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Experimental evidence of a sexually transmitted infection in a wild vertebrate, the black-legged kittiwake (*Rissa tridactyla*)

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Sexually transmitted infections (STIs) in animals may have profound eco-evolutionary consequences, yet experimental studies of the sexual transmission of pathogens in wild populations are lacking. Here to identify sexually transmitted bacteria, we experimentally manipulated ejaculate transfer in black-legged kittiwakes (*Rissa tridactyla*) by blocking insemination after pairs had commenced copulating. We found that a *Corynebacterium* pathogenic strain was cleared from the cloaca of females five times more frequently in the experimental group, indicating it had been sexually transferred. A typical feature of STIs is that they reduce fertility, and in our kittiwake population, infected females suffered significantly higher hatching failure than uninfected females. Nevertheless, infected females achieved the same reproductive success as uninfected females by laying earlier and producing more eggs, suggesting reproductive compensation, a common strategy adopted by infected animals and plants. Our results provide new insights into the fitness consequences of STIs in a wild species and may stimulate further research on their evolutionary implications.

ADDITIONAL KEYWORDS: bacteria – birds – *Corynebacterium* – pathogen – reproductive success – sexual transmission.

INTRODUCTION

Sexually transmitted infections (STIs) infect taxonomically diverse hosts and occur in virtually all sexually reproducing animal groups, including mammals, birds, insects and gastropods (Sheldon, 1993; Lockhart *et al.*, 1996; Knell & Webberley, 2004), as well as in plants (Wennström *et al.*, 2003). They differ from other infectious diseases by being less likely to cause mortality, more likely to reduce fertility and occurring at more stable frequencies over time (Lockhart *et al.*, 1996). Sexually transmitted parasites have unique implications in evolutionary ecology, as they may cause intense selective pressures on mating system, behaviours and reproductive physiology (Hamilton & Zuk, 1982; Sheldon, 1993; Lockhart *et al.*, 1996; Kokko *et al.*, 2002; Knell & Webberley, 2004; McLeod & Day, 2014; Ashby & Boots, 2015; Zuk, 2015). However, most studies of STIs have focused on domestic animals or wild species in captivity (Smith & Dobson, 1992; Lockhart *et al.*, 1996), where selection might be relaxed. Research on wild species in their natural habitats is therefore needed to identify mechanisms of selection that shape the coevolutionary dynamics between STIs and their hosts.

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In a previous study in wild black-legged kittiwakes (*Rissa tridactyla*; Helfenstein *et al.*, 2004), we used an experimental method that blocked ejaculate transfer after pairs had commenced copulating (White *et al.*, 2008), which revealed that cloacal bacterial communities of mates became increasingly dissimilar after inseminations were blocked (White *et al.*, 2010). This result indicated that males transfer bacteria to their mates via copulation (White *et al.*, 2010).

Here, we used a similar experimental design to search for sexually transmitted bacteria causing STIs in kittiwakes. First, we identified the bacterial phylotypes that are transferred during copulations, i.e. those that had been shared by mates before the start of the experiment and had become extinct in the female cloaca after inseminations were blocked. Second, we examined whether some of these sexually transmitted bacteria were associated with reduced reproductive performance.

MATERIAL AND METHODS

This study was conducted in the 2006, 2009 and 2010 breeding seasons on a wild population of black-legged kittiwakes nesting on an abandoned US Air Force radar tower on Middleton Island (59°26′N, 146°20′W), Gulf of Alaska. For the blocked-insemination experiment, we captured 70 randomly chosen kittiwake pairs during the prelaying phase. They were captured after they had commenced copulating, so that males had previously transferred their cloacal bacteria to their mates (White *et al.*, 2010). We sampled the cloacal bacteria of males and females by flushing the cloaca with 1 mL of saline solution. The males of 33 pairs were then fitted with the anti-insemination device, i.e. a ring placed over the cloaca and held in place with a harness. The remaining 37 males were fitted with the control device that allowed normal cloacal contact and insemination. All kittiwake pairs were used only once in the experiment. Method details are described elsewhere (White *et al.*, 2008). We recaptured each pair when the first egg was laid and resampled their cloacal bacteria. The experimental devices were then removed from the males. Experiments were conducted in accordance with US laws and under permits from the US Fish and Wildlife Service and State of Alaska.

