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LETTER

Sexually transmitted bacteria affect female cloacal assemblages in a wild bird

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Abstract

Sexual transmission is an important mode of disease propagation, yet its mechanisms remain largely unknown in wild populations. Birds comprise an important model for studying sexually transmitted microbes because their cloaca provides a potential for both gastrointestinal pathogens and endosymbionts to become incorporated into ejaculates. We experimentally demonstrate in a wild population of kittiwakes (*Rissa tridactyla*) that bacteria are transmitted during copulation and affect the composition and diversity of female bacterial communities. We used an anti-insemination device attached to males in combination with a molecular technique (automated ribosomal intergenic spacer analysis) that describes bacterial communities. After inseminations were experimentally blocked, the cloacal communities of mates became increasingly dissimilar. Moreover, female cloacal diversity decreased and the extinction of mate-shared bacteria increased, indicating that female cloacal assemblages revert to their pre-copulatory state and that the cloaca comprises a resilient microbial ecosystem.

Keywords

ARISA, black-legged kittiwake, cloacal bacteria, gastrointestinal microbiota, mating behaviour, sexual selection, sexually transmitted diseases, transmission.

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INTRODUCTION

The prevalence of infectious diseases in animals underlines the importance of understanding the mechanisms that promote microbe transmission in wild populations. Sexual transmission is considered an important mode of horizontal microbe transmission (Smith & Dobson 1992; Lockhart *et al.* 1996), yet its dynamics remain largely unknown in natural conditions. Birds comprise an important model for studying sexually transmitted microbes. The avian cloaca serves the dual functions of excretion and gamete transfer, creating the potential for intestinal pathogens being incorporated into ejaculates, which may facilitate the spread of sexually transmitted diseases (Sheldon 1993; Lombardo 1998). Alternatively, this dual function may provide the opportunity to gain beneficial microbes via copulation given that the gastrointestinal tract harbours a substantial proportion of mutualistic symbiotic bacteria (Lombardo *et al.* 1999; Ley *et al.* 2008b). The reproductive tract can be an additional source of sexually transmitted beneficial bacteria (e.g. Hupton *et al.* 2003). Although bacterial transmission differs from the transmission of other types of microorganisms in several ways (Nelson *et al.* 2005), understanding the sexual transmission of bacteria in birds may provide a framework for examining behavioural vectors of pathogens of medical and economical importance, such as avian and swine influenza, and West Nile virus.

In a study of captive zebra finches *Taeniopygia guttata*, Kulkarni & Heeb (2007) infected the cloaca of either the male or female mate with one bacterial strain and found that

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it was sexually transmitted from the male to the female at over four times the frequency of the reverse. Because numerous types of bacteria are likely to be sexually transmitted, this issue might best be studied at the cloacal community level. Bacterial communities have been identified and described in the semen and cloacae of wild birds using culture-based techniques (Lombardo *et al.* 1996; Stewart & Rambo 2000; Westneat & Rambo 2000; Hupton *et al.* 2003; Maul *et al.* 2005). In two of these studies, correlations between mates in the composition of cloacal bacterial assemblages suggest the occurrence of sexual transfer (Lombardo *et al.* 1996; Stewart & Rambo 2000). However, such correlations do not exclude alternative explanations for mate similarity such as joint exposure to faecal microbes in the nest, assortative mating and non-sexual horizontal transmission (Faustino *et al.* 2004), for instance allopreening or courtship feeding.

The study of bacterial communities has been enhanced by recent molecular techniques. Whereas only a minority of bacterial species can be cultured (Zoetendal *et al.* 2004), molecular methods allow straightforward identification of bacterial assemblages and detect bacterial species that are present even in relatively low concentrations (Chambers *et al.* 2001). The automated ribosomal intergenic spacer analysis (ARISA) technique describes bacterial communities by amplifying a sequence of the ribosomal genome that varies in length according to taxonomic groups (Ranjard *et al.* 2000). This technique has been utilized by several researchers to study cloacal communities hosted by wild birds (Lucas & Heeb 2005; Banks *et al.* 2009; Ruiz-Rodriguez *et al.* 2009), although not in the context of sexual transmission. Experiments are required to examine the effect of copulations on bacterial communities. However, to our knowledge, there have been no manipulations of sexual transmission of bacteria in wild populations.

We studied the mechanisms of sexual transmission and its effects on female bacterial communities by combining a molecular approach and an experiment in black-legged kittiwakes *Rissa tridactyla*. Kittiwakes are monogamous, colonial seabirds that are a model system for studying sexual transfer of bacteria for the following reasons. Pairs copulate for 30 days prior to egg-laying (Helfenstein *et al.* 2004), providing high potential for sexual transmission of bacteria. Furthermore, the fact that they are behaviourally and genetically monogamous (Helfenstein *et al.* 2004) makes it unlikely that sexually transmitted bacteria originate from extra-pair mates. Additionally, post-copulatory sperm ejection by female kittiwakes (Helfenstein *et al.* 2003; Wagner *et al.* 2004) is consistent with the possibility that pathogens are sexually transmitted. Although there is evidence that this behaviour is aimed at avoiding the detrimental effects of old sperm on hatching (Wagner *et al.* 2004; White *et al.* 2008), it may also improve female condition by reducing the amount of potentially harmful bacteria transferred during copulation (Lombardo *et al.* 1999). Finally, a method has been applied in this species that successfully blocks inseminations (White *et al.* 2008), allowing us to manipulate sexual transmission in natural conditions.

