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Source: Journal of Wildlife Diseases, 36(3) : 460-468

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-36.3.460

Journal of Wildlife Diseases, 36(3), 2000, pp. 460–468

MICROBES IN TREE SWALLOW SEMEN

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ABSTRACT: A frequently hypothesized but poorly studied cost of multiple mating in birds is that exposure to pathogenic sexually transmitted microbes (STM's) can lower reproductive success. Conversely, female birds may benefit from high frequencies of copulation and multiple copulation partners if they receive cloacal inoculations of beneficial STM's that can either protect them against future encounters with pathogens and/or serve as therapy against present infection. We examined the semen of 30 male tree swallows (*Tachycineta bicolor*) in 1998 to determine the presence and prevalence of potential pathogenic and beneficial STM's. Semen was collected directly from males after applying gentle pressure to the cloaca and we used standard microbiological techniques to identify microbes. We found that 19 of 30 samples contained one or more types of microbes. In these 19 positive samples, we isolated both pathogenic and beneficial microbes from 11, only pathogenic microbes from seven, and only beneficial microbes from one. This variation among males suggests that females would benefit from considering a particular male's potential as a donor of either pathogenic or beneficial STM's as a criterion for mate choice. There were few significant differences between males with pathogen-infected semen and those without pathogens in their semen in measures of size, morphology, and ectoparasite score and feather damage. Likewise, there were few significant differences between males with beneficial *Lactobacilli* spp. in their semen and those without *Lactobacilli* spp. in their semen in measures of size, morphology, and ectoparasite score and feather damage. We were unable to determine if there was a relationship between microbe presence and prevalence on reproductive performance.

Key words: Pathogens, semen microbes, sexually transmitted microbes, sexually transmitted diseases, tree swallow, *Tachycineta bicolor.*

INTRODUCTION

It is important to identify possible causes of sexually transmitted diseases (STD's) and determine their prevalence in wild bird populations because of their far-reaching effects. Sexually transmitted diseases can influence population dynamics (Anderson and May, 1991) and the evolution of sexual behavior (Hamilton, 1990) because they influence individual morbidity, mortality, and fertility, and may affect patterns of mating. As evidence of the possible important effects of STD's on population dynamics and behavior consider that (1) pelvic inflammatory disease in humans caused by sexually transmitted *Chlamydia trachomatis* is a major cause of human female infertility (McCormack, 1994), (2) acquired immune deficiency syndrome (AIDS) caused by sexually transmitted human immunodeficiency virus (HIV) is the leading cause of death among people aged 25 to 44 in the United States (Centers for Disease Control, 1995), and (3) the risk of contracting AIDS has influenced patterns of sexual behavior in the United States (Stine, 1993).

In stark contrast to our knowledge about STD's in humans, we know virtually nothing about the effects of STD's in wild bird populations. In fact, all of the incidences of confirmed STD's in birds occur in domesticated species of economically important waterfowl (Anseriformes) and fowl (Galliformes) (Sheldon, 1993, Table 1; Lockhart et al., 1996, Table 3). Several lines of evidence suggest that STD's may be important in wild birds. First, the existence of avian STD's in domestic and cage birds (Sheldon, 1993) is direct evidence that birds inoculate each other with pathogenic microbes during copulation. Furthermore, Perek et al. (1969) showed by experiment that male domestic cockerels (*Gallus domesticus*) with semen contaminated with bacteria infected the females with which they copulated. Second,

