelli is proportional to the number of the ocelli in the train. Furthermore, we did not find a significant relationship between the degree of degradation in the different parts of the ocelli and other secondary sexual trait in this study.

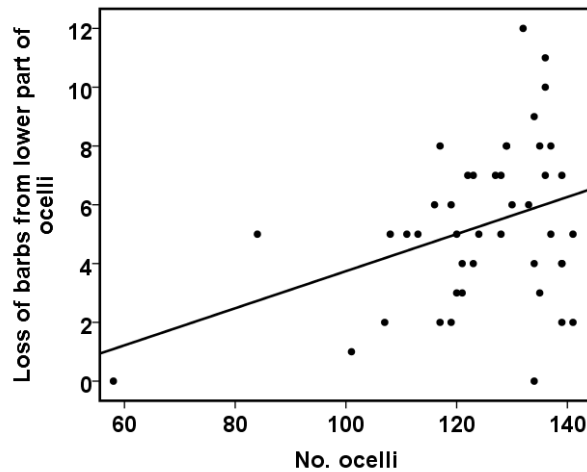


Fig. 11. Degree of loss of barbs from the lower part of ocelli in relation to the number of ocelli in 45 peacocks. The line is the linear regression line.

Train length and growth and its predictors

Train length was not significantly correlated with the total number of colonies, mean number of bacterial colonies, number of species and Simpson's diversity index.

The abundance of different bacterial taxa recovered from the three differently coloured parts of the ocelli on TSA medium was related to train length ($F = 5.45$, $df = 9, 29$, $r^2 = 0.71$, $P = 0.0008$). Five taxa showed a significant negative relationship, while the remaining four showed a significant positive relationship (Table 6).

Table 6. Abundances of different bacterial taxa from differently coloured parts of ocelli in relation to train length in 30 peacocks.

Part of ocelli	Bacterial sp.	<i>F</i>	<i>P</i>	slope	SE
Brown	HP-B/C (<i>Bacillus megaterium</i>)	4.80	0.0405	-91.78	41.90
	HP-G3 (<i>Solibacillus silvestris</i>)	5.58	0.0284	115.22	48.78
Green	HP-CF (<i>Bacillus licheniformis</i>)	5.17	0.0342	-96.81	42.59
	HP-D (<i>Paenibacillus</i> sp.)	4.80	0.0404	-130.42	59.50
	HP-F (Unidentified bacterium)	11.53	0.0029	204.87	60.32
	HP-G1/G2 (<i>Bacillus pumilus</i>)	6.35	0.0203	141.97	56.33
Blue	HP-A3/A4 (<i>Bacillus subtilis</i>)	6.46	0.0194	147.80	58.61
	HP-B/C (<i>Bacillus megaterium</i>)	8.70	0.0079	-175.79	59.58
	HP-E (<i>Bacillus mycoides</i>)	8.11	0.0099	-277.14	97.32

Train growth was positively related to the total number of colonies of bacteria from TSA medium for the blue part, while Simpson's diversity index based on bacteria from FMA medium for the green part was negatively correlated with train growth ($F = 4.37$, $df = 2, 29$, $r^2 = 0.24$, $P = 0.023$). Furthermore, three bacterial taxa recovered from TSA medium for the green and blue part, respectively, were positively related to train growth, and another two bacterial taxa from the same medium for the brown and blue part, respectively, were negatively related to train growth ($F = 6.00$, $df = 5, 29$, $r^2 = 0.56$, $P = 0.001$; Table 7).

Table 7. Abundance of different bacterial taxa from differently coloured parts of ocelli in relation to train growth in 30 peacocks.

Part of ocelli	Bacterial sp.	<i>F</i>	<i>P</i>	slope	SE
Brown	HP-B/C (<i>Bacillus megaterium</i>)	7.05	0.0138	-104.99	39.53
Green	HP-G1/G2 (<i>Bacillus pumilus</i>)	8.59	0.0073	156.24	53.30
Blue	HP-A3/A4 (<i>Bacillus subtilis</i>)	11.40	0.0025	255.08	75.55
	HP-E (<i>Bacillus mycoides</i>)	6.99	0.0142	-284.29	107.56
	HP-G1/G2 (<i>Bacillus pumilus</i>)	4.37	0.0472	85.29	40.78

Spur length and its predictors

There was a positive relationship between mean spur length and train length ($F = 5.21$, $df = 1, 45$, $r^2 = 0.11$, $P = 0.027$, estimate (SE) = 0.010 (0.004); Fig. 12). Likewise, there was a positive relationship between spur growth from one year to the next and train growth ($F = 6.13$, $df = 1, 45$, $r^2 = 0.12$, $P = 0.017$, estimate (SE) = 0.006 (0.002); Fig. 13).

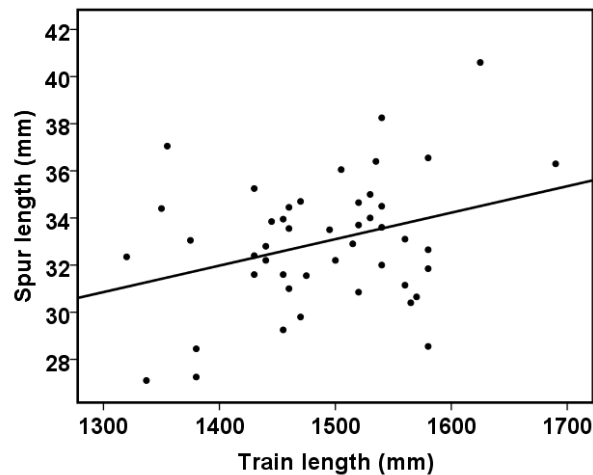


Fig. 12. Mean spur length (mm) in relation to train length (mm) in 46 peacocks. The line is the linear regression line.

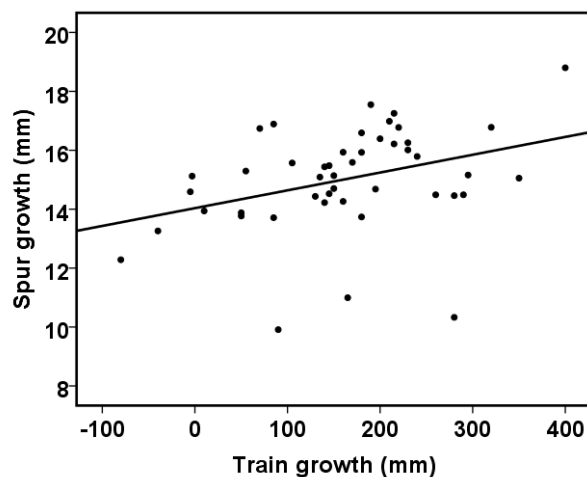


Fig. 13. Spur growth (mm) from one year to the next in relation to train growth (mm) in 46 peacocks. The line is the linear regression line.

Spur length was positively related to Simpson's diversity index based on microorganisms from TSA and FMA medium for the brown and blue parts of the ocelli, respectively ($F = 8.47$, $df = 1$, 29 , $P = 0.0073$, estimate (SE) = 90.48 (31.09); $F = 8.99$, $df = 1$, 29 , $P = 0.0059$, estimate (SE) = 24.85 (8.29)). Furthermore, the number of bacteria from FMA medium for the blue part was negatively related to mean spur length ($F = 6.58$, $df = 1$, 29 , $P = 0.0164$, estimate (SE) = -8.42 (3.28); whole model statistics; $F = 6.07$, $df = 3$, 29 , $r^2 = 0.41$, $P = 0.0028$). Two bacterial taxa recovered from the brown part of ocelli on TSA and FMA medium, respectively, were negatively related to spur length. In contrast, the abundance of one bacterial taxon from the blue part recovered on TSA medium was positively related to mean of spur length ($F = 4.95$, $df = 3$, 29 , $r^2 = 0.36$, $P = 0.0075$; Table 8). Spur growth was correlated in different directions to the abundance of five bacterial taxa recovered from the three coloured parts of the ocelli on TSA medium ($F = 4.26$, $df = 5$, 29 , $r^2 = 0.47$, $P = 0.0064$; Table 9).

Table 8. Abundance of different bacterial taxa from differently coloured parts of ocelli in relation to spur mean length in 30 peacocks.

Area	Bacterial sp.	<i>F</i>	<i>P</i>	Slope	SE
Brown	HP-DF (<i>Bacillus subtilis</i>)	3.46	0.0742	-7.05	3.79
	HP-B/C (<i>Bacillus megaterium</i>)	6.59	0.0164	-2.97	1.16
Blue	HP-G3 (<i>Solibacillus silvestris</i>)	4.26	0.0492	3.44	1.67

Table 9. Abundance of different bacterial taxa from differently coloured parts of ocelli in relation to spur growth in 30 peacocks.

Part of ocelli	Bacterial sp.	<i>F</i>	<i>P</i>	slope	SE
Brown	HP-B/C (<i>Bacillus megaterium</i>)	4.62	0.0419	2.44	1.13
	HP-D (<i>Paenibacillus</i> sp.)	14.39	0.0009	- 4.34	1.14
	HP-G3 (<i>Solibacillus silvestris</i>)	4.32	0.0484	2.56	1.23
Green	HP-F (Unidentified bacterium)	10.24	0.0038	3.94	1.23
Blue	HP-B/C (<i>Bacillus megaterium</i>)	11.03	0.0029	- 3.84	1.15

3. Is sex ratio in the peacock related to the expression of secondary sexual characters?

We tested whether the sex ratio of hatchlings was biased depending on the expression of secondary sexual characters in the peacock using a sample of 4977 eggs. In order to eliminate the possibility that hatching success in females may not be random and lead to bias in the estimate of sex ratio for different females. We tested this assumption, but found no such non-random effect in the two years of study (first year: $F = 0.59$, $df = 1, 140$, $r^2 = 0.004$, $P = 0.44$, second year: $F = 1.59$, $df = 1, 152$, $r^2 = 0.010$, $P = 0.21$).

Fisher's z - transformation of the chi-squared value for the sex ratio for the nine secondary sexual characters that were included in this study ranged from 0.001 to 0.037 with a mean (SE) of 0.017 (0.003) (Table 10). The mean Pearson correlation coefficient for the sex ratio was significantly larger than zero implying that the sex ratio was biased albeit only to a small degree ($t = 5.90$, $df = 8$, $P = 0.0004$). This implies that there is a slight sex ratio bias. In addition, the observed sex ratio was significantly smaller than the

hypothesized mean sex ratio of 0.04 for birds in general according to the data analyzed by Ewen *et al.* (2004; $t = -8.34$, $df = 8$, $P < 0.0001$). This implies that the sex ratio bias in peacocks is significantly smaller than the average bias across all studies of birds.

Table 10. Chi Square values, sample sizes, correlation coefficients (r) and Fisher's z -transformations (z) of tests for an even sex ratio in relation to the expression of nine secondary sexual characters.

Character	Chi Square	Sample size	r	z
Train length (both years)	1.1575	3803	0.01745	0.01745
Spur length (both years)	0.7282	3803	0.01384	0.01384
Ocelli area - second year	0.3453	1853	0.01365	0.01365
Lower barb loss - second year	2.4924	1853	0.03668	0.03669
Upper barb loss - second year	0.5307	1853	0.01692	0.01692
Brown degradation –second year	0.0011	1853	0.00077	0.00077
Blue degradation- second year	0.4489	1853	0.01556	0.01557
Green degradation-second year	0.4217	1853	0.01509	0.01509
Number of ocelli - first year	0.8359	1909	0.02093	0.02093

4. Do peacock signals reveal abundance and diversity of microorganisms?

Bacterial abundance and diversity in relation to the reflectance of the three different colour patches in the ocelli

Fisher's z - transformed correlation coefficients between the reflectance of the three different colour patches in the ocelli of the train of the peacock at two different angles and the bacterial abundance and diversity ranged from -0.627 to $+0.845$ with a mean (SE) of $+0.033$ (0.011). This mean estimate differed significantly from zero ($t = 3.09$, $df = 401$, $P =$

0.0021). This effect size was small, but consistent and showed evidence of a general relationship between coloration and abundance and diversity of microorganisms. In addition, the mean Fisher's z -transformed correlation coefficient in this study was much smaller than the mean estimate of 0.51 that we calculated from data in Table 1 of Dakin and Montgomerie (2013) ($t = -44.25$, $df = 401$, $P < 0.0001$), where they found that the green area affected mating success of the peacock in a North American feral population.

Fisher's z -transformed correlation coefficient was not significantly correlated significantly with the angles of incident light ($F = 0.624$, $df = 1$, 401 , $P = 0.43$), or the three colour variables (θ , ϕ , and achieved r) ($F = 2.845$, $df = 2$, 401 , $P = 0.06$).

Microorganisms or their abundance in ocelli (coded as 1, 0) did not correlate significantly with Fisher's z -transformed correlation coefficient ($F = 1.07$, $df = 1$, 401 , $P = 0.30$). Furthermore, Fisher's z -transformed correlation coefficient did not vary significantly among the 11 bacterial taxa ($F = 0.50$, $df = 11$, 257 , $P = 0.90$) nor with the abundance of these bacteria ($F = 1.27$, $df = 7$, 143 , $P = 0.27$). A reduced model between abundance of bacteria for each of the three colours separately revealed that just the abundance of bacteria in the part of the feather with brown colour was significantly correlated with Fisher's z -transformed correlation coefficient (Fig. 5; Table 11; brown: $F = 6.12$, $df = 7$, 47 , $P = 0.0001$; green: $F = 0.95$, $df = 7$, 47 , $P = 0.48$; blue: $F = 0.50$, $df = 7$, 47 , $P = 0.83$).

Table 11. Fisher’s z- transformation of Pearson’s product-moment correlations between brown colour of the ocelli and various measures of abundance and diversity of microorganisms when cultured in Feather Meal Agar (FMA) and Tryptic Soy Agar (TSA). Values in bold are statistically significant at the 5% level. The model had the statistics $F = 6.19$, $df = 7, 47$, $P < 0.0001$.

Term	Slope	SE	<i>t</i>	<i>p</i>
log Simpson diversity index of bacteria on FMA medium	0.186	0.081	2.30	0.03
log mean bacteria on FMA medium	0.319	0.081	3.95	0.0003
log No. species of bacteria on FMA medium	0.070	0.080	0.86	0.40
log total no. colonies of bacteria on FMA medium	-0.384	0.081	-4.75	< 0.0001
log Simpson diversity index of bacteria on TSA medium	-0.132	0.081	-1.64	0.11
log mean no. bacteria on TSA medium	0.016	0.080	0.2	0.84
log no. bacteria species on TSA medium	0.034	0.081	0.42	0.68

Fisher’s z- transformed correlation coefficients for the relationship between measures of colouration and abundance of microorganisms were the units of analysis. Subsequently, we tested whether these z-values differed among three categories of colour (brown, green and blue), and there was a highly significant difference among the three categories ($F = 8.74$, $df = 2, 401$, $P = 0.0002$).

A Tukey's test revealed a significant difference at the 0.05 level between the abundance of bacteria in the brown part of the feather and green and blue colour. A statistical model that included colour, microorganisms and their interaction with Fisher’s z- transformed correlation coefficient as

response variable revealed a significant effect for brown colour and its interaction with a variable reflecting whether the analysis was based on microorganism diversity or their abundance (Table 12; $F = 5.07$, $df = 5, 401$, $P = 0.0002$).

Table 12. Fisher’s z- transformation of Pearson’s product-moment correlation between the abundance and the diversity of microorganisms and colouration of ocelli and the interaction between colour whether the test was based on microorganisms diversity or their abundance. The model had the statistics $F = 5.07$, $df = 5, 401$, $P = 0.0002$. Values in bold are statistically significant at the 5% level.

Term	Slope	SE	<i>t</i>	<i>P</i>
colour[blue]	-0.017	0.015	-1.066	0.29
colour[brown]	0.051	0.016	3.310	0.001
microorganisms [0]	-0.012	0.011	-1.101	0.27
colour[blue]*microorganisms [0]	0.025	0.015	1.589	0.12
colour[brown]*microorganisms [0]	-0.039	0.016	-2.518	0.012

5. Feather bacteria may influence daily growth increments of peacock ocelli feathers

Bacterial abundance and diversity in relation to daily growth increments

Two bacterial taxa that were recovered on TSA medium from the brown area were significantly related to the mean width of daily growth increments (whole model statistics: $F = 7.34$, $df = 2, 29$, $r^2 = 0.35$, $P = 0.0028$), while the number of *Bacillus licheniformis* (HP-A1) was positively related to the mean daily growth increment ($F = 5.78$, $df = 1, 29$, $P = 0.023$, estimate (SE) = 0.09 (0.04)) while *Bacillus pumilus* (HP-G1/G2) showed a

negative relationship ($F = 12.05$, $df = 1, 29$, $P = 0.002$, estimate (SE) = -0.10 (0.03)) (Table 13).

Table 13. Relationship between the abundance of two bacterial taxa recovered in TSA medium from the brown area of ocelli and daily growth increments in ocelli of 30 peacocks ($F = 7.34$, $df = 2, 29$, $r^2 = 0.35$, $P = 0.0028$).

Term	Estimate	SE	<i>t</i>	<i>P</i>
<i>Bacillus licheniformis</i>	0.09	0.037	2.40	0.0233
<i>Bacillus pumilus</i>	- 0.10	0.028	- 3.47	0.0018

In the blue area, just four bacterial taxa among the eight bacterial taxa that had been isolated on TSA medium were significantly related to mean daily growth increments. Four bacterial taxa showed heterogeneous relationships with mean daily growth increments being positively related to the abundance of *Bacillus licheniformis* (HP-A1) and *Paenibacillus* sp. (HP-D), while the abundance of Unidentified bacterium (HP-F) and *Bacillus pumilus* (HP-G1/G2) were negatively related to the width of daily growth increments ($F = 4.04$, $df = 4, 29$, $r^2 = 0.39$, $P = 0.012$, Table 14).

Table 14. Relationship between the abundance of different bacterial taxa recovered in TSA medium from the blue area of ocelli and daily growth increments in feathers of 30 peacocks ($F = 4.04$, $df = 4, 29$, $r^2 = 0.39$, $P = 0.012$).

Term	Estimate	SE	<i>t</i>	<i>P</i>
<i>Bacillus licheniformis</i>	0.13	0.04	3.04	0.0054
<i>Paenibacillus</i> sp.	0.10	0.04	2.47	0.0209
Unidentified bacterium	- 0.09	0.04	- 2.24	0.0340
<i>Bacillus pumilus</i>	- 0.09	0.04	- 2.22	0.0354

Just two bacterial taxa isolated on FMA medium from the differently coloured parts of the ocelli were significantly related to the mean width of daily growth increments. *Micromonospora* sp. (HP-GF) from the brown and blue area were significantly negatively related to rate of feather growth (Fig. 14; $F = 5.58$, $df = 1, 29$, $r^2 = 0.17$, $P = 0.025$, estimate (SE) = $-0.11(0.05)$); Fig. 15: $F = 4.41$, $df = 1, 29$, $r^2 = 0.14$, $P = 0.045$, estimate (SE) = $-0.14(0.06)$), while the abundance of *Bacillus licheniformis* (HP-CF) from the green area was positively related to mean width of daily feather growth increments (Fig. 16: $F = 5.95$, $df = 1, 29$, $r^2 = 0.18$, $P = 0.021$, estimate (SE) = $0.19(0.08)$). Thus, different bacterial taxa were related to the rate of feather growth with both positive and negative effects.

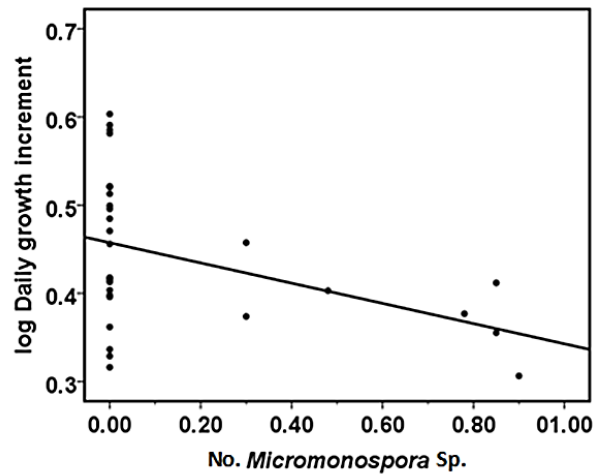


Fig. 14. Daily growth increments in peacock feathers in relation to the abundance of *Micromonospora* sp. in the brown part of ocelli for 30 peacocks. The line is the linear regression line.

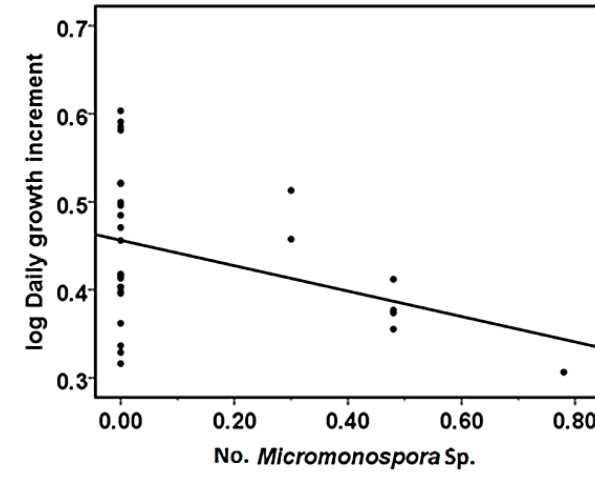


Fig. 15. Daily growth increments in relation to the abundance of *Micromonospora* sp. in the blue part of ocelli for 30 peacocks. The line is the linear regression line.

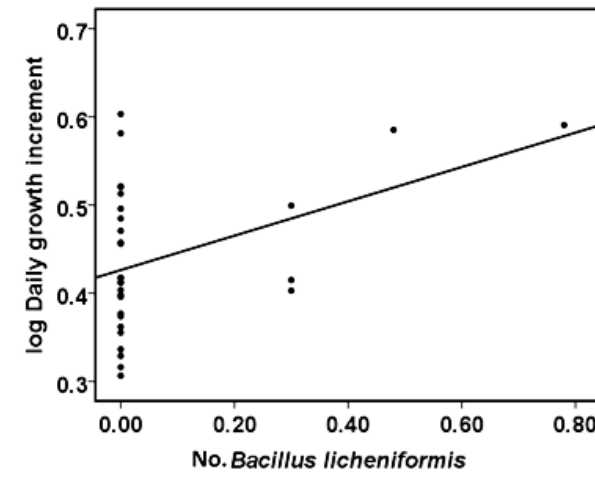


Fig. 16. Daily growth increments in relation to the abundance of *Bacillus licheniformis* in the green part of ocelli for 30 peacocks. The line is the linear regression line.

Three colour variables in relation to feather growth increments

The three colour variables (θ , ϕ , and achieved r) that were measured under different incident light angles (60° and 30°) for the differently coloured patches of the ocelli (brown, green, and blue) showed considerable variation among individuals. The coefficients of variation for reflectance spectra were mostly high, providing both males and females with considerable variation for evaluation of potential competitors and mates (Table 15).

Table 15. Mean, SE, range and coefficient of variation (CV) for the three colour variables (θ , ϕ , and achieved r) that were measured under different incident light angles (30° in (A) and 60° in (B)) for the different coloured patches of ocelli (brown, green, and blue) of 46 individual peacocks.

(A)

30°		Mean	SE	Minimum	Maximum	CV (%)
Brown	θ	-0.18	0.01	-0.37	0.06	47.64
	ϕ	-0.84	0.01	-0.94	-0.75	5.92
	Achieved r	0.64	0.00	0.58	0.69	2.68
Green	θ	0.69	0.43	-3.13	3.12	417.93
	ϕ	-0.37	0.01	-0.55	-0.23	19.42
	Achieved r	0.78	0.00	0.72	0.84	3.18
Blue	θ	-2.59	0.06	-2.80	0.00	15.46
	ϕ	0.40	0.03	0.00	0.80	45.60
	Achieved r	0.34	0.01	0.00	0.46	22.43

(B)

60°		Mean	SE	Minimum	Maximum	CV (%)
Brown	θ	-0.25	0.02	-0.58	-0.04	48.16
	ϕ	-0.88	0.01	-1.20	-0.74	10.24
	Achieved r	0.61	0.01	0.52	0.67	5.96
Green	θ	-2.83	0.01	-3.11	-2.69	3.48
	ϕ	-0.15	0.01	-0.32	-0.01	55.68
	Achieved r	0.75	0.01	0.60	0.82	6.43
Blue	θ	-2.35	0.11	-2.73	0.00	31.82
	ϕ	0.56	0.04	0.00	1.12	47.71
	Achieved r	0.50	0.03	0.00	0.87	38.91

Among the three colour variables (θ , ϕ , and achieved r) that were measured at two different angles for the three coloured patches of peacock ocelli, we found that only the value of θ and achieved r at 30° of the blue patch were negatively related to mean feather increments, respectively ($F = 9.11$, $df = 1$, 45 , $P = 0.0043$, estimate (SE) = -0.13 (0.04); $F = 10.34$, $df = 1$, 45 , $P = 0.0025$, estimate (SE) = -0.73 (0.23); whole model statistics: $F = 5.62$, $df = 2$, 45 , $r^2 = 0.21$, $P = 0.0068$). This implies that feather colour was related to the rate of feather growth.

GENERAL DISCUSSION

Peacocks spend most daylight hours walking with the train either dragging through the vegetation or dragging on the ground. This leaves ample opportunities for transfer of microorganisms from the soil and the vegetation to train feathers. Because many feather degrading microorganisms are originally soil living (Clayton 1999; Shawkey *et al.* 2003; Sangali and Brandelli 2000; Lucas *et al.* 2003; Riffel *et al.* 2003), dragging train feathers through the soil or the vegetation provides an opportunity for transfer of such microorganisms to the plumage. Bacterial communities may differ according to soil composition, and assemblages of these bacteria in the plumage may thus be habitat dependent, leading to differences even between individuals of the same bird species (Burt and Ichida 1999; Burt and Ichida 2004; Bisson *et al.* 2007).

We collected all feather samples on the same day thereby avoiding any problems of seasonality affecting the findings. However, this study also had a number of limitations. For example, the samples were collected several years ago, and this implies that the composition of the microbial

community may have changed over time. However, this study has identified real individual differences as the peacocks and their feathers were all kept under the same controlled conditions. Whilst the numbers of bacteria may have changed over time the feathers were isolated after ‘infection’ following moult so the communities must at some level reflect the individual responses to that infection.

Peacocks have large numbers of ocelli in their trains with different parts of the ocelli differing in colour from brown and green to blue. These ocelli are surrounded by long barbs that differ in strength of attachment. First, we showed that the diversity and the abundance of bacteria differed among differently coloured parts of ocelli. Second, the number of barbs broken at the lower part, but not the upper part of ocelli, was related to the force required to break a barb. The force required to break a barb was related to the abundance and diversity of bacteria in ocelli. Thus, peacocks with large ocelli lost relatively few barbs, and the degree of feather degradation in differently coloured parts of the ocelli depended on bacteria. These findings imply that differently coloured components of ocelli reflect different degrees of resistance to bacteria.

We found a negative relationship between the area of ocelli and both total number of bacteria and taxa diversity based on TSA medium (suitable growth medium for most heterotrophic bacteria). These relationships may reflect individual condition, if only healthy individuals are able to cope with the effects of these bacteria while still developing and maintaining large exaggerated ocelli (Saag *et al.* 2011a, b; Gunderson *et al.* 2009), and/or there are individual differences in anti-microbial defences. Since individual peacock feathers were collected after a winter of being in the same paddock, it is unlikely that these relationships resulted from differences in infection as

a result of habitat differences. The mean number of bacteria on FMA medium (highly selective medium for keratinolytic bacteria) was positively related to the area of ocelli. In other words, an increase in the number of bacterial taxa may result in competition for limiting resources and thereby reduce any deleterious effects on hosts (as shown for the role of competition for establishment of a healthy skin micro-flora (Tannock 1995; Davis 1996)).

There were heterogeneous relationships between the abundance of different bacterial taxa in different parts of the ocelli and the area of ocelli (Table S6). Since many bacterial taxa involved in this relationship are known to have the ability to secrete either antimicrobial substances or proteolytic enzymes (Omura *et al.* 2001; Burt and Ichida 1999; Gunderson 2008), this may affect both the bacterial community and feather properties, thereby accounting for the heterogeneous results.

The force required to break barbs was related in different ways to the abundance of three bacterial taxa from FMA medium in different parts of the ocelli. The two bacterial taxa that were positively related to the mean force required to break barbs was *Bacillus licheniformis*, which is known to have the ability to degrade feathers (Williams *et al.* 1990), but also to produce antimicrobial substances (Haavik 1974; Simlot *et al.* 1972). These antimicrobial substances are active against bacteria of the genera *Bacillus*, *Corynebacterium*, *Enterococcus* and *Mycobacterium*, amoebae (Gálvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). Likewise, *Micromonospora* species are also known to have the ability to produce keratinase that degrades feathers (El-Bondkly and El-Gendy 2010). Furthermore, *Micromonospora* species can produce many of the best-known antibiotics, including aminoglycoside antibiotics, gentamicin and netamicin

(Bérdy 2005). *Micromonospora* species also produce many other biologically active molecules like anti-fungal substances (Nolan and Cross 1988; Ismet *et al.* 2004), anti-tumour compounds (Igarashi *et al.* 2007) and vitamin B12 (Wagman *et al.* 1969), which enhance keratin quality and production. These bacterial taxa recovered from the brown area (which constitutes the larger part of the ocelli of peacock feathers) may lead to the establishment of an equilibrium bacterial community that prevents establishment of other pathogenic bacteria. Meanwhile the abundance of *Streptomyces marokkonensis* was negatively related to the mean force required to break barbs. *Streptomyces marokkonensis* is known to inhibit the growth of pathogenic yeasts and phytopathogenic fungi (Bouizgarne *et al.* 2009).