We compared the changes in community structure and diversity of cloacal bacteria between females in an experimental and a control group. In the experimental group, males were fitted with a contraceptive device (CD; a ring placed around the cloaca and maintained with a harness; Michl *et al.* 2002; White *et al.* 2008) preventing cloacal contact and insemination. Blocking inseminations allowed us to distinguish sexual transmission from alternative modes of horizontal transmission between mates. In the control group, males were fitted with devices that allowed normal cloacal contact and insemination. Thus, differences in cloacal bacterial communities between both groups could be identified as resulting exclusively from insemination and cloacal contact.

In our study, we first sampled pairs after copulations had commenced. Consequently, at the start of the experiment, we expected a positive correlation between mates in their number of bacterial strains, as found by Stewart & Rambo (2000) in house sparrows *Passer domesticus*. We also expected bacterial community composition to be more similar between mates than among non-mates, as Lombardo *et al.* (1996) reported in tree swallows *Tachycineta bicolor*. The experimental blocking of copulations in kittiwakes leads to the predictions that the number of strains will no longer be correlated between mates, and that the similarity between mates in bacterial community composition will decrease over time. These predictions are based on the assumptions that: (1) mate similarity is caused by an asymmetrical sexual transfer of bacteria from males to females (Kulkarni & Heeb 2007) and (2) once this transfer is prevented, many inseminated bacteria will disappear from the female cloaca after a period of time due to post-infection clearance kinetics (e.g. Kulkarni & Heeb 2007; Bergstrom *et al.* 2008; Hoffmann *et al.* 2009). These two assumptions also lead to the prediction that the diversity in the female cloaca caused by sexual transfer will decrease over time after inseminations are blocked.

MATERIAL AND METHODS

Study population

The study was conducted in the breeding season (May–July) of 2006 on a population of black-legged kittiwakes nesting on an abandoned U.S. Air Force radar tower on Middleton Island (59°26′ N, 146°20′ W), Gulf of Alaska. Nest sites created on the upper walls are viewable from inside the tower through sliding one-way mirrors. This enabled us to capture and easily monitor the breeders.

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Experimental procedure

We captured 19 randomly chosen pairs early in the breeding season, during the nest building period, on average 17 days before the date of laying of their first egg (range: 9–29 days). All experimental males and females were sampled for their cloacal bacterial assemblages (see below), measured and weighed. Males were caught on average 5 days after their mates (range: 0–20 days) and were then randomly assigned one of two treatments. Ten males were fitted with CDs (a ring placed over the cloaca and maintained with a harness; see White *et al.* 2008 for details) which are effective in preventing cloacal contact and inseminations while being inconspicuous and allowing normal behaviour (White *et al.* 2008). Another nine males were fitted with control devices that did not prevent males from inseminating their mates (White *et al.* 2008). Males fitted with either the control or experimental devices attended their nests less frequently than non-treated males during the first 5 days following capture (GLM, $F_{1,62} = 6.28$, $P = 0.01$). After 5 days though, all males had resumed normal nest attendance behaviour (treated vs. non-treated: GLM, $F_{1,62} = 0.06$, $P = 0.80$). For this reason, all statistical tests of the experiment were performed with and without durations of device wear of 5 days and less. Because the exclusion of these data points did not change the significance of our results, we conservatively report only the statistics with the entire data set, unless stated otherwise.

All females were recaptured soon after they had laid their first egg (on average 1 day after, range: 0–5 days), were weighed and had their cloacae sampled. We were unable to resample males because they were extremely difficult to recapture at the time of egg-laying. However, we were able to recapture them during chick-rearing and remove their devices. We further sampled the cloacae of another 145 individuals present on the tower as part of a routine monitoring and banding program.

Bacterial sampling

We sampled the bacteria present in the cloaca by flushing it with 1 mL of sterile saline solution (Phosphate buffer saline; Sigma, St Louis, MI, USA, pH 7.4). This was performed by gently inserting the tip of a sterile needleless syringe 5 mm into the cloaca, injecting the saline solution and drawing it out again. The sample was immediately inoculated into a sterile Venoject^{TM} (Terumo, Tokyo, Japan) vacuum glass tube and stored at −20 °C. For each capture event, birds were sampled twice (*c.* 10 min apart) to ensure maximum bacterial collection. Before each sampling, the exterior of the cloaca was cleaned with alcohol to avoid contamination from bacteria outside the cloaca. At each capture session, we also collected control samples by injecting the saline solution directly into sterile tubes to control for any possible contamination of the saline solution.

