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Tamara K. Mills

University of Alaska Anchorage

Michael P. Lombardo

Grand Valley State University, lombardm@gvsu.edu

Patrick A. Thorpe

Grand Valley State University

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MICROBIAL COLONIZATION OF THE CLOACAE OF NESTLING TREE SWALLOWS

TAMARA K. MILLS,¹ MICHAEL P. LOMBARDO,² AND PATRICK A. THORPE

Department of Biology, Grand Valley State University, Allendale, Michigan 49401, USA

ABSTRACT.—Microbes have the potential to be important selective forces in many aspects of avian biology. Microbes can affect fitness as a result of either their pathogenic or beneficial effects on host health. Little is known about the chronology of microbial colonization of nestlings or the effects of microbes on fledgling condition. We set out to (1) characterize the time course of microbial colonization of the cloacae of nestling Tree Swallows (*Tachycineta bicolor*), (2) examine the relationship between cloacal microbes and fledgling condition, and (3) determine if nest mates had similar assemblages of cloacal microbes. We repeatedly measured nestlings and sampled their cloacal microbes on nestling days 2, 3, 5, 7, 12, 16, and 19. We detected cloacal microbes in nestlings as early as nestling day 2. Colonization of nestlings by microbes began soon after hatching. Nestlings were colonized by more types of microbes and carried heavier loads of most types of microbes as they got older. Cloacal microbes did not affect fledging success. However, plate scores for gram-negative enteric lactose fermentors, which include *E. coli*, *Salmonella* spp., and *Shigella* spp., were positively correlated with a greater degree of wing asymmetry. This relationship suggests that microbes affect fledgling survival because wing asymmetry hinders flying ability, a critical survival skill for these aerial insectivores. Patterns in the assemblages of cloacal microbes within broods suggested host-genetic influences on the colonization of nestlings by microbes, but they also may have reflected the facts that nest mates were fed by the same adults and were raised in the same nests. Received 4 June 1998, accepted 29 January 1999.

MICROBES (viruses, bacteria, and fungi) have the potential to be important selective forces in the evolution of many aspects of avian biology (Hamilton and Zuk 1982, Hamilton 1990, Zuk 1991, Sheldon 1993, Moore and Clayton 1997). However, only recently have they been considered in studies of avian population dynamics (but see Pinowski et al. 1991, Nuttall 1997). Despite their importance, knowledge about the prevalence of microbes and their effects on the general health of wild bird populations is limited. For these reasons, we studied Tree Swallows (*Tachycineta bicolor*) to (1) characterize the time course of microbial colonization of the cloacae of nestlings, (2) examine the relationship between cloacal microbes and fledgling condition, and (3) determine if nest mates had similar assemblages of cloacal microbes.

Our limited knowledge of the effects of microbes on avian biology is surprising for several reasons. First, numerous microbial species

have been isolated from domestic and wild birds (e.g. Brittingham et al. 1988, Sheldon 1993, Lombardo et al. 1996, Nuttall 1997). Second, infectious diseases and intoxication by bacterial by-products can be important sources of mortality and reduced fitness in wild bird populations (e.g. Hudson and Dobson 1991, Pinowski et al. 1991, Nuttall 1997). Third, the transmission of microbes between individuals (between mates or between parents and offspring) has the potential to have an important effect on host fitness and should be an important force in the evolution of behavior (Troyer 1982, Sheldon 1993, Lockhart et al. 1996).

Although the pathogenic effects of many microbes are well known, some microbes can be beneficial (Nurmi and Rantala 1973, Fuller 1989). These beneficial effects are well documented in commercial animal husbandry, where suspensions of microbes obtained from healthy adults are used to inoculate newborns or juveniles. Inoculated individuals grow more rapidly, are less likely to harbor potentially pathogenic species, and immune systems that function better than do uninoculated individuals (e.g. Fuller 1989). The beneficial effects of microbes in birds also have been demonstrated

¹ Present address: Department of Biology, University of Alaska-Anchorage, 311 Providence, Anchorage, Alaska 99508, USA.