Feather barb loss from the lower part of ocelli differed from that for the upper part of ocelli, and there was a positive association between the degree of loss from the lower part and the mean force required to break a barb. This could be explained as individuals investing more in feather quality to compensate and stop the deleterious effects of bacteria to preserve at least a minimum number of barbs following bacterial degradation. Furthermore, we detected a negative relationship between the degree of barb loss from the lower part and area of ocelli, which may indicate the superior quality of individuals that grow large ocelli while still maintaining a maximum number of barbs in the lower part of the ocelli.

MANOVA results revealed that the brown area of ocelli was more affected by degradation than the blue and green areas. This degradation in the brown area was negatively correlated with the area of ocelli and positively with the loss of barbs from the lower part of ocelli. These results may reflect individual quality where poor quality males suffer more from

degradation and barb loss, which in turn affect the ability to produce large healthy ocelli. The brown area which constitutes the larger part of ocelli may represent an important clue for peahens to assess male quality through degree of damage and/or barb loss from the lower part of ocelli.

Leclaire *et al.* (2014) showed in an experiment on feral pigeons that individuals with low bacterial loads on their feathers had brighter iridescent neck feathers. They suggested that the load of feather bacteria may play an important role in alteration of iridescent feather condition and signalling of colour in pigeons. Our results showed a positive correlation between bacterial load based on TSA and FMA media with the degree of degradation in all three differently coloured parts of the ocelli, consistent with the study by Leclaire *et al.* (2014). The MANOVA showed that the degree of degradation in differently coloured parts of the ocelli was related to the abundance of different kinds of bacteria. This indicates a significant difference in within-subject effects among different colours of the ocelli, suggesting that the relationship differed for brown, green and blue parts. This implies that the three colours differ in their ability to become degraded by different kinds of bacteria. We do not have an explanation for the differential sensitivity of the brown area to degradation among different ocelli parts, but we assume that this could be related to the physical properties of this area (Zi *et al.* 2003). This suggestion requires future experiments to test degradability of the three colours of ocelli *in vitro* involving different bacterial taxa.

Peacocks like males of many other species have evolved multiple exaggerated secondary sexual traits to attract females during the breeding season, and such multiple traits may provide valuable information to potential partners about their phenotypic and genetic quality. The most

obvious trait in peacock is the magnificent decorated long train that contains large numbers of ocelli differing in colour from brown and green to blue, but also other traits like spurs and crest may contribute to signal information about health and condition (Møller and Petrie 2002; Dakin 2011; Loyau *et al.* 2005b).

The present study suggests that both heterotrophic and keratinolytic bacteria are correlated with the expression of ornaments in the peacock with the degree of barb loss and feather degradation from ocelli reliably reflecting the abundance and the diversity of bacteria.

These novel findings suggest that female peafowl, but also conspecific males may use the expression of secondary sexual characters to assess the condition of conspecifics and hence the probability of a favourable mate choice or gains in fights among peacocks at the leks. We documented a negative relationship between the number of ocelli in the train of the peacock and the mean number of bacteria recovered from brown and blue parts of ocelli based on TSA medium (favourable for the growth of most heterotrophic bacteria) and FMA medium (favourable the growth of just keratinolytic bacteria), respectively. These results follow previous studies on peacocks and other species suggesting that microbes negatively affect the expression of feather ornaments (Hill *et al.* 2004; Shawkey *et al.* 2009; Loyau *et al.* 2005b). On the other hand, we found that the diversity of bacterial taxa derived from FMA medium of the brown area was positively correlated with the number of ocelli. Numerous experimental studies and theoretical predictions for animals and plants suggest that a highly diverse bacterial community has a positive impact on host health by being more resistant to pathogen invasions (Case 1990; Kennedy *et al.* 2002; Dillon 2005; van Elsas *et al.* 2012). Furthermore, the abundance of *Paenibacillus*

sp. recovered from the blue area was negatively related to the number of ocelli in the peacock's train. *Paenibacillus* sp. are widespread in many different habits (water, soil, waste), and it has the ability to produce many types of extra-cellular enzymes like cellulose (Park *et al.* 2012), nitrogenase (Köberl *et al.* 2011), xylanase (Dheeran *et al.* 2012) and keratinase (Paul *et al.* 2013), which have negative impacts on feather condition.

Dakin and Montgomerie (2011) reported 105 to 177 ocelli among studies, and Petrie *et al.* (1991) concluded that their observations were not caused by feather loss, because the number of ocelli was recorded at the start of the mating season, while the loss of individual feathers otherwise seems to be a primary source of variation in number of ocelli. Breakage and loss of ocelli feathers is common during the breeding season, and males are often observed with broken ocelli feathers that have not yet fallen out (R. Dakin, personal communication). Feather barb loss from the lower and the upper part of ocelli were differently related to the number of ocelli. Many studies have emphasized the importance of the number of ocelli for mating success (Petrie *et al.* 1991; Petrie and Halliday 1994; Dakin and Montgomerie 2011, 2013). Thus, the positive relationship in our study between the degree of barb loss from the lower part of the ocelli and the number of ocelli may be explained as a trade-off between the production of many and weak or fewer, but stronger ocelli. Alternatively, there may be a trade-off between investment in anti-bacterial defence and sanitation behaviour.

There were heterogeneous relationships between the abundance of nine bacterial taxa (recovered from TSA medium) from different parts of ocelli and train length (Table S6). Most of these bacteria have the ability to either secrete antimicrobial substances or proteolytic enzymes or both (Haavik 1974; Simlot *et al.* 1972; Agrahari and Wadhwa 2012; Malanicheva

et al. 2012; Paul *et al.* 2013; Hasan *et al.* 2009; Rajput and Gupta 2013; Leifert *et al.* 1995; Jeevana Lakshmi *et al.* 2013). Interestingly, one of the nine taxa, *Solibacillus silvestris*, is known to have degrading activity for N-Acylhomoserine lactones (AHL). AHL are used as quorum-sensing signal molecules by many bacterial taxa to synchronize certain kinds of behaviour such as biofilm formation, virulence, and antibiotic resistance, based on the local density of the bacterial population (Morohoshi *et al.* 2012; Miller and Bassler 2001). Thus, enzymes secreted from this bacterial community may impact both bacterial community and feather properties, but it could also help clarify our heterogeneous results.

We found a positive association between train growth and total number of colonies from the blue part of ocelli. This positive relationship may be due to mutualistic action, where mutualists (originating primarily from the soil) promoted by the presence of secretions from the uropygial gland can occupy the space and prevent the dominance of pathogenic feather degrading bacteria (Pillai *et al.* 2001). Secondary sexual traits that reliably reflect individual quality of signallers might be costly to produce or maintain (Grafen 1990a, b; Kodric-Brown and Brown 1984; Zahavi 1975, 1987). Thus, individuals can allocate and redirect limiting resources from train growth to other secondary sexual traits. In contrast, we found a negative relationship between train growth and the diversity of bacterial taxa based on FMA medium for the green area of ocelli. The green area has an important positive effect on mating success of peacocks (Dakin and Montgomerie 2013; Loyau *et al.* 2007). Thus, one possible interpretation of this relationship is the degradation ability that characterizes the different bacterial taxa that are able to grow on FMA medium and their negative impact on feathers of hosts.

Five taxa from the three differently coloured parts of ocelli were either positively or negatively related to train growth (Table S2). This diversity of relationships between these five taxa and train growth could be linked to bacterial characteristics. Most of these bacteria are known to have the ability to secrete different kinds of extracellular products (antibiotic, proteolytic and keratinolytic enzymes). These products may alter the integrity of feather structure as well as the associated bacterial community, either in beneficial or harmful ways, and that would be reflected in effects on overall growth rate of individuals.

Peacocks just as other lekking species show aggressive behaviour when establishing display territories during breeding (Rands *et al.* 1984; Bradbury 1981). Peacocks use their spurs as weapons while fighting competitors (intrasexual selection) (Harikrishnan *et al.* 2010). This weapon is considered an extravagant secondary sexual trait that plays a role in signalling to individuals of the opposite sex (von Schantz *et al.* 1989; Møller 1992). Our study revealed that spur length was positively correlated with train length, and in addition spur growth was positively correlated with train growth. These findings suggest that the length of both the train and the spurs reflect male intrinsic quality. This is consistent with studies that cite the importance of train length in female choice (Petrie *et al.* 1991; Loyau *et al.* 2005b). Likewise, peacocks and several other galliform birds that have longer spurs have a higher probability of winning in intersexual competition eventually resulting in higher mating success (Kelly 1975; von Schantz *et al.* 1989; Steffen *et al.* 1990; Wittzell 1991; Mateos and Carranza 1995).

The Simpson diversity index for bacteria on the brown and blue areas of ocelli recovered from TSA and FMA medium, respectively, was positively related to mean length of spurs. That may be explained by

beneficial enzymatic activity of bacteria inhabiting these differently coloured areas and their impact on feather properties and the community of microorganisms (Gunderson 2008). In contrast, we found a negative relationship between the number of bacterial taxa from the area of the blue part of ocelli based on FMA medium and spur length. This inconsistency in results for Simpson's diversity index and the number of bacterial species with the mean length of the spur may be due to differences in the methods of estimation of diversity. Simpson's diversity index depends on both richness (measured as the number of different kinds of organisms present in a particular area) and evenness (population size of each of the species present) of bacterial taxa, while the number of species takes no account of the number of individuals of each species present in the community.

Three bacterial taxa were correlated differently with the mean length of the spur (Table S3), while an additional five taxa were also correlated with spur growth (Table S4). The taxa that were negatively correlated with mean length of the spur and spur growth are known for their harmful effect on structural properties of feathers. Interestingly, *Solibacillus silvestris* is positively associated with mean spur length and spur growth. This is consistent with the positive relationship between the abundance of this taxon and train length. That may reflect the potential role of this taxon in shaping the bacterial community in the ocelli of peacocks and in turn the expression of secondary sexual characters.

Sexual attractiveness of a male may affect the optimal sex ratio of his sons. If male attractiveness is inherited by sons, then sons of a highly attractive male may be of greater reproductive value than daughters. In contrast, daughters of unattractive males may be of relatively higher reproductive value than sons. If that were the case, it would be adaptive for

fathers and mothers to bias their sex ratio in response to the secondary sexual characters of the father (Weatherhead and Robertson (1979). In the third part of this study, the correlation between nine secondary sexual characters in peacocks with the sex ratio of the offspring by using a very large sample size (Table 10) revealed that the correlation coefficient (r) was significantly less than the value of 0.04 that was found by Ewen *et al.* (2004) in their meta-analysis.

In birds the role of sexual selection in adaptive sex ratio manipulation has been particularly controversial. Several studies suggest that females of sexually dichromatic birds may adaptively manipulate the sex ratio of their offspring in relation to male ornamentation (Krackow 1995; Emlen 1997; Komdeur *et al.* 2002; West and Sheldon 2002). For instance, females may produce more sons when copulating with a highly attractive male. One of the classical studies (Burley 1981b, 1986) suggesting adaptive sex-ratio manipulation in zebra finches (*Taeniopygia guttata*) found that leg-band colours influenced male attractiveness, and offspring of the more attractive parental sex were produced in excess. Therefore, Burley suggested that parents improve their fitness by increasing investment in more attractive offspring. Pike and Petrie (2005) conduct an experiment on peacocks which have magnificent long and highly ornamented trains. They suggested that when peahens are mated to peacocks with their attractiveness experimentally reduced by removal of eyespot feathers from their trains, they tended to produce significantly more daughters than when mated with the same males after restoring the degree of ornamentation of their trains. This suggests that peahens have a degree of control over the sex of their offspring.

While the literature may superficially suggest that there is plenty of evidence consistent with sex ratio manipulation, Ewen *et al.* (2004) conducted an exhaustive meta-analytical study and found no evidence for the general occurrence of avian primary sex ratio adjustment across all studies. Thus, facultative control of offspring sex is not a characteristic biological phenomenon in breeding birds given that the effect size measured in terms of Pearson's product-moment correlation coefficient weighted by sample size was only on average $r = 0.04$. In evolutionary biology studies, effect sizes typically account for 5-10% of the variance (Møller and Jennions 2002). This implies that sample sizes required to demonstrate a small effect accounting for 1% of the variance will require an enormous sample size that is only rarely acquired in empirical studies (Møller and Jennions 2002).

Many morphological signals like horns, antlers and plumage ornaments reveal important aspects of an individual's phenotype. These traits are typically used by males as sexual signals, and, therefore, linked to reproductive success. Sexual signals play a significant role in competition for and attraction of mates (Andersson 1994). Colourful plumage is considered one of the most important visual signals in birds, because it can serve as an reliable signal of individual quality with functions in both intrasexual (Smith and Harper 1988; McGraw and Hill 2000) and intersexual communication (Kodric-Brown and Brown 1984; Hill 2006). Bird feathers are inhabited by numerous types of parasitic microorganisms that can affect allocation of time and energy and hence development of feather signals. These parasitic microorganisms may induce changes in host phenotypes that have major consequences for host survival and fitness (Clayton and Moore 1997; Schmid-Hempel 2011). A potentially important group of

microorganisms that inhabit bird feathers is feather degrading bacteria (Burt and Ichida 1999; Gunderson 2008; Ruiz- Rodríguez *et al.* 2009).

The current study showed that the presence of bacteria in peacock ocelli could lead to slight changes in the colour of ocelli. The results for peacocks reported here were generally weak, implying that they differed significantly from the results reported by Dakin and Montgomerie (2013) for a feral population of North American peacocks. That study reported strong correlations between the three different colours of peacock ocelli especially the green area with mating success. These differences in effect sizes could be related to populations of peacocks having been subject to multiple genetic bottlenecks with consequences for the level of standing genetic variation and hence the phenotypic variability from which females may be able to make their mate choice.

The results reported here indicate that the two angles of the incident light that we used in this study for measuring the three different colour variables of the ocelli (θ , ϕ , and achieved r) was not significantly correlated with Fisher's z -transformed correlation coefficient, which is a measure of the strength of the correlation between the abundance of different bacterial taxa and the three colour variables. Fisher's z -transformed correlation coefficient did not differ significantly among the abundance of bacteria nor with the different bacterial taxa in the ocelli. These results are in accordance with the findings of Jacob *et al.* (2014) in an experimental study on a wild breeding population of great tits. They manipulated overall bacterial densities in the nest hence modifying the bacterial loads of feathers of adult birds. They did not find any significant influence of bacterial load of feathers on feather coloration of different body parts of great tits. Our results differ from those of Shawkey *et al.* (2007) and Leclaire *et al.* (2014), where

Shawkey *et al.* in structurally coloured (non-iridescent) rump feathers of eastern bluebirds found that bacterial load and feather degrading bacteria in lab experiments led to alterations in structural plumage colour through degradation. Leclaire *et al.* (2014) found in their experimental study while increasing or decreasing bacterial load in captive feral pigeons that feather bacteria can degrade feathers and lead to alterations in the coloration of iridescent neck feathers. These conflicting results may be due to differences in experimental manipulation of the bacterial community of feathers, with consequences for competition and synergism (Faust *et al.* 2012; Morgan *et al.* 2013). The bacterial community could be regulated through differences in behaviour like bathing and preening (Shawkey *et al.* 2003; Saranathan and Burtt 2007; Møller *et al.* 2009; Clayton *et al.* 2010). Furthermore, these differences could be related to physical and chemical properties of feathers in different taxa.

This study indicated that the three different colours of ocelli in peacocks were significantly correlated with Fisher's z- transformed correlation coefficients differing between analyses based on microorganisms or their abundance and diversity. These results are consistent with what we have shown in the first part of this study, where we found that the brown area was more sensitive to bacterial degradation than the two other colours of the ocelli. We hypothesized that this might be related to the difference in physical properties of these three areas (Zi *et al.* 2003). The change in colour of the brown area might be caused by bacterial degradation. Dakin and Montgomerie (2013) found that the green area was correlated with mating success in a North American feral population of peacocks. Dakin and Montgomerie (2013) speculated that the brown and the blue coloured areas could enhance the appearance of the green area. Brou *et al.* (1986)

emphasized that the colour of an object depends on the colours of objects in its immediate surroundings. Thus, an alteration in colour of the surrounding areas (brown and blue) could affect the appearance of the important cue of the medullary green area and consequently influence the assessment of the green area by the female.

We refer previously in this discussion to extravagant secondary sexual characters from feathers being vulnerable to the effects of microorganisms from the surrounding environment, and we also showed that there were differences in bacterial communities between brown, green and blue parts of ocelli. In this part of the study we tested the hypothesis that differently coloured parts of the ocelli of peacocks that harboured different bacterial communities may affect the growth of feathers during the annual moult and that mean daily rate of feather growth may relate to the abundance of certain bacteria. Here we discuss the heterogeneity in these relationships among different colour variables (θ , ϕ , and achieved r), but also among different bacterial taxa.

Time and energy allocated to maintenance of plumage may reduce the negative effects of bacteria on feathers at the expense of other vital functions like growth and reproduction. Peacocks spend a substantial part of their time budget (15%) on maintenance (Walther 2003). Leclaire *et al.* (2014) showed that individuals with elevated bacterial load will increase the quantity of preen secretion and preening time, which is an energetically costly behaviour in terms of production and time (Goldstein 1988; Redpath 1988).

This study revealed heterogeneous relationships between the abundance of different bacterial taxa in different parts of the ocelli and the rate of feather growth. Bacteria recovered from the brown part of the ocelli based on TSA medium showed that two bacterial taxa were related to the

mean width of daily growth increments (Table 13). The mean abundance of *Bacillus licheniformis* was positively related to the mean width of feather increments, while the abundance of *Bacillus pumilus* showed a negative correlation. Both bacterial taxa are known to produce keratinase that can degrade feather keratin (Williams 1990; Burt and Ichida 1999; El-Refai *et al.* 2005; Ramnani *et al.* 2005; Rajput and Gupta 2013). Furthermore, these bacterial taxa can produce a wide range of extracellular substances that show antibiotic activity. *Bacillus licheniformis* produces different kinds of antimicrobial substances (Callow and Work 1952; Simlot *et al.* 1972; Haavik 1974). These antimicrobial substances have a wide range of effects against bacterial taxa (*Bacillus sp.*, *Corynebacterium sp.*, *Enterococcus sp.* and *Mycobacterium sp.*), amoebae (Gálvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). *Bacillus pumilus* also produce such antimicrobial substances, but it mainly acts as an antifungal substance against bacterial taxa (Leifert *et al.* 1995; Bottone and Peluso 2003; Sawale *et al.* 2014). The positive relationship between *Bacillus licheniformis* and mean band width indicates that an increase in the abundance of this bacterium will increase with daily growth increments implying high quality feathers (Grubb 2006). In contrast, an increase in the abundance of *Bacillus pumilus* reduces band width possibly due to the harmful effects of degradation caused by these bacteria. This is consistent with studies showing that infection of birds with malaria (Marzal *et al.* 2013; Coon *et al.* 2016) and mites (Pérez-Tris *et al.* 2002) reduces growth rate of feathers. Since both bacteria are known to secrete degrading enzymes and antibiotic substances, the difference in relationship between feather band growth and abundance of bacteria could be due to differences in bacterial characteristic and their extracellular materials.

Bacterial recovery based on TSA medium showed that four bacterial taxa (*Bacillus licheniformis*, *Bacillus pumilus*, *Paenibacillus sp.* and Unidentified bacterium) from the blue part of the ocelli were related to mean width of daily growth increments (Table 14). We found the same relationship for *Bacillus licheniformis* and *Bacillus pumilus* with mean feather band width, while *Paenibacillus sp.* was related positively and Unidentified bacterium negatively to mean band width. *Paenibacillus sp.* are widespread in many different habitats (water, soil, waste), and they are known to produce a wide range of antimicrobial substances that can affect a wide spectrum of microorganisms (mostly Gram-positive and Gram-negative bacteria) and even anaerobic pathogens as it can act as an antifungal agent (Girardin *et al.* 2002; Guo *et al.* 2012; Kai *et al.* 2013). The prevalence of *Paenibacillus sp.* could interact synergistically with *Bacillus licheniformis* to minimize the harmful effects of degradation caused by other bacterial taxa.

The isolation of bacteria from brown and blue parts of the ocelli based on FMA medium showed that the abundance of *Micromonospora sp.* is negatively related to the width of daily growth increments. *Micromonospora sp.* are widespread in soil and water, and they are known to have the ability to produce keratinase that can degrade feather keratine (El-Bondkly and El-Gendy 2010). Thus, this bacterial taxon could deteriorate feather integrity and consequently lead to allocation of more energy and time to feather maintenance. *Bacillus licheniformis* recovered from FMA medium showed a positive relationship with mean width of daily growth increment resembling what we found for TSA medium.

The relationship between the three different colour variables and the width of feather growth increments revealed that the blue patch is more

closely linked to band width than other two coloured patches. The results indicate that θ which represents the visible light and the value of achieved r that represents colour saturation are negatively related to feather growth increments. In other words, the decrease in these variables is associated with an increase in the rate of feather growth. One possible explanation for this is that the reduction in the colour variable could occur because these birds increased daily feather growth to compensate for the negative influence of bacteria on feather integrity. These findings parallel to some extent those of Griggio *et al.* (2009), who found that blue tits (*Cyanistes caeruleus*), when induced to molt at a faster rate, tended to produce feathers with reduced brightness. Structural colouration and pigment-based colouration can serve as honest advertisements of individual quality (Keyser and Hill 1999; Keyser and Hill 2000; Matrková and Remeš 2012). Different studies found a relationship between structural colouration and feather growth rate in different bird species (Keyser and Hill 1999; Doucet 2002). Further experiments in vivo or in vitro are necessary in order to clarify how these bacterial taxa interact and how they interact with the individual host.

CONCLUSIONS

In conclusion, this study revealed a heterogeneous distribution of bacteria among differently coloured parts of peacock ocelli which could affect feather integrity, expression of secondary sexual traits and feather colouration, subsequently affecting the behaviour of peacocks. The bacterial community on different parts of ocelli in peacocks showed a combination of positive and negative relationships among bacterial taxa. The different bacterial taxa secrete either keratinase, antibacterial substances or a combination. Keratinase has the ability to alter feather integrity and cause

damage to feathers hence possibly negatively affecting flight. On the other hand, antimicrobial substances secreted by bacteria may lead to establishment of an equilibrium bacterial community that prevents establishment of other pathogenic bacteria which in turn may benefit the host. I list five major conclusions:

1. I demonstrated heterogeneous distributions of abundance of bacteria among the three coloured parts of ocelli, the degree of feather degradation in differently coloured parts of the ocelli depending on bacteria, peacocks with large ocelli losing relatively few barbs, and the force required to break barbs being related to the diversity of bacteria in differently coloured parts of the ocelli. Hence ocelli are reliable signals of diversity and abundance of bacteria in peacocks.

2. The number of ocelli was related to bacterial abundance and diversity, but also to the number of barbs lost from the lower part of ocelli. The presence of certain types of bacteria such as *Paenibacillus* sp. was associated with a reduction in the number of ocelli, while *Solibacillus silvestris* was associated with train and spur characteristics. These findings are consistent with the multiple message hypothesis stating that different secondary sexual characters in peacocks reliably reveal information on the abundance and the diversity of bacteria, allowing choosy females to pick males that have specific bacterial communities in their plumage and on their spurs.

3. We found a small effect of the expression of secondary sexual characters on bias in brood sex ratio towards production of more sons than daughters when males were particularly attractive. This study also showed that mean effect size was actually significantly smaller than the effect size in the general literature as reported by Ewen *et al.* (2004).

4. Our study demonstrated that the bacterial community in the ocelli could lead to slight changes in colouration of the three different coloured parts of ocelli with the main changes being in the brown area. These findings emphasise the importance of the brown area of the ocelli in sexual selection in the peacock.

5. The diverse bacterial community in the differently coloured parts of the ocelli of peacocks may differentially influence the growth of feathers. Our results are consistent with studies indicating that feather growth rate is related to the abundance of endoparasites or ectoparasites. The presence of *Bacillus licheniformis* and *Paenibacillus* sp. was associated with wider growth increments implying that these bacterial taxa enhance feather growth by successfully competing against harmful bacterial taxa. In contrast, the prevalence of other bacterial taxa like *Micromonospora* sp. and *Bacillus pumilus* was linked to a reduction in feather growth rate probably through alterations in the integrity of feathers by degradation. This may subsequently increase the allocation of energy and time to the maintenance of feather quality. Alternatively, harmful bacteria may give rise to a trade-off between anti-bacterial defences and development of high quality and strong train feathers that are essential for mate acquisition during the annual lekking season.

PERSPECTIVE

Parasites are ubiquitous and they can greatly influence the future reproductive success of hosts through sexual selection because only healthy individuals are able to produce exaggerated secondary sexual characters while being able to resist debilitating parasites (Hamilton and Zuk 1982).

This general hypothesis for sexual selection may similarly apply to peacocks. Several studies have highlighted the potential role of bacteria in animal behaviour and communication (Archie *et al.* 2007; Sharon *et al.* 2010; Ezenwa *et al.* 2012). Many bacteria that live in the plumage have the ability to degrade feather keratin (Burt and Ichida 1999; Gunderson 2008) that may alter the structure and condition of ornamented feathers (Shawkey *et al.* 2007), potentially leading to a reduction in essential functions like thermoregulation, aerodynamic efficiency and feather-based communication among individuals (Swaddle *et al.* 1996; Clayton 1999; Shawkey *et al.* 2007). Communication may be impaired by a reduction in feather colouration either by consumption or modification of feather microstructures or pigments due to microbial action (Shawkey and Hill 2004).

This study showed that the bacterial community in peacock feathers was highly complex and diverse among the three different parts. Furthermore, different parts of ocelli feathers were susceptible to degradation by microorganisms to different extents. This complexity of the bacterial community and susceptibility of differently coloured ocelli parts to degradation must be taken into consideration in future studies.

The different bacterial taxa in this study are known to secrete various types of extracellular enzymes that may affect the bacterial community of ocelli and hence lead to variation in individual feather condition and coloration and in turn potentially affect peacock display. One possible way to investigate the interaction between the different bacterial taxa and is to perform *in vitro* and *in vivo* experiments. *In vitro* experiments could be achieved by using different bacterial taxa to study the interaction between them. Furthermore, by using these different bacterial taxa we could test the degradability of the three different colours of ocelli and their ability to

change the colour parameters. An in vitro study could be performed through a captive breeding experiment testing the influence of excessive or reduced bacterial load on the quality and characteristics of peacock train feathers in relation to their behaviour and expression of the secondary sexual characters. This could be achieved by manipulating the different components of the bacterial community by using antiseptic substances to remove or reduce the feather bacterial community.

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AUTHOR CONTRIBUTIONS

APM and MP collected all peacock feather samples. JE estimated the force required to break barbs surrounding the ocelli. AC measured the feather reflectance spectra. HM sexed the offspring. HAM conducted all the lab analyses. The statistical analyses were made by HAM and APM. HAM wrote the text with input from APM.

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Chapter 1:
Darwin's problem of gradation in peacock ocelli:
Degradation of ocelli by microorganisms

**Haider Al-Murayati,^a Zaid Al Rubaiee,^a Petrie Marion^b and
Anders Pape Møller^{a*}**

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

^b Institute for Health and Society and Newcastle University Institute for
Ageing, Newcastle University, Campus for Ageing and Vitality
Newcastle upon Tyne, NE4 5PL, UK

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Correspondence to HAM:

Tel: (+33) 1 69155688

Fax: (+33) 1 69155688

E-mail: haideryousif1967@gmail.com

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ABSTRACT

Darwin (1871) noted that Peacocks *Pavo cristatus* have ocelli that are surrounded by partially transparent rings caused by an absence of barbules making the ocelli appear to be isolated from the train. These translucent zones may make the feathers susceptible to breakage either mechanically or as a result of the action of microorganisms. We tested whether there was a difference in abundance and diversity of bacteria in the three differently coloured parts of ocelli, whether the degree of feather degradation differed among differently coloured parts of ocelli, and whether the degree of barb loss was related to the force required to break barbs. There was a heterogeneous distribution of bacteria among differently coloured parts of ocelli, the degree of feather degradation in differently coloured parts of the ocelli depended on the abundance and the diversity of bacteria, the force required to break barbs was related to the diversity of bacteria in differently coloured parts of the ocelli, and the peacocks with large ocelli lost relatively few barbs. These findings are consistent with the hypothesis that bacteria may play a significant role in damage to and degradation of differently coloured parts of the ocelli of peacocks, and that the phenotype of ocelli may reveal reliable information about infestation with microorganisms to females and competitor males.

Key words: feather bacteria; barb breakage; feather barbs; feather breakage; feather degradation; ocelli.

INTRODUCTION

Charles Darwin (1859) first suggested that exaggeration of elaborate traits like the train of the peacock *Pavo cristatus*, and bright plumage and naked patches of skin in many birds could not possibly have evolved by natural selection since they could decrease survivorship. Subsequently he proposed that such characters may have become exaggerated because they provided individuals with the most exaggerated traits with an advantage in terms of mating success. Darwin also noted that sexual selection may act through two mechanisms: Either intrasexual competition between members of one sex, usually males, for access to mates, or choice by members of the same sex (intersexual selection, usually by females) for members of the other sex (Darwin 1871).

Charles Darwin was fascinated by peacocks and their elongated decorated trains, and he expressed admiration for the ocelli of these feathers describing them as one of the most beautiful objects in the world. Ocelli comprise a centric iridescent deep blue part with indentation in the lower section along the line of the shaft of the feather, a blue part enclosed by a rich green section, and this in turn by a wide bronze-brown area. He noticed that the base of the barbs at the top of the ocelli is derived from the missing barbules that make two clear translucent zones surrounding the ocelli thereby making it clearly separate from the rest of the feather (illustrated in Fig. 1). Darwin speculated that these clear zones may be related to the development of the ocelli (Darwin 1871). However, he did not notice that these translucent zones also made the feathers particularly susceptible to breakage (Fig. 1). Such breakage could affect the appearance of the ocelli

and thereby reveal the causes of such breakage, either mechanical or caused by microorganisms.