Automated ribosomal intergenic spacer analysis

To characterize the structure and the diversity of bacterial communities present in each cloaca sample, we performed automated ribosomal intergenic spacer analyses (ARISA; Ranjard *et al.* 2000). This DNA-fingerprinting method, used in microbial ecology, is based on the amplification of the internal transcribed spacer (ITS) region lying between the 16S and 23S rRNA genes in the ribosomal operon. The ITS region is extremely variable in both sequence and length for different prokaryotic species, thus the DNA amplification profile obtained with ARISA for a given bacterial community sample allows straightforward estimation of bacterial diversity, avoiding biases inherent of classical culture-based techniques (Lucas & Heeb 2005).

After thawing the cloacal samples, we extracted bacterial DNA using the Qiagen DNeasy® Blood & Tissue Kit and the standard protocol designed for the purification of total DNA from gram-positive bacteria (Qiagen, Venlo, Netherlands, July 2006). The 16S-23S rRNA intergenic spacer was amplified using the following FAM (6-carboxyfluorescein)-labelled primers S-D-Bact-1522-b-S-20 (5′-[6FAM] TGCGGCTGGATCCCCTCCTT-3′) and L-D-Bact-132-a-A-18 (5′-CCGGGTTTCCCCATTCGG-3′) (Ranjard *et al.* 2000). The PCR amplification was performed in 20 μL mixtures containing 200 μM dNTPs, 5% (v:v) Dimethyl Sulfoxide (DMSO), 0.5 μM of each primer and 0.25 units GoTaq DNA-polymerase with the corresponding 5× PCR buffer (Promega, Fitchburg, WI, USA) and used the following program: initial denaturation at 94 °C for 5 min, 35 cycles consisting of denaturizing at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min and a final elongation at 72 °C for 10 mins.

We then carried out 2% Nusieve agarose (Cambrex, East Rutherford, NJ, USA) gel electrophoreses for 1 h at 100 V with the PCR products to determine whether the amplification had successfully occurred. If the bands revealed in the profiles were highly saturated ($n = 163$, 38%), we performed a 20-fold dilution of the PCR product in preparation for the genetic analyser. If they showed a low level of saturation ($n = 191$, 44%), we did not dilute the PCR product. If no bands were visible in the profile ($n = 79$, 18%), we concentrated the DNA extract by evaporation, and then repeated amplification as described before.

Either diluted or undiluted PCR product of 2 μL was then mixed with 8 μL of ionized formamide and 0.1 μL GeneScan-2500 Rhodamine X-labelled size standard (Applied Biosystem, Carlsbad, CA, USA). The mixtures were denaturized at 95 °C for 5 min before separation with

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a capillary sequencer ABI 3730 (Applied Biosystem) during 3 h in the POP-7 polymer and with 7.5 kV run-voltage.

For each cloacal sample, the sequencer produced an ARISA profile in which each peak corresponds to one phylotype, or 'operational taxonomic unit' (OTU). In the cloacal samples, the sequencer detected fragments ranging from 147 to 1085 bp in size, representing a cumulated total of 190 different OTUs. Furthermore, for each fragment size category, we considered the intensity of the signal detected as a proxy for the abundance of individual OTUs (Yannarell & Triplett 2005). The ARISA method therefore allowed us to estimate the number of OTUs present in the cloaca of each individual bird and their relative abundance. No fragments were detected in control samples, indicating that the saline solution was not contaminated during the experiment.

Analyses of bacterial communities

To analyse the structure and diversity of the bacterial communities present in the cloacae, we used PRIMER v6.1 (Clarke & Gorley 2006), a specialized software used in community ecology. Duplicate samples were highly repeatable (Shannon diversity index, GLM, $F_{1,140} = 5.05$, $P < 0.0001$, correlation coefficient $r = 0.42$, $n = 282$ duplicate samples) allowing intraindividual and interindividual comparisons of cloacal communities. Consequently, we averaged the values of duplicate samples to give one ARISA profile per capture event. Before comparing cloacal communities, we standardized the relative abundances of each OTU by dividing their specific abundance by the total abundance summed over all OTUs of the ARISA profile, thus giving a proportional abundance. This avoids any biases due to differences in the quantity of DNA extracted and amplification efficiency.

To compare the OTU composition of two cloacal samples and measure their similarity, we used the zero-adjusted Bray-Curtis coefficient (Clarke *et al.* 2006), which gives a value between 0 and 100, with 0 representing two communities sharing no OTUs and 100 representing two identical bacterial communities. We used either binary data, i.e. presence or absence of OTU, or relative abundance of each OTU.

