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The evolution of size of the uropygial gland: mutualistic feather mites and uropygial secretion reduce bacterial loads of eggshells and hatching failures of European birds

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Abstract

Potentially, pathogenic bacteria are one of the main infective agents against which a battery of chemical and physical barriers has evolved in animals. Among these are the secretions by the exocrine uropygial gland in birds. The antimicrobial properties of uropygial secretions may prevent colonization and growth of microorganisms on feathers, skin and eggshells. However, uropygial gland secretions also favour the proliferation of feather mites that feed on secretions and microorganisms living on feathers that would otherwise reach eggshells during incubation if not consumed by feather mites. Therefore, at the interspecific level, uropygial gland size (as an index of volume of uropygial secretion) should be positively related to eggshell bacterial load (i.e. the risk of egg infection), whereas eggshell bacterial loads may be negatively related to abundance of feather mites eating bacteria. Here, we explore these previously untested predictions in a comparative framework using information on eggshell bacterial loads, uropygial gland size, diversity and abundance of feather mites and hatching success of 22 species of birds. The size of the uropygial gland was positively related to eggshell bacterial loads (mesophilic bacteria and *Enterobacteriaceae*), and bird species with higher diversity and abundance of feather mites harboured lower bacterial density on their eggshells (*Enterococcus* and *Staphylococcus*), in accordance with the hypothesis. Importantly, eggshell bacterial loads of mesophilic bacteria, *Enterococcus* and *Enterobacteriaceae* were negatively associated with hatching success, allowing us to interpret these interspecific relationships in a functional scenario, where both uropygial glands and mutualistic feather mites independently reduce the negative effects of pathogenic bacteria on avian fitness.

Introduction

Parasitic infections are one of the major forces driving the evolution of animals, which selects for reciprocal physiological and behavioural responses to parasitism that impede parasitic proliferations on hosts and therefore reduce the likelihood of infection and its negative fitness consequences (Price, 1980; Wakelin, 1996; Moore, 2002; Playfair & Bancroft, 2004). Bacteria constitute a major cause of mortality in free-living and domesticated animals alike and also in humans (Salyers & Whitt, 2002). Potentially, pathogenic bacteria are one of the main infective agents for which a battery of chemical and physical barriers have evolved in animals (Salyers

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& Whitt, 2002). A defensive mechanism in birds that is particularly important in preventing feather degradation and skin infection by pathogenic microorganisms consists of self-cleaning through the use of secretions from the uropygial gland when preening (Jacob & Ziswiler, 1982; Bandyopadhyay & Bahttacharyya, 1996; Shawkey *et al.*, 2003; Reneerkens *et al.*, 2008). Uropygial secretions are known to include wax, oils and volatile compounds with antimicrobial properties (Jacob & Ziswiler, 1982; Martín-Vivaldi *et al.*, 2010; Rajchard, 2010). In accordance with this role of uropygial glands as preventing feather degradation by bacteria, feathers of birds with surgically removed uropygial gland showed higher levels of degradation and higher density of fungi and bacteria than in control birds (review in Jacob & Ziswiler, 1982). Moreover, a negative relationship between density of feather-degrading bacteria and uropygial gland size has been described for the barn swallow *Hirundo rustica* (Møller *et al.*, 2009). Previous studies have shown that large uropygial glands produce a greater volume of secretion than small glands (Martín-Vivaldi *et al.*, 2009; Pap *et al.*, 2010), which justify the use of gland size as a proxy for volume of uropygial secretion (Galván *et al.*, 2008; Møller *et al.*, 2009). Here, we use gland size as a proxy for volume of secretion and use own data for testing and supporting this important assumption at the interspecific level (see Materials and Methods). Not only volume of uropygial secretion, but also chemical composition would affect antimicrobial properties of preen waxes which may vary both within (i.e. seasonally, sexually, age, condition) (Reneerkens *et al.*, 2002, 2008; Versteegh *et al.*, 2006) and among species (Jacob & Ziswiler, 1982). Current knowledge of interspecific variation in antimicrobial properties of secretions is scarce and does not allow comparative analyses. Thus, we used interspecific variation in size of the uropygial gland (i.e. volume of secretion; see Materials and Methods) as a proxy of antimicrobial efficiency of secretion of different species, an assumption that is supported by the detected association between gland size and selection pressures due to bacterial infection of eggs [i.e. hatching success (Møller *et al.*, 2010a)] and feathers [that increases the probability of predation (Møller *et al.*, 2010b)].