numerous species of pathogenic microbes have been isolated from the cloacae of wild birds (e.g., Cooper et al., 1980; Brittingham et al., 1988; Lombardo et al., 1996). The avian cloaca is involved in both excretion and gamete transfer creating the possibility of potentially pathogenic intestinal microbes becoming incorporated into ejaculates and transmitted to females (Sheldon, 1993). Furthermore, commensal microbes that normally reside in the gastrointestinal tract may evolve pathogenicity when introduced into the reproductive tract as a consequence of natural selection on other microbial traits (e.g., reproductive rate) and not necessarily virulence per se (Levin and Svanborg Edén, 1990). This possibility increases the number of potential causes of STD's in birds (Sheldon, 1993). Morrill and Robertson (1990) showed that microspheres inserted into the cloacae of male tree swallows (*Tachycineta bicolor*) were later transmitted to females during copulation demonstrating that non-semen cloacal contents can be transmitted to females during copulation. Finally, our observation that the assemblages of cloacal microbes found in pair-bonded swallows were more alike in size and microbial composition than the assemblages found in other sampled adults (Lombardo et al., 1996) implies that tree swallows transmit cloacal microbes during copulation. Tree swallows are well suited for studying the horizontal transmission of STM's because they have some of the highest frequencies of extra-pair paternity known for songbirds (Lifjeld and Robertson, 1992; Lifjeld et al., 1993; Dunn et al., 1994a, b; Barber et al., 1996) with 87 percent of nests in a Canadian population containing young fathered by extra-pair males during some years (Kempenaers et al., 1999).

While STD's may be important selective forces in the evolution of avian mate choice (Hamilton, 1990) and mating systems (Sheldon, 1993; Lombardo, 1998), some sexually transmitted microbes (STM's) may have beneficial effects on the health of their recipients (Hutchenson et al., 1991) and therefore reproductive success. Elsewhere, we have hypothesized that females may directly benefit from high frequencies of copulation and multiple copulation partners if they receive a cloacal inoculation of beneficial STM's that can either serve as therapy against present infection and/or protect them against future encounters with pathogens (Lombardo et al., 1999). The benefits associated with the horizontal transmission of beneficial STM's may be a selective force helping to mold the evolution of mate choice and copulation behavior in birds.

Our aim was to identify and determine the prevalence of STM's in tree swallow semen. Previously, we isolated potential pathogens such as *Campylobacter jejuni*, *Salmonella* spp., *Shigella* spp., *Streptococcus* spp., multiple fungi and potential beneficial microbes such as *Lactobacilli* spp. (Bokkenheuser, 1993) from the cloacae of adult tree swallows (Lombardo et al., 1996). Females that could detect males that carried pathogenic or beneficial STM's would be favored if they either avoided or pursued copulations with males in these respective categories and could possibly use a male's physical or behavioral characters as a signal of his potential as a donor of pathogenic or beneficial STM's (Lombardo et al., 1999). Therefore, one of our goals was to determine if there were relationships between microbe presence and prevalence and male size, morphology, ectoparasite score, and feather damage.

MATERIALS AND METHODS

Tree swallows are small (ca. 20 g) cavity nesting aerial insectivores that are common throughout eastern North America. In 1998, we collected semen from 30 male Tree Swallows that bred in some of the 100 wooden nest boxes mounted on metal poles erected in grids in an old field on the campus of Grand Valley State University (Ottawa County, Michigan, USA; 42°57′N, 85°53′W).

We captured male swallows at their nest boxes while they delivered food to nestlings. Each swallow was banded with a U.S. Fish and Wildlife Service numbered aluminum band and giv-

en a unique color-mark on its breast, tail, throat, or wing feathers using water-proof marking pens and acrylic paints to facilitate individual identification. For each swallow, we measured the length of the left and right tarsi and the exposed culmen with an electronic digital caliper to 0.01 mm; the unflattened left and right wing chords to 1 mm with a ruler with a stop fixed to one end, and weight to the nearest 0.2 g with an Avinet spring scale. The degree of tail forking was determined by measuring the length of tail feathers from the notch in the center of the tail to the tip of the outer tail feathers on each side of the tail to the nearest 1 mm with a ruler while the tail was held with the outer edges of each outer tail feather parallel to each other. The degree of asymmetry of tarsi, wing chords, and tail forks were calculated following Møller (1990) as the absolute value of (the difference between the lengths of the characters on the left and right side) divided by (the mean of the lengths of the characters on the left and right sides).