The peacock was a particularly difficult puzzle for Darwin, and the complexity of the ocelli feathers had caused him to be almost sick with worry of this exaggerated display, as he expressed in a letter to Asa Gray at 3 April, 1860: “The sight of a feather in a peacock’s tail, whenever I gaze at it, makes me sick!” (Burkhardt *et al.* 1994). In particular, he was completely aware that the evolution of the train was at the expense of the survival of individuals, and that an increase in train size would make males easier prey for any predator, but also increase the mating success of such males (Gadagkar 2003). Indeed, peacocks with longer trains, in a free ranging UK population, were better able to survive predation attempts by foxes *Vulpes vulpes* than males with short trains, but they also experienced higher mating success (Petrie 1992). Thus, Darwin proposed that the evolution of the extravagant plumage in polygynous birds in general was the outcome of sexual selection and most likely by female choice (Darwin 1871).

Peacocks are resident polygynous birds in South-East Asia where they prefer deciduous open forest, but they can also be found in captivity and have the ability to adapt easily to colder climates if provided with a simple shelter (Jackson 2006). Outside the breeding season peacocks live in flocks, while during the breeding season they aggregate at open communal display grounds so-called leks. There they maintain their territories calling to attract females from a distance and then displaying their erect trains to females when they arrive on the lek. Females arrive at these display grounds for several days before eventually choosing to copulate with a single male (Rands *et al.* 1984; Harikrishnan *et al.* 2010). Subsequently, peahens

construct a nest on the ground away from these display sites, where they lay eggs which they then incubate for 28-30 days. Peacocks play no part in post-mating reproduction and never interact with the offspring.

Darwin (1871) suggested that females would prefer males with more ornamented trains as confirmed by observations (Petrie *et al.* 1991; Loyau *et al.* 2005a) and experiments (Petrie and Halliday 1994, Dakin and Montgomerie 2011). However, the structure and size of ocelli (Møller and Petrie 2002; Dakin and Montgomerie 2013), the length of feathers in the train (Manning and Hartley 1991; Yasmin and Yahya 1996; Loyau *et al.* 2005b), the crest (Dakin 2011), the direction of the expanded train relative to the sun (Dakin and Montgomerie 2009), calls (Yasmin and Yahya 1996; Dakin and Montgomerie 2014; Yorzinski and Anoop 2013) and the shaking of the train feathers that produces two faintly distinct types of infrasonic mating calls (Freeman 2012) all have been reported to contribute to male mating success. However, there is considerable variation in these preferences among populations (Dakin and Montgomerie 2011).

Already Darwin pondered the question why not all males developed the most exaggerated secondary sexual characters, suggesting that the train was an attractive trait rather than being of any utility. Because male peacocks do not provide females with any material benefits, peacocks have been hypothesized to provide genetic benefits to choosy females (Davies 1978; Payne 1984). If the most ornamented males are in better condition before, but also after development of their secondary sexual characters such males might signal their superior quality (Zahavi 1975). Subsequent analyses have shown that male peacocks may reliably signal their condition (Møller and Petrie 2002), quality (Loyau *et al.* 2005b) or disease resistance

(Møller and Petrie 2002; Hale *et al.* 2009). Indeed, the suggestion is that peahens gain indirect genetic benefits for their offspring since peacocks with elaborate trains leave more surviving offspring (Petrie 1994).

Parasites have been hypothesized to play a crucial role in sexual selection because only healthy individuals are able to produce exaggerated secondary sexual characters and still remain resistant to debilitating parasites (Hamilton and Zuk 1982). This general hypothesis for sexual selection may equally apply to peacocks. Studies conducted so far have not investigated whether peacocks with the most exaggerated secondary sexual characters have fewer parasites, and there is still a deficit of information on the role played by pathogenic parasites that may balance the costs of exaggerated ornamentation. Microorganisms are ubiquitous and hence may play a key role in this process. Indeed, a recent study by Leclaire *et al.* (2014) showed in feral pigeons that individuals with low bacterial loads on their feathers had brighter iridescent neck feathers

The objectives of this study were to assess the extent to which the structure of the ocelli of peacocks reflects the abundance and the diversity of microorganisms. Specifically, we tested whether male peacocks with larger ocelli have a lower abundance and diversity of microorganisms, in particular pathogenic microorganisms, and whether the size of ocelli reveals susceptibility to feather degradation as judged from missing barbs. In general, bird feathers are coloured either by incorporation of pigments like melanins, carotenoids and porphyrines into the keratin matrix or by the structural arrangement of the feather (known as structural colouration) (Fox and Vevers 1960). That is also the case for the colouration of peacocks. Zi *et al.* (2003) showed that the colouration of peacock train feathers comes from

the partial photonic bandgap of the 2D photonic-crystal like structure in the cortex material. The difference in lattice constant or the number of periods leads to a shift in the midgap frequency of the partial photonic bandgap. In other words, it is the number of periods that control the production of additional colours, eventually leading to additive, mixed colouration. Therefore, differently coloured parts of the ocelli are likely to differ in their abilities to resist bacterial degradation because of differences in micro-structure. We isolated bacteria from the ocelli in order to determine their identity, diversity and abundance, and we subsequently analysing the relationship between diversity and abundance of bacteria and size of ocelli, feather degradation and force required to break barbs of the feathers with ocelli.

MATERIALS AND METHODS

Feather samples used were collected in the spring of 1999 at the start of the breeding season by APM and MP from peacocks involved in a captive breeding experiment conducted over two years (1998 and 1999) in a commercial peacock farm in Norfolk, UK (see Hale *et al.* 2009 for further details). Individuals had overwintered in large outdoor pens outside of the breeding season, where they were provided with shelter, water and commercial poultry feed *ad libitum* and free access to invertebrates in the soil and to green vegetation. Because all individuals were kept in the same farm and fed a similar diet provided by the same persons, all peacocks were subject to the same level of contamination with microorganisms suggesting that all individuals would be equally infected if it were not for differences in resistance to microorganisms and differences in anti-microbial defences. All individuals were subsequently distributed in pens that held one peacock and

four randomly chosen peahens, with the location of all individuals also being randomized between pens which were different in both breeding seasons.

The study was conducted under UK Home Office Licence, and there were no indications of negative effects of any of the procedures adopted during the study that adversely affected the peacocks.

Feathers were removed from birds by using examination gloves and scissors, and they were subsequently placed in dry clean plastic bags. All samples were stored under identical conditions until processed.

We randomly chose one feather from each male (in total 46 individuals). Feathers were photographed with a digital camera, and all ocelli measurements were taken from the pictures by using the 'ruler' tool in Adobe Photoshop CC software. HAM measured ocelli size in mm as the width (measured at the widest point) and the height measured at the greatest vertical diameter (Fig. 1). All measurements were made blindly with respect to identity and phenotype of individuals.

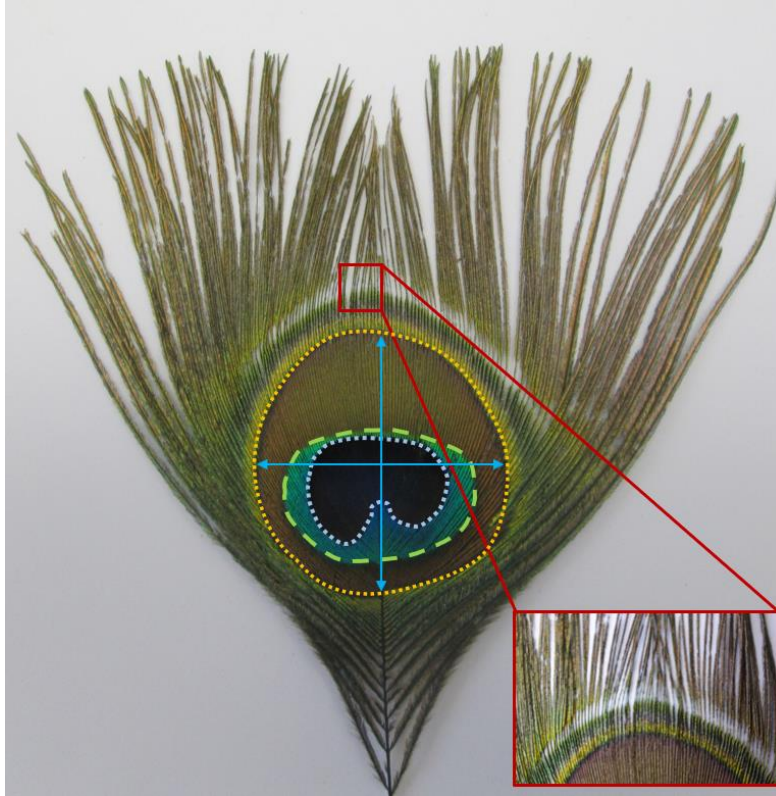


Fig. 1. Ocellus of a peacock showing the brown, green and blue parts. Orange dotted line indicates the area where HAM measured the size of ocelli (mm), while the width and height were measured at the widest points of the ocelli (light blue arrow). The red box shows a higher magnification of the clear translucent zones that surround the upper part of ocelli.

Estimating feather degradation of ocelli

HAM used the following scale in order to estimate feather degradation: (A) Counts of the number of barbs missing from the lower part of ocelli feathers. (B) Degree of loss from the upper part of barbs was scored as follows (Fig. 2): 0 = No loss due to degradation, 1 = no loss with degradation, 2 = one third of barbs from the upper part were lost or degraded, 3 = two thirds of barbs from the upper part were lost or degraded, and 4 = more than two thirds of barbs from the upper part were lost or degraded. (C) Ocelli

degradation levels were subsequently ranked from 0 (no degradation) to 3 (maximum degradation) for the area of each of the three coloured patches (brown, green and blue in Fig. 3). HAM scored the level of degradation as follows: 0 = No degradation, 1 = slight degradation (less than 1-3 small spots of degradation), 2 = medium degradation (large area of degradation), and 3 = high degree of degradation (more than half of the area degraded).

Measurements and scores of feather characters (degree of damage to different ocelli colours and area of ocelli) for 30 individuals were repeated twice on different days without any recent prior knowledge of the first set of measurements and scores. These repeated measurements and scores were used for estimating repeatability (R) (Becker 1984; Falconer and Mackay 1996), and repeatability was high in all cases. Repeatability for different characters in peacocks on different days is reported in Table S1.

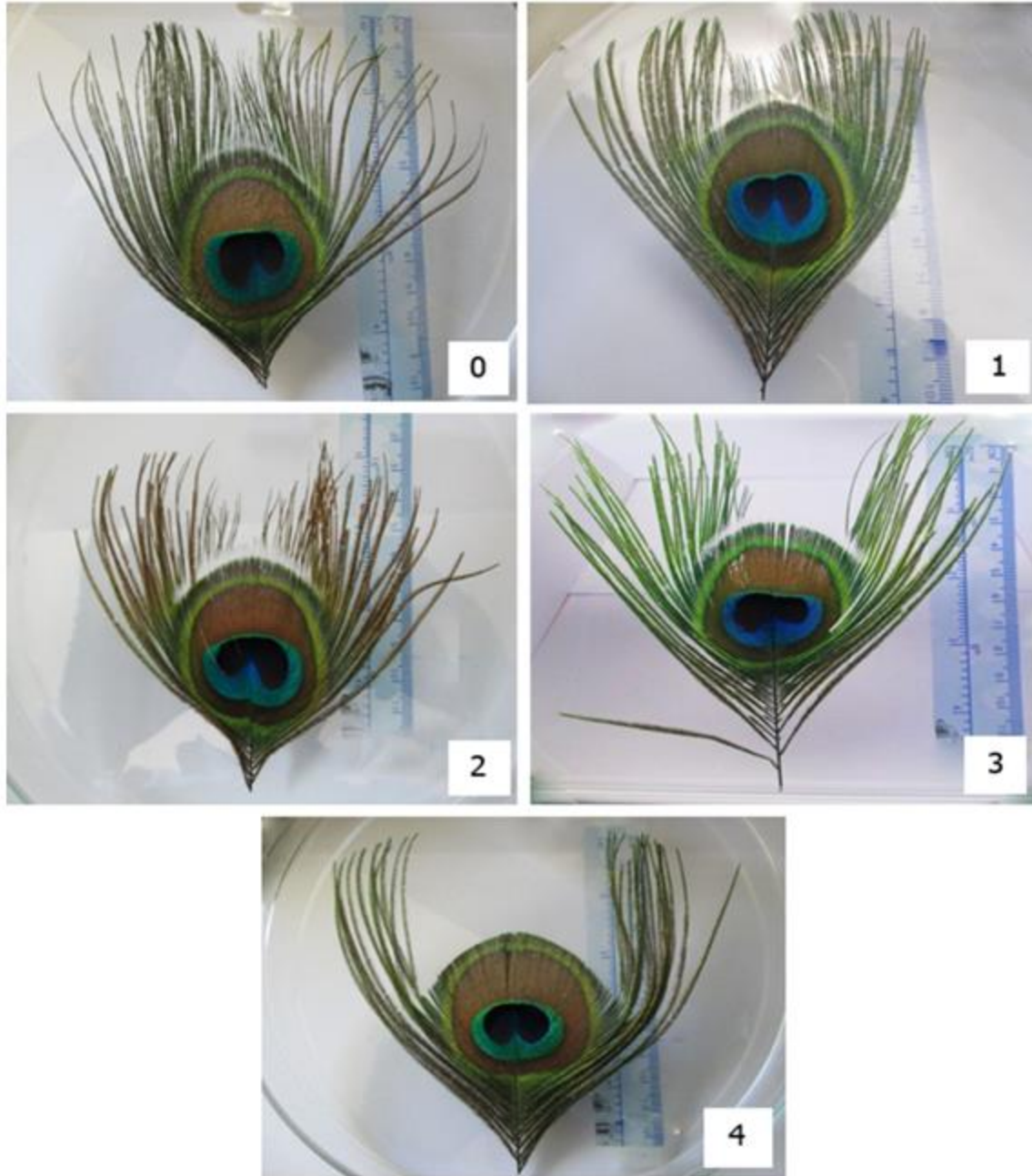


Fig. 2. Degree of loss of barbs from the upper part of ocelli: 0 = No loss due to degradation, 1 = no loss with degradation, 2 = one third of barbs from the upper part was lost or degraded, 3 = two thirds of barbs from the upper part were lost or degraded, and 4 = more than two thirds of barbs from the upper part were lost or degraded.

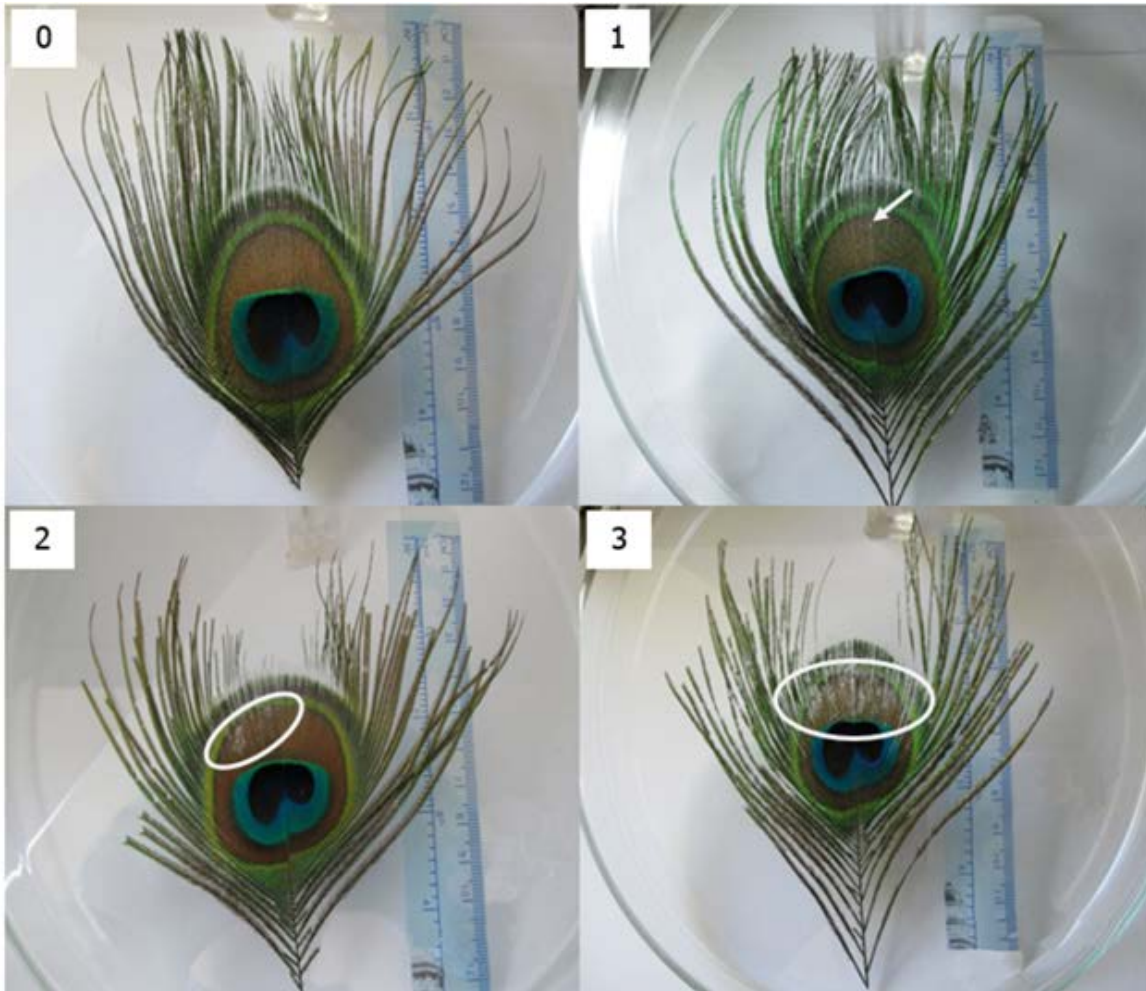


Fig. 3. Levels of feather degradation:0 = No degradation, 1 = slight level degradation (less than 1-3 small spots of degradation), 2 = medium level degradation (large area of degradation), and 3 = high degree of degradation (with more than half of the area degraded).

Estimating the force required to break feather barbs

We estimated the force required to break a feather barb that surrounds the ocelli by adopting a previously used method (Møller *et al.* 2006). First, Johannes Erritzøe (JE) fastened the end of the barb to a clamp. Subsequently, the barb was attached to a Pesola spring balance, which was pulled slowly until the barb broke, carefully reading the number on the spring balance when the barb broke. We repeated this exercise three times for a feather to allow for estimation of repeatability and for calculating more precisely the force required (repeatability (R) for force required to break a feather barb is reported in Table S2). It is well-known that traits measured with error can be measured more precisely by measuring the same trait repeatedly. Finally, we calculated the mean of the three estimates as a best estimate of the required force for inducing breakage. JE was unaware of the purpose of the study, and he had no prior knowledge of any of the other variables when estimating the forces. Therefore, the measurements were made blindly with respect to the objectives of the study.

Bacterial isolation

For bacterial isolation HAM choose feather samples randomly from 30 individuals of overall 46 individuals that include in this study (the remaining 16 males were not included for logistic reasons), in addition to a second sample from 10 randomly chosen individuals to estimate repeatability of total number of bacterial and the number of bacterial species (Becker 1984; Falconer and Mackay 1996). The degree of repeatability was high in all cases except for number of species recovered from TSA plates of the brown and blue parts of the ocelli (repeatability of total number of bacteria and the number of bacterial species on TSA and FMA media from differently

coloured parts of the ocelli are reported in Tables S3A and B). Each coloured part of peacock feathers was cut by using a sterilized scalpel and with the help of forceps in the laboratory. HAM used a pre-weighed 2 ml eppendorf tube which after receiving the feather sample was weighed again to calculate the mass of the feather and to determine the volume of sterile phosphate buffer saline (pH 7.2) to be added to the tube (each mg feather sample equalled 100 μ l PBS). This was followed by 3 vortex periods 1 min each. Free-living bacteria were washed out from the feathers and collected in PBS solution (Saag *et al.* 2011a). To quantify the cultivable and feather-degrading bacteria, duplicates were made by spreading 100 μ l of the resulting PBS with a sterile spreader loop on two different growth media: (1) Tryptic soy agar (TSA), which is a rich medium on which heterotrophic bacteria can grow, thus enabling us to assess total cultivable microorganism load of feathers. (2) Feather meal agar (FMA), which is a medium highly selective for keratinolytic bacteria with the unique source of carbon and nitrogen being keratin. Hence, only bacteria that are able to digest it can proliferate, allowing for quantification of the feather-degrading bacterial load. The FMA contains 15 g L⁻¹ feather meal, 0.5 g L⁻¹ NaCl, 0.30 g L⁻¹ K₂HPO₄, 0.40 g L⁻¹ KH₂PO₄, and 15 g L⁻¹ agar (Williams *et al.* 1990; Sangali and Brandelli 2000; Shawkey *et al.* 2003, 2007, 2009). (3) Control plate inoculated with the same volume (100 μ l) of sterilized distilled water in order to detect any contamination of media (negative controls).

Fungal growth was inhibited by adding cycloheximide to TSA and FMA media (Smit *et al.* 2001). Plates were incubated at 28°C for 3 days in the case of TSA, and for 14 days in the case of FMA. After incubation, HAM counted the number of colony-forming units (CFU) of each morphotype per plate by using dissecting microscope, and distinguished the

morphotypes on the basis of colony colour, shape, size, and presence or absence of glutinous aspects.

Isolate preservation

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

DNA extraction

Genomic DNA was extracted from each isolate by using two different protocols: (A) Freeze-thaw protocol. (1) With a wooden toothpick, HAM transferred and squashed a part of a bacterial colony in a 0.5 ml eppendorf tube containing 50 µl Tris (10 µM, pH 8.0). (2) Start of freezing and thawing by putting the eppendorf tube in liquid nitrogen for one to two minutes until completely frozen, then quickly placing the tube in a hot water bath until completely thawed. This process is known as a freeze-thaw cycle, and HAM repeated the same process for a total of three cycles. HAM made sure that he mixed the sample tube between each cycle. (3) To ensure complete cell lysis HAM put the tubes in a microwave at 270W for 5 to 6 s followed by 10 s of waiting, repeated these three times. (4) HAM centrifuged the sample tube for five minutes at 12,000 x g in a microcentrifuge. Using a micropipette, he transferred the supernatant that contains the DNA into a new clean, sterile microcentrifuge tube and discarded the pellet that contains cellular debris. HAM stored the tubes in -20 °C for the Polymerase Chain Reaction (PCR). (B) DNA Extraction kit: HAM used the Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA) to extract DNA according to the protocol that is supplied with the kit.

PCR amplification of bacterial 16SrRNA gene

DNA isolated from samples was used as a template for PCR to amplify the bacterial 16S rRNA gene by using the forward primers: 16S rDNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 16S rDNA-63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 25 µl containing 16 µl ultrapure water, 4 µl 5x buffer, 0.4 µl dNTPs 10nM, 1 µl 10 µM 27F, 1 µl 10 µM 1492R, 0.2 µl Go Taq DNA polymerase and 3-5 µl genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s, annealing for 30 sec. at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Agarose electrophoresis

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

DNA sequencing

PCR products were sent for DNA sequencing in to Beckman Coulter Genomics (Takeley, Essex CM22 6TA, United Kingdom). The sequence results were processed by using the web-based blasting program, basic local

alignment search tool (BLAST), at the NCBI site (<http://www.ncbi.nlm.nih.gov/BLAST>), and the data were compared with the NCBI/Gene bank database.

Statistical analyses

We analysed repeatability of measurements and scores for randomly chosen subsamples of individuals that were measured twice, using equations in Becker (1984) and Falconer and Mackay (1996) for estimating repeatabilities and their SE.

We used multivariate analysis of variance (MANOVA) with a repeated measures design when analysing the relationship between the degree of degradation of green, blue and brown parts of ocelli and abundance of bacteria. MANOVA is typically used when two or more response variables are related to one or more predictor variables.

The relationship between the area of ocelli and the degree of feather degradation in differently coloured parts of the ocelli was made using standard least squares regression. All statistical analyses were made using JMP (SAS 2012).

RESULTS

Bacterial community from ocelli

We found a heterogeneous abundance of bacteria in different parts of the ocelli (Tables S4A and B). In addition, there were variable numbers of bacterial taxa for differently coloured parts of the ocelli. From the eight bacterial isolates that were recovered from TSA medium, HP-F isolates (Unidentified bacterium) form the largest percentage in brown and green parts of the ocelli (19% and 20%, respectively), while the largest group for

the blue area was 19% for *Paenibacillus* sp. (HP-D). As for FMA medium HAM contained seven bacterial isolates for which *Streptomyces marokkonensis* (HP-AF) was the most dominant bacterial isolate in brown, green and blue areas of the ocelli (29, 35 and 27%, respectively) (Tables S5A and B). The different bacterial taxa were widely distributed across the phylogenetic tree of bacteria (Fig. S1 phylogenetic tree).

The area of ocelli ranged from 12.74 to 22.46 cm² with a mean \pm SE of 17.58 ± 0.36 cm², $n = 46$ males, almost a two-fold difference. The total number of colonies and the Simpson diversity index based on microorganisms from TSA medium for the green and the blue parts, respectively, were negatively related to the area of ocelli. The mean number of colonies based on FMA medium was positively correlated with the area of the blue part of the ocelli ($F = 7.27$, $r^2 = 0.46$, $df = 3,29$, $P = 0.001$). The abundance of different bacterial taxa was related to the area of ocelli ($F = 6.98$, $r^2 = 0.68$, $df = 7,29$, $P = 0.0002$). Four taxa showed a significant negative relationship with the area of ocelli, while three showed a significant positive relationship (Table S6).

Force required to break barbs

The force required to break a barb from train feathers of 46 peacocks ranged from 25.7 to 91.0 g with a mean \pm SE: 54.6 ± 2.37 g. The mean force required to break a barb was related to the abundance of three bacterial taxa from FMA medium, two specific for the brown area and one for the blue area ($F = 8.40$, $r^2 = 0.49$, $df = 3,29$, $P = 0.0005$). The abundance of two taxa was positively related to the mean force while one was negatively related (Table S7). A second series of analyses including both bacteria from TSA and FMA media showed qualitatively similar results (results not shown).

Loss of barbs from ocelli and its predictors

Peacocks varied in loss of barbs from ocelli with most individuals having a score of 2 from the upper part of ocelli (Table S8). The number of barbs lost from the lower part of ocelli ranged from 0 to 12 with a mean \pm SE: 5.24 ± 0.40 , $n = 46$ males.

The degree of barb loss differed between the upper and the lower part of the ocelli, greater barb loss being associated with a stronger force required to break a barb, while the interaction between upper and lower part of the ocelli and the force required to break a barb was not statistically significant (Table 1). Finally, we found that loss of barbs from the lower part of the ocelli was negatively related to the area of ocelli controlled statistically for the degree of loss from the upper part of the ocelli (Table 2). This implies that peacocks with large ocelli lost relatively fewer barbs.

Table 1. Degree of barb loss in relation to mean force required to break a barb, whether barb loss was from the lower or the upper part of the ocelli, and the interaction between these two predictors

Character	<i>F</i>	Estimate \pm SE	<i>P</i>
Mean force	5.20	0.03 \pm 0.01	0.026
Upper/Lower	61.00	1.62 \pm 0.21	< 0.0001
Mean force*Upper/Lower	3.57	0.02 \pm 0.01	0.062

Table 2. Relationship between loss of barbs from the lower part of ocelli and the area of ocelli and the degree of loss of barbs from the upper part of ocelli in 46 male peacocks

Character	<i>F</i>	Estimate ± SE	<i>P</i>
Area of ocelli	4.57	− 0.33 ± 0.16	0.038
Degree of barb loss	4.98	0.82 ± 0.37	0.032

Feather degradation and its predictors

Degradation levels of the brown area of ocelli was the largest, while the green area had the lowest level of degradation, with the degradation score being intermediate for the blue part of the ocelli (Table S9).

We found a significant negative relationship between the area of ocelli (cm²) and the degree of degradation for the brown area ($F = 6.97$, $r^2 = 0.14$, $df = 1,45$, $P = 0.011$, estimate ± SE: 0.99 ± 0.38 ; Fig. 4).

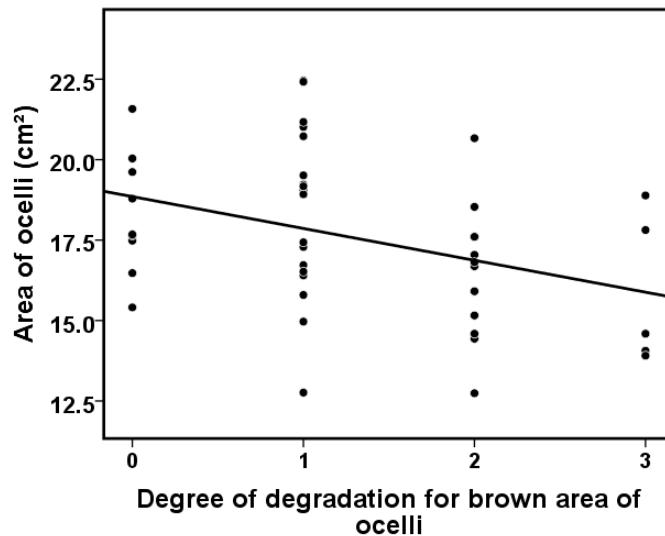


Fig. 4. Area of ocelli (cm²) in relation to degree of degradation of the brown part of ocelli for 46 peacocks. The line is the linear regression line.

We used MANOVA to test for differences of degradation among brown, green and blue parts of ocelli in relationships with area of ocelli (Table 3). For between-subjects there was no significant effect of area of ocelli. However, for within-subject effects there was a significant difference among colours, implying that the relationship was different for brown, green and blue parts of ocelli. This effect differed among areas of ocelli as shown by the significant interaction between coloration and area (Table 3). These results show that the brown area is more affected by degradation than the blue and green areas.

Table 3. Multivariate analysis of variance (MANOVA) of the relationship between degree of degradation of differently coloured parts of ocelli feathers of peacocks by area of ocelli

Between subjects	Term	<i>F</i>	df	<i>P</i>
	Area of ocelli	2.25	1, 44	0.14
Within subjects				
	Feather colour	9.46	1.94, 85.43	0.0002
	Feather colour*Area of ocelli	4.75	1.94, 85.43	0.0118

Values in bold are statistically significant ($P < 0.05$).

