



First Evidence of a Relationship Between Female Major Histocompatibility Complex Diversity and Eggshell Bacteria in House Sparrows (*Passer domesticus*)

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Bacteria are known to exert positive and negative influences on animals' health and fitness. Bacteria, in particular those inhabiting the skin and inner organs of vertebrates, are horizontally or vertically transmitted. Specifically, mothers of bird species can transfer bacterial strains to their offspring when the egg is passing the reproductive tract, as the eggshell rubs against the wall of the uterus. In this context, the female immune system might play an important role in influencing the vertical transmission of bacteria. Here, we investigate the relationship between the major histocompatibility complex (MHC) and cultivable eggshell bacteria originating putatively from the female urogenital tract in a captive population of house sparrows (*Passer domesticus*). We predict that females with a more variable MHC will transfer fewer bacteria onto the eggshells. Our results show a negative relationship between the number of functional MHC class I alleles and bacteria originating in the urinary tract and growing on a selective medium. This is the first study to find a correlation between female MHC diversity and eggshell bacteria.

Keywords: eggshell bacteria, MHC diversity, bacterial abundance, bacterial culture, *Passer domesticus*

INTRODUCTION

Animal health is significantly influenced by bacteria (Org et al., 2015). The bacteria an individual harbors can be due to (i) horizontal transmission, which means they are spread from host to host in the same generation; or (ii) vertical transmission, which means they are spread between generations, namely from parents to offspring; or (iii) the host bacteria can originate from the environment.

Bacterial communities can help during digestion, e.g., extracting energy and nutritional compounds (Hooper and Gordon, 2001; Ley et al., 2008), producing vitamins (Hill, 1997), or teaching and training the host immune system (Cebra, 1999; Macpherson and Harris, 2004). However, bacteria can also have a negative effect; for instance, in birds, eggshell bacteria may penetrate the egg and cause problems during embryo development and subsequent survival (Pinowski et al., 1994; D'Alba et al., 2010). Benign or pathogenic bacteria, which are commonly

found in the reproductive system, are known to be vertically transmitted to the host's offspring (Ruiz-de-Castañeda et al., 2011a; Tangkham et al., 2016; You et al., 2019).

In birds, early contact of offspring with bacteria occurs in the ovary and the oviduct of the mother. This happens either before oviposition, through direct contamination of the yolk, albumen, or eggshell membranes (Timoney et al., 1989; Keller et al., 1995; Okamura et al., 2001a,b), or during oviposition, when the egg is squeezed through the gastrointestinal system of the mother (De Reu et al., 2006; Messens et al., 2007). Contact with bacteria also occurs after oviposition, with bacteria from the surrounding environment, e.g., airborne bacteria (Kulkarni and Heeb, 2007; Goodenough and Stallwood, 2012), or bacteria from the nest material or the parent surface (skin and feather bacteria).

Several studies have reported a significant variation in eggshell bacterial composition and abundance between individuals, as well as species (Soler et al., 2011; Peralta-Sánchez et al., 2012, 2018; Schmitt et al., 2017), whereby between-species variation in eggshell bacteria is likely to have been influenced by environmental conditions. In this context, ambient temperature and humidity are well-known parameters influencing breeding conditions and, consequently, the eggshell bacterial community (Park et al., 2015; Leclaire et al., 2019). In contrast, within-species variation in eggshell bacteria is more likely to be related to individual quality, given that environmental conditions should be more similar within than between species. In particular, the immune system of an individual should influence its bacterial composition (Soler et al., 2011; Schmitt et al., 2017). Therefore, we predict a relationship between eggshell bacteria loads and the immunocompetence of a bird mother.