² Address correspondence to this author. E-mail: lombardm@gvsu.edu

in the rehabilitation of nestling Chimney Swifts (*Chaetura pelagica*; Kyle and Kyle 1993). Adult saliva containing a variety of microbes was used to inoculate artificial rearing food of nestlings. Nearly 100% of nestling swifts less than six days old died if given food lacking the adult saliva supplement, whereas nearly 100% of those given food inoculated with adult saliva were rehabilitated and released (Kyle and Kyle 1993).

Because little is known about the development of the assemblages of microbes in wild birds, we set out to determine the chronology of microbial colonization of the cloacae of nestling Tree Swallows by repeatedly sampling individuals throughout their nestling periods. To gain a better understanding of the composition of cloacal microbe assemblages, we surveyed more nestlings, more often, and for more microbes than did Lombardo et al. (1996).

Until recently (Pinowski et al. 1991, 1995; Kyle and Kyle 1993), microbial effects were seldom considered in studies of the growth and development of wild birds (O'Connor 1984), although the inoculation of altricial nestlings by microbes, either directly from (1) their parents, (2) the food their parents feed them, (3) their nest mates, (4) coprophagy, or (5) the nest itself is inevitable. Therefore, we sought to determine the relationship between microbes and fledgling condition.

Heritable resistance to pathogenic microbes and other parasites plays a central role in recent theories of sexual selection (Hamilton 1990, Hamilton and Zuk 1982, Møller et al. 1990, Clayton 1991, Zuk 1991, Andersson 1994); yet, little has been documented regarding the genetics of resistance to microbes in wild species of birds. Host lineage is known to affect the ability of the pathogenic bacterium *Campylobacter jejuni* to colonize the cecae of domestic chickens (Stern et al. 1990). Moreover, Lombardo et al. (1996) found that 86% of 22 Tree Swallow nestlings could be correctly matched with their nest mates based on their assemblages of cloacal microbes, implying the influence of host genetics on colonization of the cloaca by microbes. In addition, because nest mates are fed by the same adults and are reared in the same nest, they are exposed to the same sources of microbes. Consequently, we determined whether nest mates had similar assemblages of cloacal microbes.

METHODS

In 1995, we sampled the cloacal microbes of nestling Tree Swallows that were raised in wooden boxes mounted on metal poles erected in grids on the campus of Grand Valley State University, Ottawa County, Michigan (42°57'N, 85°53'W). We began monitoring breeding activity on 1 May 1995. Nests were checked daily for nest-building activity and egg laying. We numbered eggs in sequence with indelible ink and revisited nests on the expected day of hatching and then daily until hatching was complete. We clipped the toenails of nestlings in distinct patterns to identify individuals until they were banded with United States Fish and Wildlife Service numbered aluminum bands on nestling day 12. Nestling day (ND) 1 was the day the first egg in a clutch hatched.