To investigate the impact of bacterial infection on host fitness, we captured 177 females and 171 males during the prelaying period that were not involved in the anti-insemination experiments. We sampled their cloacal bacteria as described above. Nests were monitored daily to assess each pair's reproductive performance in the form of egg-laying, hatching and fledging.

For all sampled individuals, we characterized the cloacal bacterial assemblages using automated ribosomal intergenic spacer analysis (ARISA), which exploits the extreme inter-OTU (operational taxonomic units) variability in the length of the intergenic spacer (IGS) lying between the 16S and 23S rRNA genes (Ranjard *et al.*, 2000). ARISA entails the amplification of DNA extracted from the bacterial community of interest using a fluorescently labelled primer and subsequent high-resolution electrophoresis in an automated system. This method has been optimized for the characterization of cloacal bacterial communities in kittiwakes (White *et al.*, 2010; van Dongen *et al.*, 2013).

To search for bacteria that were potentially sexually transmitted, we examined the extinction frequencies of the 31 OTUs that we had identified in the cloacas of adult kittiwakes (van Dongen *et al.*, 2013) by comparing them between experimental and control pairs using Fisher's exact tests. Blocking inseminations allowed us to distinguish sexual transmission from alternative modes of horizontal transmission between mates, as differences in cloacal bacteria between experimental and control groups can result exclusively from insemination and cloacal contact. This method is conservative as we would be unlikely to detect the sexual transmission of certain highly prevalent bacteria that are consistently part of the cloacal community and that may only rarely become extinct. Further, many gastrointestinal bacteria are known to be beneficial and would not be expected to become extinct because it would be adaptive for hosts to retain them (Lombardo *et al.*, 1999). Additionally, our design requires OTUs to be sufficiently prevalent to provide an adequate sample size for statistical analysis.

We then genetically characterized the bacteria identified as being sexually transmitted, by sequencing 1524 bp of the 16S rRNA gene (including hypervariable regions 6–9:). We first amplified a part of both the IGS and the 16S gene, using a primer lying in the IGS (C34_IGS.R1: 5′-CACAGAAACCACAACACAGC-3′) and a universal bacterial primer located within the 16S gene (784F: 5′-AGGATTAGATACCCTGGTA-3′). We then amplified the remainder of the 16S gene using a primer lying within variable region 7 (C34_V7age_R: 5′-TCCCATGAGTCCCCACCATC-3′) and the universal primer 27F (5′-agagtttgatcctggctcag-3′).

We tested for associations between reproductive performance and infection by apparently sexually transmitted bacteria using generalized linear mixed models (GLMMs), incorporating kittiwake ID as a random effect. To control for confounding factors, we tested a range of models for each dependent variable that included different combinations of variables that we hypothesized may be correlated with the dependent

variable. Because we were interested in the effects of infection, all models included presence of the sexually transmitted bacteria as a fixed factor. To select the most parsimonious model, we used the Akaike information criterion (AIC) (Burnham *et al.*, 2011). The model with the lowest AIC value was considered the best. When the model with the lowest and second lowest AIC values differed by less than 2, we selected the model with the least number of variables (Quinn & Keough, 2002). For all analyses using GLMMs, we presented predicted means ± SE. All statistical analyses were conducted using SPSS 17.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Of the 31 OTUs identified, only one bacterium met the criteria of being of intermediate prevalence at the start of the experiment and showing experimental evidence of being sexually transmitted. *Corynebacterium* OTU 34 (named *C*34 because it was the 34th OTU we had identified, GenBank accession number KJ160259; van Dongen *et al.*, 2013) became extinct in experimental females' cloacas five times more frequently than in control females (extinction rate in experimental females: 69%, $N = 13$ females sampled; control females: 14%, $N = 14$ females sampled; Fisher's exact test: $P = 0.004$, Fig. 1). This result might be explained by the ability of females to clear certain microbes from their cloaca in the absence of further inseminations by their mates (White *et al.*, 2010). This interpretation is supported by a positive correlation between the duration of anti-insemination ring wear and the extinction frequency in experimental pairs (logistic regression - duration × treatment: Nagelkerke $r^2 = 0.381$, Wald $\chi^2 = 6.386$, $N = 27$, $P = 0.012$) but not in control pairs, suggesting that females were more effective at clearing their cloaca of *C*34 when they had more time to do so. Fourteen of the other 30 OTUs (47%) were not detected in the cloacas of females in either group, and the extinction frequencies of the remaining 16 OTUs did not differ between the two groups ($0.316 < P < 1.000$), suggesting they were not sexually transmitted or that we lacked the statistical power to detect sexual transmission.