In this context, one important element of the genome responsible for immune system functioning is the major histocompatibility complex (MHC). The MHC is a highly polymorphic gene region, which encodes proteins that present both self and foreign peptides to T cells, which are central for an appropriate adaptive immune response against pathogens (Rock et al., 2016; Goebel et al., 2017). Amino acids—in particular, polymorphic peptide-binding regions (PBR) of the MHC molecules—determine which antigens can be bound and are therefore crucial for the function of each allele (Schwensow et al., 2007). The general assumption is that individuals with a broad antigen-binding repertoire can recognize and eradicate a wider range of pathogens and therefore have fitness advantages over individuals with a narrow MHC repertoire (Penn et al., 2002). A broad antigen-binding repertoire is, however, not always beneficial; in house sparrows (*Passer domesticus*), a negative effect on hatching success was found (Lukasch et al., 2017a,b).

Major histocompatibility complex genes are known to influence the gut microbiota of animals (Toivanen et al., 2001; Lanyon et al., 2007; Bolnick et al., 2014; Lin et al., 2014; Kubinak et al., 2015). Recent evidence suggests that MHC diversity negatively affects the feather microbiota of blue petrels (*Halobaena caerulea*) (Leclaire et al., 2019) and Leach's storm petrels (*Oceanodroma leucorhoa*) (Pearce et al., 2017) and influences bacteria-based skeleton diseases in broiler chicken (Joiner et al., 2005). However, some studies revealed a positive relationship (Hernández-Gómez et al., 2018;

Khan et al., 2019), which suggests that the relationship between MHC and microbiota diversity is not yet settled.

In the present study, for a first approach, we used bacterial culture techniques to specifically address urinary tract bacteria (UTI bacteria) and bacteria growing on blood agar, reflecting a more general bacteria composition. Then, we examined whether there is a relationship between MHC diversity and the number of these cultivable eggshell bacteria, partly originating from the urogenital tract of female passerines, in particular, house sparrows (*P. domesticus*). We predicted that the number of eggshell bacteria originating from the urinary tract would be negatively related to the diversity of functional MHC alleles, whereas a more general bacteria composition, e.g., including bacteria originating from the environment, may not be. Therefore, females with more variable MHC would be expected to transfer fewer bacteria onto the eggshell surface. If this also affects pathogenic bacteria, there could be a transgenerational benefit. In contrast, in house sparrows, a strong immune system could be also negative, e.g., in terms of a reduced fertilization success (Lukasch et al., 2017a,b).

MATERIALS AND METHODS

Housing Conditions and Study Setup

The study was conducted in an aviary population of house sparrows at the Konrad-Lorenz Institute of Ethology in Vienna, Austria. In April 2014, 31 breeding pairs with functional MHC allele numbers, known from another study (Lukasch et al., 2017a,b), were set up in 10 aviaries (measuring 2 m × 4 m and 2.6 m high). All aviaries contained three to four pairs, were provided with sufficient nest boxes and nest material, and were equipped with vegetation and perches. Water and commercial food for granivorous passerines were provided *ad libitum*. These breeding conditions for our aviary population of wild house sparrows were intended to limit the impact of the environment on eggshell bacteria. To further reduce the environmental impact, eggs were collected within 1 h of laying (for details, see below).

To improve comparability, all females were measured prior to the start of laying on the same day (April 15). Tarsus length was determined to the nearest 0.1 mm using calipers, and body mass was estimated with an electronic scale to the nearest 0.1 g (Svensson, 1992). Each bird was fitted with an individual combination of color rings, to later determine the pair composition for each nest box. After the onset of reproduction, fresh commercial food for insectivorous passerines and mealworms was provided four times a day. The nest boxes of each breeding pair were examined daily until the second egg was laid, from which eggshell bacteria were immediately sampled (for methods, see below).