Each swallow was scrutinized for the presence and topographic distribution (i.e., head, back, rump, chin, breast, cloaca, wings, tail) of ectoparasitic feather lice (Insecta: Phthiraptera) and feather mites (Acari: Analgesoidea). The number of ectoparasites found on each topographic region was scored as 0 when no parasites were detected, 1 when between 1 to 10 parasites were detected, 2 when between 11 to 100 parasites were detected, and 3 when between 101 to 1,000 were detected. Each swallow was given ectoparasite scores for its body (the sum of head + back + rump + cloaca + breast + chin scores), each wing, and tail. In addition, we counted the numbers of holes produced by mites (Bruce and Johnson, 1969) in the primary and secondary wing and tail feathers on each bird.

Tree swallow cloacae contain many different kinds of microbes (Lombardo et al., 1996). To control for cloacal microbe contamination of semen samples, we first sampled the exterior of the cloaca with a sterile Dacron swab. Then with gentle pressure the cloaca was squeezed causing it to evert. The everted cloaca was surface sterilized with an alcohol swab (Perek et al., 1969). We then obtained semen from males by applying gentle manual pressure to the seminal vesicles in the cloacal protuberance (Samour et al., 1986). The expressed semen was swabbed with a sterile Dacron swab to collect microbes. The two microbe samples were placed in 3 ml of sterile thiogylcollate broth (Troy Biologicals, Inc., Troy, Michigan, USA) and held on ice until transported to the laboratory. After swabbing for microbes, a second semen sample was expressed and 5 μl collected into 200 μl of 5% formalin for sperm counting. We did not screen this sample for microbes.

Microbe samples were incubated at 35 C for 24 hr before they were plated on a variety of selective and/or differential growth media (Table 1). All media were obtained from Troy Biologicals, Inc., Troy, Michigan, USA. Immediately before plating, sample tubes were vortexed for 20 sec and the swab removed. This method was adopted as direct plating of the thioglycollate after sampling was found to result in only a few colonies on any one type of media. Microbe samples were plated using either a semi-quantitative streak or by quantitative plating. Triplicate 100 μl samples were plated on Trypticase soy agar (TSA) medium, and a mean colony count was scored after incubation. A semi-quantitative streak in four quadrants using a 10 μl calibrated loop was done for all other media. For these samples plates were scored as 0 (no colonies), 1 (growth in only the first quadrant), 2 (growth in the first and second quadrants), 3 (growth in the first through third quadrants), and 4 (growth in all quadrants). To control for contamination of the semen samples by microbes from the exterior of the cloaca we determinee the microbe score for a semen sample by subtracting the semen sample score from the score obtained from the exterior of the cloaca swab.

Trypticase soy agar was used as a nonselective medium for aerobic bacteria and blood agar (BA) (5% sheep blood) was used to detect anaerobic bacteria. Blood Agar plates were incubated using BBL GasPak™ pouches. Tomato juice agar (TJA) was used to detect *Lactobacilli* spp. and other acidophilic microbes. Sabouraud dextrose agar (SAB) was used to detect fungi. Eosin methylene blue agar (EMB) was used to detect gram negative enterics microbes; lactose fermentors are dark on this medium whereas lactose nonfermentors are white. Phenylethyl alcohol agar (PEA) was used to detect gram positive microbes. Thiosulfate citrate bile salts agar (TCBS) was used to detect *Vibrio* spp. microbes which grow as large yellow or blue/green centered colonies. Xylose Lysine Desoxycholate agar (XLD) plates were used to detect *Salmonella* and *Shigella* sp.; these species grow as red colonies. Cefsulodin-Irgasan®-novobiocin agar (CIN) was used to detect *Yersinia* spp. Plates were incubated at 35 C and scored either after 24 hr (all plates) or 48 hr (SAB plates).

The mean plate score for each type of media calculated from all samples was used to estimate the variation among males in infection with specific microbes. The mean of all plate scores across all types of media from individual male samples (i.e., mean semen sample score)

TABLE 1. Microbes in the semen of male tree swallows (n = 30) in Michigan.