The amount of secretion from the uropygial gland is also known to be positively related to the abundance of feather mites both inter- or intraspecifically (Galvan & Sanz, 2006; Galván *et al.*, 2008), and this may have beneficial effects for their hosts (Brown *et al.*, 2006; Galvan & Sanz, 2006) by the removal of old secretions from the uropygial gland and detritus accumulated on the feathers, as well as pathogenic microorganisms associated with these substances (Blanco *et al.*, 1997, 2001; Jovani & Blanco, 2000). Thus, it is possible that the antimicrobial effects of uropygial secretion on feather-degrading bacteria are not only due to the direct antimicrobial properties of secretions, but also indirectly through the positive effects on mites that feed on feather-degrading bacteria. Different groups of feather mites are adapted to inhabit certain microhabitats on the body of a bird (Dabert & Mironov, 1999), and thus, diversity of feather mites may also contribute to reduced bacterial loads. If that was the case, we should expect bird species with higher density of mites on the feathers to also harbour fewer bacteria, a prediction consistent with a mutualistic role of feather mites that has so far never been tested.

Uropygial glands and their secretion may also modify bacterial environment of eggshells and therefore increase embryo viability (Møller *et al.*, 2010a). Antimicrobial substances of uropygial gland secretion (Jacob & Ziswiler, 1982) may reach eggshell surfaces when feathers of incubating hosts come in contact with eggs, and therefore, these substances would protect embryos from trans-shell infections of microorganism. Moreover, bacteria on eggshells of birds may derive from those growing on the skin or feathers of incubating adults (Soler *et al.*, 2010), and they may therefore escape the effect of preening and uropygial secretions. Finally, the size of the uropygial gland may affect eggshell bacterial loads through the effect of its secretions on the abundance of feather mites, which hypothetically affect bacterial loads of feathers that come in contact with eggshells. Therefore, this scenario would predict a negative relationship between eggshell bacterial loads and size of the uropygial gland and the abundance and diversity of feather mites. In this scenario, feather mites would be truly mutualistic improving the fitness of their hosts, while hosts provided food for their feather mites.

Studies by Cook *et al.* (2003, 2005a) have shown that bacteria are an important cause of egg mortality in birds under natural conditions in tropical, but this is apparently not the case in temperate environments (Wang *et al.*, 2011). Moreover, extensive data from poultry demonstrate similar negative effects of bacteria growing on eggshells (Baggott & Graeme-Cook, 2002). Eggshell bacterial loads are also known to be related to environmental conditions of nest (Godard *et al.*, 2007; Peralta-Sánchez, 2011; Wang *et al.*, 2011) and, consequently, should reflect bacterial environments of nests and the probability of trans-shell infection of embryos. Thus, Møller *et al.* (2010a) predicted and found support for a positive relationship between uropygial gland size and hatching success of different species of birds caused by the known antimicrobial properties of uropygial secretions and the scenarios described above. However, the relationship between size of the uropygial gland and bacterial loads of eggshells has never been tested, although such a relationship would suggest a role of bacteria in explaining the association between gland size and hatching success. Here, we fill this gap and explore the association between size of the uropygial gland and eggshell bacterial loads of different species of birds.

If selection pressure due to eggshell bacterial loads contributes to the evolution of size of the uropygial

gland, we should expect that bird species with higher density of bacteria on their eggshells had evolved larger glands (Prediction 1 in Fig. 1). In this case, species with higher rates of hatching failures due to trans-eggshell bacterial contamination should be those with larger uropygial glands (Prediction 2 in Fig. 1).