There was a significant positive relationship between the loss of barbs from the lower part of the ocelli and degree of degradation of the brown area ($F = 12.71$, $r^2 = 0.22$, $df = 1,45$, $P = 0.001$, estimate \pm SE: 1.42 ± 0.40 ; Fig. 5). In contrast, there was no such relationship between the loss of barbs from the lower part of the ocelli and degradation of the blue and the green area ($F = 5.49$, $r^2 = 0.11$, $df = 1,45$, $P = 0.02$, estimate \pm SE; 1.03 ± 0.44); $F = 1.44$, $r^2 = 0.03$, $df = 1,45$, $P = 0.24$, estimate \pm SE = 0.89 ± 0.74).

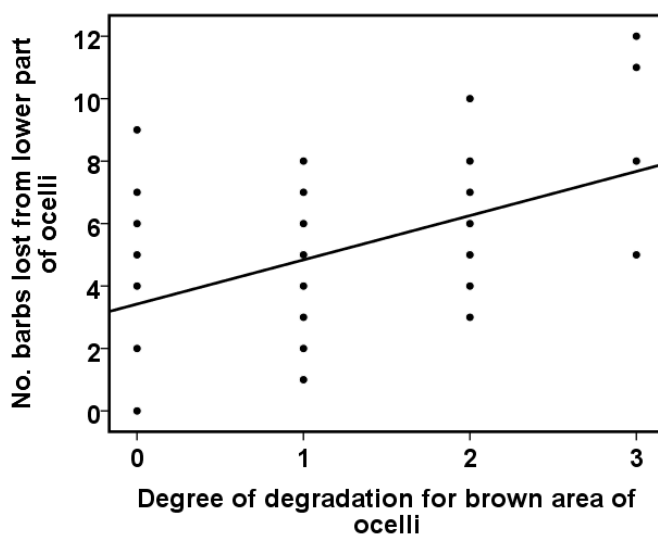


Fig. 5. Loss of barbs from the lower part of ocelli in relation to degree of degradation of the brown part of ocelli in 46 peacocks. The line is the linear regression line.

Mean number of bacteria based on TSA medium were positively correlated with degradation degree in the brown and blue area of ocelli (brown area: $F = 4.67$, $r^2 = 0.14$, $df = 1,29$, $P = 0.039$, estimate \pm SE: 1.03 ± 0.48 ; blue area: $F = 0.5.36$, $r^2 = 0.16$, $df = 1,29$, $P = 0.028$, estimate \pm SE = 0.99 ± 0.43). In contrast, the degradation degree in the green area of ocelli was positively correlated with the total number of colonies based on FMA medium ($F = 6.74$, $r^2 = 0.20$, $df = 1,29$, $P = 0.015$, estimate \pm SE: 0.52 ± 0.20).

A MANOVA revealed that the abundance of different kinds of bacteria was related to the degree of degradation of differently coloured parts of the ocelli (Table 4). There were statistically significant differences among differently coloured parts of ocelli between individuals, there was a significant within individual effect among colours, and the interaction

between colour and abundance of bacteria was significant for four out of 11 bacterial taxa (Table 4).

Table 4. Multivariate analysis of variance (MANOVA) of the relationship between degradation of differently coloured parts of ocelli feathers of peacocks by different microorganisms

Between subjects	Term	<i>F</i>	df	<i>P</i>
	<i>Bacillus mycoides</i> (HP-E)	0.32	1, 18	0.58
	<i>Bacillus megaterium</i> (HP-B/C)	0.51	1, 18	0.49
	<i>Bacillus pumilus</i> (HP-G1/G2)	0.44	1, 18	0.52
	<i>Bacillus licheniformis</i> (HP-A1)	0.04	1, 18	0.85
	<i>Bacillus subtilis</i> (HP-DF)	5.62	1, 18	0.029
	<i>Bacillus licheniformis</i> (HP-CF)	0.05	1, 18	0.82
	<i>Bacillus licheniformis</i> (HP-CF)	0.86	1, 18	0.37
	<i>Bacillus megaterium</i> (HP-BF)	1.05	1, 18	0.32
	<i>Streptomyces thermocarboxydus</i> (HP-EF)	0.05	1, 18	0.83
	<i>Streptomyces marokkensis</i> (HP-AF)	8.40	1, 18	0.0096
	<i>Streptomyces thermocarboxydus</i> (HP-EF)	1.88	1, 18	0.19
Within subjects				
	Feather color	13.38	2, 36	< 0.0001
	Feather color * <i>Bacillus mycoides</i> (HP-E)	0.64	2, 36	0.53
	Feather color * <i>Bacillus megaterium</i> (HP-B/C)	10.18	2, 36	0.0003
	Feather color * <i>Bacillus pumilus</i> (HP-G1/G2)	11.73	2, 36	0.0001
	Feather color * <i>Bacillus licheniformis</i> (HP-A1)	0.04	2, 36	0.96
	Feather color * <i>Bacillus subtilis</i> (HP-DF)	0.10	2, 36	0.90
	Feather color * <i>Bacillus licheniformis</i> (HP-CF)	1.56	2, 36	0.22
	Feather color * <i>Bacillus licheniformis</i> (HP-CF)	1.20	2, 36	0.31
	Feather color * <i>Bacillus megaterium</i> (HP-BF)	7.96	2, 36	0.0014
	Feather color * <i>Streptomyces thermocarboxydus</i> (HP-EF)	2.58	2, 36	0.090
	Feather color * <i>Streptomyces marokkensis</i> (HP-AF)	5.88	2, 36	0.0062
	Feather color * <i>Streptomyces thermocarboxydus</i> (HP-EF)	2.02	2, 36	0.15

Values in bold are statistically significant ($P < 0.05$).

DISCUSSION

Peacocks have large numbers of ocelli in their trains with different parts of the ocelli differing in colour from brown and green to blue. These ocelli are surrounded by long barbs that differ in strength of attachment. First, we showed that the diversity and the abundance of bacteria differed among differently coloured parts of ocelli. Second, the number of barbs broken at the lower part, but not the upper part of ocelli, was related to the force required to break a barb. The force required to break a barb was related to the abundance and diversity of bacteria in ocelli. Thus, peacocks with large ocelli lost relatively few barbs, and the degree of feather degradation in differently coloured parts of the ocelli depended on bacteria. These findings imply that differently coloured components of ocelli reflect different degrees of resistance to bacteria.

We collected all feather samples on the same day thereby avoiding any problems of seasonality affecting the findings. However, this study also had a number of limitations. For example, the samples were collected several years ago, and this implies that the composition of the microbial community may have changed over time. However, this study has identified real individual differences as the peacocks and their feathers were all kept under the same controlled conditions. Whilst the numbers of bacteria may have changed over time the feathers were isolated after ‘infection’ following moult so the communities must at some level reflect the individual responses to that infection.

Peacocks spend most daylight hours walking with the train either dragging through the vegetation or dragging on the ground. This leaves ample opportunities for transfer of microorganisms from the soil and the

vegetation to train feathers. Because many feather degrading microorganisms are originally soil living (Clayton 1999; Shawkey *et al.* 2003; Sangali and Brandelli 2000; Lucas *et al.* 2003; Riffel *et al.* 2003), dragging the train feathers through the soil or the vegetation provides one opportunity for transfer of such microorganisms to the plumage. Bacterial communities may differ according to soil composition, and assemblages of these bacteria in the plumage may thus be habitat dependent, leading to differences even between individuals of the same bird species (Burt and Ichida 1999; Burt and Ichida 2004; Bisson *et al.* 2007).

We found a negative relationship between area of ocelli and both total number of bacteria and taxa diversity based on TSA medium (suitable growth medium for most heterotrophic bacteria). These relationships may reflect individual condition, if only healthy individuals are able to cope with the effects of these bacteria while still developing and maintaining large exaggerated ocelli (Saag *et al.* 2011a, b; Gunderson *et al.* 2009) and/or there are individual differences in anti-microbial defences. Since the individual peacock's feathers were collected after a winter of being in the same paddock, it is unlikely that these relationships resulted from differences in infection as a result of habitat differences. The mean number of bacteria on FMA medium (highly selective medium for keratinolytic bacteria) was positively related to the area of ocelli. In other words, an increase in the number of bacterial taxa may result in competition for limiting resources and thereby reduce any deleterious effects on hosts (as shown for the role of competition for establishment of a healthy skin micro-flora (Tannock 1995; Davis 1996)).

There were heterogeneous relationships between the abundance of different bacterial taxa in different parts of the ocelli and the area of ocelli

(Table S6). Since many bacterial taxa involved in this relationship are known to have the ability to secrete either antimicrobial substances or proteolytic enzymes (Omura *et al.* 2001; Burt and Ichida 1999; Gunderson 2008), this may affect both the bacterial community and feather properties, thereby accounting for the heterogeneous results.

The force required to break barbs was related in different ways to the abundance of three bacterial taxa from FMA medium in different parts of ocelli. The two bacterial taxa that were positively related to the mean force required to break barbs was *Bacillus licheniformis*, which is known to have the ability to degrade feathers (Williams *et al.* 1990), but also to produce antimicrobial substances (Haavik 1974; Simlot *et al.* 1972). These antimicrobial substances are active against bacteria of the genera *Bacillus*, *Corynebacterium*, *Enterococcus* and *Mycobacterium*, amoebae (Gálvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). Likewise, *Micromonospora* species are also known to have the ability to produce keratinase that degrades feathers (El-Bondkly and El-Gendy 2010). Furthermore, *Micromonospora* species can produce many of the best-known antibiotics, including aminoglycoside antibiotics, gentamicin and netamicin (Bérdy 2005). *Micromonospora* species also produce many other biologically active molecules like anti-fungal substances (Nolan and Cross 1988; Ismet *et al.* 2004), anti-tumour compounds (Igarashi *et al.* 2007) and vitamin B12 (Wagman *et al.* 1969), which enhance keratin quality and production. These bacterial taxa recovered from the brown area (which constitutes the larger part of the ocelli of peacock feathers) may lead to the establishment of an equilibrium bacterial community that prevents establishment of other pathogenic bacteria. Meanwhile the abundance of *Streptomyces marokkonensis* was negatively related to the mean force

required to break barbs. *Streptomyces marokkonensis* is known to inhibit the growth of pathogenic yeasts and phytopathogenic fungi (Bouizgarne *et al.* 2009).

Feather barb loss from the lower part of ocelli differed from that for the upper part of ocelli, and there was a positive association between the degree of loss from the lower part and the mean force required to break a barb. This could be explained as individuals investing more in feather quality to compensate and stop the deleterious effects of bacteria to preserve at least a minimum number of barbs following bacterial degradation. Furthermore, we detected a negative relationship between the degree of barb loss from the lower part and area of ocelli, which may indicate the superior quality of individuals that grow large ocelli while still maintaining a maximum number of barbs in the lower part of the ocelli.

MANOVA results revealed that the brown area of ocelli was more affected by degradation than the blue and green areas. This degradation in the brown area was negatively correlated with the area of ocelli and positively with the loss of barbs from the lower part of ocelli. These results may reflect individual quality where poor quality males suffer more from degradation and barb loss, which in turn affect the ability to produce large healthy ocelli. The brown area which constitutes the larger part of ocelli may represent an important clue for peahens to assess male quality through degree of damage and/or barb loss from the lower part of ocelli.

Leclaire *et al.* (2014) showed in an experiment on feral pigeons *Columba livia* that individuals with low bacterial loads on their feathers had brighter iridescent neck feathers. They suggested that the load of feather bacteria may play an important role in alteration of iridescent feather condition and signalling of colour in pigeons. Our results showed a positive

correlation between bacterial load based on TSA and FMA media with the degree of degradation in all three differently coloured parts of the ocelli, consistent with the study by Leclaire *et al.* (2014). The MANOVA showed that the degree of degradation in differently coloured parts of the ocelli was related to the abundance of different kinds of bacteria. This indicates a significant difference in within-subject effects among different colours of the ocelli, suggesting that the relationship differed for brown, green and blue parts. This implies that the three colours differ in their ability to become degraded by different kinds of bacteria. We do not have an explanation for the differential sensitivity of the brown area to degradation among different ocelli parts, but we assume that this could be related to the physical properties of this area (Zi *et al.* 2003). This suggestion requires future experiments to test degradability of the three colours of ocelli in vitro involving different bacterial taxa.

The bacterial community in peacock feathers was highly complex, and this complexity must be taken into consideration in future studies. The interactions between these different bacterial taxa, and potentially with individuals themselves, can be analysed by investigating how these bacterial communities can affect peacocks either through degradation of feathers or by acting as opportunistic pathogens. This may lead to variation in individual feather condition and coloration and in turn potentially affect peacock display. Thus, removal or manipulation of the bacterial community may be the way forward, as an experimental change in the condition of peacocks may lead to alterations in quality and characteristics of their train feathers.

In conclusion, our study demonstrated heterogeneous distributions of the abundance of bacteria among the three coloured parts of ocelli, the

degree of feather degradation in differently coloured parts of the ocelli depending on bacteria, peacocks with large ocelli losing relatively few barbs, and the force required to break barbs being related to the diversity of bacteria in differently coloured parts of the ocelli. Hence ocelli are reliable signals of the diversity and the abundance of bacteria in peacocks.

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Chapter 2:

Why do peacocks have so many different signals?

**Haider Al-Murayati ^a, Zaid Al Rubaiee ^a, Marion Petrie ^b and
Anders Pape Møller ^a**

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

^b *Institute for Health and Society and Newcastle University Institute for
Ageing, Newcastle University, Campus for Ageing and Vitality
Newcastle upon Tyne, NE4 5PL, UK*

Word count: 8690

Correspondence to HAM:

Tel: (+33) 1 69155688

Fax: (+33) 1 69155688

E-mail: haideryousif1967@gmail.com

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ABSTRACT

The evolution of exaggerated characters in one sex, usually males, remains an enigma because the underlying factors limiting their expression are poorly understood. Here we suggest that the diversity and the abundance of bacteria may reduce the amount of resources allocated to production of extravagant ornamental characters and hence constitute a neglected factor. We studied the relationship between the prevalence and the abundance of the bacterial community in differently coloured parts of the ocelli of the peacock's *Pavo cristatus* train and their relationship to the expression of secondary sexual characters (number of ocelli, train length, train growth, spur length and spur growth). The bacterial community in the green, blue and brown coloured parts of the peacock's train varied among the three areas of the ocelli. The number of ocelli was negatively related to bacterial abundance and diversity. Likewise, the number of barbs lost from the lower part of ocelli was positively related to bacterial abundance and diversity. The presence of only few bacterial taxa such as *Paenibacillus* sp. and *Solibacillus silvestris* was related to the length of the train and the spurs. These findings are consistent with the hypothesis that different secondary sexual traits provide a partial picture of the overall condition of male peacocks.

Keywords: bacteria; feather barbs; feather breakage; ocelli; spur; tail.

INTRODUCTION

Animals have evolved an amazing variety of different signals for communication with each other. Signals fall into three main categories: vocal, olfactory or chemical, and visual signals. Furthermore, electric signals are detected in some fish species that can release pulses (Andersson 1994). These signals are traits that have evolved to transfer information for signallers in attempts to manipulate their behaviour to their own advantage. Signals between the same species (conspecifics) are used in different social situations (attraction of mates, defence of the territory against competitors and warnings other conspecifics in case of the presence of a predator). Interspecific signals are less common. The type of signal that has been used by animals relies on the type of messages that are transferred, the environment, and the sender of the signal (Drickamer *et al.* 2002).

Most males, but also females in the animal kingdom have multiple sexual ornaments that are used to advertise their 'quality' during mating, and they frequently use them simultaneously rather than depending on one single type of signal at a time. Multiple signal displays may involve a number of different visual, auditory and olfactory components. For example, birds use feather ornamentation, vocalizations and display behaviour (Zuk *et al.* 1990, 1992; Redondo and Castro 1992; Edmunds 1974). All these signals appear to be used as part of a male's courtship display to females (Møller and Pomiankowski 1993; Andersson 1994).

Several hypotheses have been proposed to explain the existence of multiple ornaments. Møller and Pomiankowski (1993) presented three explanations for this diversity of signals: **The multiple message hypothesis** suggests that each ornament signals a specific feature of individual condition, and that in turn each signal provides an overall assessment of

individual condition. Alternatively, these multiple signals may reflect individual condition during their lifespan, where some traits are undergoing distinctive changes over long periods, like the ornamented plumes of a bird or the antlers of a deer which grow once a year. In contrast, the inflatable bare skin patches of grouse or the colourful patches in primates are more likely to reflect current body condition. In brief, this hypothesis assumes that different signals act as indicators of different aspects of individual quality.

The redundant signal hypothesis (also known as the back-up signal hypothesis) suggests that each secondary sexual trait provides a partial picture of overall male condition (Møller and Pomiankowski 1993). Thus, females make a better estimation of overall male condition by inspecting several traits, where each is correlated with individual condition. If each ornament reflected male quality with a certain level of error, the female could be misled if choice was based on one ornament and choose a male in poor rather than good condition. Thus, females tend to assess multiple traits to improve their mate choice decisions by minimizing the errors that might occur if they relied on just one trait. In this way, a female will reduce the number of mates, and the time and the energy that is spent on inspection of a group of potential mates, thereby reducing mate choice costs (Candolin 2003). This is considered an important factor in the evolution of multiple preferences for different secondary sexual characters (Iwasa and Pomiankowski 1995).

The unreliable signal hypothesis suggests that many multiple sexual traits actually may not provide a good indication of current male condition (Møller and Pomiankowski 1993). These traits could have evolved by taking advantage of pre-existing female preferences (Ryan 1990), and through time they lost the correlation with individual condition. Subsequently these

signals do not currently reliably reflect male quality. Another explanation for the presence of such unreliable signals is that they could be easy to produce and maintain. Therefore, it would be an unreliable character on which females make the assessment leading to the use of multiple signals to avoid mis-judgment of male quality.

Andersson *et al.* (2002) proposed **the multiple receiver hypothesis**, which suggests that the presence and the evolution of costly multiple traits may be due to inter- and intrasexual selection, in which different signals are selected by separate receivers (males and females relying on the use of different signals).

Darwin (1871) suggested that females prefer to mate with certain males depending on different cues that may signal their quality. Several studies have revealed that female choice can be based on morphological (males with bright feathers or long tails) and behavioural traits (males that defend high quality territories or other resources essential for reproduction) (Burley 1981; Johnstone 1996; Lozano 2009; Dolnik and Hoi 2010; Hoi and Griggio 2012). Females of many animals habitually visit several males before choosing one (Lill 1974, 1976; Gronell 1989; Trail and Adams 1989; Dale *et al.*, 1990; Petrie *et al.* 1991; Bensch and Hasselquist, 1992; Byers *et al.* 1994; Fiske and Kålås 1995).

Peahens *Pavo cristatus* also follow this sampling behaviour during the breeding season. Males forming leks (on small display territories), where they advertise their extravagant traits in an attempt to attract a potential mate. Females visit a number of leks and a number of males within a lek before deciding on the most suitable mate. The suitable male for a choosy female usually has a highly ornamented train as confirmed by observations (Petrie *et al.* 1991; Loyau *et al.* 2005b) and experiments (Petrie and Halliday

1994; Dakin and Montgomerie 2013). Train ornamentation is related to the structure and size of ocelli (Møller and Petrie 2002; Dakin and Montgomerie 2013), but also to the length of feathers in the train (Manning and Hartley 1991; Yasmin and Yahya 1996; Loyau *et al.* 2005a). Furthermore, other traits such as the crest (Dakin 2011), the angle in which the train is spread out towards the sun (Dakin and Montgomerie 2009), calls (Yasmin and Yahya 1996; Dakin and Montgomerie 2014; Yorzinski and Anoop 2013), and infrasonic calls that are produced by shaking of the train feathers (Freeman 2012) may contribute to male mating success. However, there is considerable variation in these preferences among populations (Dakin and Montgomerie 2011).

Recently, several studies have emphasized the potential role of bacteria in animal behaviour and communication (e.g. Archie *et al.* 2007; Sharon *et al.* 2010; Ezenwa *et al.* 2012). Feather degrading bacteria are widespread among wild birds, with their ability to break down feather structures (Burt and Ichida 1999) leading to a reduction in thermoregulation, aerodynamic efficiency and communication among individuals with more bacteria (Swaddle *et al.* 1996; Clayton 1999; Shawkey *et al.* 2007). Communication may be impaired by a reduction in feather colouration either by consumption or modification of feather microstructures or pigments via microbial action (Shawkey and Hill 2004). Most secondary sexual characters are costly to produce and maintain (Andersson 1994), and the prevalence of different bacterial taxa may reduce the ability to produce and maintain extravagant ornamental characters.

Here we suggest that the abundance and the diversity of bacteria in the different coloured parts of the ocelli of the train play important roles in the expression of secondary sexual characters like the number of ocelli in the

train, the size of different parts of these ocelli, spur length and train length. Thus, different secondary sexual characters may signal different properties of individual quality, which in turn may allow females to assess male quality and hence decide the quality of their future partner.

MATERIALS AND METHODS

Marion Petrie and Anders Pape Møller collected the peacock train feathers during a breeding experiment conducted during 1998-1999 in a commercial farm in Norwich, UK (see Hale *et al.* 2009 for further details). All individuals were kept overwinter in large outdoor pens and provided with water and feed ad libitum. Free access to soil and vegetation was provided. Since the experiments were conducted in the same farm, under the same conditions and by the same persons, we assume that all individuals were equally contaminated with microorganisms. At the start of the mating and breeding season individuals were randomly assigned to pens, with each peacock allocated with four peahens chosen at random. This work was conducted under Home Office Licence (UK), and there were no recorded negative effects of the experiments on peacocks.

Ocelli (n=10) at the tip of train feathers were cut from the train of each male and kept in dry plastic bags, samples were transported under cold condition to the laboratory and stored until processing.

The total number of ocelli in the train of peacocks from photographs of fully expanded trains. Because the count was taken from photographs, and it is difficult to get a photograph where the train is completely visible, this measure is an estimate of the number of ocelli rather than an accurate count. Train length was recorded in mm (measured for the longest train feather by

using measuring tape), while spur length was measured from base to tip (mm) with digital callipers. Train and spur growth from one year to the next was calculated as the difference in length between the two years.

From each of the 46 individual males in this study, one ocelli train feather was chosen randomly and photographed with a digital camera to facilitate precision measurements of ocelli directly from the picture by using the 'rule' tool in Adobe Photoshop CC software (see Figure 1 in Chapter 1). All measurements were done by HAM to avoid inter-observer variation, and the size of ocelli (measured in mm) was calculated as follows: the product of the width at the widest point multiplied by the height at the greatest vertical distance. All measurements were done blindly without prior knowledge about individual identity and phenotype.

Estimating feather degradation of ocelli

To estimate degradation of ocelli of the train, HAM adopted three different methods: First, HAM counted the number of barbs that were missing from the lower part of ocelli feathers as revealed by remaining stubs of barbs. Secondly, the degree of barb loss from the upper part of the ocelli was estimated according to the following scale: 0 = no loss due to degradation; 1 = no loss with degradation; 2 = one third of barbs from the upper part were lost or degraded; 3 = two thirds of barbs from the upper part were lost or degraded; and 4 = more than two thirds of barbs from the upper part were lost or degraded (see Figure 2 in Chapter 1).

The third scale was the estimation of degradation level in different parts of the ocelli (brown, green and blue), with degradation ranked on a four-point scale: 0 = no degradation; 1 = slight degradation (less than 1-3

small spots of degradation); 2 = medium degradation (large area of degradation); and 3 = high degree of degradation (more than half of the area degraded) (see Figure 3 in Chapter 1).

To estimate the precision of our measurements and scoring of the ocelli characters, repeatability (R) (Becker 1984; Falconer and Mackay 1996) was calculated from 30 individuals for which measurements were repeated twice on different days without prior knowledge of the first set of measurements and scoring. HAM found that the repeatability was high in all cases (see table S1).

Bacterial isolation

Among the 46 individuals that were included in this study, feather samples from 30 individuals were chosen randomly by HAM to isolate and identify the bacterial community (the remaining 16 individuals were not included for logistic reasons). Repeatability (Becker 1984) for 10 additional feather samples chosen randomly from the same set of 30 individuals revealed that the repeatability of total and specific number of bacteria recovered on growth medium was high in all cases except for the number of species recovered on TSA (Tryptic Soy Agar) medium for brown and blue parts of the ocelli (repeatability of total number of bacteria and the number of bacterial species on TSA and FMA (Feather Meal Agar) media from differently coloured parts of the ocelli as reported in Table S3 A and B in Chapter 1). The differently coloured parts of the ocelli were cut under septic conditions with a sterilized sharp blade and with the help of pincers. The small piece of barbs from each coloured part was mounted in pre-weighed eppendorf tubes. The eppendorf tubes were weighed again to estimate the mass of the feather barbs so we could calculate the desired amount of sterile

phosphate buffer saline (pH 7.2) to be added to each sample (each mg feather sample equalled 100 µl PBS) so this amount of buffer equalled the amount of feather. Three-time vortex cycles of 1 min each were used to facilitate detachment of free-living bacteria from barbs to the PBS solution (Saag *et al.* 2011). Two different culture media were used to quantify the cultivable and feather-degrading bacteria: (1) Tryptic soy agar (TSA) is a rich medium on which most heterotrophic bacteria can grow and this allows us to estimate the total cultivable bacterial load. (2) Feather meal agar (FMA) is a highly selective medium for keratinolytic bacteria that contains one source for carbon and nitrogen, which is keratin allowing quantification of the load of feather-degrading bacterial that can consume keratin (Williams *et al.* 1990; Sangali and Brandelli 2000; Shawkey *et al.* 2003, 2007, 2009).

Duplicate plates from these media were made by spreading 100 µl PBS that contains the washed bacteria with a sterile spreader loop, while a third plate (for each of the different medium) was used as a control, which was inoculated with the same volume of sterilized PBS. Cycloheximide was added to the growth medium to prevent the growth of fungi (Smit *et al.* 2001). Cultivation temperature was 28°C for 3 days and 14 days, respectively, for TSA and FMA medium. After incubation and with the aid of a dissecting microscope, HAM counted the total number of colony forming units (CFU) for each morphotype per plate. The morphological characters used to distinguish the different morphotypes were colour, shape, size, and presence or absence of mucoid textures.

Isolate preservation

From identified pure culture a slant of nutrient agar and 40% glycerol stocks was prepared and stored at 4°C and -80°C, respectively.

DNA extraction

Two different protocols were used by HAM to extract bacterial DNA. The freeze-thaw protocol summarized as follows was used: A well identified colony was transferred to a 0.5 ml Eppendorf tube containing 50 μ l Tris (10 μ M, pH 8.0) and crushed well with a wooden stick, followed by three cycles of fast freezing and thawing. To increase the chance of cell lysis, the tubes were put in a microwave three times at 270 W for 5 to 6 s followed by 10 s of waiting. The supernatant that contains the bacterial DNA after centrifugation (5 min at 12,000 x g) was transferred to clean DNA free micro-centrifuge tube and stored at -20 °C until processing. The second protocol was by the Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA).

PCR amplification of bacterial 16SrRNA gene

HAM amplified the bacterial 16S rRNA gene by using the forward primers: 16S rDNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3') or 16S rDNA-63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). DNA isolated from samples was used as a template. PCR was carried out in a total volume of 25 μ l containing 16 μ l ultrapure water, 4 μ l 5x buffer, 0.4 μ l dNTPs 10nM, 1 μ l 10 μ M 27F, 1 μ l 10 μ M 1492R, 0.2 μ l Go Taq DNA polymerase and 3-5 μ l genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s, annealing for 30 s at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Agarose electrophoresis

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

DNA sequencing

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

Statistical analyses

All statistical analyses were made using JMP (SAS 2012). We analysed repeatability of measurements and scores for randomly chosen subsamples of individuals that were measured twice, using equations in Becker (1984) and Falconer and Mackay (1996) for estimating repeatabilities and their SE.

The relationship between the different bacterial taxa from differently coloured parts of the ocelli with different secondary sexual characters was made using standard least squares regression.

Simpson's index (a measure of diversity independent of sample size) was calculated to determine the diversity of microorganisms in the differently coloured parts of the ocelli, which takes into account the number

of species present, as species richness and evenness increase, so does diversity (Magurran 2004).

RESULTS

Summary statistics for secondary sexual characters

The different secondary sexual characters varied considerably among peacocks (Table 1). Coefficients of variation were generally high thus providing males and females with considerable variation for evaluation of potential competitors and mates (Table 1). The largest coefficients of variation were in train growth and barb loss (Table 1).

Table 1. Mean, SE, range and coefficient of variation (CV) for secondary sexual characters in peacocks.

Character	Mean	SE	Minimum	Maximum	CV (%)	N
No. ocelli	125	2.37	58	147	12.72	45
Barb loss from lower part of ocelli	5.24	0.40	0	12	51.77	46
Barb loss from upper part of ocelli	1.91	0.14	0	3	48.62	44
Train length (mm)	1488	11.71	1320	1690	5.34	46
Train growth	162	15.00	-80	300	62.80	46
Spur length (mm)	32.98	0.40	27.1	40.6	8.23	46
Spur growth (mm)	15.01	0.26	9.91	18.8	11.75	46

Bacterial community on ocelli

Bacteria isolated from the three differently coloured parts of the peacock's ocelli revealed a diversity in abundance and numbers of bacterial taxa (see Table S4 A and B).

Eight different bacterial isolates were recovered from TSA medium and isolation from FMA medium showed the presence of seven bacterial isolates. From the TSA medium and in the brown and green area, HP-F isolates of unidentified bacterium was the largest group (19% and 20%, respectively). *Paenibacillus* sp. (HP-D) formed the largest percentage in the blue area (19%). FMA medium was dominated by *Streptomyces marokkonensis* (HP-AF) in all three differently coloured parts (brown 29%, green 35 % and blue 27%) (see Table S5 A and B). The different bacterial taxa were widely distributed across the phylogenetic tree of bacteria (see Figure S1).