We examined bacterial immigration and extinction rates between first and second captures of females. Immigration rate was calculated as the number of new OTUs at second capture divided by total number of OTUs at second capture, and extinction rate as the number of OTUs no longer present at second capture divided by total number of OTUs at first capture.

For each cloacal sample, we also calculated diversity indices using the PRIMER software: the Shannon diversity index [$H' = -\text{sum}\ p_i \ln(p_i)$, where p_i is the proportion of the total abundance arising from the ith species] and the Simpson index ($1 - \text{lambda}' = 1 - [\text{Sum}_i\ N_i(N_i - 1)]/[N(N - 1)]$, where N_i is the number of individuals of species i).

Statistical analyses

Statistical analyses were carried out using the SAS® package (©SAS Institute Inc. 1999, Cary, NC, USA). For analyses of changes in similarity and diversity, we used the GLM procedure (ANCOVA) with treatment, duration of treatment and their interaction as main factors. To account for potential seasonal effects on bacterial communities, we included date of capture as a covariate in the initial models and conserved it only if it was significant, as reported in the Results section. For the analysis of male–female correlations in the number of OTUs according to time, we used a mixed model (GLMM procedure) including individual female as a random effect to avoid pseudoreplication. For all analyses, we verified the normality of the distribution of data and the homogeneity of variance across groups. Sample values are expressed as mean ± SE throughout.

RESULTS

General cloacal assemblage structure

From a total sample size of 165 kittiwakes, we identified 190 OTUs of which only two (335 and 436 bp in length) were present in all individuals and eight in over 75% of all individuals. In contrast, 151 OTUs (79%) showed low prevalence (< 20%) including 118 OTUs (62%) with very low prevalence (< 5%; Fig. 1). In the 38 birds in our experiment, 154 different OTUs were identified with a very similar pattern of prevalence (5 OTUs with a prevalence of over 75% and 109 with a prevalence below 20%). The number of OTUs detected per cloacal sample averaged 28.60 ± 1.47.

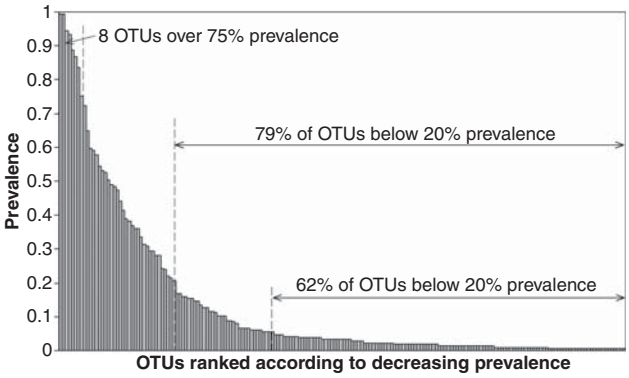

Figure 1 Prevalence of the 190 cloacal operational taxonomic units (OTUs) detected in all the birds sampled ($n = 165$). OTUs are presented in order of decreasing prevalence.

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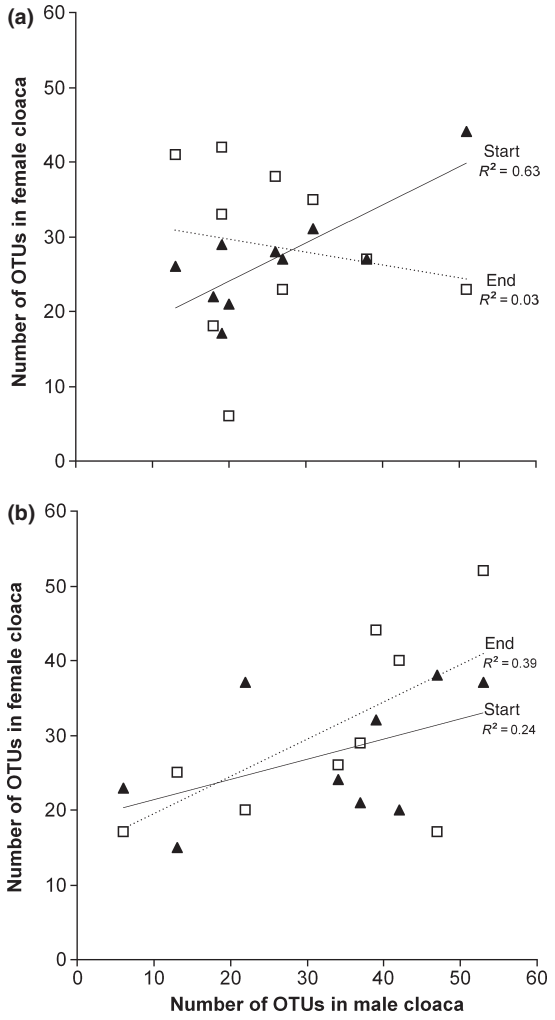

Figure 2 Number of operational taxonomic units (OTUs) in the female cloaca in relation to that of the male in (a) contraceptive device (CD) pairs and (b) control pairs at the start of the treatment (filled triangles, solid line) and the end of the treatment (empty squares, dashed line).