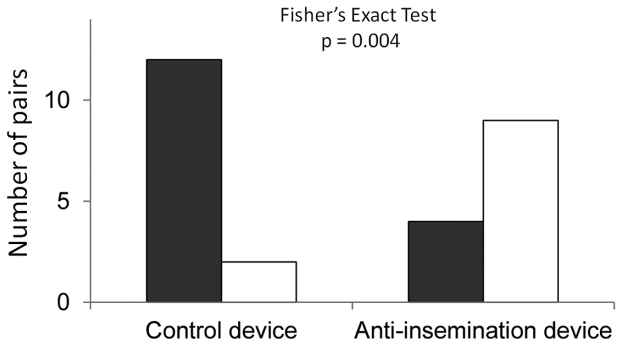

Figure 1. Extinction rate of *C*34 according to treatment. Number of pairs in which *C*34 was retained (grey bars) or became extinct (white bars) in the female cloaca when her mate was fitted with an anti-insemination ring or control device.

We found that *C*34 belongs to the genus *Corynebacterium*, which includes many medically relevant species, several of which are known zoonoses (Bernard, 2012). Phylogenetic analyses revealed that the closest known relatives of *C*34 are *Corynebacterium ciconiae* and *C. trachiae* (Supporting Information Fig. S1), two bacteria isolated from the trachea of storks (*Ciconia* sp.) (Fernández-Garayzábal *et al.*, 2004; Kämpfer *et al.*, 2015). The next closest relative of *C*34 is *Corynebacterium propinquum* (Supporting Information Fig. S1), a pathogen known in humans (Díez-Aguilar *et al.*, 2013), that can be sexually transmitted (Abdolrasouli & Roushan, 2013). *C*34 also clusters closely with the pathogenic *C. pseudodiphtheriticum* (Díez-Aguilar *et al.*, 2013) and more distantly with the causative agent of diphtheria (*C. diphtheriae*) and the pathogenic *C. pseudotuberculosis* (Supporting Information, Fig. S1) (Bernard, 2012). Other species of *Corynebacterium* are associated with infertility in humans (Riegel *et al.*, 1995) and other mammals (Hartigan, 1980).

In female kittiwakes that were not involved in the anti-insemination experiment, *C*34 was detected in the cloacas of 53.4% of them. Eggs laid by infected females were significantly less likely to hatch than eggs laid by uninfected females (predicted proportion of eggs that hatched: infected females = 0.72 ± 0.04% of eggs, uninfected females = 0.80 ± 0.04% of eggs; Table 1, Fig. 2A; Table S3). Sexually transmitted diseases are commonly associated with infertility in humans, animals and plants (Sheldon, 1993; Hurst *et al.*, 1995; Lockhart *et al.*, 1996; Knell & Webberley, 2004; Apari *et al.*, 2014), but studies in wild vertebrates are scarce. Our results add to the evidence from wild koalas (*Phascolarctos cinereus*; Polkinghorne *et al.*, 2013) and wild chimpanzees (*Pan troglodytes*; Keele *et al.*, 2009), where STIs were reported to cause reproductive failure in females.