A negative relationship between eggshell bacterial loads and size of the uropygial glands would also be possible if species under intense selection were able to reduce eggshell bacterial load to the level of species under low intensity of selection. However, this possibility is unlikely given that it has been shown that there exist interspecific positive relationships between size of immune organs and intensity of parasite-induced selection (Møller & Erritzøe, 1996, 1998; Møller *et al.*, 1998). A negative relationship between eggshell bacterial loads and uropygial gland size could also be predicted if the effect of gland size was not directly selected to reduce or eliminate bacteria on the eggshells, but instead it was an indirect effect of selection of larger glands due to its direct effects reducing or eliminating bacteria in other parts of the body (i.e. feathers) (Piault *et al.*, 2008). Particularly interesting is the possibility that the relationship between gland size and abundance and diversity of feather mites (Galván *et al.*, 2008) indirectly resulted in negative relationship between size of the uropygial gland and eggshell bacterial loads (Prediction 3 in Fig. 1). The value of these predictions relies on the assumption that interspecific variation in eggshell bacterial loads is negatively related to hatching success (see Fig. 1 for a complete view of the hypothetical scenarios linking size of the uropygial gland and hatching success). Therefore, we also tested this relationship (Prediction 4 in Fig. 1). We investigated all these predictions in a comparative framework with 22 species of birds for which we collected information on eggshell bacterial loads, size of uropygial glands and the number of species of feather mites. For a subsample of eight species, we also collect information from the literature on mean densities of feather mites.

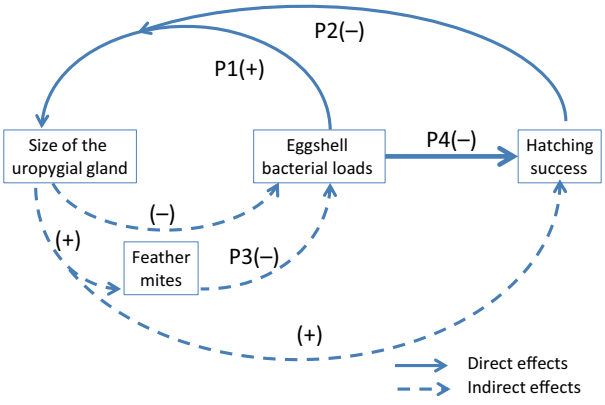

Fig. 1 Diagram showing hypothetical associations between size of the uropygial gland (i.e. volume of secretion), eggshell bacterial loads and hatching success. Directions of solid lines in the upper part of the diagram show that selection pressures favouring the increase in uropygial gland size would be lower for species with higher hatching success, but higher for species with larger eggshell bacterial loads. In this case, the negative predicted association between size of the uropygial gland and hatching success (P4) would be a direct effect of the antimicrobial properties of secretion reducing eggshell bacterial loads given the negative predicted association between bacterial density of eggshells and hatching success (P3). Dotted lines show possible indirect effects of gland size on both eggshell bacterial loads and hatching success mediated by the association between uropygial gland size and abundance and diversity of feather mites first, and by the predicted negative association between feather mites and eggshell bacterial load (P2) later.

Materials and methods

Study sites and nest locations

Bacterial communities on eggshells of nests were sampled during the breeding seasons 2007–2008 and 2006–2008 at Kraghede (Denmark, 57° 12′ N; 10° 00′ E) and Guadix (Spain 37° 18′ N; 3°11′ W), respectively. For a detailed description of the Danish and Spanish study area, see respectively Møller (1987), Martín-Vivaldi *et al.* (2006) and Soler & Avilés (2010).

JMP-S and APM in Denmark and JMP-S, JJS and MM-V in Spain made extensive systematic searches for nests in suitable habitats throughout the breeding season. We relied on extensive help from amateurs with a good knowledge of birds in locating nests, and we requested help in locating nests of all common breeding species. Adult birds carrying nest material in their beak were particularly used as a means of locating nests with fresh eggs. Most nests were therefore located during nest building by intensively searching suitable habitat in the study area and by checking nest boxes. We deliberately attempted not to touch nests or disturb the surrounding vegetation to avoid increasing the risk of nest predation. When a nest was detected during egg laying, on the basis of the typical clutch size of the species, we estimated date of clutch completion and visited the nests the following day to perform eggshell bacterial samplings. Nests were again visited at hatching. The number of nest checks was in this way minimized to reduce any unnecessary predation due to investigators.