Changes in bacterial assemblage similarity within pairs

Correlation in number of OTUs

We investigated the relation between the number of OTUs in males and females. At the start of the experiment, the number of OTUs in the male and female cloaca was highly positively correlated (GLM, $F_{1,16} = 9.63$, $P = 0.007$; Fig. 2) with no significant difference between the treatment groups ($F_{1,16} = 0.44$, $P = 0.52$) or between mates (pairwise t-test, $t_{18} = -0.76$, $P = 0.45$). We then examined how this correlation changed according to the treatment and found, when correcting for the duration of the treatment, that the number of OTUs was no longer correlated in CD pairs (GLMM, $F_{1,7} = 0.46$, $P = 0.52$; significant interaction between number of OTUs in females and treatment event: $F_{1,8} = 5.11$, $P = 0.05$, with female as random variable; Fig. 2a) but remained correlated in the control pairs (GLMM, $F_{1,9} = 7.15$, $P = 0.02$; no interaction between number of OTUs in females and treatment event: $F_{1,7} = 0.59$, $P = 0.47$; Fig. 2b).

Bacterial community similarity coefficient

We calculated the similarity in cloacal bacterial assemblages among individuals using binary data (i.e. presence/absence of OTUs in each cloacal sample). When comparing the assemblages of all individuals (all possible combinations except breeding pairs, $n = 722$) at the start of the treatment, we found an average similarity of $50.30 \pm 0.44\%$. In comparison, males and females within a breeding pair had significantly more cloacal bacterial OTUs in common (mean: $57.70 \pm 3.27\%$, $n = 19$, GLM, $F_{1,740} = 7.12$, $P = 0.008$) reflecting that in most pairs bacterial transfer through copulation had occurred before the start of the experiment. We also measured the within-pair similarity of two unmanipulated pairs opportunistically captured seconds after the end of copulation and found some of the highest levels of similarity in our data set, 67 and 71%.

The within-pair similarity of the CD and control pairs did not differ before the experiment, indicating that pairs were chosen at random (GLM, $F_{1,18} = 1.39$, $P = 0.25$). Within-pair similarity was negatively related to the duration of the interval between female and male capture (GLM, $F_{1,18} = 5.67$, $P = 0.03$).

When we examined the similarity between cloacal assemblages of females re-sampled after the treatment with that of their mates, we found that control females shared more OTUs with their mates ($52.07 \pm 2.52\%$) than did CD females ($46.87 \pm 4.65\%$), although this difference was not significant (GLM, $F_{1,18} = 0.96$, $P = 0.33$). However, when we considered the change in similarity (i.e. the difference in male–female similarity before and after the treatment), duration of treatment (the time between fitting of device on the male and female recapture) had a strong negative effect in the CD group (GLM, $F_{1,9} = 11.33$, $P = 0.0098$; Fig. 3a) but not in the control group (GLM, $F_{1,8} = 0.24$, $P = 0.64$; Fig. 3b). The interaction between duration and treatment was not significant using the entire data set (GLM, $F_{1,18} = 1.59$, $P = 0.23$) but was significant when short durations were excluded from the analysis (GLM, $F_{1,16} = 5.62$, $P = 0.03$; see Material and Methods). Thus, the cloacal assemblages of CD females became increasingly dissimilar to those of their mates with time, whereas similarity did not change in the control group. In the same way, when considering within-female assemblage similarity (pre-treatment vs. post-treatment) according to the duration of the treatment, we found that it decreased significantly with time in CD females (GLM, $F_{1,8} = 9.94$, $P = 0.016$),

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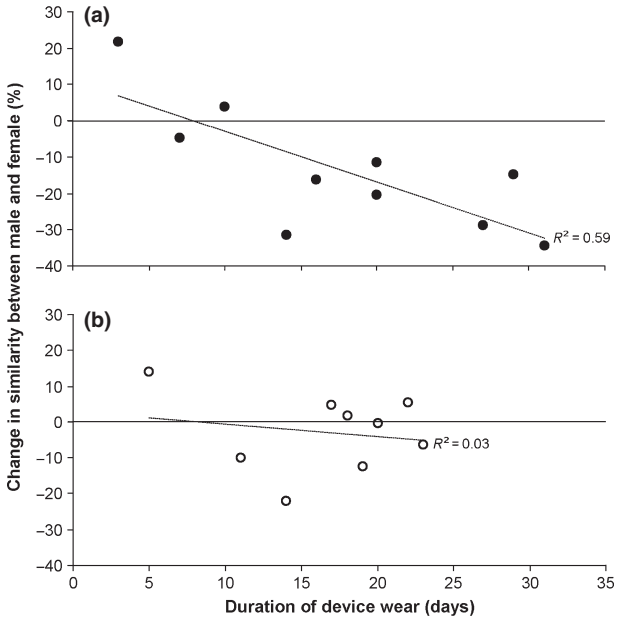

Figure 3 Change in similarity between the cloacal bacterial assemblages of the male and the female within a breeding pair according to the duration of device wear in (a) contraceptive device (CD) pairs and (b) control pairs. The *Y*-axis represents: Bray-Curtis coefficient ($Male_{before\ treatment}$ / $Female_{after\ treatment}$) − Bray-Curtis coefficient ($Male_{before\ treatment}$ / $Female_{before\ treatment}$) and the *X*-axis number of days between fitting of the device and female recapture.