The mean number of bacterial colonies in the brown area (recovered from TSA medium) and blue area (recovered from FMA medium) were negatively related to the number of ocelli in the train ($F = 8.11$, $df = 1, 28$, $P = 0.009$, estimate (SE) = - 18.97 (6.66); $F = 8.00$, $df = 1, 28$, $P = 0.0023$, estimate (SE) = - 31.60 (11.17)). In addition, the diversity index based on microorganisms from FMA medium for the brown area was positively correlated with the number of ocelli in the train ($F = 11.47$, $df = 1, 28$, $P = 0.009$, estimate (SE) = 96.66 (28.54); whole model statistics: $F = 6.34$, $df = 3, 28$, $r^2 = 0.43$, $P = 0.002$). Furthermore, among the fifteen bacterial isolates in this study, *Paenibacillus* sp. (HP-D) was the only species from the blue area that was significantly negatively related to the number of ocelli of the

train ($F = 7.08$, $df = 1,28$, $r^2 = 0.21$, $P = 0.013$, estimate (SE) = -11.44 (4.3); Fig. 1).

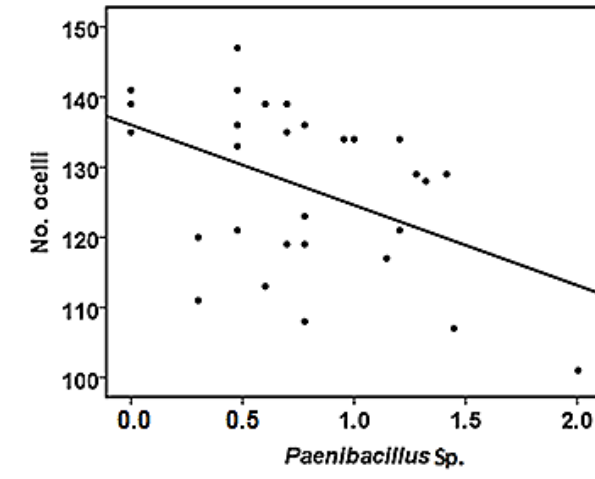


Fig. 1. Number of ocelli in relation to the abundance of *Paenibacillus* sp. in the blue part of ocelli for 30 peacocks. The line is the linear regression line.

Degradation of ocelli and its predictors

The degree of barb loss from the lower part of the ocelli was positively associated with the number of ocelli in the peacock's train ($F = 6.82$, $df = 1, 44$, $r^2 = 0.14$, $P = 0.0123$, estimate (SE) = 0.06 (0.02); Fig. 2), while the relationships between the degree of barb loss from the upper part of the ocelli and the number of ocelli was not statistically significant (results are not shown). This indicates that the number of barbs lost from the lower part of ocelli is proportional to the number of the ocelli in the train. Furthermore, we did not find a significant relationship between the degree of degradation in the different parts of the ocelli and other secondary sexual trait in this study.

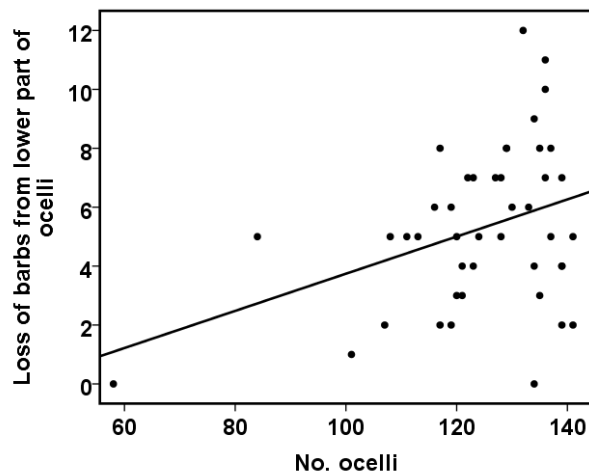


Fig. 2. Degree of loss of barbs from the lower part of ocelli in relation to the number of ocelli in 45 peacocks. The line is the linear regression line.

Train length and growth and its predictors

Train length was not significantly correlated with the total number of colonies, mean number of bacterial colonies, number of species and Simpson's diversity index.

The abundance of different bacterial taxa recovered from the three differently coloured parts of the ocelli on TSA medium was related to train length ($F = 5.45$, $df = 9, 29$, $r^2 = 0.71$, $P = 0.0008$). Five taxa showed a significant negative relationship, while the remaining four showed a significant positive relationship (Table 2).

Train growth was positively related to the total number of colonies of bacteria from TSA medium for the blue part, while Simpson's diversity index based on bacteria from FMA medium for the green part was

negatively correlated with train growth ($F = 4.37$, $df = 2, 29$, $r^2 = 0.24$, $P = 0.023$). Furthermore, three bacterial taxa recovered from TSA medium for the green and blue part, respectively, were positively related to train growth, and another two bacterial taxa from the same medium for the brown and blue part, respectively, were negatively related to train growth ($F = 6.00$, $df = 5, 29$, $r^2 = 0.56$, $P = 0.001$; Table 3).

Table 2. The abundances of different bacterial taxa from differently coloured parts of ocelli in relation to train length in 30 peacocks.

Part of ocelli	Bacterial sp.	<i>F</i>	<i>P</i>	slope	SE
Brown	HP-B/C (<i>Bacillus megaterium</i>)	4.80	0.0405	-91.78	41.90
	HP-G3 (<i>Solibacillus silvestris</i>)	5.58	0.0284	115.22	48.78
Green	HP-CF (<i>Bacillus licheniformis</i>)	5.17	0.0342	-96.81	42.59
	HP-D (<i>Paenibacillus</i> sp.)	4.80	0.0404	-130.42	59.50
	HP-F (Unidentified bacterium)	11.53	0.0029	204.87	60.32
	HP-G1/G2 (<i>Bacillus pumilus</i>)	6.35	0.0203	141.97	56.33
Blue	HP-A3/A4 (<i>Bacillus subtilis</i>)	6.46	0.0194	147.80	58.61
	HP-B/C (<i>Bacillus megaterium</i>)	8.70	0.0079	-175.79	59.58
	HP-E (<i>Bacillus mycoides</i>)	8.11	0.0099	-277.14	97.32

Table 3. The abundance of different bacterial taxa from differently coloured parts of ocelli in relation to train growth in 30 peacocks.

Part of ocelli	Bacterial sp.	<i>F</i>	<i>P</i>	slope	SE
Brown	HP-B/C (<i>Bacillus megaterium</i>)	7.05	0.0138	-104.99	39.53
Green	HP-G1/G2 (<i>Bacillus pumilus</i>)	8.59	0.0073	156.24	53.30
Blue	HP-A3/A4 (<i>Bacillus subtilis</i>)	11.40	0.0025	255.08	75.55
	HP-E (<i>Bacillus mycoides</i>)	6.99	0.0142	-284.29	107.56
	HP-G1/G2 (<i>Bacillus pumilus</i>)	4.37	0.0472	85.29	40.78

Spur length and its predictors

There was a positive relationship between mean spur length and train length ($F = 5.21$, $df = 1, 45$, $r^2 = 0.11$, $P = 0.027$, estimate (SE) = 0.010 (0.004); Fig. 3). Likewise, there was a positive relationship between spur growth and train growth ($F = 6.13$, $df = 1, 45$, $r^2 = 0.12$, $P = 0.017$, estimate (SE) = 0.006 (0.002); Fig. 4).

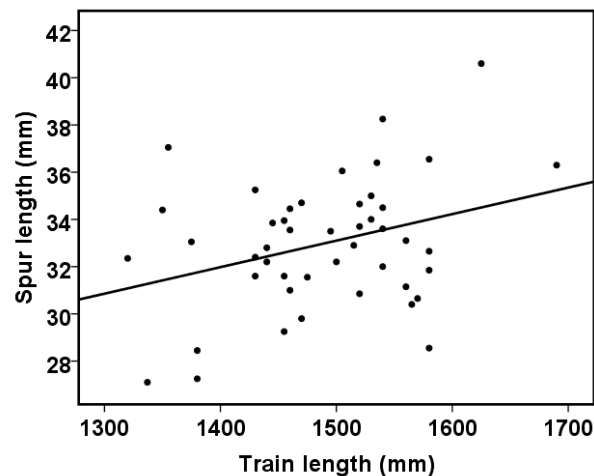


Fig. 3. Mean spur length (mm) in relation to train length (mm) in 46 peacocks. The line is the linear regression line.

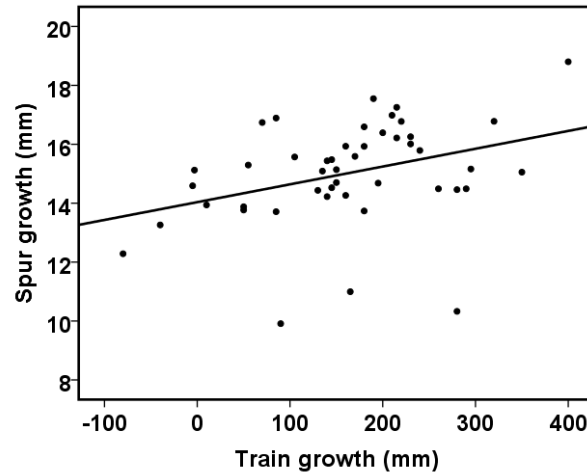


Fig. 4. Spur growth (mm) from one year to the next in relation to train growth (mm) in 46 peacocks. The line is the linear regression line.

Spur length was positively related to Simpson's diversity index based on microorganisms from TSA and FMA medium for the brown and blue parts of the ocelli, respectively ($F = 8.47$, $df = 1, 29$, $P = 0.0073$, estimate (SE) = 90.48 (31.09); $F = 8.99$, $df = 1, 29$, $P = 0.0059$, estimate (SE) = 24.85 (8.29)). Furthermore, the number of bacteria from FMA medium for the blue part was negatively related to mean spur length ($F = 6.58$, $df = 1, 29$, $P = 0.0164$, estimate (SE) = -8.42 (3.28); whole model statistics; $F = 6.07$, $df = 3, 29$, $r^2 = 0.41$, $P = 0.0028$). Two bacterial taxa recovered from the brown part of ocelli on TSA and FMA medium, respectively, were negatively related to spur length. In contrast, one bacterial taxon from the blue part recovered on TSA medium was positively related to mean of spur length ($F = 4.95$, $df = 3, 29$, $r^2 = 0.36$, $P = 0.0075$; Table 4). Spur growth was correlated in different directions to the abundance of five bacterial taxa recovered from the three

coloured parts of the ocelli on TSA medium ($F = 4.26$, $df = 5, 29$, $r^2 = 0.47$, $P = 0.0064$; Table 5).

Table 4. The abundance of different bacterial taxa from differently coloured parts of ocelli in relation to spur mean length in 30 peacocks.

Area	Bacterial sp.	<i>F</i>	<i>P</i>	Slope	SE
Brown	HP-DF (<i>Bacillus subtilis</i>)	3.46	0.0742	-7.05	3.79
	HP-B/C (<i>Bacillus megaterium</i>)	6.59	0.0164	-2.97	1.16
Blue	HP-G3 (<i>Solibacillus silvestris</i>)	4.26	0.0492	3.44	1.67

Table 5. The abundance of different bacterial taxa from differently coloured parts of ocelli in relation to spur growth in 30 peacocks.

Part of ocelli	Bacterial sp.	<i>F</i>	<i>P</i>	slope	SE
Brown	HP-B/C (<i>Bacillus megaterium</i>)	4.62	0.0419	2.44	1.13
	HP-D (<i>Paenibacillus</i> sp.)	14.39	0.0009	- 4.34	1.14
	HP-G3 (<i>Solibacillus silvestris</i>)	4.32	0.0484	2.56	1.23
Green	HP-F (Unidentified bacterium)	10.24	0.0038	3.94	1.23
Blue	HP-B/C (<i>Bacillus megaterium</i>)	11.03	0.0029	- 3.84	1.15

DISCUSSION

Males of many species have evolved multiple secondary sexual characters to attract females during the breeding season, and such multiple traits may provide valuable information to potential partners about their phenotypic and genetic quality. The peacock is the best example of a species with many exaggerated traits. The most obvious trait is the magnificent decorated long train that contains large numbers of ocelli differing in colour from brown and green to blue, but also other traits like spurs and crest may contribute to signal information about health and condition (Møller and Petrie 2002; Dakin 2011; Loyau *et al.* 2005b).

The present study suggests that both heterotrophic and keratinolytic bacteria are correlated with the expression of ornaments in the peacock with the degree of barb loss and feather degradation from ocelli reliably reflecting the abundance and the diversity of bacteria. We avoided any problems of seasonality by collecting all feather samples on the same day at the beginning of the breeding season. Another limitation of our study was the long duration of feather storage (several years), which could lead to changes in the bacterial community. However, we see no reason why this storage should have caused any consistent bias among males with specific phenotypes. All peacock feathers were kept under identical conditions, implying that the bacterial community isolated from the feathers were the outcome of infection that occurred during initial feather growth in chicks or following subsequent moults. Thus, the bacterial community should be a reflection of individual responses to this infection.

These novel findings suggest that female peafowl, but also conspecific males may use the expression of secondary sexual characters to assess the condition of conspecifics and hence the probability of a favourable mate choice or gains in fights among peacocks at the leks. We documented a negative relationship between the number of ocelli in the train of the peacock and the mean number of bacteria recovered from brown and blue parts of ocelli based on TSA medium (favourable for the growth of most heterotrophic bacteria) and FMA medium (favourable the growth of just keratinolytic bacteria), respectively. These results follow previous studies on peacocks and other species suggesting that microbes negatively affect the expression of feather ornaments (Hill *et al.* 2004; Shawkey *et al.* 2009; Loyau *et al.* 2005b). On the other hand, we found that the diversity of bacterial taxa derived from FMA medium of the brown area was positively correlated with the number of ocelli. Numerous experimental studies and theoretical predictions for animals and plants suggest that a highly diverse bacterial community has a positive impact on host health by being more resistant to pathogen invasions (Case 1990; Kennedy *et al.* 2002; Dillon 2005; van Elsas *et al.* 2012). Furthermore, the abundance of *Paenibacillus* sp. recovered from the blue area was negatively related to the number of ocelli in the peacock's train. *Paenibacillus* sp. are widespread in many different habits (water, soil, waste), and it has the ability to produce many types of extra-cellular enzymes like cellulose (Park *et al.* 2012), nitrogenase (Köberl *et al.* 2011), xylanase (Dheeran *et al.* 2012) and keratinase (Paul *et al.* 2013), which have negative impacts on feather condition.

Dakin and Montgomerie (2011) reported 105 to 177 ocelli among studies, and Petrie *et al.* (1991) concluded that their observations were not caused by feather loss because the number of ocelli was recorded at the start

of the mating season the loss of individual feathers seems to be a primary source of variation in number of ocelli. Breakage and loss of ocelli feathers is common during the breeding season, and males are often observed with broken ocelli feathers that have not yet fallen out (R. Dakin, personal communication). Feather barb loss from the lower and the upper part of ocelli were differently related to the number of ocelli. Many studies have emphasized the importance of the number of ocelli for mating success (Petrie *et al.* 1991; Petrie and Halliday 1994; Dakin and Montgomerie 2011, 2013). Thus, the positive relationship in our study between the degree of barb loss from the lower part of the ocelli and the number of ocelli may be explained as a trade-off between the production of many and weak or fewer, but stronger ocelli. Alternatively, there may be a trade-off between investment in anti-bacterial defence and sanitation behaviour.

There were heterogeneous relationships between the abundance of nine bacterial taxa (recovered from TSA medium) from different parts of ocelli and train length (Table S6). Most of these bacteria have the ability to either secrete antimicrobial substances or proteolytic enzymes or both (Haavik 1974; Simlot *et al.* 1972; Agrahari and Wadhwa 2012; Malanicheva *et al.* 2012; Paul *et al.* 2013; Hasan *et al.* 2009; Rajput and Gupta 2013; Leifert *et al.* 1995; Jeevana Lakshmi *et al.* 2013). Interestingly, one of the nine taxa, *Solibacillus silvestris*, is known to have degrading activity for N-Acylhomoserine lactones (AHL). AHL are used as quorum-sensing signal molecules by many bacterial taxa to synchronize certain kinds of behaviour such as biofilm formation, virulence, and antibiotic resistance, based on the local density of the bacterial population (Morohoshi *et al.* 2012; Miller and Bassler 2001). Thus, enzymes secreted from this bacterial community may

impact both bacterial community and feather properties, but it could also help clarify our heterogeneous results.

We found a positive association between train growth and total number of colonies from the blue part of ocelli. This positive relationship may be due to mutualistic action, where mutualists (originating primarily from the soil) promoted by the presence of secretions from the uropygial gland can occupy the space and prevent the dominance of pathogenic feather degrading bacteria (Pillai *et al.* 2001). Secondary sexual traits that reliably reflect individual quality of signallers might be costly to produce or maintain (Grafen 1990a, b; Kodric-Brown and Brown 1984; Zahavi 1975, 1987). Thus, individuals can allocate and redirect limiting resources from train growth to other secondary sexual traits. In contrast, we found a negative relationship between train growth and the diversity of bacterial taxa based on FMA medium for the green area of ocelli. The green area has an important positive effect on mating success of peacocks (Dakin and Montgomerie 2013; Loyau *et al.* 2007). Thus, one possible interpretation of this relationship is the degradation ability that characterizes the different bacterial taxa that are able to grow on FMA medium and their negative impact on feathers of hosts.

Five taxa from the three differently coloured parts of ocelli were either positively or negatively related to train growth (Table S2). This diversity of relationships between these five taxa and train growth could be linked to bacterial characteristics. Most of these bacteria are known to have the ability to secrete different kinds of extracellular products (antibiotic, protoylitic and keratinolytic enzymes). These products may alter the integrity of feather structure as well as the associated bacterial community,

either in beneficial or harmful ways, and that would be reflected in effects on overall growth rate of individuals.

Peacocks just as other lekking species show aggressive behaviour when establishing display territories during breeding (Rands *et al.* 1984; Bradbury 1981). Peacock use their spurs as weapons while fighting competitors (intrasexual selection) (Harikrishnan *et al.* 2010). This weapon is considered an extravagant secondary sexual trait that plays a role in signalling to individuals of the opposite sex (von Schantz *et al.* 1989; Møller 1992). Our study revealed that spur length was positively correlated with train length, and in addition spur growth was positively correlated with train growth. These findings suggest that the length of both the train and the spurs reflect male intrinsic quality. This is consistent with studies that cite the importance of train length in female choice (Petrie *et al.* 1991; Loyau *et al.* 2005b). Likewise, peacocks and several other galliform birds that have longer spurs have a higher probability of winning in intersexual competition eventually resulting in higher mating success (Kelly 1975; von Schantz *et al.* 1989; Steffen *et al.* 1990; Wittzell 1991; Mateos and Carranza 1995).

The Simpson diversity index for bacteria on the brown and blue areas of ocelli recovered from TSA and FMA medium, respectively, was positively related to mean length of spurs. That may be explained by beneficial enzymatic activity of bacteria inhabiting these differently coloured areas and their impact on feather properties and the community of microorganisms (Gunderson 2008). In contrast, we found a negative relationship between the number of bacterial taxa from the area of the blue part of ocelli based on FMA medium and spur length. This inconsistency in results for Simpson's diversity index and the number of bacterial species with the mean length of the spur may be due to differences in the methods of

estimation of diversity. Simpson's diversity index depends on both richness (measured as the number of different kinds of organisms present in a particular area) and evenness (population size of each of the species present) of bacterial taxa, while the number of species takes no account of the number of individuals of each species present in the community.

Three bacterial taxa were correlated differently to the mean length of the spur (Table S3), while an additional five taxa were also correlated with spur growth (Table S4). The taxa that were negatively correlated with mean length of the spur and spur growth are known for their harmful effect on structural properties of feathers. Interestingly, *Solibacillus silvestris* is positively associated with mean spur length and spur growth. This is consistent with the positive relationship between the abundance of this taxon and train length. That may reflect the potential role of this taxon in shaping the bacterial community in the ocelli of peacocks and in turn the expression of secondary sexual characters.

Due to the high diversity of the bacterial community in differently coloured parts of the ocelli of peacocks and the heterogeneous relationships with different secondary sexual characters, we need to experimentally manipulate the different components of the bacterial community to test for differential effects of different bacterial taxa on the expression of the train and spurs in peacocks.

In conclusion, the number of ocelli was related to bacterial abundance and diversity, but also to the number of barbs lost from the lower part of ocelli. The presence of certain types of bacteria such as *Paenibacillus* sp. was associated with a reduction in the number of ocelli, while *Solibacillus silvestris* was associated with train and spur characteristics. These findings are consistent with the multiple message hypothesis stating that different

secondary sexual characters in peacocks reliably reveal information on the abundance and the diversity of bacteria, allowing choosy females to pick males that have specific bacterial communities in their feather and on their spurs.

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Chapter 3:

Is sex ratio in the peacock related to the expression of secondary sexual characters?

**Haider Al-Murayati ^a, Zaid Al Rubaiee ^a, Marie Hale ^b,
Marion Petrie ^c and Anders Pape Møller ^a**

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

^b *School of Biological Sciences, University of Canterbury, Christchurch, New Zealand*

^c *Institute for Health and Society and Newcastle University Institute for Ageing, Newcastle University, Campus for Ageing and Vitality*

Newcastle upon Tyne, NE4 5PL, UK

Word count: 4803

Correspondence to HAM:

Tel: (+33) 1 69155688

Fax: (+33) 1 69155688

E-mail: haideryousif1967@gmail.com

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ABSTRACT

It is frequently argued that females mating with attractive mates should produce more sons because these sons will inherit their father's traits of attractiveness and hence increase their reproductive success through the mating success of their sons. Adaptive sex ratio manipulation by females in breeding birds has become a major focus in evolutionary biology in recent years and several empirical and theoretical studies have addressed this hypothesis, with inconsistent results that have led to considerable confusion. The inconsistency in results in this field is mainly attributed to sampling error. In the present study, and by using a large data set to avoid problems of sampling error, we used multiple secondary sexual traits of the peacock *Pavo cristatus* that are thought to be involved in female choice and play an important role in sexual selection to test whether the expression of these traits was correlated with the brood sex ratio of the peacock. We found a weak positive correlation between the expression of secondary sexual characters slightly biasing the sex ratio in peacock broods towards sons. The observed sex ratio was significantly smaller than that reported in the meta-analysis by Ewen *et al.* (2004), implying that it is possible to demonstrate small, but significant effect sizes.

Keywords: feather barbs; ocelli; peacock; sex ratio; spur length; train length.

INTRODUCTION

The proportion of males to females in a population of any sexually reproducing species is defined as the sex ratio. The primary sex ratio is the ratio at the time of fertilization, the secondary sex ratio is the ratio at the time of birth or hatching, and the tertiary sex ratio is the ratio at sexual maturity (Allaby 2003).

Every offspring has a mother and a father and both contribute equally to the genes in the offspring in the next generation. On this basis, Fisher (1930) presented his equal allocation theory, which refers to frequency-dependent selection favouring an unbiased sex ratio at the population level, when males and females have an equal reproductive cost, with parents investing evenly in the production of sons and daughters.

Hamilton (1967) first recognized how certain conditions can interfere with assumptions underlying the equal allocation theory of Fisher. Hamilton emphasized that equal allocation could not be expected in species where within-group interactions have a differential effect on the fitness of males and females (i.e. local mate competition where brothers compete for a limited number of mates, so parental fitness will increase if extra daughters are produced, as shown in studies on parasitic wasps (Hamilton 1967)). Consequently, selection should favour a sex ratio that is biased towards the sex experiencing the lower intensity of competition with close relatives.

Trivers and Willard (1973) suggested that individuals could be selected to adjust the sex of their offspring in response to environmental conditions. When environmental conditions have a differential effect on the fitness of the two sexes of offspring, parents should bias the sex ratio of their offspring towards the sex that has a larger contribution to parental fitness (Trivers and Willard 1973). Such facultative bias by individual parents can

take place regardless of strong selection for equal investment in daughters and sons in the population.

In many animal species, females choose their mates based on signals of individual quality (Andersson 1994). Weatherhead and Robertson (1979) proposed that females should adjust the sex ratio of their offspring in response to the attractiveness or quality of their mate. This idea is comparable to the classic Trivers and Willard (1973) argument, the sole difference being that it is mate quality rather than maternal condition that influences offspring fitness. The differential allocation hypothesis suggests that females may manipulate reproductive allocation efforts for their offspring on the basis of attractiveness of their mates (Burley 1988; Sheldon 2000). Thus, if male attractiveness contributes to offspring fitness (either by providing maximum paternal care or high genetic quality), females that choose to mate with highly attractive males may produce more offspring or offspring of superior quality than those that mate with less attractive males.

The attractiveness hypothesis suggests that females that gain matings with particularly attractive males should produce more sons that inherit the sexually attractive traits of their sires (Burley 1986; Cockburn *et al.* 2002). Numerous studies based on birds [e. g. zebra finches *Taeniopygia guttata* (Burley 1981, 1986), great tits *Parus major* (Kölliker *et al.* 1999) and peahens (Pike and Petrie 2005)], fish (Karino and Sato 2009; Sato and Karino 2010), and mammals (Røed *et al.* 2007) have indicated that mating with attractive males will lead to changes in the bias of the sex ratio in favour of producing more male offspring. According to this hypothesis, the reproductive success of sons depends on the attractiveness of their sires, while daughter reproductive success is relatively constant and not related to the attractiveness of their sires (Cockburn *et al.* 2002). Thus, females can

enhance their fitness by producing chicks with male-biased sex ratios if they mate with attractive males.

However, many studies on birds did not find a significant relationship between male sexual attractiveness and biased sex ratio. For example, female barn swallows (*Hirundo rustica*) seem not to adjust their sex ratio to the length of their mate's tail (Saino *et al.* 1999) which is considered a signal of male viability (Møller 1994) affected by female choice (Møller 1988). Recently, Romano *et al.* (2015) found in their study of the barn swallow that sex ratio bias toward production of sons was associated with maternal plumage darkness and paternal tail length. In blue tits (*Parus caeruleus*), Dreiss *et al.* (2006) used colour ornamentation of the male as a sexual trait to investigate adaptive sex ratio manipulation by females, but found no significant relationship between colouration and sex ratio, while there was a positive relationship between song characteristics of fathers and the proportion of sons in their broods. Korsten *et al.* (2006) found little evidence of sex ratio bias in a Dutch population of blue tits. In contrast, Sheldon found evidence of sex ratio bias in another population of blue tits. However, Ewen *et al.* (2004) in an exhaustive meta-analysis showed that primary sex ratio manipulation in birds does not exhibit variability beyond that which could be expected due to sampling error. Thus, that study did not provide any evidence that facultative primary sex ratio adjustment is a biologically important phenomenon.

The peacock's highly elaborated train is the typical example of a male trait believed to have evolved in response to female choice. The number of ocelli in the train is an important predictor of male mating success (Petrie *et al.* 1991; Loyau *et al.* 2005; Dakin and Montgomerie 2011), where a reduction in the number of ocelli leads to a decrease in mating success

(Petrie and Halliday 1994; Dakin and Montgomerie 2013). Moreover, the degree of train elaboration is a heritable trait that is positively related to offspring survival, with a stronger effect in sons than in daughters (Petrie 1994). Thus, females change the sex ratio of their offspring by producing significantly more daughters when mated with less attractive males, suggesting that peahens have the ability to control the sex of their offspring (Pike and Petrie 2005).

Hamilton and Zuk (1982) proposed that females may assess male quality from their degree of ornamentation, which represents a particularly reliable indicator of parasite load of the bearer, because only healthy individuals are able to produce exaggerated secondary sexual characters and still remain resistant to debilitating parasites. Many parasites that inhabit the plumage have the ability to degrade keratin (Burt and Ichida 1999; Gunderson 2008) that may alter the structure and condition of ornamented feathers (Shawkey *et al.* 2007). Thus, if sons are able to inherit the attractiveness of their fathers, subsequently sons of attractive males might be of higher reproductive value than daughters of such males (Ellegren *et al.* 1996). It would then be adaptive for females paired to attractive males to bias the sex ratio of their offspring towards males.

Given the reported conflicting results on sex ratio bias in relation to expression of secondary sexual characters, we aimed, by using a large data set in the present study, to avoid the problem of sampling error emphasised by Ewen *et al.* (2004). Furthermore, hatching success in females may not be random, which may lead to bias in the estimate of sex ratio for different females. Therefore, we tested whether there was such an effect in two successive years using a different set of females in each year.

The objective of this paper was to explore if there was a relationship between primary sex ratio and expression of secondary sexual characters such as train and spur length, number of ocelli, and degree of feather degradation as caused by feather degrading microorganisms and offspring sex ratio in the peacock.

MATERIALS AND METHODS

Captive breeding experiment

We conducted a captive breeding experiment in which a total of 46 adult males (fathers) each were randomly mated with four females, in total 184 adult females (mothers). Each mother was allocated to a single male for the entire breeding season, so each mother was mated with only one male. Eggs were collected daily, numbered and weighed on a digital top pan balance to the nearest 0.1 g. Any mother that died during the experiment was replaced and mothers and fathers were allocated to pens at random at the start of the breeding season. During the experiment, only four females were replaced (2% of total females) and all replacements occurred during the first four weeks of the 20-week breeding season. Blood samples were taken from each individual for MHC and microsatellite genotyping, and parentage and sex of offspring were determined genetically.

At the start of the breeding season, on the day adults were allocated to pens, body weight (g) was recorded and spur length (mm) and tarsus length (mm) were measured with digital calipers for all adults, and train length (length of the longest feather in mm), number of ocelli was the number of ocelli counted from photographs taken of each male. Because the count was taken from photographs, and it is difficult to get a photograph where the

train is completely visible, this measure is an estimate of number of ocelli rather than an accurate count. The peafowl were provided with water and fed a poultry layers' pellet that did not contain any antibiotics. The breeding experiment was conducted over two years (in 1998 and 1999) in a commercial peacock farm in Norfolk, UK (see Hale *et al.* 2009 for further details), with mothers and fathers randomly reassigned to mates between the two years. The random allocation of four mothers to each father was designed to reduce the impact of any maternal effects in the analyses of reproductive output, including any impact of previous mate history.