Bacteria Sampling and Cultivation Procedure

We followed a procedure already used for other bird species (Ruiz-de-Castañeda et al., 2011b), in our own previous studies (Brandl et al., 2014; Hoi et al., 2019) and the instructions of the agar manufacturer (Oxoid). After removing the egg from

the nest with sterile gloves, we swabbed the egg surface for 20 s using a sterile cotton swab soaked in sterile water. The swab was immediately stored in a transport medium (transport viscose swab with Amies transport medium, Sarstedt). Within 48 h, the swab was transferred to 1.0 ml of phosphate-buffered saline (PBS) in the laboratory. The 15-ml tube, containing 1.0 ml of PBS and the swab, was mixed by vortex agitation for 120 s. One hundred microliter subsamples of the bacterial suspensions were extracted and serially decimal diluted, ranging from no dilution up to 10^{-6} , and applied to separate plates. Usually, about four different concentrations were produced for each sample. After being incubated for 24 h, bacterial colonies originated and developed, each from a single founder bacterium. Thus, each colony-forming unit (CFU) reflected a single bacterium. Based on the number of CFUs developed, the plate with the most suitable number of colonies to separate them as distinct colonies (according to our experience up to 300 colonies/plate) was selected. The number of CFUs was then extrapolated according to the dilution on the respective plate. Finally, to reach the total number for the whole sample of 1.0 ml, this number was multiplied by 10. This ensured that two relative abundance measurements of cultivated bacteria were derived for further analyses (**Supplementary Material**).

After dilutions were completed, 0.1 ml of all samples and dilutions were spread-plated on non-selective (Columbia agar with 7% sheep blood, Oxoid) and selective (Brilliance UTI, Oxoid) media that indicated total bacterial counts. The plates were then incubated for 24 h at 37°C. After the incubation period, CFU counting, a common method reflecting bacteria density and used in several other studies (Godard et al., 2007; Peralta-Sánchez et al., 2012), was performed for both media separately.

Columbia agar with 7% sheep blood is a highly nutritious, general purpose medium for the isolation and cultivation of non-fastidious and fastidious microorganisms from a variety of clinical and non-clinical materials. The sheep blood supplement allows the culture of most culturable bacteria, regardless of their metabolic requirements¹. Columbia blood agar has been used to cultivate bacteria from different animal taxa including birds (Ruiz-de-Castañeda et al., 2011b; Merbach et al., 2019) and mammals (Infante et al., 2008; Di et al., 2020).

Brilliance UTI agar is a chromogenic medium used more specifically for the presumptive identification and differentiation of the main microorganisms that cause UTIs (Wellman-Labadie et al., 2008; Maluta et al., 2014; Barber et al., 2016) including *Enterococcus* spp., *Escherichia coli*, *Proteus*, *Morganella*, *Providencia*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and coliforms. Therefore, we used two bacterial categories (see above): (i) general bacterial abundance, which included the number of all bacteria growing on the Columbia agar with sheep blood, and (ii) UTI bacterial abundance, which are bacteria growing on the Brilliance UTI agar. These two relative abundance measurements, reflecting the number of CFUs of the two bacterial groups, have been used as a dependent variable.

We analyzed the second egg of 68 clutches in total. Due to missing data in individual subsets, 61 eggs were used for the general bacterial abundance, and 60 eggs were used for

UTI bacterial abundance. Of the 31 females used, each female contributed on average 2.2 ± 0.1 (s.e.) eggs.

MHC Genotyping

Characterization of the MHC class I exon 3 encoding parts of the peptide-binding region was carried out using 454 amplicon sequencing (Galan et al., 2010), refined for house sparrows by Karlsson and Westerdahl (2013) at Lund University Sequencing Facility (Faculty of Science), Sweden. For MHC bioinformatics and data processing after 454 sequencing was carried out, the data were extracted and assigned to samples using the program jMHC (Stuglik et al., 2011). MHC diversity was evaluated as the number of different functional alleles per individual.

The MHC genotyping of the female house sparrows was carried out in an earlier study (for details, see Lukasch et al., 2017a,b). The number of functional alleles determined in this subset of females ($N = 31$) was on average 4.258, ranging from 2 to 8. Based on their chemical properties in the peptide-binding region, these could be translated into 35 unique functional MHC alleles.