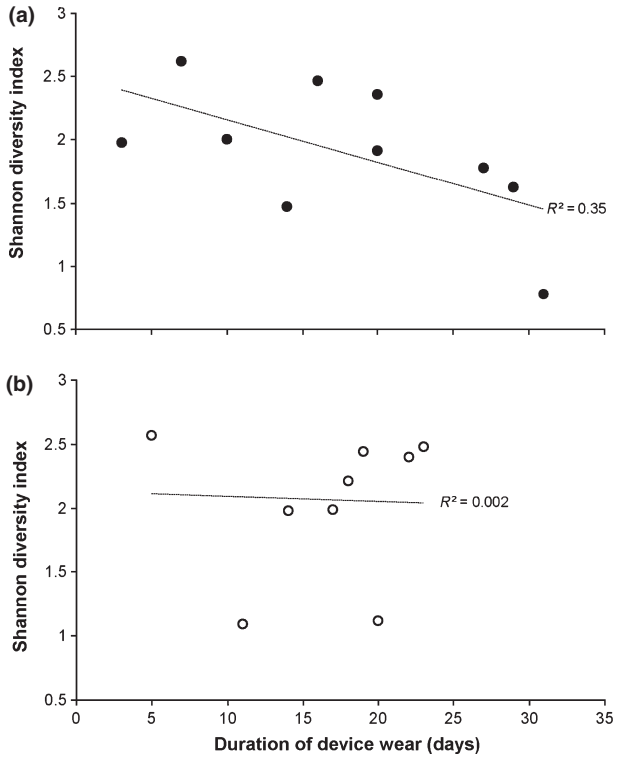

Figure 4 Diversity (Shannon index) of bacteria present in the female cloaca after the treatment according to the duration of device wear (in days) by (a) contraceptive device (CD) males and (b) control males.

whereas it did not change significantly in control females (GLM, $F_{1,8} = 0.78$, $P = 0.41$; duration × treatment interaction: $F_{1,17} = 5.40$, $P = 0.035$).

Changes in bacterial diversity

Shannon diversity index

We used the Shannon diversity index to measure the diversity of the bacterial communities of the cloaca. No significant difference in bacterial diversity was found between the control and CD females before the treatment (GLM, $F_{1,19} = 0.07$, $P = 0.80$).

The Shannon diversity of bacteria in all females after the treatment was negatively correlated with the date of female recapture (GLM, $F_{1,19} = 5.32$, $P = 0.037$). At the end of the treatment, bacterial diversity was significantly higher in control females than in CD females (GLM, $F_{1,19} = 5.15$, $P = 0.039$). We found a significant interaction between treatment and duration of treatment (GLM, $F_{1,19} = 5.95$, $P = 0.029$). More specifically, this meant that CD females underwent a strong decrease in bacterial diversity (GLM, $F_{1,9} = 6.29$, $P = 0.040$; Fig. 4a), whereas control females did not (GLM, $F_{1,9} = 1.11$, $P = 0.33$; Fig. 4b). The Simpson diversity index yielded qualitatively similar results as the Shannon index.

Bacterial immigration and extinction rate

In the previous results, we measured overall bacterial diversity regardless of which OTUs were present. In this section, we considered whether specific OTUs disappeared or appeared during the treatment. We did so by calculating for each cloacal community a mean immigration rate and a mean extinction rate, in both groups of females. There was no evidence of an effect of the treatment on the appearance of new OTUs, i.e. immigration rate in either control or CD females (control: 0.50 ± 0.07, CD: 0.50 ± 0.05; GLM, $F_{1,19} = 0.01$, $P = 0.93$). However, extinction rate increased significantly with the duration of treatment in CD females (GLM, $F_{1,9} = 5.8$, $P = 0.046$; Fig 5a) but remained constant in control females (mean: 0.46 ± 0.03; GLM, $F_{1,9} = 0.05$, $P = 0.83$; Fig 5b; duration × treatment interaction: $F_{1,16} = 7.35$, $P = 0.017$ when excluding short durations; see Materials and Methods). We further considered the fate of the OTUs that were shared by both mates at the start of the experiment. The extinction rate of these shared OTUs increased with the duration of the treatment in CD females (GLM; $F_{1,9} = 10.72$, $P = 0.01$), whereas there was no significant trend in control females (GLM, $F_{1,8} = 0.44$, $P = 0.53$).