Media used	Microbe types detected	Positive semen samples n (%)	Mean plate score ± SD
	All aerobic microbes		
Trypticase soy agar	Aerobic bacteria	16 (53.3)	1.97 ± 2.24
	Microbes known to be beneficial		
Tomato juice agar	*Lactobacilli* spp.	12 (40.0)	0.70 ± 0.99
	Microbes known to be pathogenic		
Sabouraud dextrose agar	Fungi	5 (16.7)	0.37 ± 0.85
Blood agar	Anaerobic bacteria	14 (46.7)	0.83 ± 1.05
Eosin methylene blue agar, white	Lactose nonfermentors	9 (30.0)	0.70 ± 1.18
Eosin methylene blue agar, dark	Lactose fermentors	2 (6.7)	0.13 ± 0.51
Phenylethyl alcohol agar	Gram positive bacteria	15 (50.0)	0.93 ± 1.05
Xylose lysine desoxycholate, red	*Salmonella* spp., *Shigella* spp.	1 (3.3)	0.07 ± 0.37
Xylose lysine desoxycholate, other	May include *Streptococcus* spp., *Escherichia* spp.	7 (23.3)	0.47 ± 0.90
Thiosulfate citrate bile salts, yellow	*Vibrio* spp.	0	—
Thiosulfate citrate bile salts, blue	*Vibrio* spp. and *Parahaemolyticus* spp.	0	—
Thiosulfate citrate bile salts, other	May include *Proteus* spp., *Enterocci* spp., *Pseudomonas* spp., *Aeromonas* spp.	1 (3.3)	0.03 ± 0.18
Cefsulodin-Iragasan® novobiocin	*Yersinia* spp.	8 (26.7)	0.54 ± 0.94

was used to estimate the variation among males in the intensity of infection with microbes.

We examined the data for normality and used parametric and nonparametric statistical tests to analyze data where appropriate using SPSS 8.0 for Windows (SPSS, 1997, SPSS, Inc., Chicago, Illinois, USA). Data are reported as means ± SD (standard deviation) unless otherwise stated.

RESULTS

We isolated one or more types of microbes from 19 of 30 (63%) semen samples (Table 1). We isolated both potential pathogens (e.g., *Escherichia coli, Salmonella* spp., *Shigella* spp., *Yersinia* spp.) and potential beneficial (e.g., *Lactobacilli* spp.) STM's from 11 of 19 (58%) of positive samples, only potential pathogens from seven of 19 (37%) positive samples, and only *Lactobacilli* spp. from one of 19 (5%) positive samples.

Mean plate scores of media across all samples differed (Friedman's Test, χ^2 = 10.25, df = 12, $P < 0.001$) and ranged from 0 to 1.97 (Table 1). However, there were no significant differences among semen sample scores on each type of media (Kruskal-Wallis ANOVA, all $P > 0.05$). The mean semen sample score across all media from all 30 semen samples was 0.52 ± 0.54, whereas the mean semen sample score across all media from the 19 positive samples was 0.81 ± 0.45.

There were few significant differences between males with pathogen-infected and pathogen-free semen in measures of size, morphology, ectoparasite scores and feather damage (Table 2). However, no differences between males with pathogen-laden semen and those without remain significant after a Bonferroni correction for multiple comparisons (Zar, 1984) (critical value = 0.004 for 12 comparisons, Table 2).

Females that pursue copulations with males that could inoculate them with beneficial STM's should be favored, therefore females should evolve the ability to detect males with beneficial STM's (cf. Lombardo et al., 1999). However, there were few significant differences between males with

TABLE 2. Measures of reproductive performance, male size and morphology, ectoparasite score, feather damage and the presence or absence of pathogenic and beneficial microbes in 30 tree swallow semen samples.