Infected kittiwake females tended to lay more eggs than uninfected females (predicted number of eggs laid: infected females = 1.86 ± 0.04, uninfected females = 1.79 ± 0.04; Table 1, Fig. 2B; Table S3), and the numbers of chicks fledged by infected and uninfected females did not differ significantly (predicted number of chicks fledged: infected females = 0.55 ± 0.06, uninfected females = 0.52 ± 0.06; Table 1, Fig. 2C; Table S3). Infected females may thus achieve equivalent fledging success to that of uninfected

Table 1. Models outlining associations between infection of female kittiwakes by *C*34 and various reproductive variables. Each model incorporated infection as a fixed factor (highlighted in bold) and various other factors as covariables. The most parsimonious models are shown, as determined using Akaike's information criterion

Dependent variable	Predictor	*F*	d.f.	*P*
Number of offspring fledged	**Infection**	**0.248**	**1152**	**0.619**
	Laying date	22.894	1152	<0.001
	Number of eggs hatched	128.761	1152	<0.001
	Sampling year	34.138	2152	<0.001
Number of eggs laid	**Infection**	**3.787**	**1153**	**0.053**
	Laying date	13.252	1153	<0.001
	Sampling year	6.964	2153	0.001
Proportion of eggs hatched	**Infection**	**5.612**	**1154**	**0.019**
	Number of eggs laid	44.012	1154	<0.001
	Sampling year	20.877	2154	<0.001
Laying date of first egg	**Infection**	**8.110**	**1154**	**0.005**
	Sampling year	37.320	2154	<0.001

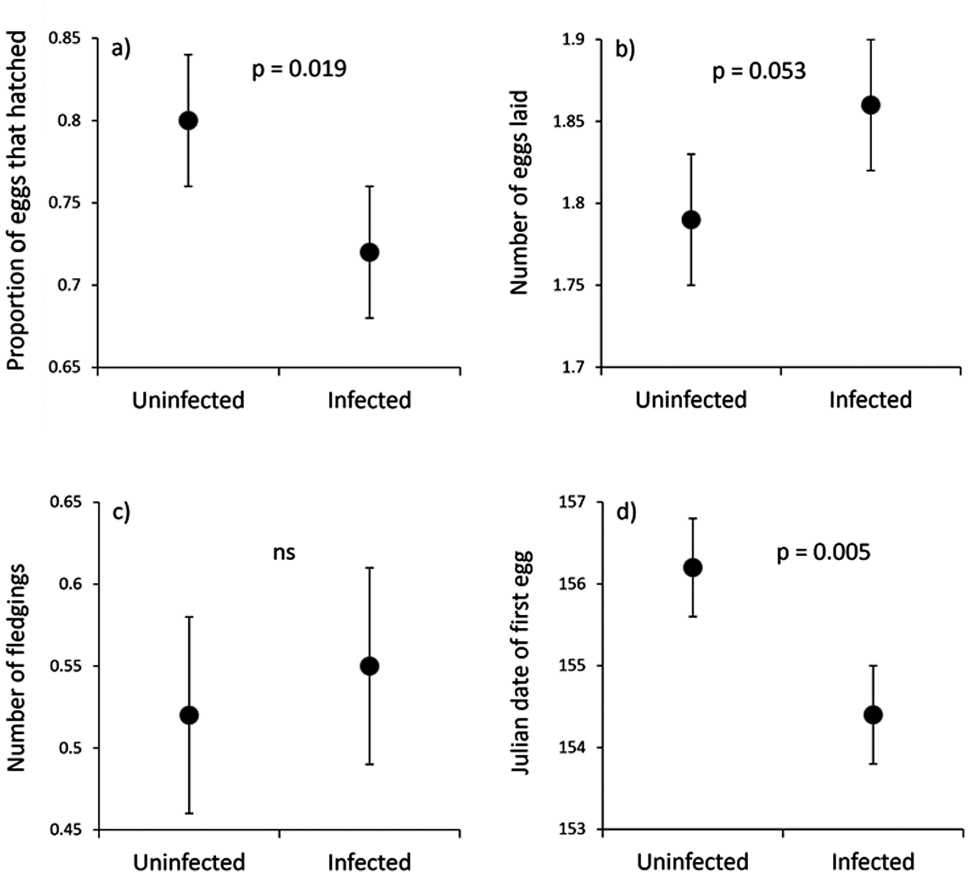

Figure 2. Impact of C34 on reproduction success. Association between female infection by *C*34 and reproductive success, including proportion of eggs hatched (A), number of eggs laid (B), number of fledglings (C) and laying date of first egg (D). Data are shown as means ± SE.

females by making greater reproductive effort, such as by increasing their clutch size. In addition, infected females laid their eggs significantly earlier than uninfected females (predicted laying Julian-date of first egg: infected females = 154.4 ± 0.6 days, uninfected females = 156.2 ± 0.6 days; Table 1, Fig. 2D; Table S3).

