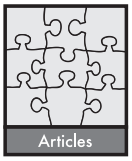

Journal of Avian Biology 45: 354–358, 2014
doi: 10.1111/jav.00353

Subject Editor: Simon Griffith. Accepted 7 January 2014

The mucous covering of fecal sacs prevents birds from infection with enteric bacteria

Juan Diego Ibáñez-Álamo, Magdalena Ruiz-Rodríguez and Juan José Soler

J. D. Ibáñez-Álamo (jia@ugr.es), Depto de Zoología, Facultad de Ciencias, Univ. de Granada, Avda. Fuentenueva s/n, ES-18071 Granada, Spain. – M. Ruiz-Rodríguez and J. J. Soler, Depto de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas (EEZA-CSIC), Ctra. Sacramento s/n, ES-04120 La Cañada de San Urbano, Almería, Spain.

Nestlings of many bird species produce fecal sacs, excrements encapsulated within a mucous covering. Although it facilitates parents' removal of feces from nests, which would improve hygienic conditions for developing nestlings, no functional (i.e. adaptive) explanation of fecal sac production has been previously investigated. We propose that the mucous covering would isolate enteric pathogenic bacteria, thereby preventing contamination of nestlings and parents. This antimicrobial hypothesis therefore predicts that density of bacteria would be drastically reduced from the inside to the outside of nestlings' droppings, and that the fecal sac covering would inhibit other bacterial grow. We tested these predictions by means of culturing bacteria obtained from different parts of the sac and inhibition tests. In accordance with the hypothesis, bacterial loads of the outside of fecal sacs were significantly lower than those estimated from the inside of the covering. In addition, we did not find evidence of antimicrobial activity of the covering, which suggests that the hypothesized bacterial isolation function is accomplished by a physical rather than a chemical protection. Bacterial density of the liquid that permeates out after 23 min does not differ with that estimated for the inside of the sac, suggesting short-term effects of fecal sacs as bacterial barrier. These findings highlight the major role of bacterial infections as a selective pressure for explaining the evolution of traits that, as the covering of fecal sacs, facilitate nest sanitation in this group of animals.

Many bird species are characterized by a period in which offspring (eggs and nestlings) stay in a fixed location, the nest, wherein they are completely dependent on their parents (Del Hoyo et al. 1992). The study of parent–offspring interactions during the nesting phase has been critical in the advances of different research areas, including the evolution of traits that reduce the probability of infection, (Loye and Zuk 1991, Royle et al. 2012). Excrements are a source of potential pathogenic microorganisms and consequently may play an important role in the evolution of such traits. Up to date, however, few studies have investigated this topic. For example, although nestlings' droppings can act as an important source of energy and nutrients for adult birds (Morton 1979, Glück 1988, Dell'Omo et al. 1998) they may facilitate the transmission of harmful microorganisms from young to their parents. Furthermore, the accumulation of excrements in (or around) the nest may facilitate nest detection and, therefore, increase probability of predation (Herrick 1900, Weatherhead 1984, Petit et al. 1989, but see Ibáñez-Álamo et al. 2013b). Independently of adaptive functioning, nest sanitation by means of feces removal is a widespread parental behavior in birds (approximately 99% of North American passerine species according to Guigueno and Sealy 2012). One of the most intriguing adaptations regarding nestlings' droppings are fecal sacs (excrements encapsulated in a mucous covering, Herrick 1900, Weatherhead 1984), which are restricted exclusively to the nestling stage of many bird species (Blair and Tucker 1941, Guigueno and Sealy 2012). Some researchers have suggested that the sac surrounding the feces will help parents to carry them away from the nest (McGowan 1995). In addition, Herrick (1900) proposed that the mucous covering could avoid 'soiling the bill', which obviously may result in contamination by microorganisms.

Microorganisms are important determinants of avian development and survival (Benskin et al. 2009, Archie and Theis 2011, Ezenwa et al. 2012) and the perspective of animal evolution in a bacterial world has recently been claimed as imperative for the life science (McFall-Ngai et al. 2013). Bacteria can have beneficial (Moreno et al. 2003) or detrimental effects in birds. Pathogenic bacteria cause several diseases in birds (Batt et al. 1996, Lombardo et al. 1996, Mills et al. 1999), some of them for instance inducing embryonic mortality (Pinowski et al. 1994), growth reduction (Potti et al. 2002) or the degradation of feathers (Gunderson 2008). Enteric bacteria of adult and nestling wild birds include pathogens (Brittingham et al. 1988,