The 4,977 collected eggs over the two years were either incubated to term (28 day) in separate compartments or incubated to day 10. Approximately half the eggs laid were incubated to day 10 and half to full term. For those eggs incubated to term any unhatched eggs were dissected and the stage of development recorded. Blood and/or tissue samples were taken from any fertilized and unhatched eggs. Blood was taken from all hatched chicks. All eggs incubated for 10 days were dissected and the state of development recorded and blood and tissue samples were taken.

Outside of the breeding season all individuals were sheltered in large outdoor aviaries, and allowed free access to invertebrates in the soil and to green vegetation. Therefore, all individuals were supposed to have similar contamination levels with microorganisms, since all were housed in the same farm and cared for by the same persons, suggesting that the difference in infection level is due to resistance and anti-microbial defences of each individual.

The study was approved by the UK home office and there were no signs of negative effects of any of the procedures adopted throughout the study that may adversely have affected the peacocks.

The feathers were removed aseptically from peacocks and kept in plastic bags and were transported under cold condition to the laboratory and stored until processed. One ocellus was chosen randomly among 10 ocelli collected from all males included in this study, with the aid of a digital camera, HAM photographed the feathers to simplify the measurement and improve accuracy of measurement of the area of feather ocelli, which was done by using the 'rule' tool in the Adobe Photoshop CC software (see Figure 1 chapter 1). Ocelli area (in mm) was estimated as the product of the two widest points in height and width of the ocelli. All measurements were done blindly without any information about individual identity and phenotype.

Ranking of degree of ocelli degradation

Three ranking categories were used by HAM to estimate the degree of damage in feather ocelli of the 46 males used in this study:

(1) By counting the missing or broken barbs at the lower part of the ocelli from the photograph.

(2) Categorizing the damage to the upper part of the ocelli on a five-rank scale (see figure 2 in chapter 1), 0 = No loss due to degradation, 1 = no loss with degradation, 2 = one third of barbs from the upper part were lost or degraded, 3 = two thirds of barbs from the upper part were lost or degraded, and 4 = more than two thirds of barbs from the upper part were lost or degraded.

(3) Degradation in the three differently coloured parts of the ocelli were ranked at unit intervals from 0 (no degradation) to 3 (maximum degradation) (see Figure 2 in Chapter 1). HAM scored the level of degradation as follows: 0 = No degradation, 1 = slight degradation (less than 1-3 small spots of degradation), 2 = medium degradation (large area of degradation), and 3 = high degree of degradation (more than half of the area degraded) (see Figure 3 in Chapter 1).

HAM repeated the measurements of the area of ocelli scoring the damage to ocelli characters on two different days without any reference to the first set of measurements. These repeated measurements and scores were performed to estimate repeatability (R) (Becker 1984; Falconer and Mackay 1996). Repeatability was high in all cases (see Table S1 for repeatability of different characters in peacocks on different days).

Offspring sexing

Offspring were sexed by MH via PCR amplification of the CHD genes using primers P2 and P8 (Griffiths *et al.* 1998) in 10 μ l reactions containing 1 x *Taq* buffer, 2mM MgCl₂, 200 μ M each dNTP, 1.0 μ M each primer, 0.3U *Taq* and 0.5 μ l template DNA. The reaction cycle was 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, 50°C for 20 seconds and 72°C for 25 seconds. PCR products were detected on 8% denaturing polyacrylamide gels. Females produced three bands, males two bands.

Statistical analyses

Repeatability and its SE was calculated for area measurements and damage scores of the feather samples (measured twice) by using equations in Becker (1984) and Falconer and Mackay (1996). We used JMP (SAS 2012) for the

statistical analyses. Sex ratio of the offspring for each male was expressed as the proportion of males to females produced by a female. Generalized linear models with binomial error distribution and a logit link was used to express the strength of association between the sex ratio and the expression of secondary sexual characters by calculating the effect sizes (r) from the value of chi square according to the following formula (Rosenthal 1994):

$$r = \sqrt{\frac{\chi^2 (1)}{N}}$$

The sample correlation r was subsequently transformed for analysis using Fisher's z -transformation (Fisher 1915)

$$z = 0.5 \times \ln\left(\frac{1 + r}{1 - r}\right)$$

The measures of effect size were weighted by sample size to account for differences in sampling effort among males.

RESULTS

Summary statistics for secondary sexual characters are reported in Table 1 (a, b, c). All characters showed considerable variation.

In order to eliminate the possibility that hatching success in females may not be random and lead to bias in the estimate of sex ratio for different females. We tested this assumption, but found no such non-random effect in

the two years of study (first year: $F = 0.59$, $df = 1, 140$, $r^2 = 0.004$, $P = 0.44$, second year: $F = 1.59$, $df = 1, 152$, $r^2 = 0.010$, $P = 0.21$).

Table 1.

(a) Summary statistics for secondary sexual characters of peacocks.

Character	Range	Mean (SE)	N
First year train length (mm)	1060 -1510	1327.6 (13.98)	46
Second year train length (mm)	1320 - 1690	1488 (11.02)	46
First year spur length (mm)	11.98 -26.22	17.97 (0.39)	46
second year spur length (mm)	27.1 - 40.6	32.98 (0.40)	46
Ocelli area - second year (cm ²)	12.74 -22.46	17.58 (0.36)	46
Lower barb loss - second year	0 -12	5.24 (0.40)	46
Number of ocelli - first year	58 - 147	125 (2.37)	45

(b) Frequency and percentage of degree of loss of barbs from the upper part of ocelli from 46 peacocks.

Degree of loss	Frequency	%
0	6	13
1	4	8
2	22	48
3	12	26

(c) Frequency and percentage of degree of degradation of differently coloured parts of ocelli from 46 peacocks.

Degree of degradation	Brown		Blue		Green	
	frequency	%	frequency	%	frequency	%
0	9	20	20	43	40	87
1	20	43	18	39	3	7
2	12	26	5	11	3	6
3	5	11	3	7	0	0

Fisher's z - transformation of the chi-squared value for the sex ratio for the nine secondary sexual characters that were included in this study ranged from 0.001 to 0.037 with a mean (SE) of 0.017 (0.003) (Table 2).

Table 2. Chi Square values, sample sizes, correlation coefficients (r) and Fisher's z -transformations (z) of the sex ratio in relation to the expression of nine secondary sexual characters.

Character	Chi Square	Sample size	r	z
Train length (both years)	1.1575	3803	0.01745	0.01745
Spur length (both years)	0.7282	3803	0.01384	0.01384
Ocelli area - second year	0.3453	1853	0.01365	0.01365
Lower barb loss - second year	2.4924	1853	0.03668	0.03669
Upper barb loss - second year	0.5307	1853	0.01692	0.01692
Brown degradation –second year	0.0011	1853	0.00077	0.00077
Blue degradation- second year	0.4489	1853	0.01556	0.01557
Green degradation-second year	0.4217	1853	0.01509	0.01509
Number of ocelli - first year	0.8359	1909	0.02093	0.02093

The mean Pearson correlation coefficient for the sex ratio was significantly larger than zero implying that the sex ratio was biased albeit only to a small degree ($t = 5.90$, $df = 8$, $P = 0.0004$). This implies that there is a slight sex ratio bias. In addition, the observed sex ratio was significantly smaller than the hypothesized mean sex ratio 0.04 for birds in general according to the data analyzed by Ewen *et al.* (2004); $t = -8.34$, $df = 8$, $P < 0.0001$). This implies that the sex ratio bias in peacocks is significantly smaller than the average bias across all studies of birds.

DISCUSSION

Sexual attractiveness of a male may affect the optimal sex ratio of his sons. If male attractiveness is inherited by sons, then sons of a highly attractive male may be of greater reproductive value than daughters. In contrast, daughters of unattractive males may be of relatively higher reproductive value than sons. If that were the case, it would be adaptive for fathers and mothers to bias their sex ratio in response to the secondary sexual characters of the father (Weatherhead and Robertson (1979).

In the current study, the correlation between nine secondary sexual characters in peacocks with the sex ratio of the offspring by using a very large sample size (see Table 2) revealed that the correlation coefficient (r) was significantly less than the value of 0.04 that was found by Ewen *et al.* (2004) in their meta-analysis.

In birds the role of sexual selection in adaptive sex ratio manipulation has been particularly controversial. Several studies suggest that females of sexually dichromatic birds may adaptively manipulate the sex ratio of their offspring in relation to male ornamentation (Krackow 1995; Emlen 1997; Komdeur *et al.* 2002; West and Sheldon 2002). For instance, females may produce more sons when copulating with a highly attractive male. One of the classical studies (Burley 1981, 1986) suggesting adaptive sex-ratio manipulation in zebra finches (*Taeniopygia guttata*) found that leg-band colours influenced male attractiveness, and offspring of the more attractive parental sex were produced in excess. Therefore, Burley suggested that parents improve their fitness by increasing investment in more attractive offspring. Pike and Petrie (2005) conduct an experiment on peacocks which have magnificent long and highly ornamented trains. They suggested that when peahens are mated to peacocks with their attractiveness experimentally

reduced by removal of eyespot feathers from their trains, they tended to produced significantly more daughters than when mated with the same males after restoring the degree of ornamentation of their trains. This suggests that peahens have a degree of control over the sex of their offspring.

While the literature may superficially suggest that there is plenty of evidence consistent with sex ratio manipulation, Ewen *et al.* (2004) conducted an exhaustive meta-analytical study and found no evidence for the general occurrence of avian primary sex ratio adjustment across all studies. Thus, facultative control of offspring sex is not a characteristic biological phenomenon in breeding birds given that the effect size measured in terms of Pearson's product-moment correlation coefficient weighted by sample size was only on average $r = 0.04$. In evolutionary biology studies, effect sizes typically account for 5-10% of the variance (Møller and Jennions 2002). This implies that sample sizes required to demonstrate a small effect accounting for 1% of the variance will require an enormous sample size that is only rarely acquired in empirical studies (Møller and Jennions 2002).

In conclusion, we found a small effect of the expression of secondary sexual characters on bias in brood sex ratio towards production of more sons than daughters when males were particularly attractive. This study also showed that mean effect size was actually significantly smaller than the effect size in the general literature as reported by Ewen *et al.* (2004).

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Chapter 4:

Do peacock signals reveal abundance and diversity of microorganisms?

**Haider Al-Murayati ^a, Zaid Al Rubaiee ^a, Marion Petrie ^b,
Alessandra Costanzo ^c, and Anders Pape Møller^a**

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

^b *Institute for Health and Society and Newcastle University Institute for Ageing, Newcastle University, Campus for Ageing and Vitality*

Newcastle upon Tyne, NE4 5PL, UK

^c *Department of Biosciences, University of Milan, via Celoria 26, I-20133 Milan, Italy*

Word count: 6700

Correspondence to HAM:

Tel: (+33) 1 69155688

Fax: (+33) 1 69155688

E-mail: haideryousif1967@gmail.com

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ABSTRACT

Birds use visual signals such as bright colours or exaggerated ornamentation for socio-sexual communication as well as species recognition. Bird feathers harbour numerous microorganisms, some of which are able to degrade feather keratin such as the feather degrading bacteria, which can affect feather integrity and alter feather coloration. The train ocelli of the peacock (*Pavo cristatus*) contain three distinct iridescent colours. Such ornaments are considered to be “honest” signals of quality because they are costly to produce and reveal condition of the signaller. We hypothesised that the different parts of ocelli are susceptible to degradation by microorganisms to a different extent. We investigated whether there was a relationship between the abundance and the diversity of the bacterial community in the ocelli of the train of the peacock and the colouration of the brown, green and blue patches of the ocelli. We showed that the bacterial community in the ocelli was related to changes in the colouration of the three differently coloured parts with the main changes being found for the area of the brown part of ocelli. These findings emphasize the importance of the brown area in sexual selection of the peacock.

Keywords: bacteria; feather colouration; feather degradation; ocelli; peacock.

INTRODUCTION

The colourful plumage of birds has long attracted the attention of scientists (Darwin 1871; Andersson 1994), who have emphasized the importance of bright colours in visual signalling (Burt 1986; Griffith *et al.* 2006; Hill and McGraw 2006). Visual signals play an important role in avian communication, mainly derived from the discrimination of colour intensity (Cuthill 2006; Håstad and Ödeen 2008) that occurs in various avian feather ornaments (Andersson 1994). Colour characteristics of secondary sexual characters may provide reliable information about the condition of an individual due to differences in genetic quality (Hamilton and Zuk 1982; Figuerola *et al.* 1999), aggressiveness during contests (Alonso-Alvarez *et al.* 2004) or reproductive strategy (Badyaev and Hill 2002). Such colourful signals may give rise to intrasexual or intersexual selection (Andersson 1994).

The mechanisms responsible for the production of feather colouration fall into two main categories. The first one is pigmentary colours resulting from the chemical properties of pigments that are incorporated into the matrix of keratin, and the concentration of these pigments in feathers affect the absorption and the reflectance of incident light at different wavelengths. Feather pigments come in three different types: melanins, carotenoids and porphyrins. Eumelanin and pheomelanin, which are the two main forms of melanin, are responsible for the black grey and the earth-toned feather colour, respectively (Fox and Vevers 1960; Haase *et al.* 1992). Carotenoids, existing in many types based on their molecular structure (e.g. carotenes, xanthophylls), produce bright yellow and brilliant orange yellow (Goodwin 1984). Porphyrins, produced by modifying amino acids, yield to a range of colours such as pink, browns, reds, and greens, but also produce brilliant

greens and reds (Proctor and Lynch 1993). Furthermore, these pigments may interact to produce several other hues (McGraw 2006).

The second main type of colouration is structural. Therefore, depending on the arrangement and the differential refraction properties of the nanostructures of the feather barbules they produce an amazing array of green, blue, violet and ultraviolet colours (Kinoshita *et al.* 2008). The most common components in these structures are keratin, melanin (as a granule, called melanosomes) and air (Prum 2006). Structural colourations can be classified as either iridescent or non-iridescent (Shawkey *et al.* 2009a). In iridescent feathers and within the cortex of the barbules, melanin rods are arranged to create two-dimensional (2D) photonic crystal-like structures at the sub-micron scale (Yoshioka and Kinoshita 2002; Zi *et al.* 2003; Li *et al.* 2005). The colours are produced by thin-film interferences in feather barbules, that act like a prism splitting the light into rich component colours. As the viewing angle changes, the refracted light becomes visible in a shimmering, glowing iridescent display. In contrast, colours in non-iridescent feathers are produced by 3D spatial periodicity in feather barbs (the tiny air pockets in the barbs of feathers can scatter incoming light), and the change in the observer angle does not lead to a change in colours (Proctor and Lynch 1993). Furthermore, feather structures of many bird species can reflect light in the ultraviolet range (many birds can discriminate a greater diversity of colours than humans, including ultraviolet wavelengths) (Holley 2015).

Numerous microorganisms like bacteria and fungi are prevalent in the biosphere and constitute the largest part of our planet (Gilbert *et al.* 2012; McFall-Ngai *et al.* 2013). Bacteria are ubiquitous microorganisms that frequently live in association with other microbes. Recently, numerous

studies emphasised the potential role played by the entire assemblage of microorganisms (generally referred as the microbiome (Morgan *et al.* 2013)), as selective agents influencing the evolution of host life history traits (Dillon *et al.* 2005; Ezenwa 2012). Birds carry a large diversity of potential pathogens on their plumage (Hubálek 2004). Shawkey *et al.* (2005) suggested that birds may acquire their bacterial community either from soil or water. Among the potential pathogenically diverse bacterial groups that are carried by bird feathers are feather-degrading bacteria, a polyphyletic assemblage of microorganisms (Burt and Ichida 1999; Whitaker *et al.* 2005; Shawkey *et al.* 2007; Gunderson *et al.* 2009; Shawkey *et al.* 2009b). Correlative evidence suggests that these bacteria are active in the plumage and capable of degrading β -keratin, a protein that constitutes the majority of feather mass (Muza *et al.* 2000; Sangali and Brandelli 2000; Lucas *et al.* 2003; Gunderson *et al.* 2009; Shawkey *et al.* 2009b). Such degradation may impair flight because damaged feathers deteriorate aerodynamics and hence flight ability with consequences for communication, predator-prey interactions and the ability to capture prey.

Feather colouration may affect the bacterial community, as well-supported studies emphasise the importance of melanin as one of the mechanisms to increase resistance against bacterial degradation by keratinolytic bacteria such as *Bacillus pumilus*, *Bacillus licheniformis* and other *Bacillus* species (Burt and Ichida 1999; Goldstein *et al.* 2004; Grande 2004; Gunderson *et al.* 2008). Several scientists have suggested that feather-degrading bacteria could reduce feather-based communication by modifying feather colouration. Shawkey *et al.* (2007) and Gunderson *et al.* (2009) showed that the presence of feather-degrading bacilli has an adverse consequence for the plumage of eastern bluebirds (*Sialia sialis*), because

they altered feather colour (non-iridescent), reduced body condition and lowered reproductive success. Likewise, Leclaire *et al.* (2014) experimentally manipulated the feather bacterial load on captive feral pigeons (*Columba livia*) and found that individuals of both sexes with lower bacteria load on their feathers had more brightly iridescent neck feathers. In contrast, Jacob *et al.* (2014) in their experimental study on great tits (*Parus major*) found no significant relationship between increased bacterial load and a reduction in feather colour (brightness and UV chroma).

The peacock (*Pavo cristatus*) has developed upper tail coverts into a highly elaborate train that is characterized by the fascinating ocelli that have three iridescent colours (a dark blue centre that is surrounded by a greenish zone followed by a broad brown zone) (see Figure 1 in chapter 1). Already Darwin (1871) was bothered by the multiple signals of peacocks and the functional basis for the evolution of these signals. How is it possible that mate preferences for these different characters have evolved in the context of sexual selection and most likely female mate preferences? The train of the peacock is believed to have evolved in response to female choice, and several studies suggest that peahens prefer males with more ornamented trains (Petrie *et al.* 1991; Petrie and Halliday 1994; Loyau *et al.* 2005; Takahashi *et al.* 2008; Dakin and Montgomerie 2011, 2013). Peacocks have feathers that are degraded to a different degree in different parts of the ocelli, suggesting that different parts of ocelli are susceptible to degradation by microorganisms to a different extent. Feather degradation occurs as a consequence of feather degrading bacteria from the soil or vegetation that adhere to the plumage (Lucas *et al.* 2003). We have previously shown that the three different colours of the ocelli are subject to feather degradation to a different extent, raising the possibility that the three different parts of ocelli

also differ in diversity or abundance of microorganisms (Al-Murayati *et al.* 2016 (unpublished results)).

The aim of this study was to explore if there is a relationship between the abundance and the diversity of the bacterial community in the ocelli of the train of the peacock and the characteristics of the colouration of the brown, green and blue parts of the ocelli.

MATERIALS AND METHODS

Field study and samples

A peacock breeding experiment was conducted throughout two year (1998-1999) in a commercial farm in Norwich (United Kingdom) by Marion Petrie and Anders Pape Møller (see Hale *et al.* 2009 for further details). Outside the breeding season, all individuals were housed in large outdoor cages with suitable food (commercial poultry feed) and water (*ad libitum*) that not contained any antibiotics.

All individuals were cared for by the same people and they were allowed to roam freely in the farm so they were directly in full contact with soil and vegetation. For that reason, we assume that all individuals included in this study were exposed to a similar amount of microorganisms present in the surrounding fields, and that variation in susceptibility to infection may be due to the general condition of each individual. At the mating season, each peacock in this study were allocated with four randomly chosen peafowl so each mother was mated with single father for the entire breeding season.

Ten ocelli train feathers were collected aseptically from each peacock and kept in dry plastic bags. Samples are transported under cold conditions to the laboratory and stored until processing. This experiment was done

under home office licence (UK), and there are no adverse effects from any methodology of this experiment on the peacocks.

Bacterial isolation

One sample of ocelli feathers was chosen randomly by HAM from 30 individuals out of a total of 46 individuals (the remaining samples are not included in this study due to logistical purpose) to isolate and identify bacteria in two different media: Tryptic soy agar (TSA) is a rich medium on which most heterotrophic bacteria can grow and this allowed us to estimate the total cultivable bacterial load, and feather meal agar (FMA), which is a highly selective medium for keratinolytic bacteria that contains one source of carbon and nitrogen (keratin) allowing us to quantify the load of feather-degrading bacteria (Williams *et al.* 1990; Sangali and Brandelli 2000; Shawkey *et al.* 2003, 2007, 2009b). An additional set of 10 feather samples was chosen randomly from the same set of 30 individuals to estimate repeatability of abundance and diversity of bacteria (Becker 1984; Falconer and Mackay 1996).

The differently colored parts of the ocelli were cut into pieces under aseptic conditions with the aid of a sterilized sharp blade and pincers. The barb pieces were placed in sterilized pre-weighed eppendorf tubes. Sterilized phosphate buffer saline (pH 7.2) was added to each eppendorf tube in proportion to the weight of the barb (each mg equalled 100 μ l PBS). To facilitate the detachment of bacteria from barbs into the PBS solution, a three-time vortex cycles of 1 min each was used (Saag *et al.* 2011).

An inoculum of 100 μ l PBS that contained the washed bacteria were spread on two different media with a sterile spreader and a third plate inoculate with sterilized PBS as a control. Cycloheximide was added to

prevent fungal growth (Smit *et al.* 2001). The plates were incubated at 28°C, and the incubation period for TSA and FMA was 3 and 14 days respectively. At the end of the incubation period, the plates were subject to a count of the total number of colony forming units (CFU) under a dissecting microscope. Furthermore, counts of each morphotype were recorded according to the morphological characters of the colonies (colour, shape, size, and presence or absence of mucoid textures). Stocks of nutrient agar slants were prepared from well identified pure colonies and stored at 4°C.

DNA extraction and amplification of bacterial 16SrRNA gene

Bacterial DNA extraction was made using two protocols. The first protocol depended on freezing and thawing of samples in order to lyse the bacterial cells. With the use of the tip of a wooden stick, a small part of a bacterial colony was transferred to an eppendorf tube (0.5 ml) containing 50 µl Tris (10 µM, pH 8.0) and crushed well. The tubes closed well and were transferred immediately to a liquid nitrogen jar until completely frozen, and then the tube was transferred to a hot water bath (60°C) until completely thawed out (this procedure was repeated three times). To increase the probability of cell lysis the tubes were microwaved three times at 270 W for 5 to 6 s followed by 10 s of waiting. After the microwave step, the samples were centrifuged for 5 min (12,000 x g), the supernatant was transferred to a free DNA tube and stored at -20°C for further processing. The second protocol was done by using a Soil DNA isolation kit (MOBIO Laboratories, Inc. USA).

The bacterial 16S rRNA gene were amplified by PCR by using the following forward primers: 16S rDNA-27F (5'-

AGAGTTTGATCCTGGCTCAG-3') or 16S rDNA-63f (5-CAG GCC TAA CAC ATG CAA GTC-3) and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). The DNA of bacterial isolate served as a template (PCR mixture (25 µl): 16 µl ultrapure water, 4 µl 5x buffer, 0.4 µl dNTPs 10nM, 1 µl 10 µM 27F, 1 µl 10 µM 1492R, 0.2 µl Go Taq DNA polymerase and 3-5 µl genomic DNA). The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s, annealing for 30 seconds at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed.

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

DNA sequencing and bacterial identification

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

Reflectance measurements

To measure the reflectance spectra of the different coloured patches of the ocelli (brown, green, and blue) under different incident light angles (60° and 30°) (Fig. 1), Alessandra Costanzo used an Avantes dh 2000 spectrophotometer with a deuterium-halogen light to measure coloration of ocelli (<http://www.avantes.com/products/light-sources/item/237-avalight-d-h-s-deuterium-halogen-light-sources>). Collimating lenses (<http://www.avantes.com/products/fiber-optics/item/256-collimating-lens>) were mounted onto the ends of the reflection probes (<http://www.avantes.com/products/fiberoptics/item/245-reflection-probe-standard>) for both illumination (3 cm from the feather surface) and measurement (6 cm from the feather surface for 60° measurement and 3 cm from the feather surface for 30° measurement) of a spot of feather from the ocelli about 2 mm in diameter. The measurement was performed in a dark room. We took on average 5 scans at 800 ms integration time for the 60° measurements, and on average 05 scans at 150 ms integration time for the 30° measurements. The difference in the integration time did not affect the result of the measurements. Recalibration of the dark and white standards was done every 15 minutes. Each sample was measured twice, remounting it in the apparatus between measurements. We measured again those samples that deviated by more than 3 SD from the mean value. Reflectance spectra (Fig. 2) were then processed according to the Tetrahedral colour space model (Stoddard and Prum 2008) to adopt the spectral sensitivity of the peacock. Feather colour was thus described by three colour variables: θ , which represents the visible light, ϕ , which accounts for the ultraviolet component and achieved r , which is a measure of colour saturation.

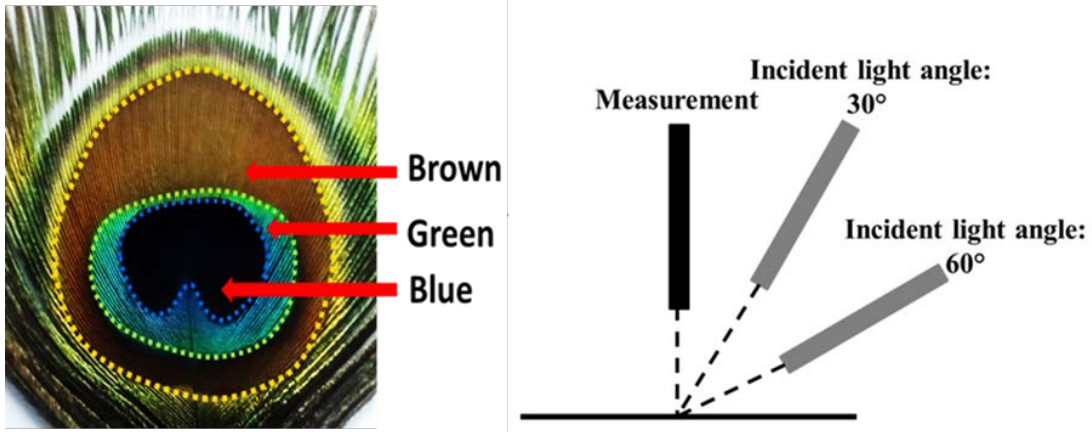


Fig. 1. On the left, the three parts of the ocelli measured (brown, blue and green), marked with a white dot. The measurements were always performed on the left side of the feather when viewed from the front. On the right, the reciprocal position (30° and 60°) of measurement and light source probes (modified by Dakin and Montgomerie, 2013).

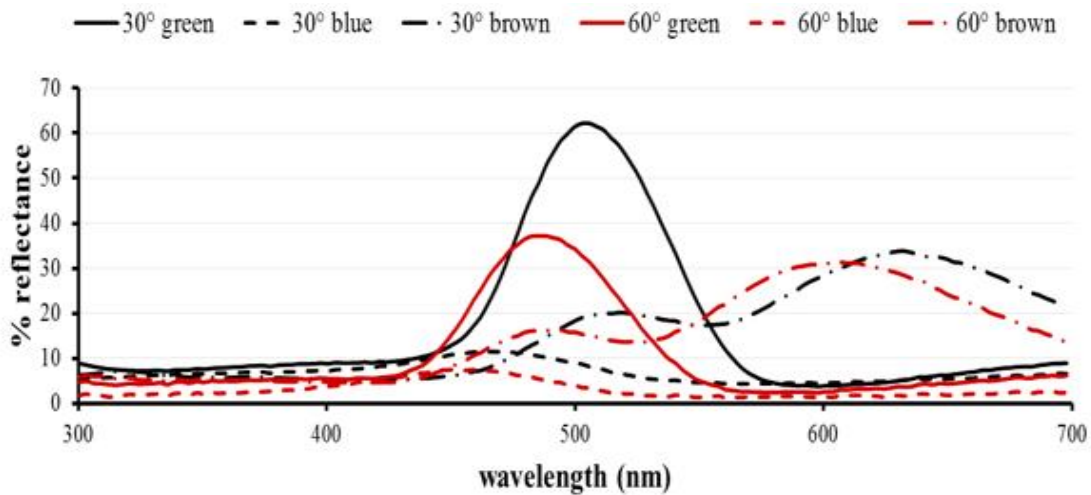


Fig. 2. Reflectance spectra of the brown, blue and green parts of the ocelli, measured at illumination angles of 30° and 60° (black and red curves, respectively) from the female’s typical viewing position in front of the male during courtship. Curves represent the average of the measurements taken from a single feather from each of the 46 individuals’ ocelli.

Statistical analyses

We estimated repeatability of measurements and scores for randomly chosen subsamples of individuals that were measured twice, using equations in Becker (1984) and Falconer and Mackay (1996) for estimating repeatabilities and their SE.

Simpson's index (a measure of diversity independent of sample size) was calculated to determine the diversity of microorganisms in the differently coloured parts of the ocelli, which takes into account the number of species present, as species richness and evenness increase, so does diversity (Magurran 2004).

To express the strength of the association between the colour variables (θ , ϕ , and α) at different incident light angles (60° and 30°) and the abundance of bacteria and the different bacterial species, Pearson's product-moment correlation coefficients (r) were calculated from the (t) ratio of the partial effect for each variable according to the following formula (Rosenthal 1994):

$$r = \sqrt{\frac{t^2}{t^2 + df}}$$

The sample correlation (r) was subsequently transformed for analysis by Fisher's z -transformation (Fisher 1915):

$$z = 0.5 \times \ln\left(\frac{1 + r}{1 - r}\right)$$

The relationship between the three-colour variables at different incident light angles for all coloured patches of the ocelli and the abundance of bacteria and the taxa diversity was made using standard least squares analyses.