Statistical Analyses

Generalized linear mixed models with a negative binomial error distribution were fit to test the effect of the female number of functional MHC alleles for general bacterial abundance and UTI bacterial abundance from the eggshell surface. Female identity and aviary identity were used as random effects. Other potential factors, namely the start of egg laying and the female body condition, which is known to be related to the immune system (Dannemiller et al., 2017), were also included in the model. For the start of egg laying, a continuous series of numbers was used, taking January 1 as the first day. For the female body condition, we used the residuals from a linear regression between body mass not explained by size (tarsus length). A model with negative binomial distribution was used because this method performs better than log-transformation of count data.

Both models were fit using R version 3.6.0 (R Core Team, 2019), package lme4 (Bates et al., 2015). To calculate pseudo- R^2 values for non-linear regression models, we used the R package MuMIn. To ensure that the results were not biased by a correlation between variables, we calculated the variance inflation factors (VIF) of the models (Zuur and Ieno, 2016). None of the variables exceeded two, which is the recommended threshold (Zuur et al., 2010). Model assumptions such as normality of residuals, overdispersion, and zero inflation were tested by the package DHARMA (Hartig, 2020). Normality of residuals was visually tested using a Q-Q plot and the Kolmogorov-Smirnov test (general bacterial abundance model, $p = 0.32$; UTI bacteria abundance model, $p = 0.70$). Models for count data following a negative binomial or Poisson distribution do not assume homogeneity of variance. However, they have other additional assumptions that we tested for, such as overdispersion (general bacterial abundance model, $p = 0.35$; UTI bacteria abundance model, $p = 0.23$) and zero inflation (general bacterial abundance model, $p = 0.97$; UTI bacteria abundance model, $p = 1$). All of these assumptions were met in both models.

¹www.biomerieuxdiagnostics.com

RESULTS

When examining MHC diversity in terms of the number of functional alleles (fMHCsum) in relation to the general bacterial numbers, we found no significant effect (**Table 1a** and **Figure 1A**, pseudo- $R^2 = 0.19$). The parameter estimates predicted a 27.3% decrease in bacterial numbers, with an increase in the number of functional alleles. However, this effect was only near significance ($p = 0.077$, see **Table 1a**). The female body condition (residual body mass not explained by tarsus length) did not influence bacterial numbers at all; however, there was a significant seasonal effect, namely that bacterial numbers increase with time during the season (**Table 1**).

In contrast, a significant negative effect was found when examining MHC diversity in terms of the number of functional alleles (fMHCsum) in relation to UTI bacterial abundance (**Table 1b** and **Figure 1B**, pseudo- $R^2 = 0.31$). The abundance of UTI bacteria found on the eggshell of a female decreased by 30.2% with every extra functional allele. Again, the season was revealed to be important and the female body condition was not. Similar to the general bacterial numbers, the positive parameter estimate suggested an increase in UTI bacteria over the season (**Table 1**).

