

Article

Not so black, not so white: differences in microorganism load of contiguous feathers from white stork chicks

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Abstract

Many organisms are characterized by strikingly contrasting black and white coloration, but the function of such contrasts has been inadequately studied. In this article, we tested the function of black and white contrasting plumage in white stork *Ciconia ciconia* chicks. We found greater abundance and diversity of microorganisms on black compared with adjacent white feathers. In addition, nest size was positively correlated with the abundance and diversity of microorganisms on white feathers. Flight initiation distance (FID), defined as the distance at which adult white storks took flight when approached by a human, was negatively correlated with most measurements of microorganism abundance. Breeding success was generally positively correlated with the abundance and diversity of microorganisms on black feathers. The feather growth rate was positively correlated with some and negatively correlated with other measurements of microbial abundance and diversity. Finally, chick growth was negatively correlated with the number of microbial species on black feathers and positively with the abundance and diversity of microorganisms on white feathers. These findings are consistent not only with the role of microorganisms in the maintenance of a benign microbial environment which differs between black and white feathers, but also with the hypothesis that several taxa of microorganisms found in black and white plumage are virulent, with negative effects on the fitness of their hosts.

Key words: accumulation of microorganisms, *Ciconia ciconia*, fungi, microbiome

Microorganisms are ubiquitous, colonizing the tegument of the bodies of animals, and thus may affect their hosts. In particular, abundant benign microorganisms protect host individuals from other pathogenic microorganisms (e.g., Hubálek 1976, 1978; Brook 1999; Riley and Wertz 2002; Benskin et al. 2009, 2015; Van Veelen et al. 2017; Javůrková et al. 2019a). Microorganisms play a vital role in the microbiome of their hosts (Benskin et al. 2009, 2015; Ezenwa et al. 2012; Jacob et al. 2015), but also mediate the hosts'

life-history traits (Salyers and Whitt 2002; Benskin et al. 2009, 2015; Al Rubaiee et al. 2018), including the risk of predation and parasitism (Møller et al. 2011). In particular, hair, feathers, and nesting material as an organic structure may contain large numbers of diverse microorganisms that play an important role in mediating life history (Shawkey et al. 2007; Gunderson 2008; Gunderson et al. 2009; Saag et al. 2011). The re-used nests of birds may be a source of bacteria that colonize and develop within nestlings (Zablótni

et al. 2020). Bacteria and fungi are well known for their negative impact on the fitness of birds through increasing the degradation and impairing the condition of feathers (Kent and Burt 2016).

Therefore, animals have evolved defense mechanisms that limit the colonization and development of fungi and bacteria on their body surfaces. For instance, Javůrková et al. (2019b) found that a large proportion of feather-degrading bacteria in the feather microbiome of birds is composed of bacteriocin-producing bacteria (i.e., potentially beneficial bacteria). Another mechanism of this type is the production of melanin and its deposition in skin, hair, or feathers (e.g., Mackintosh 2001). Feather coloration may affect the microbial community; several studies have shown that increased melanization of plumage may be selected to minimize microbial damage, as darker feathers are more resistant than white (Shawkey et al. 2007; Peele et al. 2009; Saag et al. 2011; Ruiz-De-Castañeda et al. 2012). Melanin plays an important role in reducing the effects of oxidative stress and boosting immune support capacity (Moreno and Møller 2006). It may also play a significant role in the maintenance of functional plumage. Because eumelanin granules not only protect the plumage of birds from wear (Burt 1986; Jacquin et al. 2011) but also prevent microorganisms from destroying the plumage of nestlings, plumage may serve parents as an indicator of the quality of their offspring. Therefore, we predicted that black feathers are characterized by a higher level of resistance to microbial destruction. Hence, we expected lower levels of relative abundance and diversity of microorganisms in black than adjacent white feathers in the same individual bird. Nevertheless, this difference had never been tested using birds with black and white feathers.

Feather-degrading bacteria and fungi are common in birds with black and white feathers, with the latter being less resistant to feather-degrading microorganisms than the former (Ruiz-De-Castañeda et al. 2012). Due to differences in the susceptibility of black and white feathers to degradation, feathers should differ in their ability to signal inherent plumage quality. Adjacent black and white feathers are common among nestling and adult birds alike; the difference in coloration between feathers may constitute a signal (Fitzpatrick 1998a, 1998b), which may not be restricted to sexual selection, but may also signify offspring quality (Wright and Leonard 2002).