Lombardo et al. 1996, Westneat and Rambo 2000) and, consequently, the manipulation of chicks' excrements by adults or the contact with nestling skin would imply an increased risk of infection. We propose a new hypothesis within this theoretic framework, the antimicrobial hypothesis, which states that the mucous covering of fecal sacs will prevent infection of parents and/or nestlings by harmful microorganisms contained within nestlings' excrements. This protection could be provided by two different, not mutually exclusive, mechanisms. On the one hand, the mucous covering could act as a barrier isolating enteric bacteria inside, avoiding thereby the contamination of birds during the contact, similarly to the physical defensive function of the eggshell that protects the embryo from bacterial infection (Wellman-Labadie et al. 2008a, b). On the other hand, fecal sacs may contain antimicrobial compounds, as found for example in uropygial secretions (Martín-Vivaldi et al. 2010), conferring chemical protections to adults and nestlings.

The main objective of this study was to investigate whether the mucous covering of fecal sacs has an antimicrobial function. Evidence of antimicrobial properties would suggest that fecal sacs impede trans-sac bacterial contamination of adults and nestlings. To investigate several predictions of this new hypothesis, we carried out different approximations using the common blackbird *Turdus merula* as the model species. According to the antimicrobial hypothesis, bacterial loads of the inside of fecal sacs should be higher than those of the outside (prediction 1) due to the presence of the mucous covering between these two parts of nestlings' droppings (i.e. physical barrier functioning). Evidence supporting this prediction would imply that adults manipulating fecal sacs would face a reduced risk of infection in comparison with those handling excrements not covered by mucous sacs. Moreover, if the antimicrobial function of this mucous layer is accomplished chemically by the presence of antimicrobial compounds (i.e. chemical antimicrobial functioning), we could predict that: a) intact fecal sacs would inhibit bacterial growing at a higher rate than excrements without covering (prediction 2). Furthermore, given that some bacteria should have been inhibited by this layer after contact, b) bacterial loads inside the sac should be higher than in the liquid that passes through the covering some time after its production (prediction 3). These two possible functionings of fecal sacs are not mutually exclusive.

Methods

Study area and field work

This study was conducted in a population of common blackbirds located in the Valley of Lecrín, south of Spain (36°56′N, 3°33′ W; 580 m a.s.l.) from April to May 2012. The study area is dominated by orange groves in which blackbirds usually nest (see Ibáñez-Álamo and Soler 2010 for a more detailed description of the population). We used the common blackbird as the model species given that their nestlings produce fecal sacs and adults remove them from their nests (Ibáñez-Álamo et al. 2013a, b).

We actively searched for blackbird nests since the beginning of the field season (beginning of March). All nests were visited regularly and fecal sacs obtained directly from middle aged chicks (mean ± SE: 6.6 ± 0.4 d old; n = 46) to standardize for possible differences in microbiota due to age (Mills et al. 1999). Fecal sacs were taken by using new latex gloves washed with 96% ethanol for each nest to maintain sterile conditions and avoid inter-nest contamination. A sterilized plastic container (60 ml) was placed just below the cloaca of each nestling, and fecal sacs fell inside directly (blackbird nestlings easily defecate when handled, unpubl.). Containers with fecal sacs were conserved at ambient temperature until arrival at the laboratory (for up to 8 h, usually for no more than 4–5 h) when they were stored in a refrigerator at 5°C until their processing (within the next 24 h).

Bacterial growing from fecal sacs

In order to test the first and third prediction, we used a first group of containers (n = 53). We took three samples from each excrement by using sterile swabs: 1) from the outside, 2) the inside of the sac, and 3) from the liquid that percolated from the sac to the container after a mean of 23.75 ± 2.82 min (n = 53). To homogenize the quantity of sample taken from each part, we made a single touch (i.e. time in contact 1 s) with the swab over the external or internal surface of the excrement, or over the liquid. We used a different sterile stick to open each sac. Containers were opened under sterile conditions in the lab.

The swab was then introduced in an eppendorf tube containing 1 ml of sterile phosphate buffer (pH 7.2, 0.2 M) and vigorously agitated in the vortex. Then, 100 µl of the mix was spread onto a general culture medium for mesophilic bacteria and in two specific media for *Enterococcus* and *Enterobacteriaceae*; both groups are typical from intestinal microbiota of birds and include several opportunistic pathogens (Brittingham et al. 1988, Lombardo et al. 1996, Westneat and Rambo 2000). A serial dilution was performed at a factor of 100 (990 µl of sterilized distillated water and 10 µl of sample) to count bacterial colonies in the petri dishes. Plates were incubated aerobically at 37°C during 72 h, and then colonies that grew in each plate were counted. Bacterial load was estimated as the number of CFU (colony forming units) per ml of buffer.