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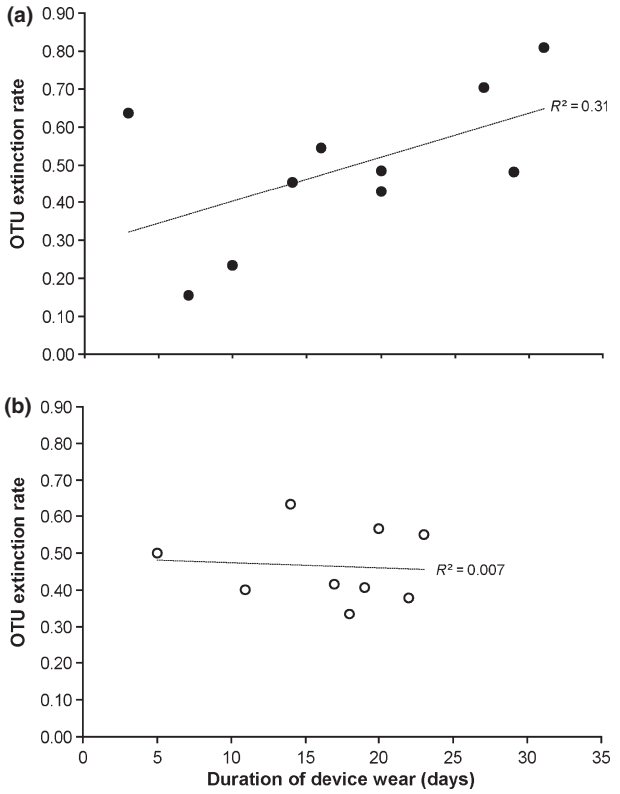

Figure 5 Extinction rate of operational taxonomic units (OTUs) present in the cloaca before the treatment in (a) contraceptive device (CD) females and (b) control females according to the duration of device wear (in days).

DISCUSSION

General assemblage structure

We identified 190 different bacterial OTUs, most of which showed very low prevalence. The presence of numerous rare strains in the cloacae of black-legged kittiwakes indicates wide variation in cloacal assemblages among individuals. It is well established that bacterial communities hosted in the gut of vertebrates show a high degree of interindividual variability in community composition. Studies on bacterial communities found in avian cloacae (Lucas & Heeb 2005; Klomp *et al.* 2008; Banks *et al.* 2009; Ruiz-Rodriguez *et al.* 2009), avian crops (Godoy-Vitorino *et al.* 2008), the gut of fish (Burr *et al.* 2005) and mammals (Ley *et al.* 2008a), including humans (Backhed *et al.* 2005; Ley *et al.* 2008b) all show that individuals harbour a unique internal microbiota forming a 'microbial signature'. The fact that over 25% of all OTUs identified in this study were unique to individuals suggests that such a cloacal microbial signature also occurs in kittiwakes, providing the potential for detecting sexual transfer of cloacal bacteria.

Changes in female cloacal assemblage composition

At the start of the experiment, mates shared more cloacal OTUs than they did with other individuals, as was found in tree swallows (Lombardo *et al.* 1996). Furthermore, we found a positive correlation between mates in the number of OTUs, as reported by Stewart & Rambo (2000) in house sparrows. Both previous studies offered these patterns as evidence of transfer of cloacal bacteria during copulation. By experimentally preventing inseminations, we were able to examine whether sexual transmission is the cause of such similarity between mates. When inseminations were prevented, the correlation in numbers of OTUs no longer existed, whereas it was maintained in the control group (Fig. 2). In another analysis, we found that the cloacal communities of experimental females grew increasingly dissimilar to those of their mates, whereas similarity was unchanged in control pairs (Fig. 3). Given that insemination was the only manipulated means of transmission, our experiment provides direct evidence that mate similarity is maintained by sexual transmission of bacteria.

The analysis of bacterial diversity of cloacal communities provided further insight on the effects of inseminations on the female cloacal microbiota. When copulations were prevented, bacterial diversity decreased (Fig. 4a), the proximate reason being an increase in OTU extinction rate (Fig. 5a). We found that the vast majority of OTUs that became extinct from the female cloaca were those that females shared with their mates before the devices were fitted. The disappearance of such mate-shared bacterial OTUs may thus explain the decrease in female cloacal diversity as well as the decrease in male–female similarity.

An additional question is whether male communities are also affected by copulation. We were unable to address this because of the difficulty of re-trapping males during the experiment. However, there is experimental evidence in birds that sexual transfer is predominantly from males to females (Kulkarni & Heeb 2007), as expected by the directional flow of the ejaculate. In our control pairs, female communities after treatment remained similar to those of their mates before treatment (Fig. 3b). This suggests that male communities were fairly stable and that little female to male transfer occurred. Nevertheless, female to male transfer of bacteria needs to be examined to achieve a better understanding of the dynamics of bacterial transmission.