DISCUSSION

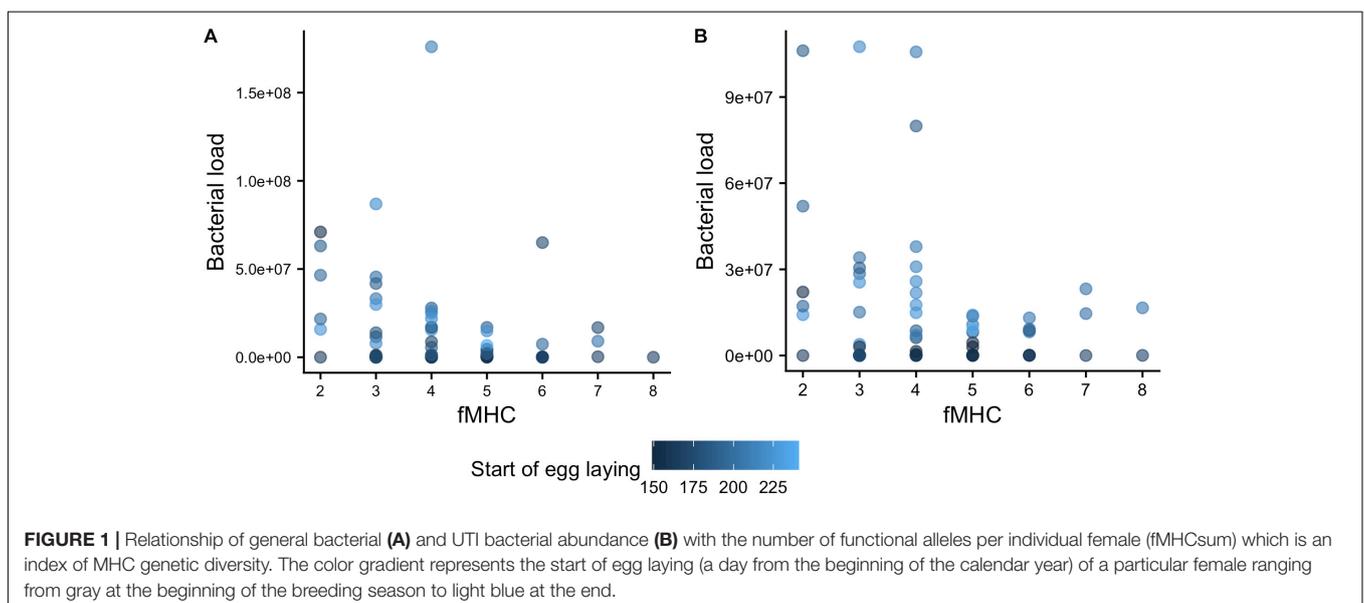
Our results support the prediction that eggshell bacterial numbers, cultivated on different media, are negatively related to the number of fMHC alleles and that this effect of female fMHC seems stronger with regard to UTI bacteria likely originating from the female urogenital tract. There seems to be a similar trend in general bacterial numbers, although a significant effect could only be found in the case of UTI bacteria. This is the first study that has revealed evidence of a relationship between female MHC diversity and eggshell bacteria. These findings suggest that maternal MHC plays a role in hatching success and offspring survival (Lukasch et al., 2017a). However, further research is needed to determine which eggshell bacteria are particularly important in this context.

The non-significant relationship between general bacterial numbers and female functional MHC diversity may be due to the fact that general bacterial numbers also include bacteria originating from the environment outside the female body. We did not expect a strong relationship between general bacteria in relation to the female immune system. The weaker impact of bacteria growing on blood agar is probably also due to bacterial

TABLE 1 | Relationship between the MHC diversity on general bacterial (a) and UTI bacterial abundance (b).

	(a) General bacterial abundance				(b) UTI bacterial abundance			
	Estimate	s.e.	z-value	p	Estimate	s.e.	z-value	P
(Intercept)	10.50	2.09	5.03	<0.001	7.77	1.87	4.16	<0.001
fMHCsum	-0.32	0.18	-1.77	0.08	-0.36	0.16	-2.23	0.03
Fcond	-0.13	0.16	-0.85	0.40	-0.08	0.15	-0.51	0.61
Date	0.04	0.01	3.79	<0.001	0.05	0.01	5.70	<0.001

For explanation of general bacterial abundance and UTI bacterial abundance, see Materials and Methods section. The number of functional alleles per individual female (fMHCsum) was used as an index of MHC genetic diversity. Additionally, female condition (residual body mass not explained by body size represented by tarsus length, Fcond) and season using date (date) were also included as confounding covariables. Parameter estimate (estimate), standard error (s.e.), z-value, and significance values (p) are given.



samples being taken immediately after laying, where eggs were only briefly exposed to the environment. Consequently, it is likely that eggshell bacteria originating from the environment have less time to settle and their occurrence is more stochastic. The type of nest boxes, nest box position, and nest material provided are also the same. Hence, no striking differences in environmental conditions are to be expected between nest boxes, whereas variation in bacterial numbers between nest boxes is huge (see **Supplementary Material** and **Figure 1**). Thus, environmentally transmitted eggshell bacteria should mainly originate from the body surface of the mother (Soler et al., 2012; Jacob et al., 2014). It has been shown that bacteria transmitted to the eggshell, originating from the parental skin or feathers, can be affected by uropygial gland wax composition, which in turn is influenced by the female immune system (Soler et al., 2012; Jacob et al., 2014) and nest material.