Antimicrobial properties of fecal sacs

A second group of samples (n = 69) from different nestlings were used for the inhibition tests to investigate our second prediction. Half of the sacs (n = 35) were directly placed on plates with brain heart infusion (BHI, see below) medium mixed with the indicator bacteria (antagonistic plates). The rest of the collected sacs (n = 34) were opened by breaking the covering with a sterile stick and the whole contents placed in plates as we did for the complete fecal sac.

Antagonistic plates were prepared with two indicator bacteria from separated taxonomic groups, *Enterococcus faecalis* MRR-10 and *Bacillus licheniformis* D-13, both from our laboratory collection. The former is a typical commensal

of the intestine, frequently detected in bird's cloaca (Moreno et al. 2003), while the latter is a keratinolytic bacteria typically found in birds plumage (Burtt and Ichida 1999). Indicator bacteria were cultured in (BHI) overnight, and 100 µl of each culture were inoculated in 15 ml of BHI-B (1.8% BHI, 0.8% agar in 0.1 M pH 7 phosphate buffer). This solution was poured onto sterile petri dishes, and after solidification, sacs were laid on. Plates were prepared just before the experiments.

After 12 h of incubation at 28°C, plates were checked for inhibition halos (a transparent zone that would indicate inhibition of the indicator bacteria) around sacs. In the case of the existence of halos, they were measured from the limit of the sac until where the indicator bacteria started to grow.

Statistical analyses

We carried out repeated-measures ANOVAs to detect differences in bacterial loads among the three different parts of each sac and test prediction 1 (inside vs outside) and 3 (inside vs liquid). CFU ml^{-1} in each medium was the dependent variable, and as explanatory ones we used the part of the sac sampled, consistently included as the within-group factor, and age as a covariable, while considering their interaction too. Recent literature has detected a change in bacterial communities of cloacal samples in relation with nestling ages even for short-term periods (González-Braojos et al. 2012). Thus, we decided to include age as an additional independent factor to statistically control for differences in bacterial community associated with age. The assumptions underlying the use of these analyses were systematically checked and the $\log_{10}$ transformation was applied for bacterial loads. Analyses were performed by using the Statistica 7.0 software.

We used a generalized linear mixed model to determine if there were differences in antibacterial activity between opened and closed fecal sacs (prediction 2). The zone of inhibition was fitted to a Poisson distribution. We selected the best model according to the Akaike information criteria among those built including state of the fecal sac (open or close), nestling and nest identity. The best model included fecal sac state as a fixed factor and nest identity as a random factor. We used R 2.15 (lme4 package) for these analyses.

Results

Bacterial loads from the outside of recently collected fecal sacs were three orders of magnitude lower than that estimated for the inside or for the percolated liquid after more than 20 min, which support prediction 1. It occurs independently of the culture media (TSA: $F_{2,52} = 4.04$, $p = 0.02$; KF: $F_{2,64} = 12.50$, $p < 0.001$; HK: $F_{2,64} = 5.62$, $p = 0.006$; Tukey HSD post hoc tests, $p = 0.0001$ in all cases Fig. 1). Bacterial loads of samples from the inside of the sac and the permeated liquid did not differ for any of the three media (Tukey HSD post hoc tests, TSA: $p = 0.59$; KF: $p = 0.10$; HK: $p = 0.79$), which did not fit with prediction 3. Nestling age or its interaction with the origin of samples was not significant in any case (results not shown) and was therefore removed from the final model.

In relation to antimicrobial tests, the size of the inhibition halo produced by opened and closed fecal sacs when tested against *B. licheniformis* ($Z_3 = 0.94$, $p = 0.35$; $n = 49$; mean ± SE for opened sacs = 0.58 ± 0.21 mm; closed sacs = 0.84 ± 0.26 mm) or *E. faecalis* ($Z_3 = 0.30$, $p = 0.76$; $n = 20$; opened sacs = 0.50 ± 0.22 mm; closed sacs = 0.60 ± 0.22) did not differ significantly, providing no support for prediction 2.