RESULTS

Bacterial community of ocelli

Repeatability of the abundance of bacteria recovered in the two media was high in all cases except for the number of species recovered from TSA medium of the brown and blue parts of the ocelli (repeatability of total number of bacteria and the number of bacterial species on TSA and FMA media from differently coloured parts of the ocelli are reported in Table S3 A and B in chapter 1).

We found a heterogeneous abundance of bacteria in different parts of the ocelli (see Tables S4 A and B). In addition, there were variable numbers of bacterial taxa for differently coloured parts of the ocelli. From the eight bacterial isolates that were recovered from TSA medium, HP-F isolates of Unidentified bacterium. formed the largest percentage in brown and green parts of the ocelli (19% and 20%, respectively). The largest group for the blue area was 19% for *Paenibacillus* sp. (HP-D). As for FMA medium HYA contained seven bacterial isolates for which *Streptomyces marokkonensis* (HP-AF) was the most dominant bacterial isolate from brown, green and blue areas of the ocelli (accounting for 29, 35 and 27%, respectively) (see tables S5A and B). The different bacterial taxa were widely distributed across the phylogenetic tree of bacteria (See Fig. S1).

Fisher's z- transformed correlation coefficients between the reflectance of the three different colour patches in the ocelli of the train of the peacock at two different angles and the bacterial abundance and diversity ranged from -0.627 to $+0.845$ with a mean (SE) of $+0.033$ (0.011). This mean estimate differed significantly from zero ($t = 3.09$, $df = 401$, $P = 0.0021$). This effect size was small, but consistent and showed evidence of a general relationship between coloration and abundance and diversity of

microorganisms. In addition, the mean Fisher's z -transformed correlation coefficient in this study was much smaller than the mean estimate of 0.51 that we calculated from data in Table 1 of Dakin and Montgomerie (2013) ($t = -44.25$, $df = 401$, $P < 0.0001$), where they found that the green area affected mating success of the peacock in a North American feral population.

Fisher's z -transformed correlation coefficient was not correlated significantly with the angles of incident light ($F = 0.624$, $df = 1$, 401 , $P = 0.43$), or the three colour variables (θ , ϕ , and achieved r) ($F = 2.845$, $df = 2$, 401 , $P = 0.06$).

Microorganisms or their abundance in ocelli (coded as 1, 0) did not correlate significantly with Fisher's z -transformed correlation coefficient ($F = 1.07$, $df = 1$, 401 , $P = 0.30$). Furthermore, Fisher's z -transformed correlation coefficient did not vary significantly among the eleven-bacterial taxa ($F = 0.50$, $df = 11$, 257 , $P = 0.90$) nor with the abundance of these bacteria ($F = 1.27$, $df = 7$, 143 , $P = 0.27$). A reduced model between abundance of bacteria for each of the three colours separately revealed that just the abundance of bacteria in the part of the feather with brown colour was significantly correlated with Fisher's z -transformed correlation coefficient (Fig. 1; Table 1; brown: $F = 6.12$, $df = 7$, 47 , $P = 0.0001$; green: $F = 0.95$, $df = 7$, 47 , $P = 0.48$; blue: $F = 0.50$, $df = 7$, 47 , $P = 0.83$).

Fisher's z -transformed correlation coefficients for the relationship between measures of colouration and abundance of microorganisms were the units of analysis. Subsequently, we tested whether these z -values differed among three categories of colour (brown, green and blue), and there was a

highly significant difference among the three categories ($F = 8.74$, $df = 2$, 401 , $P = 0.0002$).

Table 1. Fisher's z- transformation of Pearson's product-moment correlations between brown colour of the ocelli and various measures of abundance and diversity of microorganisms when cultured in Feather Meal Agar (FMA) and Tryptic Soy Agar (TSA). Values in bold are statistically significant at the 5% level. The model had the statistics $F = 6.19$, $df = 7, 47$, $P < 0.0001$.

Term	Slope	SE	<i>t</i>	<i>p</i>
log Simpson diversity index of bacteria on FMA medium	0.186	0.081	2.30	0.03
log mean bacteria on FMA medium	0.319	0.081	3.95	0.0003
log No. species of bacteria on FMA medium	0.070	0.080	0.86	0.40
log total no. colonies of bacteria on FMA medium	-0.384	0.081	-4.75	< 0.0001
log Simpson diversity index of bacteria on TSA medium	-0.132	0.081	-1.64	0.11
log mean no. bacteria on TSA medium	0.016	0.080	0.2	0.84
log no. bacteria species on TSA medium	0.034	0.081	0.42	0.68

A Tukey's test revealed a significant difference at the 0.05 level between the abundance of bacteria in the brown part of the feather and the green and blue colour. A statistical model that included colour, microorganisms and their interaction with Fisher's z- transformed correlation coefficient as the response variable revealed a significant effect for brown colour and its interaction with a variable reflecting whether the

analysis was based on microorganism diversity or their abundance (Table 2; $F = 5.07$, $df = 5$, 401, $P = 0.0002$).

Table 2. Fisher’s z- transformation of Pearson’s product-moment correlation between the abundance and the diversity of microorganisms and colouration of ocelli and the interaction between colour whether the test was based on microorganisms diversity or their abundance. The model had the statistics $F = 5.07$, $df = 5$, 401, $P = 0.0002$. Values in bold are statistically significant at the 5% level.

Term	Slope	SE	<i>t</i>	<i>P</i>
colour[blue]	-0.017	0.015	-1.066	0.29
colour[brown]	0.051	0.016	3.310	0.001
microorganisms [0]	-0.012	0.011	-1.101	0.27
colour[blue]*microorganisms [0]	0.025	0.015	1.589	0.12
colour[brown]*microorganisms	-0.039	0.016	-2.518	0.012

DISCUSSION

Many morphological signals like horns, antlers and plumage ornaments are important aspects of an individual’s phenotype. These traits are typically used by males as sexual signals, and, therefore, linked to reproductive success. Sexual signals play a significant role in competition for and attraction of mates (Andersson 1994).

Colourful plumage is considered one of the most important visual signals in birds, because it can serve as an honest signal of individual quality with functions in both intrasexual (Smith and Harper 1988; McGraw and Hill 2000) and intersexual communication (Kodric-Brown and Brown 1984; Hill 2006). Bird feathers are inhabited by numerous types of parasitic microorganisms that can affect allocation of time and energy and hence

development of feather signals. These parasitic microorganisms may induce changes in host phenotypes that have major consequences for host survival and fitness (Clayton and Moore 1997; Schmid-Hempel 2011). A potentially important group of microorganisms that inhabit bird feathers is feather degrading bacteria (Burt and Ichida 1999; Gunderson 2008; Ruiz-Rodríguez *et al.* 2009).

The current study showed that the presence of bacteria in peacock ocelli could lead to slight changes in the colour of ocelli. The results for peacocks reported here were generally weak, implying that they differed significantly from the results reported by Dakin and Montgomerie (2013) for a feral population of North American peacocks. That study reported strong correlations between the three different colours of peacock ocelli especially the green area with mating success. These differences in effect sizes could be related to populations of peacocks having been subject to multiple genetic bottlenecks with consequences for the level of standing genetic variation and hence the phenotypic variability from which females may be able to make their mate choice.

The results reported here indicate that the two angles of the incident light that we used in this study for measuring the three different colour variables of the ocelli (θ , ϕ , and achieved r) was not correlated significantly with Fisher's z-transformed correlation coefficient, which is a measure of the strength of the correlation between the abundance of different bacterial taxa and the three colour variables. Fisher's z-transformed correlation coefficient did not differ significantly among the abundance of bacteria nor with the different bacterial taxa in the ocelli. These results are in accordance with the findings of Jacob *et al.* (2014) in an experimental study on a wild breeding population of great tits (*Parus major*). They manipulated overall

bacterial densities in the nest hence modifying the bacterial loads of feathers of adult birds. They did not find any significant influence of bacterial load of feathers on feather coloration of different body parts of great tits. Our results differ from those of Shawkey *et al.* (2007) and Leclaire *et al.* (2014), where Shawkey *et al.* in structurally coloured (non-iridescent) rump feathers of eastern bluebirds found that bacterial load and feather degrading bacteria in lab experiments led to alterations in structural plumage colour through degradation. Leclaire *et al.* (2014) found in their experimental study while increasing or decreasing bacterial load in captive feral pigeons that feather bacteria can degrade feathers and lead to alterations in the coloration of iridescent neck feathers. These conflicting results may either be due to differences in experimental manipulation of the bacterial community of feathers, with consequences for competition and synergism (Faust *et al.* 2012; Morgan *et al.* 2013). The bacterial community could be regulated through differences in behaviour like bathing and preening (Shawkey *et al.* 2003; Saranathan and Burt 2007; Møller *et al.* 2009; Clayton *et al.* 2010). Furthermore, these differences could be related to physical and chemical properties of feathers in different taxa.

This study indicated that the three different colours of ocelli in peacocks were significantly correlated with Fisher's z- transformed correlation coefficients differing between analyses based on microorganisms or their abundance and diversity. These results are consistent with what we have shown in our previous study (Al-Murayati *et al.* 2016 (unpublished results)), where we found that the brown area was more sensitive to bacterial degradation than the two other colours of the ocelli. We hypothesized that this might be related to the difference in physical properties of these three areas (Zi *et al.* 2003). The change in colour of the brown area might be

caused by bacterial degradation. Dakin and Montgomerie (2013) found that the green area was correlated with mating success in a North American feral population of peacocks. Dakin and Montgomerie (2013) speculated that the brown and the blue colours areas could enhance the appearance of the green area. Brou *et al.* (1986) emphasized that the colour of an object depends on the colours of objects in its immediate surround. Thus, an alteration in colour of the surrounding areas (brown and blue) could affect the appearance of the important cue of the medullary green area and consequently influence the assessment of the green area by the female.

In conclusion, our study demonstrated that the bacterial community in the ocelli could lead to slight changes in colouration of the three different coloured parts of ocelli with the main changes being in the brown area. These findings emphasise the importance of the brown area of the ocelli in sexual selection in the peacock.

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Chapter 5:

Feather bacteria may influence daily growth increments of peacock ocelli feathers

**Haider Al-Murayati ^a, Zaid Al Rubaiee ^a, Marion Petrie ^b,
Alessandra Costanzo ^c, and Anders Pape Møller^a**

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

^b *Institute for Health and Society and Newcastle University Institute for Ageing,
Newcastle University, Campus for Ageing and Vitality*

Newcastle upon Tyne, NE4 5PL, UK

^c *Department of Biosciences, University of Milan, via Celoria 26, I-20133 Milan,
Italy*

Word count: 7598

Correspondence to HAM:

Tel: (+33) 1 69155688

Fax: (+33) 1 69155688

E-mail: haideryousif1967@gmail.com

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ABSTRACT

Animals may affect the integrity of their integument by the presence of microorganisms acquired from the surrounding environment. Pathogens such as microorganisms may exert intense selection on their hosts by reducing fecundity and survivorship. Bacteria are common within the body of all living organisms, but also on skin, scales, hair and feathers. Several bacterial taxa that live on feathers have the ability to degrade feather keratin and cause damage to feather structure. Development and maintenance of scales, feathers and hair are energy and time consuming because it is essential for fundamental functions of these structures. Rates of daily growth increments of feathers can be readily quantified from the alternating light and dark bands on feathers. We studied the relationship between the prevalence and the abundance of the bacterial community in differently coloured parts of the ocelli of the peacock's *Pavo cristatus* train and the rate of daily growth increments. We also studied the relationship between three different colour variables (θ , ϕ , and achieved r) for the different ocelli patches of the ocelli and the daily feather growth increments. The bacterial community in differently coloured parts of peacock ocelli differed significantly. The abundance of *Bacillus licheniformis* and *Paenibacillus* sp. was positively associated with wider daily growth increments, while the abundance of *Micromonospora* sp. and *Bacillus pumilus* was associated with reduced daily growth increments. The three colour variables showed considerable variation among individuals, although only the colour of the blue patch was negatively related to the width of feather increments. These findings are consistent with the hypothesis that different parts of ocelli harbour different kinds of bacteria differing in impact on their peacock hosts. These differences imply that the differently coloured parts of the ocelli reveal information on feather growth and the microbiome of signalling peacocks.

Keywords: bacteria; daily growth increments; feather; moult; ocelli, peacocks.

INTRODUCTION

All multi-cellular organisms are colonised by microorganisms both internally and externally (Marshall 2007), and this microbiome constitutes at the same time a means of defence, but also the potential for colonisation and development of colonies of pathogens that may cause disease and eventually reduce fecundity and viability (Peterson 1996). This relationship between diversity and abundance of microorganisms and their hosts relies to a large extent on the numerous habitats generated by scales, feathers and hair. The presence of these structures may enhance the defence against microorganisms by impairing the normal function of these tegumentary structures. Thus, thermoregulation, signalling and locomotion may all deteriorate due to the action of microorganisms. Regular moult and replacement of scales, feathers and hair may re-establish a surface that is not damaged by microorganisms, but also re-store the function of the surface. These structures include the generation of a microbiome that is mainly composed of taxa with benign relationships with their hosts.

Fish, reptiles, birds and mammals grow surface structures with bands that reflect the diurnal patterns of development with alternating light and dark bands that reveal temporal growth increments. In birds, feather growth is considered as a reflection for the quality of the individuals (Grubb 1995, 2006; Clarkson 2011; Saino *et al.* 2012, 2013). Each set of dark and light bands show the growth during a twenty-four-hour cycle, with the dark band representing growth during the day and the light bands representing growth during night (Riddle 1907, 1908; Michener and Michener 1938; Wood 1950; Murphy and King 1991). The width of these growth bands represents the rate of feather growth, and wider daily growth increments reflect faster feather growth. A number of studies has shown that feather growth is related to the nutritional status of the individual at the time when the feather was

produced, and this has been used as an estimator of overall condition and an assessment for ecological stressors (Grubb 1995). Several studies have shown a link between width of growth increments and body condition, implying that such growth increments can serve as useful tools in the study of physiological trade-offs (e.g. the better nutritional status of the individual, the wider the daily growth increments (Riddle 1908; Wood 1950; Grubb 1991; Grubb *et al.* 1998; Saino *et al.* 2014)).

The plumage of birds contains numerous microorganisms. Some taxa belonging to this bacterial community are opportunistic pathogens that may have detrimental effects on hosts (Scott 2001; Cogen *et al.* 2008), while others are the normal microflora with a beneficial and mutualistic relationship with the host (Tannock 1995). An important bacterial group is the feather-degrading bacteria (FDB), a polyphyletic assemblage of bacteria (Burt and Ichida 1999; Whitaker *et al.* 2005; Shawkey *et al.* 2007; Gunderson *et al.* 2009; Shawkey *et al.* 2009). Many studies suggest that these bacteria have the ability to degrade β -keratin that constitutes the majority of feather mass (Muza *et al.* 2000; Sangali and Brandelli 2000; Lucas *et al.* 2003; Gunderson *et al.* 2009; Shawkey *et al.* 2009). These microorganisms may by deteriorating feather structure reduce fitness of their hosts by reducing thermoregulatory efficiency, flight performance and visual signalling (Swaddle 1996; Clayton 1999; Shawkey *et al.* 2007).

Birds have in order to maintain feather condition and health evolved a complex system of behavioural and physiological defences like preening, sun bathing, dust bathing, use of secretions from the uropygial glands and immunity in order to reduce bacterial populations (Burt and Ichida 1999; Clayton 1999; Shawkey *et al.* 2003; Gunderson 2008; Proksch *et al.* 2008; Reneerkens *et al.* 2008). This is a significant investment in terms of time because birds devote on

average 9% of their daily time budget on maintenance behaviour (Cotgreave and Clayton 1994). Not surprisingly these defence mechanisms are energetically costly, and hence the benefits from such defences are traded against the costs (Croll and McLaren 1993; Carey 1996; Goldstein 1998; Hasselquist and Nilsson 2012; King and Swanson 2013).

Extravagant secondary sexual traits, such as the well-known peacock's train, are known to be honest signals allowing assessment of male quality (Andersson 1994). The elaborate train feathers are costly to produce and maintain because this long structure may be dragged on the ground exposing them to different kinds of microorganisms. Walther and Clayton (2005) showed that ornamented species devoted significantly more time to maintenance behaviour than non-ornamental species. For example, peacocks spent 15% of their total time budget on maintenance behaviour, while they spent just 7% on train display and 25% of their total grooming time on preening their trains (Walther 2003).

Scientists have long been fascinated by the bright colours of birds (Darwin 1871; Andersson 1994), highlighting the importance of bright colours in visual signalling (Burt 1986; Griffith *et al.* 2006; Hill and McGraw 2006). Visual signals play a major role in avian communication, primarily derived from the discrimination of colour intensity (Cuthill 2006; Håstad and Ödeen 2008) that occurs in numerous avian feather ornaments (Andersson 1994). Colour characteristics of secondary sexual characters may provide credible evidence about the condition of an individual due to differences in genetic quality (Hamilton and Zuk 1982; Figuerola *et al.* 1999), aggressiveness during contests (Alonso-Alvarez *et al.* 2004) or reproductive strategy (Badyaev and Hill 2002). Such colourful signals may give rise to intrasexual or intersexual selection (Andersson 1994).

Numerous studies suggest that feather-degrading bacteria may reduce feather-based communication by modifying feather colouration. Bluebirds (*Sialia sialis*) suffer from alteration of feather colour, reducing body condition and lowering reproductive success as a consequence of the presence of feather-degrading bacilli (Gunderson *et al.* 2009; Shawkey *et al.* 2007). Similarly, an experimental study conducted by Leclaire *et al.* (2014) showed that manipulation of bacterial load of feathers in captive feral pigeons (*Columba livia*) led to changes in the neck iridescent feathers (individuals of both sexes with lower bacteria load had more brightly coloured feathers). In contrast, in another experimental study performed on great tits (*Parus major*), Jacob *et al.* (2014) found no such relationship between bacterial load and feather colouration.

The objectives of this study were to investigate (1) the relationship between the abundance and the diversity of the bacterial community in peacock ornamental tail feathers and the daily rate of feather growth during the annual moult; and (2) the relationship between characteristics of the colouration of the brown, green and blue parts of the ocelli and daily growth increments. We did so by investigating a sample of feathers collected from a population of peafowl kept at a commercial farm as part of a long-term experiment to investigate the functional significance of the exaggerated train of peacocks.

MATERIALS AND METHODS

Anders Pape Møller and Marion Petrie conducted a captive breeding experiment during 1998-1999 in a commercial peacock farm (Norwich, UK) (see Hale *et al.* 2009 for further details). During the experiment, all individual peafowl were housed in large outdoor paddocks supplied with sufficient commercial poultry feed and water (*ad libitum*) free of any antibiotics. The peacocks were handled by the

same people, and they were allowed to range freely on the farm so they had free access to soil and vegetation making all individuals experience the same level of contamination by microorganisms as in the surrounding environment. Variation in susceptibility to infection among individuals could be attributed to the difference in resistance and general condition of each individual. At the beginning of the breeding season, each peacock was allocated to four peahens chosen randomly and kept in pens, with fathers and mothers randomly reassigned to mates between the two years in different pens.

The study was approved by the UK Home Office and the procedures adopted during the experiment did not show any negative effects on individuals included in this study.

At the beginning of the breeding season (spring 1999), 10 ocelli feathers were removed aseptically from each of the 46 peacocks by using a pair of sterile examination gloves and sharp scissors, and the removed feathers were placed in dry clean plastic bags. All samples were transported in a cool box to the laboratory and stored under the same conditions until processed.

A single ocellus feather was chosen randomly from the 10 feather samples that were collected from each individual (in total 46 individuals) to isolate bacteria and measure daily growth increments. All measurements were made blindly with respect to identity and phenotype of individuals.

Growth increment measurement

The width of daily growth increments was measured according to Takaki *et al.* (2001). HAM used the back side of the feathers (with the aid of a faint light) to measure the width of bands (Fig. 1). The feather was placed on white plain paper

fixed to a polystyrene board. HAM stuck insect pins through the paper at the distal edge of each growth band on the ocelli. After removal of the feather, HAM measured the distances between the first and last pin marks with a digital calliper to the nearest 0.01 mm (Mitutoyo CD- 6" BS). The mean width of each band was estimated by dividing the measured distance by the number of bands measured. All measurements were made blindly with respect to ornamentation or other phenotypic characters.

The measurements of the width of daily feather growth for 46 individuals were repeated twice on different days without any recent prior knowledge of the first set of measurements. All bands were measured by HAM to eliminate any variation in measurements due to among-observer variability.



Fig. 1. The back side of peacock train feathers showing daily growth increments (indicated by white arrows).

Bacterial isolation

HAM randomly selected feather samples for bacterial isolation from 30 of the 46 individuals that were included in this study (the other 16 individuals were not included for logistic reason). Furthermore, a second set of 10 feather samples was selected randomly from the same set of 30 individuals to estimate repeatability of bacterial isolation (Becker 1984; Falconer and Mackay 1996). Two different kinds of media were used for bacterial cultivation, Tryptic Soy Agar (TSA) and Feather Meal Agar (FMA). TSA is a rich medium on which most heterotrophic bacteria can grow allowing us to estimate the cultivable bacterial load. Meanwhile, HAM used FMA to quantify the load of feather-degrading bacteria because it is a highly selective growth medium where just keratinolytic bacteria can grow (containing only one source of carbon and nitrogen which is the keratin) (Williams *et al.* 1990; Sangali and Brandelli 2000; Shawkey *et al.* 2003, 2007, 2009). Cycloheximide was added to the both medium to prevent fungal growth (Smit *et al.* 2001).

Under septic conditions, small pieces from each differently coloured part of the ocelli were cut with a sterilized sharp blade and with the aid of sterilized forceps. The feather pieces were transferred to a sterilized pre-weighed eppendorf tube (2 ml), phosphate buffer saline (pH 7.2) were added corresponding to the sample feather (each mg feather sample equalled 100 μ l PBS). The eppendorf tube was vortexed three times (each cycle lasting 1 min) to enhance the detachment of bacteria from feather pieces (Saag *et al.* 2011). The two media were inoculated with 100 μ l PBS with a sterilised spreader, and a third plate inoculate with sterilized PBS as a control. Plates were incubated at 28°C for 3 days in the case of TSA, and for 14 days in the case of FMA. The colony-forming units (CFU) of each morphotype per plate were counted by using dissecting microscope, and HAM

distinguished the morphotypes on the basis of colony colour, shape, size, and presence or absence of glutinous aspects.

DNA extraction

For extraction of genomic DNA from bacterial isolate HAM use two different procedures.

A-Freeze-thaw protocol:

1- with a wooden toothpick a small part of a bacterial colony was transferred to an eppendorf tube (0.5 ml) containing 50 μ l Tris (10 μ M, pH 8.0) and crushed well.

2- To lyse the bacterial cells, a freezing and thawing cycle was performed three times by using liquid nitrogen and warm water bath alternately. Furthermore, and to ensure complete bacterial lysis, the tubes were put in a microwave at 270W for 5 to 6 s followed by 10 s of waiting for three times.

3- To collect the supernatant that contains the DNA the eppendorf tubes were centrifuged for five minutes at 12,000 x g in a microcentrifuge. The supernatant was transferred carefully without touching the pellet that contains cellular debris to a DNA free tube and stored at -20°C .

B- DNA Extraction kit:

Power Soil DNA isolation kit (MOBIO Laboratories, Inc. USA). Extraction of DNA was performed according to the protocol that is supplied with the kit.

PCR amplification of bacterial 16SrRNA gene

Amplification of the bacterial 16S rRNA gene were done by PCR. DNA isolated from samples was used as a template. The forward primers are the: 16S rDNA-27F

(5'-AGAGTTTGATCCTGGCTCAG-3'), 16S rDNA-63f (5'-CAGGCCTAACACATGCAAGTC-3') and the reverse primer 16S rDNA-1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 25 µl containing 16 µl ultrapure water, 4 µl 5x buffer, 0.4 µl dNTPs 10nM, 1 µl 10 µM 27F, 1 µl 10 µM 1492R, 0.2 µl Go Taq DNA polymerase and 3-5 µl genomic DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, denaturation at 94°C for 15 s, annealing for 30 s at 55°C and 1.30 min of primer extension at 72°C. The cycle of denaturation, annealing and elongation was repeated 35 times. A final elongation at 72°C for 7 min was then performed. The PCR products were sent to sequencing by Beckman Coulter Genomics.

Agarose electrophoresis

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

DNA sequencing

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

Reflectance measurements

To measure the reflectance spectra of the different coloured patches of the ocelli (brown, green, and blue) under different incident light angles (60° and 30°) (see Fig. 1 in chapter 4), Alessandra Costanzo used an Avantes dh 2000 spectrophotometer with a deuterium-halogen light to measure coloration of ocelli (<http://www.avantes.com/products/light-sources/item/237-avalight-d-h-s-deuterium-halogen-light-sources>). Collimating lenses (<http://www.avantes.com/products/fiber-optics/item/256-collimating-lens>) were mounted onto the ends of the reflection probes (<http://www.avantes.com/products/fiberoptics/item/245-reflection-probe-standard>) for both illumination (3 cm from the feather surface) and measurement (6 cm from the feather surface for 60° measurement and 3 cm from the feather surface for 30° measurement) of a spot of feather from the ocelli about 2 mm in diameter. The measurement was performed in a dark room. We took on average 5 scans at 800 ms integration time for the 60° measurements, and on average 05 scans at 150 ms integration time for the 30° measurements. The difference in the integration time did not affect the result of the measurements. Recalibration of the dark and white standards was done every 15 minutes. Each sample was measured twice, remounting it in the apparatus between measurements. We measured again those samples that deviated by more than 3 SD from the mean value. Reflectance spectra (see figure 2 chapter 4) were then processed according to the Tetrahedral colour space model (Stoddard and Prum 2008) to adopt the spectral sensitivity of the peacock. Feather colour was thus described by three colour variables: θ , which represents the visible light, ϕ , which accounts for the ultraviolet component and achieved r , which is a measure of colour saturation.

Statistical analyses

Repeatability estimation for feather band growth measurements and bacterial isolation from ocelli on two different media according to the equations in Becker (1984) and Falconer and Mackay (1996).

The relationship between the width of growth increments and the abundance of different bacterial taxa in differently coloured parts of the ocelli including the three colour variables was made using standard least squares regression. All statistical analyses were made using JMP (SAS 2012).

RESULTS

Growth increments

The repeatability of measurements of daily growth increments in train feathers of 46 individual peacocks was high at R (SE) 0.88 (0.033), $F = 15.78$, $df = 45, 46$, $P < 0.0001$. The width of daily growth increments in peacock ocelli ranged from 1.91 to 4.56 mm with a mean (SE) of 2.73 (0.09) mm.

Bacterial community of ocelli

The degree of repeatability for bacterial recovery on two different media showed a high value in all cases except for the number of species recovered from TSA plates of the brown and blue areas of the ocelli (repeatability of total number of bacteria and the number of bacterial species on TSA and FMA media from differently coloured parts of the ocelli are reported in Tables S3A and S3B).

Bacterial recovery on the two different media showed heterogeneity in the abundance of bacteria in different parts of the ocelli (Tables S4A and S4B). Furthermore, there was a variable number of bacterial taxa for differently coloured areas of the ocelli. Among the eight bacterial taxa recovered on TSA medium, HP-

F isolates (Unidentified bacterium) formed the largest percentage in the brown and the green area of the ocelli (19% and 20%, respectively), while the largest group in the blue area was 19% for *Paenibacillus* sp. (HP-D). Meanwhile, in FMA medium HYA recovered just seven bacterial taxa where *Streptomyces marokkonensis* (HP-AF) was the most predominant bacterial taxon in brown, green and blue areas of the ocelli (29, 35 and 27%, respectively) (Tables S5A and S5B). The different bacterial taxa were widely distributed across the phylogenetic tree of bacteria (Fig. S1)

Two bacterial taxa that were recovered on TSA medium from the brown area were significantly related to the mean width of daily growth increments (whole model statistics: $F = 7.34$, $df = 2, 29$, $r^2 = 0.35$, $P = 0.0028$), the number of *Bacillus licheniformis* (HP-A1) was positively related to the mean daily growth increment ($F = 5.78$, $df = 1, 29$, $P = 0.023$, estimate (SE) = 0.09 (0.04)) while *Bacillus pumilus* (HP-G1/G2) showed a negative relationship ($F = 12.05$, $df = 1, 29$, $P = 0.002$, estimate (SE) = - 0.1 (0.03)) (Table 1).

Table 1. Relationship between the abundance of two bacterial taxa recovered in TSA medium from the brown area of ocelli and daily growth increments in ocelli of 30 peacocks.

Term	Estimate	SE	<i>t</i>	<i>P</i>
<i>Bacillus licheniformis</i>	0.09	0.037	2.40	0.0233
<i>Bacillus pumilus</i>	- 0.10	0.028	- 3.47	0.0018

In the blue area, just four bacterial taxa among the eight bacterial taxa that had been isolated on TSA medium were significantly related to the mean daily growth increment. Four bacterial taxa showed heterogeneous relationships with mean daily growth increments being positively related to the abundance of *Bacillus licheniformis* (HP-A1) and *Paenibacillus* sp. (HP-D), while the abundance of Unidentified bacterium (HP-F) and *Bacillus pumilus* (HP-G1/G2) were negatively related to the width of daily growth increments ($F = 4.04$, $df = 4$, 29 , $r^2 = 0.39$, $P = 0.012$, Table 2).

Table 2. Relationship between the abundance of different bacterial taxa recovered in TSA medium from the blue area of ocelli and daily growth increments in feathers of 30 peacocks.