Are female cloacal communities resilient?

It would also be interesting to examine the initial composition and diversity of the cloacal communities of mates and the changes that may occur after they start copulating. As we were unable to sample kittiwakes before copulations commenced, we lack direct evidence that the cloacal

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assemblages of mates were significantly different before the first copulations. However, three lines of evidence suggest that they probably do differ. (1) We found high interindividual variability in cloacal bacterial community composition. (2) As is typical for pelagic seabirds, kittiwakes spend most of the year on the open sea where mates are unlikely to have physical contact. (3) When sexual transmission was experimentally prevented, pairs lost 35% of cloacal similarity in < 30 days (Fig. 3a). It is also plausible that before copulations began, female cloacal diversity was lower than at the start of the experiment, as suggested by the decrease in female bacterial diversity when re-inseminations were prevented (Fig. 4a).

When copulations commence, ejaculates likely convey new bacterial strains to the female cloaca, adding on to the indigenous bacterial species, thus causing the higher levels of diversity and similarity observed at the start of our experiment. The decrease in overall diversity and the extinction of mate-shared OTUs (Fig. 5a) suggests that when copulations cease, the female cloacal bacteria return to a lower diversity assemblage specific to the female. It has been shown that mammalian gastrointestinal bacterial communities possess a certain level of resilience following perturbation (De La Cochetiere *et al.* 2005; Antonopoulos *et al.* 2009). It appears that the same process may be occurring in the cloacae of individual kittiwakes, where the female cloacal bacterial community reverts to its former state after the changes caused by sexual transfer of male-specific bacteria. Our findings thus extend to the cloaca the view that the gut comprises a resilient ecosystem of microorganisms (e.g. Zoetendal *et al.* 2006). In this perspective, bacteria transferred by the male are equivalent to species introduced into a new, potentially inhospitable environment already occupied by indigenous species. The increasing extinction rate of apparently introduced species suggests that the female cloaca may comprise a stable ecosystem. This is all the more noteworthy as female cloacal communities receive input not only from ingested material as does the rest of the gastrointestinal tract, but also from inseminations and cloacal contacts.

Sexually transmitted bacteria and host condition

The indigenous gastrointestinal microbiota performs a variety of beneficial functions for their host. These include a central role in digestion and nutrition (Sears 2005), in the immune system (Sansonetti & Di Santo 2007) and in prevention of colonization by pathogens in the gastrointestinal tract (Servin 2004). The addition of new bacterial strains, the increase in overall bacterial diversity and the change in the bacterial community structure caused by copulations is thus likely to affect female physiology and health in various ways. The addition of new species may increase the probability of being inseminated with beneficial bacteria (Lombardo *et al.* 1999) that produce bacteriocins lethal to resident pathogenic strains or that help in the synthesis and absorption of nutrients (Stevens & Hume 1998) thus providing the female with new probiotic and/or antibiotic bacteria. Alternatively, the addition of new species and the sudden increase in diversity may disrupt the normal balance of the indigenous bacterial community and may create net costs to the female. A higher number of bacterial strains was negatively related to chick growth in Magellanic penguins *Spheniscus magellanicus* (Potti *et al.* 2002). Furthermore, sexual transfer of new species may increase the probability of pathogenic bacteria becoming established in the cloaca (Sheldon 1993), disrupting the indigenous microbiota (Kuehl *et al.* 2005) and affecting host health. These potential consequences emphasize the need for further research to determine whether bacteria transferred sexually affect host condition or immunity. Such investigations would be strengthened by the identification of the bacterial species corresponding to the OTUs that impact host fitness (see Benskin *et al.* 2009 for a review of bacterial pathogens in wild birds).

Sexually transmitted bacteria, behaviour and sexual selection

The opposing fitness consequences of beneficial and costly sexually transmitted bacteria are likely to drive the evolution of mating strategies in different ways (Lombardo *et al.* 1999; Thrall *et al.* 2000; Kokko *et al.* 2002) and create multiple implications for sexual selection (Loehle 1997). Females may evolve behavioural strategies to reduce the detrimental impact of sexually transmitted bacteria, such as the post-copulatory sperm ejection behaviour exhibited by female kittiwakes (Helfenstein *et al.* 2003; Wagner *et al.* 2004). Ornamental traits may evolve to signal the likelihood that they will transmit pathogens (Hamilton 1990) or beneficial bacteria (Lombardo *et al.* 1999) during copulation. Furthermore, as cloacal assemblages are likely to be influenced by the genetic characteristics of hosts, such as their heterozygosity and Major Histocompatibility Complex (MHC) alleles, the consideration of sexual transmission of bacteria may shed new light on the processes involved in mate choice based on genetic criteria (Mulard *et al.* 2009). More generally, our findings may stimulate further research on the role of behavioural vectors in the transmission of diseases in wild populations.

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