Measure	Pathogenic microbes in semen			Beneficial microbes in semen		
	No (n = 12)	Yes (n = 18)	P[a]	No (n = 18)	Yes (n = 12)	P
Reproductive performance						
Date of first egg	129 ± 2	128 ± 1	NS[b]	128 ± 2	129 ± 1	NS
Clutch size	5.67 ± 0.65	5.83 ± 0.79	NS	5.61 ± 0.61	6.00 ± 0.85	NS
Number eggs hatch per clutch	5.33 ± 0.78	5.17 ± 0.86	NS	5.22 ± 0.89	5.25 ± 0.75	NS
Percent eggs hatch per clutch	94 ± 11	89 ± 13	NS	93 ± 13	88 ± 12	NS
Size and morphology						
Left tarsus length (mm)	12.12 ± 0.40	12.21 ± 0.52	NS	11.99 ± 0.34	12.43 ± 0.53	0.012
Exposed culmen (mm)	6.60 ± 0.31	6.53 ± 0.50	NS	6.55 ± 0.35	6.56 ± 0.54	NS
Left wing length (mm)	117.33 ± 3.03	116.18 ± 4.64[c]	NS	117.11 ± 3.60	115.91 ± 4.74[d]	NS
Wing asymmetry	0.005 ± 0.003	0.002 ± 0.003	0.012	0.004 ± 0.003	0.002 ± 0.002	NS
Left tail length (mm)	11.36 ± 2.29	11.22 ± 2.31	NS	11.06 ± 2.30[c]	11.58 ± 2.27	NS
Tail asymmetry	0.020 ± 0.015[d]	0.007 ± 0.015	0.035	0.019 ± 0.017[d]	0.002 ± 0.007	0.001
Weight (g)	20.76 ± 1.15	20.96 ± 0.98	NS	20.62 ± 0.98	21.67 ± 1.32	NS
Ectoparasite scores						
Body score	0.17 ± 0.39	0.11 ± 0.32	NS	0.22 ± 0.43	0.00	0.042
Left wing score	1.58 ± 0.51	1.72 ± 0.67	NS	1.61 ± 0.61	1.75 ± 0.62	NS
Tail score	0.25 ± 0.62	0.28 ± 0.57	NS	0.22 ± 0.54	0.33 ± 0.65	NS
Feather damage						
Holes in wings	2.58 ± 3.48	1.39 ± 3.05	NS	1.83 ± 3.01	1.92 ± 3.65	NS
Holes in tail	1.58 ± 4.03	0.39 ± 1.04	NS	1.33 ± 3.34	0.17 ± 0.58	NS

[a] From t-test.
[b] Not significant, $P > 0.05$.
[c] n = 17.
[d] n = 11.

Lactobacilli spp. in their semen and those without in measures of size, morphology, ectoparasite score and feather damage (Table 2). After Bonferroni correction for multiple comparisons, the only significant difference that remained was that the tails of males with *Lactobacilli* spp. in their semen were more symmetrical.

It was not possible to examine directly the potential consequences of either pathogen or *Lactobacilli* spp. laden semen on female reproductive performance because we (1) did no parentage studies and (2) performed a series of experiments in which we inoculated nestlings at many nests with either Protexin® (Probiotics International, Somerset, UK), a commercially available probiotic (i.e., microbes that enhance nutrition, growth, and immune function; Fuller, 1989), or a placebo. However, whether a male's semen contained either potential pathogens or *Lactobacilli* spp. had no influence on how early in the season their mates began egg laying, the number of eggs their mates laid, the number of eggs that hatched, or the proportion of eggs that hatched (Table 2).

DISCUSSION

The semen of 63 percent of the 30 male tree swallows in this study contained bacteria and/or fungi. Each male cloacal swab sample examined by Lombardo et al. (1996) contained one or more types of microbe and individuals varied in the composition of their assemblages of cloacal microbes. Together, these results demonstrate that female tree swallows are often exposed to both potentially pathogenic and/or beneficial STM's during copulation. These observations have important implications for studies of mate choice in birds.

First, these results suggest that STM's may play an important role in the evolution of avian mating systems (Sheldon, 1993; Lombardo, 1998) because they demonstrate that there is a high probability that copulating females will be exposed to potentially pathogenic STM's. Exposure to pathogenic STM's increases the costs of copulation and extra-pair copulations (EPC's) to females by negatively affecting female health (Sheldon, 1993). In addition, exposure to pathogens from semen may influence female reproductive performance by affecting offspring survivorship. For example, Reiber et al. (1995) showed by experiment that the ovaries and oviducts of female chickens inseminated with semen contaminated with strains of *Salmonella enteridis* and *S. typhimurium* became infected and the hens laid *Salmonella* spp. contaminated eggs (see also Thiagrajian et al., 1993). Thus, if exposure to pathogenic STM's from copulation is likely, then we predict that females should evolve ways to defend themselves from infection. Defense mechanisms could include, but are not restricted to, detecting and avoiding copulating with donors of pathogenic STM's, ejection of pathogen laden semen (Lombardo et al., 1999), and immune responses to the presence of pathogens.