The white and black coloration characteristic of feathers of many bird species differs in terms of effectiveness of defense against degradation. For example, differences between black and white feathers have been described in the black and white pied flycatcher *Ficedula hypoleuca* (Ruiz-De-Castañeda et al. 2012). However, the density and diversity of microorganisms have never been tested in terms of black and white coloration. Another example of a bird with black (with a high content of eumelanin) and white (without eumelanin) wing coverts is the white stork *Ciconia ciconia*. This is a large migratory bird, belonging to the family Ciconiidae, with iridescent black wing feathers (primaries, secondaries, rectrices, and wing coverts) that contrast with the bright white feathers of the head, neck, belly, back, and tail, in addition to some white wing coverts which neighbor black ones (Cramp 1977). White storks nest close to humans and build their nests on trees, roofs of buildings, chimneys, electricity pylons, and other human-made structures near meadows, ponds, and streams (Tryjanowski et al. 2006, 2009; Tobolka et al. 2013). Stork nests are often re-occupied and re-built in each breeding season by the same or a different breeding pair. After their arrival, pairs of white storks add new material to existing nests to make them suitable for egg-laying (Janiszewski et al. 2013). Hence, nests become huge and bulky over time as the result of an

accumulation of sticks, twigs, straw, and hay, as well as, sometimes, anthropogenic material such as twine, rags, plastic foil, and paper (Vergara et al. 2010; Jagiello et al. 2018). Such re-used nests, along with the accumulated feces of many individuals, are suitable environments for microbial growth (e.g., Wilharm et al. 2017; Wilharm and Skiebe 2019). Moreover, the white stork is a foraging opportunist (Kosicki et al. 2006) using anthropogenic sources of food such as landfill (Tortosa et al. 2002). Therefore, several pathogenic microorganisms have been found in storks (Olias et al. 2010, 2011; Szczepańska et al. 2015; Wilharm et al. 2017), rendering them suitable subjects for tests of the role of melanins in the control of microorganisms that colonize wild birds.

Two additional links may connect the functioning of the immune system with melanin deposition (Moreno and Møller 2006; Jacquin et al. 2011). First, melanocytes are sensitive to oxidative stress (Burt 1986; Koca et al. 2004); hence, melanocyte development may signal the performance of antioxidants in tegumentary tissues. Furthermore, melanocytes play an active role in immune function and disease resistance in humans and other organisms (Mackintosh 2001); given their important functions, direct immuno-enhancing and anti-bacterial effects (Riley 1992) are associated with their incorporation into plumage. Melanins may also play an important role as antioxidants; furthermore, they protect cells from free radicals (McGinness et al. 1970; Różanowska et al. 1999). On the other hand, melanogenesis induces the production of free oxygen radicals as melanins are themselves stable free-radicals (Nappi et al. 1995; Cesarini 1996; Hegedus 2000; Nofsinger et al. 2002). This dual role of melanins could conceivably enable melanin-based ornaments (e.g., black feathers) to provide information on antioxidant status.

The objectives of this study were to identify and quantify microorganisms in the plumage of white stork chicks. We accomplished this by testing the following predictions: (1) if black feathers are more resistant to microorganisms than white feathers, we should expect greater abundance and diversity of microorganisms in white feathers; (2) if chicks with higher resistance are characterized by the presence of fewer microorganisms, these individuals should possess higher residual reproductive value, and hence their parents should incur lower risks when approached by humans, as reflected by flight initiation distance (FID); (3) likewise, chicks with fewer microorganisms should come from broods characterized by a higher breeding success; (4) therefore, chicks that are more resistant to microorganisms should grow feathers faster than less-resistant chicks; and (5) finally, given that territories with the highest share of preferred land use (i.e., meadows and wetlands) are occupied annually (Janiszewski et al. 2013), such territories should be occupied by individuals characterized by better condition, with fewer pathogenic microorganisms.