Discussion

Our findings suggest that the mucous covering of fecal sacs protects birds from bacterial contamination, thus supporting this new antimicrobial hypothesis. Bacteria on the surface of fecal sacs were less abundant than in their

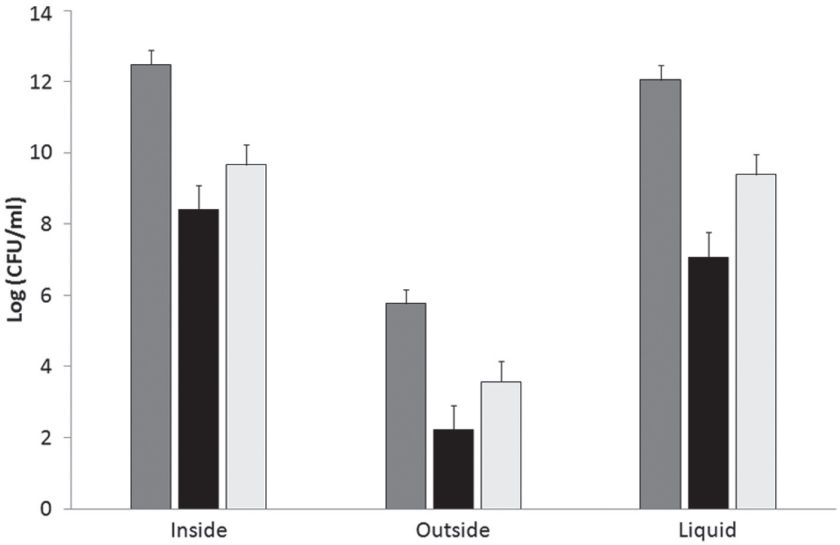

Figure 1. Mean bacterial loads (± SE) for each part of the fecal sac for Tryptone Soja Agar (TSA; dark-grey bars), Hektoen Agar (HK; light-grey bars) and Kenner-Faecal Agar (KF; black bars) culture media.

inside (prediction 1; Fig. 1). However, the barrier effect of fecal covering disappeared after 23 min as shown by bacterial loads estimated for percolated liquids (Fig. 1). This short-term antimicrobial effect could partly explain the commonly observed parental behavior of stimulating nestlings' defecation during parental visits (Dell'Omo et al. 1998, Ibáñez-Álamo et al. 2013a), and why chicks in many species only defecate in the presence of their parents (Herrick 1900, Brooke and Birkhead 1991). Otherwise, if nestlings' feces production and parental disposal of excrements are not synchronized, adult birds (and probably offspring) may become contaminated with potentially harmful bacteria.

We did not find evidence of antimicrobial properties of fecal sacs due to chemical compounds since we did not detect any significant differences in the inhibitory activity of feces with and without the covering (prediction 2). We found a little halo of inhibition in both (non-manipulated and opened) fecal sacs. Several bacterial groups, including gut bacteria, produce different kind of antimicrobial substances, mainly bacteriocins, to outcompete other bacteria (Riley and Wertz 2002). Therefore, is not surprising to find a low rate of inhibition, although it was similar between the two types of excrements. Additionally, the amount of bacteria was the same after the liquid passed over the fecal covering (prediction 3; Fig. 1), which means that there was no significant inhibition in the mucous layer. Thus, the mucous covering seems to act as a physical barrier isolating (at least temporally) intestinal bacteria within the sac. This function will be similar to that of the eggshell impeding pathogenic bacteria to infect the embryo (Board et al. 1994).

Feces manipulation entails the possibility of contact with potentially pathogenic microorganisms, and feces covering could prevent their transmission from nestlings to adults. Thus, the detected isolation effect of fecal covering sac detected here, even if temporal, seems to be adaptive, as many enteric bacteria of birds are potentially dangerous and may even provoke their death (Brittingham et al. 1988, Lombardo et al. 1996, Westneat and Rambo 2000, Potti et al. 2002). Although we have not detected evidence of antimicrobial activity against the two tested bacteria, it would be very interesting to investigate whether this isolation function of the mucous covering is also effective against other potentially harmful microorganisms like other bacterial groups, viruses, fungi or protists.