We measured nestlings and sampled the cloacal microbes of 8 nestlings at 2 nests on ND2, 9 nestlings at 2 nests on ND3, 10 nestlings at 2 nests on ND5, 24 nestlings at 6 nests on ND7, 70 nestlings at 15 nests on ND12, 24 nestlings at 6 nests on ND16, and 22 nestlings at 6 nests on ND19. The same nestlings at six nests were sampled on ND7 ($n = 24$), 12 ($n = 24$), 16 ($n = 24$), and 19 ($n = 22$). Tree Swallows typically fledge at ND20 (Robertson et al. 1992). To prevent nestlings from fledging before we could sample them, we reduced the size of the nest-box hole with a piece of tar paper on ND18 so that nestlings could not exit the box. The hole remained large enough for parents to deliver food to nestlings. The tar paper was removed 4 to 6 h after nestlings were measured and sampled on ND19. Boxes were then checked daily for fledging. Repeated handling of nestlings had little effect on length of the nestling period (see Burt 1977). Nestlings handled three or more times were as likely to fledge on ND20 (9 of 22, 41%) as were nestlings handled two or fewer times (76 of 138, 55%; Fisher exact test, $P = 0.25$, $n = 160$). On each nestling we measured the length of each tarsus with a digital caliper (± 0.1 mm) and each flattened wing chord with a stopped ruler (± 1 mm). Relative degrees of tarsus and wing chord fluctuating asymmetry (FA) were calculated following Møller (1990:1186). Fluctuating asymmetry results in part from random deviations from symmetry in otherwise symmetrical traits that arise as a consequence of the inability of the developing individual to cope with genetic and environmental stress (Parsons 1990). Thus, FA may serve as an indicator of general health (Møller 1995). We used an electronic balance to weigh nestlings to the nearest 0.05 g on ND 2, 3, 5, and 7 and a spring scale to weigh nestlings to the nearest 0.2 g on ND12, 16, and 19. We examined nestlings for the presence of ectoparasites in eight topographic regions (head, back, rump, chin, breast, cloaca, wings, tail). The ectoparasite load on each region was scored as 0 when no parasites were detected, 1 when 1 to 10 parasites were detected, 2 when 11 to 100 parasites were de-

tected, and 3 when 101 to 1,000 parasites were detected. Each nestling was then given an ectoparasite score that was the sum of the scores from each topographic region.

To obtain microbe samples, we inserted a sterile Dacron swab (5 × 2 mm; Dacronstik MW 151) into each nestling's cloaca for 10 s. The swab was transferred to 4 mL of sterile thioglycollate broth and the shaft removed. For controls, we collected field blanks by holding a sterile swab in the air outside of each sampled family's nest box for 10 s and then processed each field blank as if it were a cloacal sample. Samples were held for 1 h on an ice pack in the field and during transport to the laboratory and then stored at 4°C for 30 min before being plated. Immediately before plating, sample tubes were vortexed for 30 s with the swabs in the tubes and then the swabs were removed with sterilized forceps. To produce scorable plates, samples were diluted in sterile thioglycollate broth so that, depending on the plating medium, concentrations of samples within broth were 10^0 , 10^{-1} , 10^{-2} .

We plated samples on a variety of differential and selective growth media on different nestling days (Table 1). Three dilutions of each sample (0.1 mL of 10^0 , 10^{-1} , and 10^{-2} dilutions) of the thioglycollate resuspension were plated on single plates for AERO II (see Table 1 for definitions of acronyms) and LACT. Two dilutions (0.1 mL of 10^0 and 10^{-1}) were plated on the remaining media with the exception of PSED, VIBR, and YERS, for which only single dilutions (0.1 mL of 10^0) were plated. All samples were plated in triplicate and incubated for 24 h at 32°C. Blood agar plates were incubated in BBL GasPak pouches, and CAMPY plates were incubated in BBL microaerophilic CAMPY pouches.

After incubation, colonies on plates were counted and the plates were given the following scores: 0 = 0 colonies, 1 = 1 to 10 colonies, 2 = 11 to 100 colonies, 3 = 101 to 1,000 colonies, and 4 = more than 1,001 colonies. Plates with colonies too numerous to count were not included in the analyses (54 of 5,786, 1.02%). To check accuracy of counting, all plates were counted twice by different individuals. Plate scores assigned to nestlings for each plating medium were calculated as the mean of the plate counts of microbial colonies. Relative plate score was used as a measure of the relative size of microbe loads and was calculated as the sum of the plate scores from each plating medium divided by the maximum possible plate score sum (e.g. maximum plate score sum for 10 plates = 40). We used these semi-quantitative methods to estimate the size and diversity of microbial assemblages because the use of community ecology statistical techniques to estimate parasite diversity (e.g. indices of diversity and species richness) is problematic (e.g. Sousa 1994, Poulin 1997).