Material and Methods

Study site

The study was conducted in 2014 in Western Poland near the town of Leszno (51°51' N, 16°35' E) in a rural area of arable fields (54%) interspersed with meadows (7%), pastures (<1%), human settlements (10%), forests (17%), and other land uses, for example, set-asides, orchards, or wetlands (11%). The white stork is a solitary breeder here, but sometimes forms local aggregations of < 5 pairs, mainly in small river valleys or near lakes. The population density declined from 8.86 pairs/100 km² in 1974 to 5.27 pairs/100 km² in 2009, with a small increase to 6.72 pairs/100 km² in 2010–2013 (Tobolka et al. 2013, 2018).

Chick growth rate and breeding parameters

We collected data on the growth rate of 96 chicks from 33 broods. A total of 59 chicks from 20 broods were measured at regular intervals (7 days) in order to determine whether their growth rate depended on the number of visits. The remaining 37 chicks from 13 broods were sampled only once. During nest visits, we measured the length of the bill; the length of the head with bill and tarsus, using a caliper with an accuracy of 0.02 mm; the length of 1 wing, using a ruler with an accuracy of 0.5 mm; and body weight, using an electronic balance with an accuracy of 1 g. The growth rate was calculated as the changes in the value of particular parameters divided by the time between the first and last records.

We recorded the distance at which adults took flight from the nest (FID) when chicks were 30- to 40-days old. We used a laser rangefinder when we approached the nest with a cherry-picker and recorded the exact distance between the observer and the nest when the adult flew away (with an accuracy of 0.1 m). Only one stork was found in the nest at the time of a visit, because, when 1 parent is guarding the chicks, the second is engaged in foraging flights. During each visit, we also recorded the dimensions of the nest, such as height and diameter, to the nearest centimeter with measuring tape in order to calculate the volume (in accordance with Vergara et al. 2010).

Feather sample collection

We chose wings of white stork chicks to test differences in the microbial loads of white and black feathers because the 2 categories of feathers are characterized by adjacent positions, meaning they are exposed to similar microbial loads from the nest. Furthermore, chicks, in contrast to adults, lack uropygial glands that might otherwise provide defense against microorganisms (Jacob and Ziswiler 1982).

We collected black and white feathers from 96 stork chicks from 33 nests. We climbed to the nests when chicks were 30- to 40-days old (with no significant effect when this variable was used as a covariate in the analyses) and removed a sample of white and black feathers (adjacent wing coverts, always from the same part of the wing) using sterile gloves rinsed in absolute alcohol. Each sample was placed in sterile bags before being placed in a freezer pending treatment in the lab. After removal of the feather sample, the chicks were returned to the nest. No visible side effects of this procedure were observed on the behavior or the well-being of the chicks, nor did any chicks die following feather removal.

Feather growth rates

We recorded the width of 5 growth bars for each of the feathers of stork chicks by using an insect pin to penetrate the feather and a piece of paper prior to measuring the width with a digital caliper with a precision of 0.01 mm (Grubb 2006). Measurements of this kind are highly repeatable, as shown in previous studies (Grubb 2006).

Bacterial isolation

In the laboratory, using sterilized tweezers, each piece of each feather was introduced in a 15-mL falcon tube with sterile phosphate buffer saline (pH=7.2) at a volume equal to the length of the feather. This was followed by 3 periods of vortex shaking of 10 s each. Free-living bacteria were washed out of the feathers and collected in Phosphate-buffer saline (PBS) solution (Saag et al. 2011), followed by serial 10-fold dilution to 10^{-4} . To quantify cultivable and feather-degrading bacteria, duplicates were made by spreading

100 μ L with a sterile spreader loop on 2 different growth media, tryptic soy agar (TSA) and feather meal agar (FMA) (prepared in accordance with Sangali and Brandelli 2000), and negative controls. FMA is a highly selective medium for keratinophilic bacteria, for which keratin is the sole source of carbon and nitrogen. Hence, only bacteria that can digest keratin are able to proliferate, thus enabling quantification of the feather-degrading bacterial load (Al Rubaiee et al. 2017a, 2017b).

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

Fungal isolation

We cut the feather samples accurately into 1-cm pieces and cultured the pieces directly to Mycobiotic Agar, a selective medium for fungi. This is a standard procedure, consisting of moistening the material to be examined with 0.5 mL of sterilized PBS (Deshmukh 2004). The cultures were incubated at $28 \pm 2^\circ\text{C}$ and checked daily for fungal growth, from the third day onward, for a period of 4 weeks. The observed developing mycotic growths were recorded under a stereoscopic binocular microscope, and subsequently individually and directly transferred to a Sabouraud dextrose agar medium with chloramphenicol (50 mg/L). The resulting products were incubated further at $28 \pm 2^\circ\text{C}$ for 2 weeks to obtain a pure isolate for identification purposes (Al Rubaiee et al. 2017a, 2017b).