Independently of the more or less broad effectiveness of fecal sacs covering preventing infections, our results suggest that it would protect nestlings and/or adults. It is interesting to note that parents dispose of fecal sacs by two different mechanisms: transporting them away with their beaks or directly ingesting them (Blair and Tucker 1941, Guigueno and Sealy 2012). In the former case, the mucous covering might avoid internal infection by pathogenic bacteria hosted within their nestlings' excrements. In the latter (the case of the blackbird; Ibáñez-Álamo et al. 2013a), the protection would most likely be directed to prevent contamination of skin of adults. In both cases, however, fecal sacs would influence the bacterial environment of nests (i.e. hygienic conditions) which, likely, would affect the probability of infection of nestlings and brooding adults. In relation to this, other components of sanitation behavior have already been proposed to play a role in feather degrading bacteria-birds interactions (Lucas et al. 2005, Shawkey et al. 2007). However, this isolation function of the mucous covering will not only be restricted to detrimental bacteria but also to beneficial microorganisms (Moreno et al. 2003). Surely, the benefits associated with the protection against harmful bacteria will surpass those provided by such beneficial microbiota.

Alternatively, the bacterial isolation effect detected here could be the byproduct of other functions of the mucous covering like for example facilitating manipulation by parents during nest sanitation tasks (McGowan 1995). However, independently of the origin of this trait, the protective effects against bacterial infection should have strengthened the evolution of fecal sacs enhancing such function. More experimental works are in any case necessary for further conclusions related to the evolution of fecal sacs in birds and we hope this work contribute to encourage further research.

To sum up, our findings indicate that the mucous covering would confer protection to adults and/or nestlings against bacteria contained in excrements, although only for a short time after chicks' defecation. Furthermore, this isolation function seems to be due to a physical barrier that encapsulates bacteria within a gelatinous container rather than the presence of antibiotics. Our study provides the first adaptive explanation for the evolution of fecal sacs and offers a new perspective about parent-offspring relationships in birds. It also highlights that bacteria-birds interactions could have played a major role shaping nest sanitation, a poorly understood but important parental behavior in birds.

Acknowledgements – We thank F. Ruiz-Raya for his help in the field, G. Roncalli offered assistance for some statistical analyses and M. Martín-Vivaldi helped us with some interesting discussions about our study. Financial support to MRR and JJS was provided by the Spanish Ministerio de Educación y Ciencia/FEDER (research project CGL 2010-19233-C03-01).

References

Archie, E. A. and Theis, K. R. 2011. Animal behaviour meets microbial ecology. – Anim. Behav. 82: 425–436.

Batt, R. M., Rutgers, H. C. and Sancak, A. A. 1996. Enteric bacteria: friend or foe? – J. Small Anim. Pract. 37: 261–267.

Benskin, C. M. H., Wilson, K., Jones, K. and Hartley, I. R. 2009. Bacterial pathogens in wild birds: a review of the frequency and effects of infection. – Biol. Rev. 84: 349–373.

Blair, R. H. and Tucker, B. W. 1941. Nest sanitation. – Br. Birds 34: 206–215, 226–235, 250–255.

Board, R. G., Clay, C., Lock, J. and Dolman, J. 1994. The egg: a compartmentalized, aseptically packaged food. – In: Board, R. G. and Fuller, R. (eds), Microbiology of the avian egg. Chapman and Hall, pp. 43–62.

Brittingham, M. C., Temple, S. A. and Duncan, R. M. 1988. A survey of the prevalence of selected bacteria in wild birds. – J. Wildl. Dis. 24: 299–307.

Brooke, M. and Birkhead, T. 1991. The Cambridge encyclopedia of ornithology. – Cambridge Univ. Press.