To control for possible confounding effects of age of the breeding female on colonization of nestlings

by cloacal microbes, only nestlings reared in nests tended by after-second-year females (Hussell 1983) were sampled. Individual nestlings were treated as independent samples in analyses of relationship between microbes and nestling condition because we assumed that nestlings varied genetically and that individuals are affected most directly by the microbes residing in their own bodies and not by the mean microbe load of their nest mates. Selection for resistance to pathogenic microbes, or for compatibility with mutualistic microbes, must occur at the individual level.

RESULTS

Cloacal microbes, nestling age, and growth.—We detected cloacal microbes shortly after nestlings hatched (Fig. 1). As nestlings got older they were colonized by more types of microbes and harbored larger numbers of microbes because relative plate scores increased significantly with age (Fig. 2).

The remainder of our analyses focuses on ND19 nestlings because nestlings typically fledge on or around ND20 (i.e. condition at ND19 is a good predictor of condition at fledging), and all of the nestlings we sampled fledged. Plate scores of a variety of different plating media were significantly correlated with some measure of nestling condition on ND19 (Table 2). We calculated partial correlation coefficients for the statistically significant correlations in Table 2 to further investigate the relationship between microbes and nestling condition because plate scores for different plating media were also correlated with one another (Table 3). In our context, partial correlation examines the relationship between specific measures of nestling condition and specific plate scores while controlling for other relationships by holding them constant (Zar 1994). Partial correlation analysis resulted in only one statistically significant relationship; high plate scores for gram-negative enteric lactose fermentors (e.g. *E. coli* and *Salmonella* and *Shigella* spp.) grown on EMB agar were positively correlated with a greater degree of wing asymmetry (partial $r = 0.87$, $df = 4$, $P = 0.02$).

Family patterns in assemblages of cloacal microbes.—We compared microbial composition of broods on each day they were sampled to determine if broods differed significantly throughout the nestling period. The two broods sampled for microbes on ND 2 differed

TABLE 1. Microbe growth media and nestling day. D = differential media, S = selective media.

Microbial group	Microbial code	Medium	Medium code	D/S	Nestling day
Aerobic plate count	AERO II	Trypticase soy agar (TSA II)	TSA	D	All
Aerobic plate count	BA	TSA II with 5% sheep blood	BLOOD	D	12
<i>Campylobacter</i> spp.	CAMPY	Campylobacter agar	CAMPY	S	16, 19
Enteric organisms	EEMB	Eosin methylene blue, Levine	EMB; white = lactose nonfermentors; dark = lactose fermentors; total = white + dark	D/S	All
Fungi	FUNG	Sabouraud dextrose agar with chloramphenicol	SAB	S	All
Gram-positive organisms	GPOS	Phenylethyl alcohol agar	PEA	S	All
Lactobacilli	LACT	MRS agar	MRS	S	All
<i>Pseudomonas</i> spp. and nonfermentors	PSED	<i>Pseudomonas</i> isolation agar	PSE	S	12
<i>Salmonella</i> and <i>Shigella</i> spp.	SS	Desoxycholate citrate lactose sucrose agar	DCLS; red = <i>Vibrio</i> spp., <i>E. coli</i> , and other enterics; white = <i>Salmonella</i> spp., <i>Shigella</i> spp., and cholera <i>Vibrio</i> spp.; total = red + white	D/S	All
<i>Vibrio</i> spp.	VIBR	Thiosulfate citrate bile salts sucrose agar	TCBS	S	12
<i>Yersinia</i> spp.	YERS	Cefsulodin irgasan agar	CIN; red = <i>Yersinia</i> spp.; white = other microbes	D/S	12