Early laying is a well-documented, costly tactic to increase reproductive success in birds in that it may increase the time window for subsequent breeding activities or reduce the lag between breeding and the seasonal peak of food abundance (Verhulst & Nilsson, 2008). In our kittiwake population, there was a highly significant relationship between the date of egg-laying and the number of eggs laid (Table 1). Our finding that infected females made a greater reproductive effort suggests they had pursued a strategy of compensation, as is common in infected animals and plants (Shykoff & Kaltz, 1997; Sanz et al., 2001; Korves & Bergelson, 2003; Knell & Webberley, 2004; Jones *et al.*, 2008). Further investigating whether *C*34 infection and the associated greater reproductive effort entail long-term fitness costs in females would be crucial to determine their potential as selective pressure shaping strategies, such as monogamy or mate choice.

Some of our findings could hypothetically result from reduced fertility of males caused by *C*34. Specifically, damage to the reproductive organs or sperm could explain reduced hatching success, in which case the extra reproductive effort by females may be in compensation for lower male fertility. We examined this by performing the same analyses on 171 males and found no effects of infection by *C*34 on any variables relating to reproductive performance (Supporting Information, Tables S1 and S4). Furthermore, among the reproductive variables that were significant for females, we found significant interactions between sex and infection. These analyses indicate that reproductive performance was affected by female, and not by male, infection (Tables S2 and S5).

In conclusion, to the best of our knowledge, our study provides the first manipulation of sperm transfer to demonstrate that a bacterium potentially causing infection is sexually transmitted in a wild species in its natural habitat. The ability to do so may stimulate further research on the role of STIs in shaping sexual behaviour and the coevolution of host–parasite dynamics in wild species.

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AUTHOR CONTRIBUTIONS

J.W., E.D. and R.H.W. conceived and designed the study, J.W., S.A.H. and S.L. collected the field data, W.F.D.V.D. and H.B.B. performed the genetic and statistical analyses, and R.H.W. and W.F.D.V.D. wrote the manuscript. All authors commented on the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. 16S rRNA phylogenetic trees outlining the position of *C*34 relative to known species of *Corynebacterium*. Phylogenies were inferred using maximum likelihood and Bayesian methods. Thick-lined nodes represent those with high maximum likelihood bootstrap support (≥70%) and asterisks highlight nodes with Bayesian posterior probabilities ≥98%. The tree was rooted with *Amycolatopsis mediterranei* and *Mycobacterium leprae*. Corynebacteria isolated from birds are highlighted in red.

Table S1. Models outlining associations between infection of male kittiwakes by *C*34 and various reproduction variables. Each model incorporated infection as a fixed factor (highlighted in bold) and various other factors as covariables. The most parsimonious models are shown, as determined using Akaike's information criterion.
Table S2. Models outlining associations between the interaction between sex and infection by *C*34 on various reproduction variables. Each model incorporated sex × infection as a fixed factor (highlighted in bold) and various other factors as covariables. The most parsimonious models are shown, as determined using Akaike's information criterion.
Table S3. The most parsimonious models explaining the effect of female infection with *C*34 on various reproduction variables. The most parsimonious models were determined using Akaike's information criterion.
Table S4. The most parsimonious models explaining the effect of male infection with *C*34 on various reproduction variables. The most parsimonious models were determined using Akaike's information criterion.
Table S5. The most parsimonious models explaining the effect of the interaction between sex and infection with *C*34 on various reproduction variables. The most parsimonious models were determined using Akaike's information criterion.