Bacterial and fungal identification

Bacterial and fungal identification were accomplished using molecular characterization with the aid of a polymerase chain reaction. For additional detailed information about genetic analyses, see Al Rubaiee et al. (2018).

Statistical analyses

This study analyzed the difference in microorganism abundance and diversity using a paired design of samples of feathers from adjoining feather tracts colored either black or white. In total, feather samples were taken from 96 chicks from 33 nests for each of the statistical analyses; the pairs of white and black feather samples constituted a robust statistical method.

Generalized linear mixed models with normally distributed data (Shapiro–Wilk normality test $W > 0.97575$, $P > 0.2959$) and an identity link function were used. Life history (Nest volume, Breeding success, Feather growth rate, and Chick growth) and behavioral (Escape behavior) variables were used as response variables and microorganism abundance (Total microorganisms in FMA B (black), Total microorganisms in TSA B, Total microorganisms in FMA W (white), Total microorganisms in TSA W), and diversity (No. of species in FMA B [black], No. of species in TSA B, No. of species in FMA W [white], No. species in TSA W), with a response variable and predictor variables (listed in Tables 1 and 2). Nest ID was used as a random effect. Final models were simplified by excluding nonsignificant predictors from previous models.

We used Pearson product–moment correlations as measures of effect size rather than using an adjustment for multiple tests. Many of the relationships investigated here were characterized by small to intermediate effect sizes (*sensu* Cohen 1988), typically accounting

Table 1. Linear mixed models with a paired design for white (W) and black (B) feathers for total no. of colonies, total no. of species in FMA medium, total number of species of microorganisms, total number of species in TSA medium, and number of species in white stork chicks

Response variable	Black	White	Mean difference	SE	N	t	df	P-value	Correlation
Total no. colonies	0.740	0.569	0.171	0.041	50	4.135	49	<0.0001	0.410
Total no. of species in FMA	0.457	1.255	-0.797	0.123	50	6.498	49	<0.0001	0.638
Total no. of species in TSA	0.691	2.329	-1.638	0.145	50	-11.310	49	<0.0001	0.410
No. of species	0.448	0.388	0.060	0.023	50	2.600	49	0.012	0.141

The table also shows differences between white and black feathers, paired *t*-tests for these differences and Pearson product-moment correlations between the 2 sets of data. Numbers are log₁₀-transformed data.

Table 2. Fixed models of the relationship between nest volume, breeding success, feather growth rate, escape behavior, and chick growth in relation to microorganism predictors in white (W) and black (B) feathers from white stork nestlings

Response variable	F	Df	r ²	P-value	Predictor variable	Estimate	SE	F	P-value
Nest volume	5.599	2.26	0.25	0.0095	Total microorganisms in FMA W	0.149	0.069	4.689	0.040
					No. of species in W	2.000	0.695	8.275	0.0079
Breeding success	3.915	3.26	0.23	0.020	Total microorganisms in FMA B	0.166	0.069	5.760	0.024
					No. of species in TSA B	0.752	0.252	8.940	0.0061
					Total microorganisms in TSA B	-0.219	0.086	6.503	0.017
Feather growth rate	9.730	5.19	0.65	<0.0001	Total microorganisms in FMA B	0.691	0.162	17.978	0.0004
					No. of species in FMA B	-2.911	0.671	18.836	0.0004
					Total microorganisms in TSA B	-0.368	0.149	6.101	0.023
					Total microorganisms in TSA W	0.598	0.187	10.304	0.0046
					Total no. of species in TSA W	-0.631	0.149	17.893	0.0005
Escape behavior (FID)	3.502	4.26	0.26	0.021	Total microorganisms in FMA B	-1.737	0.699	6.126	0.020
					No. of species in TSA B	-6.149	2.553	5.808	0.024
					Total microorganisms in TSA B	2.567	0.845	9.242	0.0055
					No. of species in W	-8.314	4.3021	-3.725	0.065
Chick growth	4.919	3.25	0.30	0.008	No. of species in FMA B	-4.096	1.190	11.857	0.002
					Total microorganisms in FMA W	0.668	0.318	4.425	0.046
					Total no. of colonies in W	1.703	0.936	3.309	0.081

Partial effects, estimates (SE) and *P*-values are also reported. FMA and TSA refer to FMA and TSA medium, although B and W refer to black and white feathers, respectively.

for 1–10% of the variance. Similar levels of explained variance are typical in ecology and evolution, where scientists, even those with experimental approaches account for, on average, 5–7% of the variance (Møller and Jennions 2002). We used JMP® (SAS 2012) for all statistical analyses.