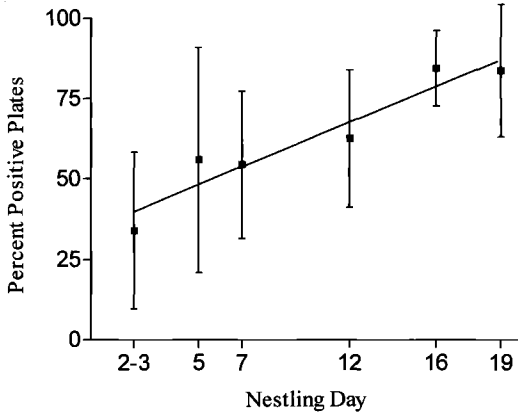


FIG. 1. Percent of positive plates and nestling day ($\bar{x} \pm SD$). Nestlings sampled on days 2-3 ($n = 17$), 5 ($n = 10$), and 7 ($n = 24$) were resampled on days 12 ($n = 70$), 16 ($n = 24$), and 19 ($n = 22$). Forty-seven nestlings were sampled only on nestling day 12; $y = 2.72x + 33.46$, $r^2 = 0.30$, $F = 70.6$, $P < 0.0001$.

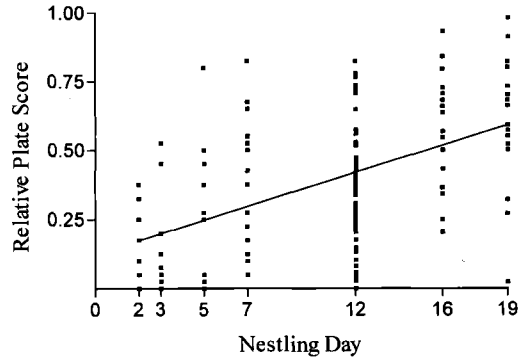


FIG. 2. Relative plate score and nestling day. Nestlings sampled on days 2 ($n = 8$), 3 ($n = 9$), 5 ($n = 10$), and 7 ($n = 24$) were resampled on days 12 ($n = 70$), 16 ($n = 24$), and 19 ($n = 22$). Forty-seven nestlings were sampled only on nestling day 12; $y = 2.45x + 12.61$, $r^2 = 0.26$, $F = 57.42$, $P < 0.001$.

only in their mean plate scores of aerobic bacteria grown on TSA agar (Wilcoxon two-sample, $Z = -2.01$, $P = 0.04$). The two broods sampled on ND3 differed only in their mean plate scores for enteric *Lactobacilli* spp. grown on EMB agar ($Z = 2.18$, $P = 0.03$). There were no significant differences in plate scores between the two broods sampled on ND 5 or between the six broods sampled on ND 7. The 15 broods sampled on ND 12 differed only in their mean plate scores for *Yersinia* spp. grown on YERS agar ($\chi^2 = 27.96$, $df = 14$, $P = 0.01$) and hemolytic anaerobic bacteria grown on BA agar ($\chi^2 = 26.36$, $df = 14$, $P = 0.02$). On ND 16, the six broods sampled differed in their mean plate

scores of *Lactobacilli* spp. grown on MRS agar ($\chi^2 = 13.86$, $df = 5$, $P = 0.02$), gram-positive organisms ($\chi^2 = 12.54$, $df = 5$, $P = 0.03$), and *Campylobacter* spp. grown on CAMPY agar ($\chi^2 = 12.20$, $df = 5$, $P = 0.03$). The six broods sampled on ND 19 differed in their mean plate scores of *Lactobacilli* spp. grown on MRS agar ($\chi^2 = 11.61$, $df = 5$, $P = 0.04$) and in *Vibrio* spp., *E. coli*, and other enteric bacteria grown on DCLS agar ($\chi^2 = 11.12$, $df = 5$, $P = 0.05$).

DISCUSSION

Nestling growth and development require an integration of a variety of factors (O'Connor 1984). Microbes are one such factor, producing both positive and negative effects on nestling

TABLE 2. Pearson correlation coefficients for plate scores and measures of nestling condition at nestling day 19. See Table 1 for plating medium codes; $n = 21$ for all condition variables except mass ($n = 22$).