Burtt, E. H. and Ichida, J. M. 1999. Occurrence of feather-degrading bacilli in the plumage of birds. – Auk 116: 364–372.
Del Hoyo, J., Elliott, A. and Sargatal, J. 1992. Handbook of the birds of the world. Volume 1. – Lynx Edicions.
Dell'Omo, G., Alleva, E. and Carere, C. 1998. Parental recycling of nestling faeces in the common swift. – Anim. Behav. 56: 631–637.
Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal behavior and the microbiome. – Science 338: 198–199.
Glück, E. 1988. Why do parent birds swallow the feces of their nestlings? – Experientia 44: 537–539.
González-Braojos, S., Vela A. I., Ruiz-de-Castañeda, R., Briones, V. and Moreno, J. 2012. Age-related changes in abundance of enterococci and Enterobacteriaceae in pied flycatcher (*Ficedula hypoleuca*) nestlings and their association with growth. – J. Ornithol. 153: 181–188.
Guigueno, M. F. and Sealy, S. G. 2012. Nest sanitation in passerine birds: implications for egg rejection in hosts of brood parasites. – J. Ornithol. 153: 35–52.
Gunderson, A. R. 2008. Feather-degrading bacteria: a new frontier in avian and host–parasite research? – Auk 125: 972–979.
Herrick, F. H. 1900. Care of nest and young. – Auk 17: 100–103.
Ibáñez-Álamo, J. D. and Soler, M. 2010. Does urbanization affect selective pressures and life-history strategies in the common blackbird (*Turdus merula* L.)? – Biol. J. Linn. Soc. 101: 759–766.
Ibáñez-Álamo, J. D., Sanllorente, O., Arco, L. and Soler, M. 2013a. Does nest predation risk induce parent birds to eat nestlings' fecal sacs? An experimental study. – Ann. Zool. Fenn. 50: 71–78.
Ibáñez-Álamo, J. D., Ruíz-Raya, F., Roncalli, G. and Soler, M. 2013b. Is nest predation an important selective pressure determining fecal sac removal? The effect of olfactory cues. – J. Ornithol. doi: 10.1007/s10336-013-1031-7
Lombardo, M. P., Thorpe, P. A., Cichewicz, R., Henshaw, M., Millard, C., Steen, C. and Zeller, T. K. 1996. Communities of cloacal bacteria in tree swallow families. – Condor 98: 167–172.
Loye, J. E. and Zuk, M. 1991. Bird–parasite interactions. Ecology, evolution and behaviour. – Oxford Univ. Press.
Lucas, F. S., Moureau, B., Jourdie, V. and Heeb, P. 2005. Brood size modifications affect plumage bacterial assemblages of European starlings. – Mol. Ecol. 14: 639–646.
Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. – Proc. R. Soc. B 277: 123–130.
McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Loío, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. – Proc. Natl Acad. Sci. USA 110: 3229–3236.
McGowan, K. J. 1995. A test of whether economy or nutrition determines fecal sac ingestion in nesting corvids. – Condor 97: 50–56.
Mills, T. K., Lombardo, M. P. and Thorpe, P. A. 1999. Microbial colonization of the cloacae of nestling tree swallows. – Auk 116: 947–956.
Moreno, J., Briones, V., Merino, S., Ballesteros, C., Sanz, J. J. and Tomás, G. 2003. Beneficial effects of cloacal bacteria on growth and fledging size in nestling pied flycatchers (*Ficedula hypoleuca*) in Spain. – Auk 120: 784–790.
Morton, M. L. 1979. Fecal sac ingestion in the mountain white-crowded sparrow. – Condor 81: 72–77.
Petit, K. E., Petit, L. J. and Petit, D. R. 1989. Fecal sac removal: do the pattern and distance of dispersal affect the change of nest predation. – Condor 91: 479–482.
Pinowski, J., Barkowska, M., Kruszewicz, A. H. and Kruszewicz, A. G. 1994. The causes of the mortality of eggs and nestlings of *Passer* spp. – J. Biosci. 19: 441–451.
Potti, J., Moreno, J., Yorio, P., Briones, V., García-Borboroglu, P., Villar, S. and Ballesteros, C. 2002. Bacteria divert resources from growth for Magellanic penguin chicks. – Ecol. Lett. 5: 709–714.
Riley, M. A. and Wertz, J. E. 2002. Bacteriocins: evolution, ecology and application. – Ann. Rev. Microbiol. 56: 117–137.
Royle, N. J., Smiseth, P. T. and Kölliker, M. 2012. The evolution of parental care. – Oxford Univ. Press.
Shawkey, M. D., Pillai, S. R., Hill, G. E., Siefferman, L. M. and Roberts, S. R. 2007. Bacteria as an agent for change in structural plumage color: correlational and experimental evidence. – Am. Nat. 169: S112–S121.
Weatherhead, P. J. 1984. Fecal sac removal by tree swallows: the cost of cleanliness. – Condor 86: 187–191.
Wellman-Labadie, O., Picman, J. and Hincke, M. T. 2008a. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. – Br. Poult. Sci. 49: 133–143.
Wellman-Labadie, O., Picman, J. and Hincke, M. T. 2008b. Antimicrobial activity of the anseriform outer eggshell and cuticle. – Comp. Biochem. Physiol. B 149: 640–649.
Westneat, D. F. and Rambo, T. B. 2000. Copulation exposes female red-winged blackbirds to bacteria in male semen. – J. Avian Biol. 31: 1–7.