Results

A greater number of microorganism colonies and species were found in black than in white feathers of white stork chicks (Table 1). No significant correlation was found between the numbers of fungi in white and black feathers (Figure 1A). Contrastingly, a significantly positive correlation was found between the number of colonies in black compared with white feathers, with a correlation coefficient of 0.410 (Figure 1B).

Next, we analyzed behavioral and reproductive variables in relation to measurements of microorganism abundance and diversity. Nest volume of the white stork was related to 2 variables: the total number of microorganisms in FMA medium from white feathers and the number of species in white feathers, which accounted for 25% of the variance (Table 2).

Breeding success of white storks, calculated as the proportion of eggs that resulted in fledglings, was related to 3 predictors that accounted for 23% of the variance (Table 2). Breeding success increased with the total number of microorganisms in FMA medium in black feathers and the total number of species in TSA medium in

black feathers, and decreased with the total number of microorganisms in TSA medium in black feathers (Figure 2A and Table 2).

We found that 3 of 4 variables (Total microorganisms in FMA B [black], No. of species in TSA B, No. of species in W [white]) showed negative relationships with FID, as expected. Contrastingly, 1 variable (Total microorganisms in TSA B) showed a positive relationship accounting for 65% of variance (Table 2).

The daily rate of feather growth explained 65% of variance based on 3 predictors (Table 2). The feather growth rate increased with the total number of microorganisms in FMA medium based on black feathers, and decreased with increasing total microorganisms in FMA medium (Figure 2C) and microorganisms in TSA medium from white feathers (Table 2).

The chick growth rate was explained by 3 variables that accounted for 30% of variance (Table 2). Chick growth declined with the number of species of microorganisms in FMA medium on black feathers (Table 2) and increased with the total number of microorganisms in FMA medium based on white feathers (Table 2). Finally, the chick bill growth rate increased with the total number of colonies from white feathers (Table 2 and Figure 2B).

Discussion

Greater numbers of colonies and species of microorganisms were found in adjacent black than in white feathers in the same feather tracts of white storks. However, the abundance of microorganisms