Medium	Nestling condition at day 19					
	Mass	Left tarsus	Tarsus FA	Left wing	Wing FA	Wing parasites
TSA	-0.42	0.09	-0.17	0.05	0.18	-0.22
CAMPY	0.17	0.46*	-0.17	0.14	-0.23	-0.22
DCLS-red	-0.04	0.27	-0.37	-0.44*	0.04	-0.13
DCLS-white	-0.12	0.38	-0.44*	0.15	0.11	-0.20
DCLS-total	-0.14	0.49*	-0.45*	-0.34	0.09	-0.09
EMB-dark	-0.08	-0.19	-0.48*	-0.04	0.56**	-0.06
EMB-white	-0.32	0.14	-0.18	-0.10	-0.07	-0.34
EMB-total	0.36	0.16	-0.23	-0.14	0.09	-0.23
MRS	-0.18	0.14	0.04	0.45*	-0.13	-0.55**
PEA	0.07	0.18	-0.05	0.32	-0.12	-0.61**
SAB	-0.21	0.18	-0.05	-0.32	-0.12	0.24

*, $P < 0.05$; **, $P < 0.01$.

TABLE 3. Pearson correlation coefficients for plate scores on different plating media on nestling day 19. See Table 1 for plating medium codes.

Medium	TSA	CAMPY	DCLS-red	DCLS-white	DCLS-total	EMB-dark	EMB-white	EMB-total	MRS	PEA
CAMPY	0.31									
DCLS-red	0.32	0.61**								
DCLS-white	0.59**	0.55**	0.43*							
DCLS-total	0.58**	0.68***	0.63***	0.89***						
EMB-dark	0.43*	0.16	0.26	0.51*	0.36					
EMB-white	0.78***	0.49*	0.40	0.60**	0.65***	0.41				
EMB-total	0.84***	0.68***	0.63***	0.65***	0.68***	0.47*	0.95***			
MRS	0.72***	0.27	0.08	0.35	0.24	0.41	0.67***	0.65***		
PEA	0.58**	0.12	0.05	0.17	0.10	0.22	0.76***	0.47	0.76***	
SAB	0.27	0.42*	0.53**	0.64***	0.67***	0.49*	0.02	0.41	0.02	-0.16

*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

health. Minor alterations in a nestling's internal and external environments that affect microbes during this period may have considerable effects on host fitness.

Nestlings were colonized by microbes soon after hatching. The number of nestlings with microbes inhabiting the cloaca increased significantly with nestling age (Fig. 1). Likewise, Malyszko et al. (1991b) found that the intestinal tracts of nestling House Sparrows (*Passer domesticus*) and Tree Sparrows (*P. montanus*) were colonized by fungi (e.g. *Candida* spp.) and bacteria (e.g. *E. coli*) soon after hatching and that the proportion of nestlings with fungal infections increased with nestling age (see Kozłowski et al. 1991). In Jackdaws (*Corvus monedula*), the proportion of Enterobacteriaceae (e.g. *E. coli*) in the total intestinal biocenosis increased with nestling age (Malyszko et al. 1991a).

Nestlings probably acquire most of their intestinal microbes from the regurgitated insects that adults feed them and from adult saliva (Kyle and Kyle 1993). The conventional view is that the avian egg shell is impervious to most microbes (Carey 1983) and that the neonate gut is sterile (Savage 1977). However, evidence from domestic poultry suggests that microbes enter eggs before and after they are laid (Thiagrajian et al. 1993, Board et al. 1994, Bruce and Drysdale 1994). Regardless, from hatching onward nestlings are exposed to environmental microbes and to microbes passed to them when they are fed by adults. Although Tree Swallow nestlings may also acquire microbes from the nest itself and from contact with their nest mates, it is likely that regurgitated food and adult saliva are the major sources of microbes. Populations of microbes that we sampled for repeatedly increased significantly with nestling age (Fig. 2). The increase in diversity of microbial composition and the growth of microbial populations with nestling age can probably best be attributed to the exposure of nestlings to a variety of food items brought by adults. However, it is important to note that the microbes of the gastrointestinal tract are part of a dynamic system (Savage 1977) whose composition may result from the outcome of interactions among different microbes.