Term	Estimate	SE	<i>t</i>	<i>P</i>
<i>Bacillus licheniformis</i>	0.13	0.04	3.04	0.0054
<i>Paenibacillus</i> sp.	0.10	0.04	2.47	0.0209
Unidentified bacterium	- 0.09	0.04	- 2.24	0.0340
<i>Bacillus pumilus</i>	- 0.09	0.04	- 2.22	0.0354

Just two bacterial taxa isolated on FMA medium from the differently coloured parts of the ocelli were significantly related to the mean width of daily growth increments. *Micromonospora* sp. (HP-GF) from the brown and blue area were significantly negatively related to rate of feather growth (Fig. 2; $F = 5.58$, $df = 1$, 29 , $r^2 = 0.17$, $P = 0.025$, estimate (SE) = -0.11(0.05); Fig. 3: $F = 4.41$, $df = 1$, 29 , $r^2 = 0.14$, $P = 0.045$, estimate (SE) = -0.14(0.06)), while the abundance of *Bacillus licheniformis* (HP-CF) from the green area was positively related to mean width of daily feather growth increments (Fig. 4: $F = 5.95$, $df = 1$, 29 , $r^2 = 0.18$, $P = 0.021$, estimate (SE) = 0.19 (0.08)).

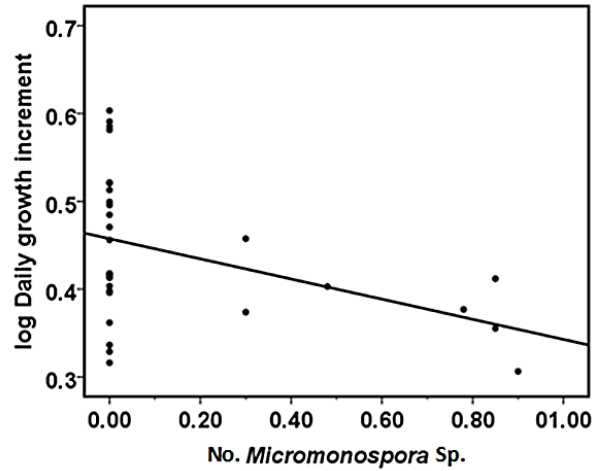


Fig. 2. Daily growth increments in peacock feathers in relation to the abundance of *Micromonospora* sp. in the brown part of ocelli for 30 peacocks. The line is the linear regression line.

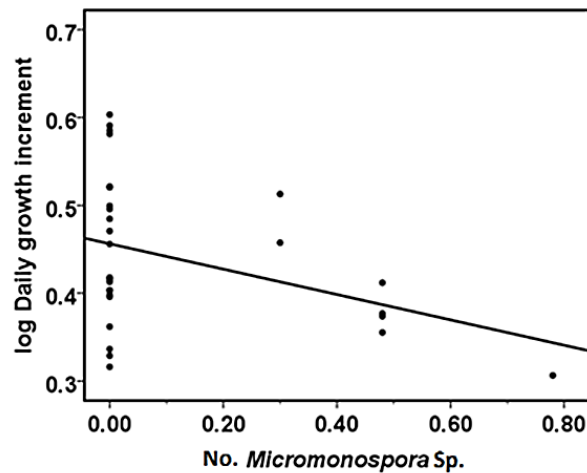


Fig. 3. Daily growth increments in relation to the abundance of *Micromonospora* sp. in the blue part of ocelli for 30 peacocks. The line is the linear regression line.

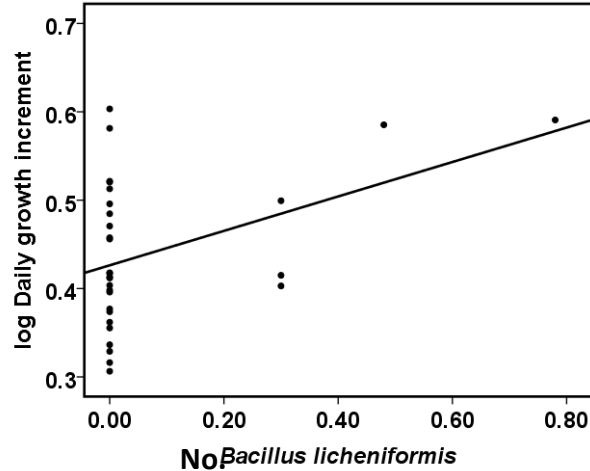


Fig. 4. Daily growth increments in relation to the abundance of *Bacillus licheniformis* in the green part of ocelli for 30 peacocks. The line is the linear regression line.

Three colour variables in relation to feather growth increments

The three colour variables (θ , ϕ , and achieved r) that were measured under different incident light angles (60° and 30°) for the different coloured patches of the ocelli (brown, green, and blue) showed considerable variation among individuals. The coefficients of variation for reflectance spectra were mostly high, providing both males and females with considerable variation for evaluation of potential competitors and mates (Table 3).

Among the three colour variables (θ , ϕ , and achieved r) that were measured at two different angles for the three coloured patches of peacock ocelli, we found that just the value of θ and achieved r at 30° of the blue patch were negatively related to the mean feather increments respectively ($F = 9.11$, $df = 1, 45$, $P = 0.0043$, estimate (SE) = -0.13 (0.04); $F = 10.34$, $df = 1, 45$, $P = 0.0025$, estimate (SE) = -0.73 (0.23); whole model statistics: $F = 5.62$, $df = 2, 45$, $r^2 = 0.21$, $P = 0.0068$).

Table 3. Mean, SE, range and coefficient of variation (CV) for the three colour variables (θ , ϕ , and achieved r) that were measured under different incident light angles (30° in (A) and (60° in (B)) for the different coloured patches of ocelli (brown, green, and blue) of 46 individual peacocks.

(A)

30°		Mean	SE	Minimum	Maximum	CV (%)
Brown	θ	-0.18	0.01	-0.37	0.06	47.64
	ϕ	-0.84	0.01	-0.94	-0.75	5.92
	Achieved r	0.64	0.00	0.58	0.69	2.68
Green	θ	0.69	0.43	-3.13	3.12	417.93
	ϕ	-0.37	0.01	-0.55	-0.23	19.42
	Achieved r	0.78	0.00	0.72	0.84	3.18
Blue	θ	-2.59	0.06	-2.80	0.00	15.46
	ϕ	0.40	0.03	0.00	0.80	45.60
	Achieved r	0.34	0.01	0.00	0.46	22.43

(B)

60°		Mean	SE	Minimum	Maximum	CV (%)
Brown	θ	-0.25	0.02	-0.58	-0.04	48.16
	ϕ	-0.88	0.01	-1.20	-0.74	10.24
	Achieved r	0.61	0.01	0.52	0.67	5.96
Green	θ	-2.83	0.01	-3.11	-2.69	3.48
	ϕ	-0.15	0.01	-0.32	-0.01	55.68
	Achieved r	0.75	0.01	0.60	0.82	6.43
Blue	θ	-2.35	0.11	-2.73	0.00	31.82
	ϕ	0.56	0.04	0.00	1.12	47.71
	Achieved r	0.50	0.03	0.00	0.87	38.91

DISCUSSION

Extravagant secondary sexual characters from feathers are vulnerable to the effects of microorganisms from the surrounding environment. Soil contains a diversity of feather degrading microorganisms (Clayton 1999; Sangali and Brandelli 2000; Lucas *et al.* 2003; Riffel *et al.* 2003; Shawkey *et al.* 2003). These bacterial communities depend on soil composition (Burt and Ichida 1999, 2004; Bisson *et al.* 2007). Here we tested the hypothesis that differently coloured parts of the ocelli of peacocks harboured different bacterial communities that affected the growth of feathers during the annual moult. We showed that there were differences in bacterial communities between brown, green and blue parts of ocelli, and that mean the daily rate of feather growth was related to the abundance of certain bacteria. Here we discuss the heterogeneity in these relationships among different colour variables (θ , ϕ , and achieved r), but also among different bacterial taxa.

Time and energy allocated to maintenance of plumage may reduce the negative effects of bacteria on feathers at the expense of other vital functions like growth and reproduction. Peacocks spend a substantial part of their time budget (15%) on maintenance (Walther 2003). Leclaire *et al.* (2014) showed that individuals with elevated bacterial load will increase the quantity of preen secretion and preening time, which is an energetically costly behaviour in terms of production and time (Goldstein 1988; Redpath 1988).

All feathers were collected on a single day thereby avoiding any seasonal effects. There may be other limitations related to this study, for instance the feather samples were stocked for a long time, which could have resulted in a change in the microbial community over time. However, there must have shown real differences among individuals at the start of the study as their feathers were all held under the same controlled conditions. Even though the number of bacteria may have changed

over time, the feathers were collected after ‘infection’ by the bacterial communities following moult, and the bacterial communities remaining must at some level represent the individual responses to such infection since it is likely that they were exposed to the same environmental conditions at the outset.

This study revealed heterogeneous relationships between the abundance of different bacterial taxa in different parts of the ocelli and the rate of feather growth. Bacteria recovered from the brown part of the ocelli based on TSA medium showed that two bacterial taxa were related to the mean width of daily growth increments (Table 1). The mean abundance of *Bacillus licheniformis* was positively related to the mean width of feather increments, while the abundance of *Bacillus pumilus* showed a negative correlation. Both bacterial taxa are known to produce keratinase that can degrade feather keratin (Williams 1990; Burt and Ichida 1999; El-Refai *et al.* 2005; Ramnani *et al.* 2005; Rajput and Gupta 2013). Furthermore, these bacterial taxa can produce a wide range of extracellular substances that show antibiotic activity. *Bacillus licheniformis* produces different kinds of antimicrobial substances (Callow and Work 1952; Simlot *et al.* 1972; Haavik 1974). These antimicrobial substances have a wide range of effects against bacterial taxa (*Bacillus sp.*, *Corynebacterium sp.*, *Enterococcus sp.* and *Mycobacterium sp.*), amoebae (Gálvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel *et al.* 2004). *Bacillus pumilus* also produce such antimicrobial substances, but it mainly acts as an antifungal substance against bacterial taxa (Leifert *et al.* 1995; Bottone and Peluso 2003; Sawale *et al.* 2014). The positive relationship between *Bacillus licheniformis* and mean band width indicates that an increase in the abundance of this bacterium will increase with daily growth increments implying high quality feathers (Grubb 2006). In contrast, an increase in the abundance of *Bacillus pumilus* reduces band width possibly due to the harmful effects of

degradation caused by these bacteria. This is consistent with studies showing that infection of birds with malaria (Marzal *et al.* 2013; Coon *et al.* 2016) and mites (Pérez-Tris *et al.* 2002) reduces growth rate of feathers. Since both bacteria are known to secrete degrading enzymes and antibiotic substances, the difference in relationship between feather band growth and abundance of bacteria could be due to differences in bacterial characteristic and their extracellular materials.

Bacterial recovery based on TSA medium showed that four bacterial taxa (*Bacillus licheniformis*, *Bacillus pumilus*, *Paenibacillus sp.* and Unidentified bacterium) from the blue part of the ocelli are related to mean width of daily growth increments (Table 2). We found the same relationship for *Bacillus licheniformis* and *Bacillus pumilus* with mean feather band width, while *Paenibacillus sp.* was related positively and Unidentified bacterium negatively to mean band width. *Paenibacillus sp.* are widespread in many different habits (water, soil, waste) and they are known to produce a wide range of antimicrobial substances that can affect a wide spectrum of microorganisms (mostly Gram-positive and Gram-negative bacteria) and even anaerobic pathogens as it can act as an antifungal agent (Girardin *et al.* 2002; Guo *et al.* 2012; Kai *et al.* 2013). The prevalence of *Paenibacillus sp.* could interact synergistically with *Bacillus licheniformis* to minimize the harmful effects of degradation caused by other bacterial taxa.

The isolation of bacteria from brown and blue parts of the ocelli based on FMA medium showed that the abundance of *Micromonospora sp.* is negatively related to the width of daily growth increments. *Micromonospora sp.* are widespread in soil and water, and they are known to have the ability to produce keratinase that can degrade feather keratine (El-Bondkly and El-Gendy 2010). Thus, this bacterial taxon could deteriorate feather integrity and consequently lead

to allocation of more energy and time to feather maintenance. *Bacillus licheniformis* recovered from FMA medium showed a positive relationship with mean width of daily growth increment resembling what we found for TSA medium.

The relationship between the three different colour variables and the feather growth increment revealed that the blue patch is more closely linked to band width than other two coloured patches. The results indicate that θ which represents the visible light and the value of achieved r that represents colour saturation are negatively related to feather growth increments. In other words, the decrease in these variables is associated with an increase in the rate of feather growth. One possible explanation for this is that the reduction in the colour variable could occur because these birds increased daily feather growth to compensate the negative influence of bacteria on feather integrity. These findings parallel to some extent those of Griggio *et al.* (2009), who found that blue tits (*Cyanistes caeruleus*) when induced to molt at a faster rate tended to produce feathers with reduced brightness. Structural colouration and pigment-based colouration can serve as honest advertisements of individual quality (Keyser and Hill 1999; Keyser and Hill 2000; Matrková and Remeš 2012). Different studies found a relationship between structural colouration and feather growth rate in different bird species (Keyser and Hill 1999; Doucet 2002). Further experiments in vivo or in vitro are necessary in order to clarify how these bacterial taxa interact and how they interact with the individual host.

In conclusion, the diverse bacterial community in the differently coloured parts of the ocelli of peacocks may differentially influence the growth of feathers. Our results are consistent with studies indicating that feather growth rate is related to the abundance of endoparasites or ectoparasites. The presence of *Bacillus*

licheniformis and *Paenibacillus* sp. was associated with wider growth increments implying that these bacterial taxa enhance feather growth by successfully competing against other harmful bacterial taxa. In contrast, the prevalence of other bacterial taxa like *Micromonospora* sp. and *Bacillus pumilus* was linked to a reduction in feather growth rate probably through alterations in the integrity of feathers by degradation. This may subsequently increase the allocation of energy and time to maintenance of feather quality. Alternatively, harmful bacteria may give rise to a trade-off between anti-bacterial defences and development of high quality and strong train feathers that are essential for mate acquisition during the annual lekking season.

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SUPPLEMENTARY MATERIAL

Fig. S1.

Phylogenetic tree of bacterial taxa isolated from the train feathers of peacocks.

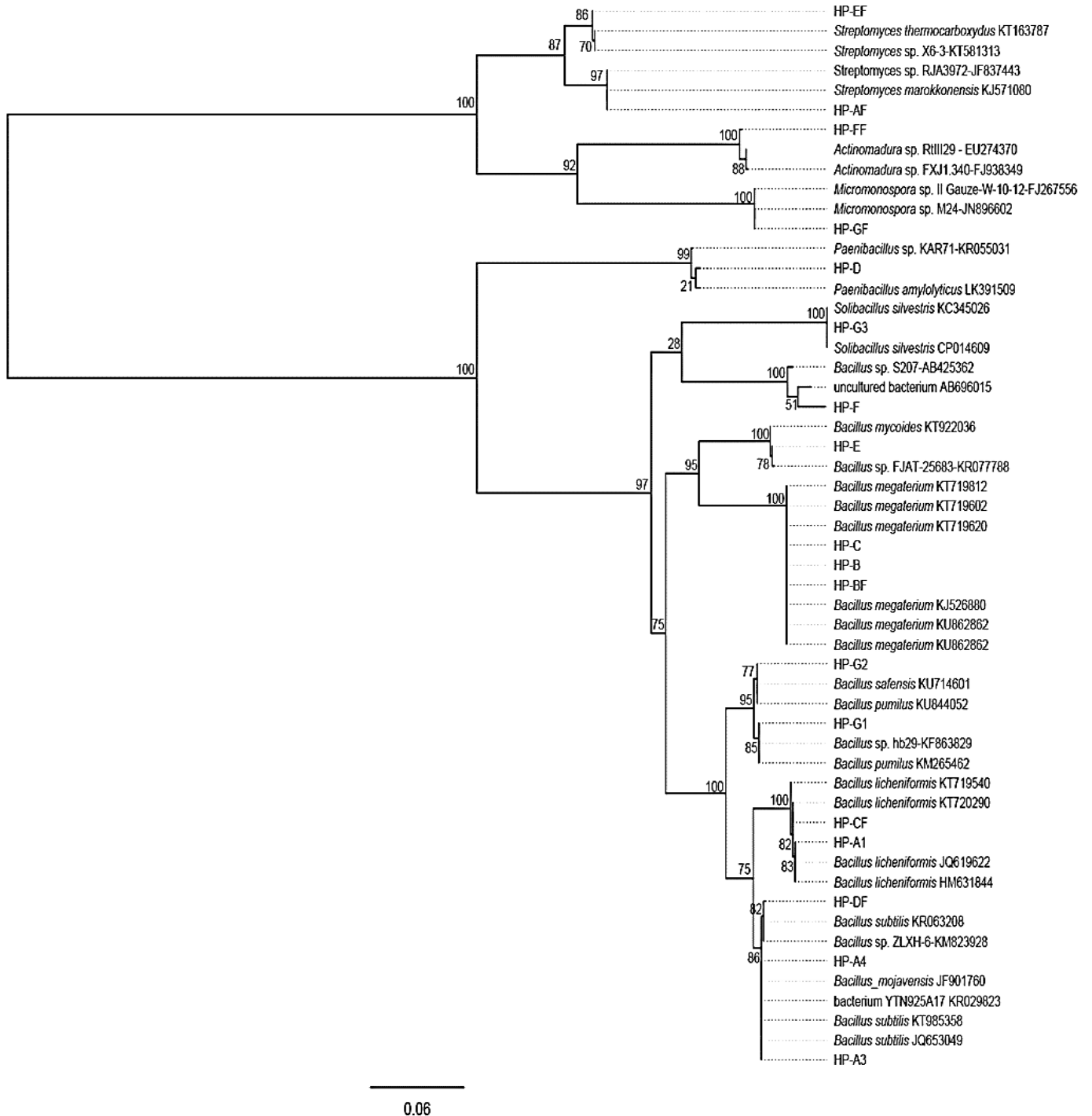


Table S1.

Repeatability (*R*) of the degree of damage to differently coloured parts of ocelli and area of ocelli of 30 peacocks.

Character	<i>R</i> ± SE	<i>F</i>	df	<i>P</i>
Brown	0.979 ± 0.008	94.517	29,59	< 0.0001
Blue	0.889 ± 0.039	17.035	29,59	< 0.0001
Green	0.904 ± 0.034	20.035	29,59	< 0.0001
Ocelli area	0.958 ± 0.015	47.648	29,59	< 0.0001

Table S2.

Repeatability (R) of force required to break barbs from feathers with ocelli from 46 peacocks.

$R \pm SE$	F	df	P
0.443 ± 0.090	3.389	45,137	< 0.0001

Table S3.

(A) Repeatability (*R*) of total number of bacteria and (B) The total number of bacterial species on TSA and FMA media for differently coloured parts of the ocelli for 30 peacocks.

(A)

Character	Medium	$R \pm SE$	F	df	P
Brown	TSA	0.528 ± 0.240	3.238	9,19	0.0406
Brown	FMA	0.917 ± 0.053	23.124	9,19	< 0.0001
Blue	TSA	0.870 ± 0.081	14.372	9,19	0.0001
Blue	FMA	0.756 ± 0.143	7.186	9,19	0.0024
Green	TSA	0.924 ± 0.049	25.416	9,19	< 0.0001
Green	FMA	0.754 ± 0.144	7.120	9,19	0.0025

(B)

Character	Medium	$R \pm SE$	F	df	P
Brown	TSA	0.452 ± 0.265	2.648	9,19	0.0726
Brown	FMA	0.545 ± 0.234	3.398	9,19	0.0350
Blue	TSA	0.398 ± 0.281	2.323	9,19	0.1027
Blue	FMA	0.886 ± 0.071	16.611	9,19	0.0001
Green	TSA	0.537 ± 0.237	3.323	9,19	0.0375
Green	FMA	0.947 ± 0.034	36.889	9,19	< 0.0001

Table S4.

Log-transformed total number of bacterial colonies (A) and total number of bacterial species (B) from TSA and FMA media for differently coloured parts of ocelli from 30 males.

(A)

Area	TSA		FMA	
	Mean \pm SE	Range	Mean \pm SE	Range
Brown	1.58 \pm 0.08	0.48-2.50	0.44 \pm 0.08	0-1.45
Green	1.34 \pm 0.08	0.60-2.32	0.25 \pm 0.07	0-1.28
Blue	1.38 \pm 0.09	0.30-2.39	0.36 \pm 0.08	0-1.66

(B)

Area	TSA		FMA	
	Mean \pm SE	Range	Mean \pm SE	Range
Brown	0.84 \pm 0.02	0.48-0.95	0.28 \pm 0.05	0-0.70
Green	0.78 \pm 0.02	0.30-0.90	0.17 \pm 0.04	0-0.70
Blue	0.77 \pm 0.03	0.30-0.95	0.25 \pm 0.05	0-0.78

Values are means, SE and range.

1 **Table S5.**

2 **Identity and abundance of bacterial species on (A) TSA and (B) FMA media. Values are means, standard**
 3 **errors, range and percentage of occurrence for differently coloured parts of ocelli from 30 peacocks.**

4

5 **(A)**

Bacterial species	Brown area			Green area			Blue area		
	Mean ± SE	Range	%	Mean ± SE	Range	%	Mean ± SE	Range	%
HP-A1 <i>Bacillus licheniformis</i>	0.62 ± 0.07	0-1.41	13	0.42 ± 0.06	0-1.15	11	0.57 ± 0.06	0-1.26	15
HP-A3/A4 <i>Bacillus subtilis</i>	0.22 ± 0.04	0-0.60	4	0.12 ± 0.04	0-0.60	3	0.18 ± 0.04	0-0.78	5
HP-B/C <i>Bacillus megaterium</i>	0.93 ± 0.09	0-2.05	19	0.67 ± 0.09	0-1.48	18	0.73 ± 0.09	0-1.88	19
HP-D <i>Paenibacillus</i> sp.	0.89 ± 0.08	0-2.03	18	0.72 ± 0.08	0-1.93	19	0.75 ± 0.09	0-2.00	19
HP-E <i>Bacillus mycoides</i>	0.14 ± 0.04	0-0.60	3	0.04 ± 0.02	0-0.48	1	0.06 ± 0.03	0-0.48	2
HP-F Unidentified bacterium	0.93 ± 0.08	0-1.76	19	0.73 ± 0.08	0-1.71	20	0.66 ± 0.09	0-1.46	17
HP-G1/G2 <i>Bacillus pumilus</i>	0.85 ± 0.09	0-1.56	17	0.69 ± 0.07	0-1.32	19	0.65 ± 0.09	0-1.45	17
HP-G3 <i>Solibacillus silvestris</i>	0.39 ± 0.06	0-1.15	8	0.32 ± 0.06	0-1.46	9	0.31 ± 0.06	0-1.18	8

6 **(B)**

Bacterial species	Brown area			Green area			Blue area		
	Mean \pm SE	Range	%	Mean \pm SE	Range	%	Mean \pm SE	Range	%
HP-AF <i>Streptomyces marokkonensis</i>	0.21 \pm 0.06	0-0.90	29	0.13 \pm 0.05	0-0.85	35	0.17 \pm 0.06	0-1.32	27
HP-BF <i>Bacillus megaterium</i>	0.18 \pm 0.05	0-0.70	26	0.06 \pm 0.03	0-0.70	18	0.14 \pm 0.05	0-1.00	23
HP-CF <i>Bacillus licheniformis</i>	0.09 \pm 0.04	0-0.95	13	0.07 \pm 0.03	0-0.78	20	0.12 \pm 0.04	0-0.78	18
HP-DF <i>Bacillus subtilis</i>	0.05 \pm 0.02	0-0.48	7	0.02 \pm 0.02	0-0.48	4	0.03 \pm 0.02	0-0.60	5
HP-EF <i>Streptomyces thermocarboxydus</i>	0.03 \pm 0.03	0-0.95	5	0.02 \pm 0.02	0-0.60	6	0.03 \pm 0.02	0-0.30	5
HP-FF <i>Actinomadura</i> sp.	0.00 \pm 0.00	0-0.00	0	0.00 \pm 0.00	0-0.00	0	0.04 \pm 0.04	0-1.08	6
HP-GF <i>Micromonospora</i> sp.	0.15 \pm 0.05	0-0.90	21	0.06 \pm 0.03	0-0.70	18	0.11 \pm 0.04	0-0.78	17

7

8 **Table S6.**

9 **The abundances of different bacterial taxa from differently coloured**
 10 **parts of ocelli in relation to area of ocelli from 30 peacocks.**

Area	Bacterial sp.	<i>F</i>	Estimate ± SE	<i>P</i>
Brown	HP-A1 <i>Bacillus licheniformis</i>	14.41	5.13 ± 1.35	0.001
	HP-G1/G2 <i>Bacillus pumilus</i>	13.34	- 2.49 ± 0.68	0.001
Green	HP-AF <i>Streptomyces marokkonensis</i>	1395	5.61 ± 1.5	0.001
	HP-CF <i>Bacillus licheniformis</i>	16.46	- 9.49 ± 2.34	0.0005
	HP-GF <i>Micromonospora</i> sp.	4.91	- 4.68 ± 2.11	0.037
Blue	HP-CF <i>Bacillus licheniformis</i>	4.80	3.41 ± 1.55	0.039
	HP-A1 <i>Bacillus licheniformis</i>	10.16	- 4.08 ± 1.28	0.004

11

12

13 **Table S7.**

14 **Mean force required to break barbs of ocelli feathers from 30 peacocks**
15 **in relation to abundance of different bacterial taxa.**

16

Area	Bacterial sp.	<i>F</i>	Estimate ± SE	<i>P</i>
Brown	HP-CF <i>Bacillus licheniformis</i>	4.28	29.19 ± 14.11	0.049
	HP-GF <i>Micromonospora</i> sp.	24.40	45.94 ± 9.30	< 0.0001
Blue	HP-AF <i>Streptomyces marokkonensis</i>	11.45	-34.58 ± 10.22	0.002

17

18 **Table S8.**
19 **Frequency (%) of degree of loss of barbs from the upper part of ocelli from 46**
20 **peacocks.**

21

Degree of loss of barbs from the upper part of ocelli	Frequency	%
0	6	13
1	4	8
2	22	48
3	12	26

22

23 **Table S9.**

24 **Frequency (%) of degree of degradation of differently coloured parts of ocelli**
25 **from 46 peacocks.**

26

Degree of degradation	Brown		Blue		Green	
	frequency	%	frequency	%	frequency	%
0	9	20	20	43	40	87
1	20	43	18	39	3	7
2	12	26	5	11	3	6
3	5	11	3	7	0	0

27

Titre : Diversité de la communauté bactérienne et caractères sexuels secondaires chez le paon

Mots clés : *Pavo cristatus* ; Diversité bactérienne ; Barbules ; Barbes ; Dégradation des plumes ; Incréments de croissance quotidienne ; Coloration des plumes ; Traîne paon ocelles ; Sexe ratio ; Caractère sexuel secondaire.

Résumé: Les plumes d'oiseaux abritent de nombreux microorganismes qui pourraient être acquis dans l'environnement, ces microorganismes pouvant exercer une sélection intense sur leurs hôtes en réduisant leur fécondité et leur survie. Plusieurs taxons bactériens qui vivent sur des plumes ont la capacité de dégrader la kératine des plumes et causent des dommages à leur structure et peuvent modifier aussi leur coloration. Les oiseaux utilisent des signaux visuels tels que des couleurs vives ou des ornements exagérés pour la communication socio-sexuelle ainsi que la reconnaissance des espèces. Seuls les individus en bonne santé sont capables de produire des caractères sexuels secondaires exagérés et restent résistants aux parasites débilissants. Le paon (*Pavo cristatus*) est une espèce polygame qui a plusieurs décorations exagérées, les caractères sexuels secondaires les plus remarquables du paon sont leur traîne décorée avec des ocelles magnifiques qui contiennent trois couleurs irisées différentes. Grâce à une technique basée sur la culture, j'ai

isolé la communauté bactérienne des plumes de différentes parties colorées des ocelles de la traîne du paon. L'étude révèle qu'il y a eu une répartition hétérogène des bactéries parmi les différentes parties colorées des ocelles. L'abondance et la prévalence des taxa bactériens spécifiques étaient liées au degré de dégradation des plumes, à l'expression de différents caractères sexuels secondaires, à des changements dans la coloration des ocelles et à l'augmentation de la croissance quotidienne des plumes. En outre, nous avons constaté un petit effet de l'expression de caractères sexuels secondaires sur la proportion sexuelle des couvées avec un biais en faveur des individus masculins.

Les travaux présentés dans cette thèse fournissent des preuves que les ocelles de plumes peuvent être considérés comme un signal fiable de la diversité et de l'abondance de bactéries chez le paon. En conséquence, ils représentent une indication pour la qualité individuelle, ce qui permet aux femelles de choisir des mâles avec une communauté bactérienne spécifique.

Title : Diversity of the bacterial community and secondary sexual characters in the peacock

Keywords : *Pavo cristatus*; Bacterial diversity; Barbules; Barbs; Feather degradation; Daily growth increments; Feather colouration; peacock train ocelli; sex ratio; secondary sexual character.

Abstract: Bird feathers harbour numerous microorganisms that could be acquired from the surrounding environment, these microorganisms may exert intense selection on their hosts by reducing fecundity and survivorship. Several bacterial taxa that live on feathers have the ability to degrade feather keratin and cause damage to feather structure and may alter the feather colouration. Birds use visual signals such as bright colours or exaggerated ornamentation for socio-sexual communication as well as species recognition. Only healthy individuals are able to produce exaggerated secondary sexual characters and still remain resistant to debilitating parasites. Peacocks (*Pavo cristatus*) is a polygamous species that have different exaggerated ornamentation, the most notable secondary sexual characters of the peacock are their long-decorated trains that comprise the magnificent ocelli which contain three different iridescent colours.

Through a culture based technique we isolate feather bacterial community from differently coloured parts of the ocelli of the peacock's train. The study reveal that there was a heterogeneous distribution of bacteria among the differently coloured parts of ocelli. The abundance and prevalence of specific bacterial taxa was related to the degree of feather degradation, expression of different secondary sexual character, changes in ocelli colouration and daily growth increment. Furthermore, we found a small effect of the expression of secondary sexual characters on biasing of brood sex ratio towards production of more sons than daughters.

The work presented in this thesis provide evidence that feather ocelli may consider as a reliable signal of the diversity and the abundance of bacteria in peacock and in consequence indication for the individual quality and that allowing the choosy females to pick males with a specific bacterial community.