In this context, it was found that bacteria originating from the environment, such as the feather microbial community in blue petrels (*H. caerulea*), can be affected by the fMHC diversity of the parents (Leclaire et al., 2019). A likely underlying mechanism is that MHC variants alter preen wax composition and, consequently, the bacterial composition, shown for seabirds (Leclaire et al., 2019) and songbirds (Slade et al., 2016).

The female body condition had no effect on eggshell bacterial abundance, probably because resource investment could be balanced between the immune system and other activities (Westneat and Birkhead, 1998).

We also discovered a seasonal effect on eggshell bacterial abundance, namely an increase in abundance of both bacterial groups when sampled later in the season. This increase was not due to a temperature increase on the egg surface, provoked by incubation, because bacterial samples were always taken immediately after laying and prior to the start of incubation (Ruiz-de-Castañeda et al., 2011a). Similarly, a positive relationship between eggshell bacterial load and the laying date in active magpie (*Pica pica*) nests was found, due to parental activities (Soler et al., 2015). However, since the egg was again taken immediately after laying, this explanation might be less important in our results.

Alternatively, the seasonal change in bacterial abundances before the start of incubation may be due to the accumulation of bacteria in the female tract (cloaca) over time. It is well known that bacteria can be sexually transmitted to a great extent (Lombardo et al., 1996). Intra- as well as extrapair copulation activity may lead to an accumulating effect and an increase in bacterial abundance. However, earlier studies in our aviary population did not reveal an obvious change in copulation behavior during that period, and genetic analyses revealed no evidence for extrapair paternity (unpublished results). An explanation could be that male paternity guarding is more efficient under aviary conditions. However, the lack of extrapair paternity does not necessarily imply a lack of extrapair copulations. Thus, bacterial accumulation in the female cloaca is a possibility, but the cloaca is regularly emptied and bacteria transferred by males should therefore be removed to a great extent. If bacteria were to accumulate in the female, one would also expect older females to harbor more bacteria. However,

there is no indication for this, and in our study, all females originated from the same age cohort, which prevents further conclusions. It is most likely that ambient conditions, e.g., temperature for bacteria development, improve and lead to a growing contamination of food and water, which may influence gut bacterial compositions. The lack of clear difference in the seasonal effect between general and UTI bacteria, at least when looking at the parameter estimate (**Table 1**), suggests an indirect environmental effect, e.g., bacteria transferred through the gut, rather than a direct ambient effect.

There is evidence that female house sparrows with higher MHC diversity are more effective in reducing a higher proportion of male-transmitted bacteria (Lukasch et al., 2017a). However, experiments would be necessary to demonstrate causal relationships between female MHC and bacterial composition. An experimental approach would also be necessary to determine bacterial effects on chick fitness. One possibility would be to compare control incubated eggs with sterilized and incubated eggs. Finally, it would be interesting to determine the importance of different bacterial strains for offspring fitness.

CONCLUSION

Female house sparrows, which harbor a more diverse MHC, lay eggs with less cultivable eggshell bacteria. This is the first evidence of a possible alternative route of maternal effects. In particular, we found evidence that the transgenerational transmission of bacteria can depend on the immune genotype of the mother. However, given that the effect of bacteria can be negative or positive, it is questionable whether a good maternal immune system is beneficial for the offspring. It may depend very much on the bacterial strains involved. Further research is needed to investigate the impact of bacteria on embryo development and whether mothers are able to selectively act against specific bacterial strains.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Commission of the Austrian Ministry of Sciences (ZI.09/0997/2011).

AUTHOR CONTRIBUTIONS

HH contributed to the study concept and preparation of the manuscript. MP contributed to the observation in the aviaries,

bacterial sampling from the eggshells, and statistical analysis. AD and JK contributed to the bacterial sampling and bacterial cultivation. BL contributed to the MHC determination. All authors were involved in the interpretation of the results and approved the revised manuscript before its submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.615667/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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