Correlations between specific types of microbes and measures of nestling condition (Table 2) suggest that microbes have important effects on growth and development of Tree Swal-

lows. To test this idea, experiments that control environmental conditions (e.g. brood size, diet, exposure to microbes) need to be conducted. Tree Swallow nestlings with high plate scores for cloacal gram-negative enteric lactose fermentors on ND19 had relatively asymmetrical wings. This result may reflect the sensitivity of wing growth in nestling Tree Swallows to these pathogens (see Van Valen 1962, Palmer and Strobeck 1986, Parsons 1992) and suggests a possible negative effect on fitness associated with harboring these microbes. Wing asymmetry can increase the cost of flight and reduce flight performance (Norberg 1990, Balmford et al. 1993, Thomas 1993) and thus could be very disadvantageous for young swallows that must depend on flight speed and maneuverability to capture aerial insects and evade predators.

Cloacal microbes did not affect fledging success in our study. However, microbes have been implicated in nestling mortality in other species (e.g. *Passer*; Kozłowski et al. 1991, Pawiak et al. 1991, Kruszewicz et al. 1995). Although all nestlings in our study fledged, they did not fledge in the same physical condition because they varied in size (tarsus and wing chord) and mass. A nestling's physical condition at fledging affects the probability of surviving to the first breeding season (Perrins 1980, Smith 1988).

The microbial composition of the cloaca, especially of young nestlings, may have been influenced by host lineage. Using a different set of plating media for comparisons, Lombardo et al. (1996) found that a discriminant analysis correctly paired 19 of 22 Tree Swallow nestlings with their nest mates based on their assemblages of cloacal microbes. The family pattern in nestling Tree Swallows suggests two explanations that are not mutually exclusive. First, nest mates may have similar cloacal microbes because they are fed by the same adults and raised in the same nest. Second, the establishment of cloacal microbes may be under host-genetic influences (Wakelin and Apanius 1997). For example, Stern et al. (1990) found that some stocks of broiler chickens were more resistant to cecal colonization by pathogenic *Campylobacter jejuni* than were others, and immune-response genes have been found in chickens (Pevzner et al. 1975) and implicated in the control of bacterial (Krejci et al. 1974, Pevzner et al. 1975) and viral (Crittenden et al. 1974)

infections. Thus, Tree Swallow broods may have differed in the size and diversity of their assemblages of microbes because they differed genetically and the nestlings in them had internal environments that favored the growth of different types of microbes.

Nestlings resembled their nest mates in the composition and size of their populations of cloacal microbes early in the nestling period but began to vary between ND5 and ND7. Lack of concordance between nest mates in their assemblages of microbes may be due to environmental conditions (e.g. food availability). Environmental quality influences growth of Tree Swallow nestlings under natural (e.g. Zach 1982, Zach and Mayoh 1982, Teather 1996) and experimental (Zach and Mayoh 1984, Wiggins 1990) conditions. Teather (1996) found age-related patterns in tarsus and primary feather FA between ND1 and ND14 and suggested that periods of maximum asymmetry are related to stress during periods of maximum growth, when energetic demands are highest (Drent and Daan 1980).

Our observations may be influenced by sample-size effects and also may be correlated with the onset of homeothermy and emergence of dorsal contour feathers. Dorsal contour feathers emerge around days 6 and 7 (Marsh 1980), and individual nestlings attain effective homeothermy at 9.5 days (Dunn 1979). These energetically expensive events may affect microbial colonization in ways that result in the differentiation of nestlings and affect patterns of nestling growth and development (Drent and Daan 1980, Teather 1996). The relationship between microbial colonization and nestling growth and development is complex and warrants further study.

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