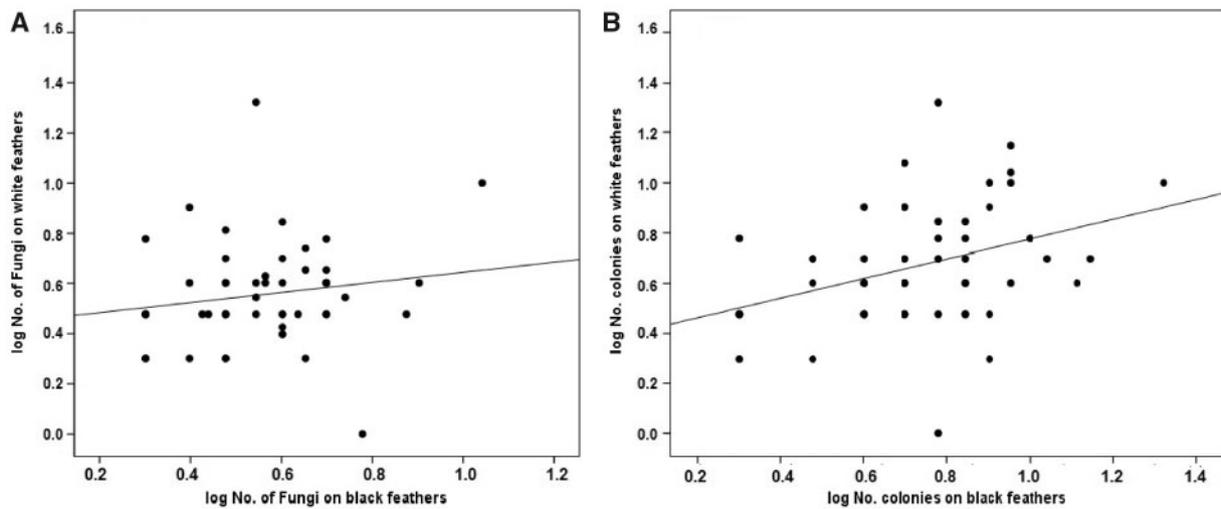


Figure 1. (A) Mean number of fungi in white feathers in relation to the number of fungi in black feathers of white stork chicks; and (B) number of colonies in white feathers in relation to the number of fungi in black feathers of white stork chicks.

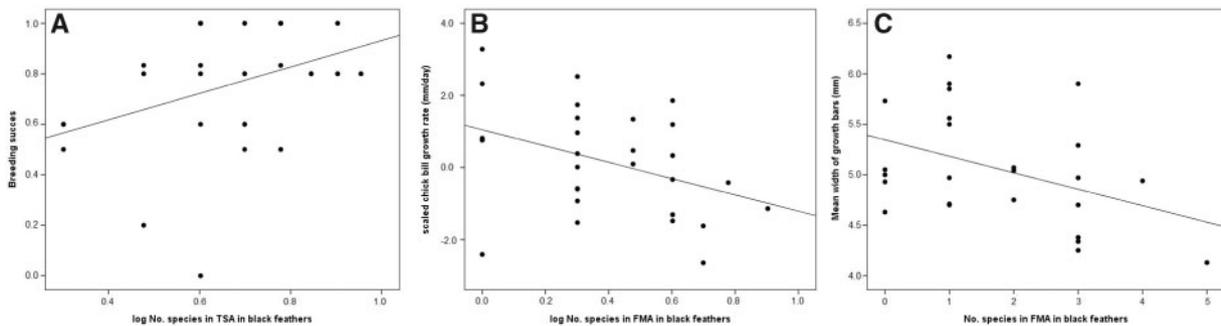


Figure 2. (A) Breeding success in relation to the number of microorganism species in TSA medium in black feathers of white stork chicks; (B) chick growth in relation to the number of microorganism species in FMA medium in black feathers of white stork chicks; and (C) width of growth bars in relation to the number of microorganism species in FMA medium in black feathers of white stork chicks.

expressed as Total number of species in FMA and Total number of species in TSA was reversed, that is, greater in white than in adjacent black feathers, as expected for feathers differing in quality due to differences in eumelanin content (Fitzpatrick 1998a, 1998b). Similar differences in microbial damage between black and white feathers have also been described in other birds (Ruiz-De-Castañeda et al. 2012); our results partially confirmed that differences in microorganismal diversity and abundance are also detectable. We were able to explain large variances in the statistical models using diversity and abundance of microorganisms in white and black plumage as response variables. This high degree of explanatory power suggests that microorganisms play a significant role in the life history of white stork chicks. However, we emphasize that all of these relationships are correlational, and therefore must consider the possibility that these relationships are due to correlations with 1 or more confounding variables. Hence, some of the results were contradictory.

The occurrence of patterns of white unmelanized patches adjoining otherwise dark melanized feathers is widespread among birds (Jones 1990; Price and Pavelka 1996; Brooke 1998; Fitzpatrick 1998a, 1998b; Kose and Møller 1999; Moreno-Rueda 2005; McGlothlin et al. 2007; Morales et al. 2007; Galván 2008; Hegyi et al. 2008; Hanssen et al. 2009). Sexual selection suggests that the reliability of sexual and social advertisements may be the result of the costs associated with the development and maintenance of