Second, the observation that males varied in the identity of microbes found in their semen supports the assumption of the beneficial STM hypothesis of avian copulation (Lombardo et al., 1999) that birds in the local population carry both pathogenic and beneficial STM's while others carry only one or the other, or neither. The variation in assemblages of tree swallow semen microbes is consistent with variation in cloacal microbes found in adult Tree Swallows (Lombardo et al., 1996) and other bird species (e.g., Cooper et al., 1980; Petrak, 1982; Brittingham et al., 1988; Flammer and Drewes, 1988; Calnek et al., 1991; Fritz et al., 1992), and semen microbes in a variety of mammals (Ibrahim et al., 1983; Tamuli et al., 1984; Miskolc, 1990; Bjurstrom and Linde-Forsberg, 1992; Howard et al., 1993; Madsen and Christensen, 1995), including humans (e.g., Granouillet et al., 1982). Indeed, human semen contains components that have antibacterial effects (e.g., Mårdh and Colleen, 1975) that may provide direct benefits to inseminated females and suggests

that the beneficial STM hypothesis has general applicability to species with internal fertilization (cf. Lombardo et al., 1999). The existence of variation among males as potential donors of pathogenic and/or beneficial STM's is critical to the beneficial STM hypothesis because variation among males gives females something from which to choose when making mate choice decisions.

The results of this study suggest that female detection of male donors of pathogenic or beneficial STM's may be difficult because of the lack of clear relationships between the presence of pathogenic microbes in semen and male size, morphology, and ectoparasite scores and feather damage. However, females may be able to identify males with beneficial microbes in their semen because the tails of these males were more symmetrical than the tails of males without beneficial microbes.

The lack of clear relationships in this study may be an artifact of our method of identifying microbes. Our methods did not permit us to identify detected microbes to the species or strain level. It may be that the relationship between the presence and loads of particular strains of STM's and the male characters that we measured were obscured because our analyses of microbe identity were not stringent enough. This possibility demands more thorough identification of STM's in birds.

Identifying the microbes found in avian semen is only a first step in the elucidation of the role of STM's in avian mating systems. However, it is important because semen microbes might affect reproductive success. For example, semen microbes might affect male fertility. However, the results of clinical studies designed to discover the relationship between the presence of semen microbes on human male infertility have been equivocal. Some studies have shown a positive association between the presence of semen microbes (e.g., *Mycoplasma hominis*) and sperm tail abnormalities (Bornman et al., 1989) while in other studies there was no association between the presence of semen microbes and male fertility (Granouillet et al., 1982; Eggert-Kruse, 1995; Willén et al., 1996). Likewise, the relationship between the presence of semen microbes on male health and fertility in boars (*Sus scrofa*) (Tamuli et al., 1984), domestic cats, and cheetahs (*Acinonyx jubatus*) (Howard et al., 1993), and horses (*Equus caballus*) (Madsen and Christensen, 1995) is unclear. These results, plus our knowledge of human (Holmes et al., 1990) and nonhuman microbial STD's (Lockhart et al., 1996) indicate that the effects of semen-borne microbes on both male and female reproduction can vary greatly among different microbes and that more data are needed on the prevalence of STM's and their effects on reproduction in birds.

ACKNOWLEDGMENTS

The Research and Development Committee and the Department of Biology at GVSU supported this study. The Institutional Animal Care and Use Committee at GVSU approved this study. M. Ballard, M. Czarnowski, M. Hebert, M. Miedema, and B. Underhill assisted in the collection of semen samples. H. W. Power, I. Stewart, and D. Westneat provided useful comments on a previous version of the manuscript.

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Received for publication 2 April 1999.