signals (Andersson 1994; Zahavi and Zahavi 1997). The costs implied by white wing bands consisting of unmelanized patches on otherwise dark melanized flight feathers may derive from the production of more resistant barbs. In terms of molecular organization (Vagási et al. 2010), it can withstand physical abrasion (Barrowclough and Sibley 1980; Burt 1986; Bonser 1995) and biotic degradation by feather-degrading bacilli (Goldstein et al. 2004; Gunderson et al. 2008; Burt et al. 2010) or feather lice (Kose and Møller 1999), even in the absence of melanization. Unmelanized feather patches would, therefore, suffer greater damage in individuals of poorer quality (Fitzpatrick 1998b). Microorganisms are known to affect the life history of their hosts, either by maintaining a community of benign organisms that protect the host (Hubálek 1976, 1978; Brook 1999; Riley and Wertz 2002; Benskin et al. 2009, 2015) or by exploiting resources that the host might otherwise have been able to draw upon (Hubálek 1976, 1978; Salyers and Whitt 2002; Benskin et al. 2009, 2015; Al Rubaiee 2018). Here, we also found evidence that microorganisms in the plumage were linked to variables either directly or indirectly associated with breeding success and feather, skeletal, and offspring growth. These findings are consistent with a role for feathers differing in eumelanin content in affecting microorganisms through effects on plumage quality.

Melanin pigments play a role in signaling (Ruiz-De-Castañeda et al. 2012), but may also maintain antioxidants and cause oxidative

stress (Peele et al. 2009). Melanins may prevent degradation of feathers and thereby assist in the maintenance of plumage, enabling efficient flight (Møller et al. 2011; Al Rubaiee et al. 2017a, 2017b). However, while the total number of species in FMA and TSA media was greater in white feathers, greater numbers of colonies and species of microorganisms were found in black compared with white feathers. This finding was heterogeneous, with some measures of microorganism abundance and diversity showing higher values for black feathers and other measures showing the opposite. We hypothesize that this diversity may help us to explain the maintenance of patterns of microorganisms linked to patterns of coloration.

Although white storks breed close to human habitations, individuals differ considerably in their escape behavior. White storks that fled great distances from humans hosted fewer microorganisms. Thus, adult white storks took greater risks when approached by a human (FID) if the plumage of chicks contained a greater number of microorganisms. This negative relationship may imply that chicks with more microorganisms are less likely to survive; hence parent birds may run smaller risks when their offspring are less likely to survive (Cooper and Blumstein 2015).

Interestingly, both adult escape behavior and chick growth are negatively correlated with abundance and diversity of microorganisms. Furthermore, a greater microbial load in black and white feathers was associated with increased reproductive success in white storks. This implies that adults may adjust their behavior to the growth rate of their young, which depends in turn on the white and black coloration of wing plumage. If chicks perform poorly in terms of feather and skeletal growth, the probability of their survival is likely to be poor (Kosicki and Indykiewicz 2011). Thus, adults likely adjust their investment in escape relative to the residual reproductive value of their young (Møller 2015). The results reported here are consistent with this scenario.

Clear links were found between coloration and abundance of microorganisms. This suggests that not only plumage color, but also pigments involved in coloration, play a role in host–parasite interactions between birds and microorganisms. Nevertheless, transfers from parents to juvenile birds may also play a significant role in the assemblage of microorganisms inhabiting particular individuals (Lee et al. 2020). It would be especially useful to conduct experiments, adding microorganisms and/or treating hosts, thereby eliminating specific microorganisms, and/or sterilizing the nest before breeding. A second approach would involve manipulating the reproductive efforts of parents, and thus the costs of maintaining low levels of pathogenic microorganisms, by means of for example, antibiotics or sterilization. A third approach would be to manipulate the availability of old nests that may have accumulated nest material, and thus microorganisms, over time, with the use of control nests incorporating either new or old nest material for nest construction (e.g., in accordance with Zablotni et al. 2020).

Although the results of this study show that differences in microbial load between black and white feathers vary (Møller and Jennions 2002), the percentage of variance explained in biological studies suggests a vital role for microorganisms in life history and feather coloration. However, a well-designed experiment will help understand this process better.

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Authors' contributions

A.P.M. designed the study. M.T. collected the samples. Z.A.L. and H.A.M. extracted and analyzed samples. A.P.M. analyzed the data and wrote the first draft. All authors took part in the writing and approved the final manuscript.

Conflict of interest statement

The authors declare no conflicts of interest.

Ethical statement

Compliance with Ethical Standards. The research was approved by Regionalny Dyrektor Ochrony Środowiska [the Regional Director of Environmental Protection] in Poznań, Poland (permit no. WPN-II.6401.167.2015.AS.2) and the Local Ethical Commission in Poznań, Poland (permit nos. 43/2010, 44/2015). The removal of feathers, as performed in our study, does not require approval from an ethics committee in Poland.

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