Estimation of eggshell bacterial loads

We sampled eggs at the beginning of incubation (i.e. 2–3 days after clutch completion). Incubation may reduce both density and diversity of bacteria on eggshells (Cook *et al.*, 2005a; Shawkey *et al.*, 2009) but, at least for some species, eggshell bacterial loads increase during the incubation period (Soler *et al.*, 2011b). Thus, by sampling soon after incubation started, we assured that

independent of interspecific differences in start of incubation, the effect of incubation on eggshell bacterial loads is controlled.

We sampled eggshells in sterile conditions mainly to prevent between-nest contamination. We wore latex gloves sterilized with ethanol and took bacterial samples by cleaning eggshells with a sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M; pH 7.2). The entire clutch was cleaned with the same swab, which was preserved in an Eppendorf tube at 4 °C containing the sterile buffer until laboratory analyses during the following 30 days. Although sample storage has traditionally been considered a major problem in environmental microbiology, Lauber *et al.* (2010) have recently shown that the relative abundance of most bacterial taxa from soil samples is largely unaffected by temperature (i.e. 20, 4, −20 and −80 °C) even after 14 days of storage. Most of our samples were analysed within this period (median = 10 days; mean (SE) = 13.4 (0.34) days). Moreover, the duration of the storage period does not differ for different species ($F_{21,30.8} = 0.71$, $P = 0.79$) after controlling for variation among years ($F_{2,\ 45.3} = 9.22$, $P = 0.0004$) and its interaction with species identity ($F_{29,896} = 9.18$, $P < 0.0001$). Finally, the duration of storage period did not affect rank position of different species as shown by a comparison of ranked values of heterotrophic bacterial loads of 21 species from which in 2006 we collected samples that were stored less than 3 days ($N = 120$) and others that were stored up to 1 month ($N = 156$) (Kendall coefficient of concordance = 0.95; average Spearman's rank correlation = 0.91, Friedman ANOVA, $\chi^2 = 38.13$, $P = 0.009$). Thus, we are confident that variation in duration of storage would not affect our results. Estimates of bacterial load were standardized to total eggshell surface sampled by taking into account the number of sampled eggs and the surface area of each egg in the nest (following Narushin, 2005).

In the laboratory, samples were collected from Eppendorf tubes after vigorously shaking the Eppendorf in vortex for at least three periods of 5 s. Serial decimal dilutions up to 10^{-6} were cultivated by spreading homogeneously 100 μL of sample (measured with a micropipette) on plates containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used tryptic soy agar (TSA), a broadly used general medium to grow mesophilic bacteria, and three specific media: Kenner faecal agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel–Johnson agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen enteric agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. Plates were incubated at 32 °C for 72 h, and afterwards, the number of colonies on each plate was counted. Bacterial density was estimated as colony forming units (CFU) per cm^2. For a more detailed description of agar media, estimates of eggshell bacterial density and repeatability estimates of intraspecific variation of bacterial growth, see Peralta-Sánchez *et al.* (2010).

Enterobacteriaceae and *Staphylococcus* sp. are saprophytic and opportunistic bacteria (Houston *et al.*, 1997; Singleton & Harper, 1998; Cook *et al.*, 2005a) that live on skin, hair and feathers of mammals and birds (Krieg & Holt, 1984). They commonly appear on avian eggshells and are known to be pathogenic for avian embryos (Bruce & Drysdale, 1994). *Enterococcus*, the third analysed group of bacteria, are also frequently found inside unhatched eggs (Bruce & Drysdale, 1994) and are opportunistic pathogens (Franz *et al.*, 1999), although some species might also have beneficial effects (Moreno *et al.*, 2003; Soler *et al.*, 2008, 2010). Moreover, estimations of eggshell bacterial loads from counts of aerobic growth on general and specific media have been used as a proxy of the bacterial environment of nests (Cook *et al.*, 2003, 2005b; Peralta-Sánchez *et al.*, 2010; Møller *et al.*, 2011; Soler *et al.*, 2011a, b) and flight feathers (Møller *et al.*, 2012) in different ecological contexts. Therefore, there are good reasons for considering the estimated eggshell bacterial loads as proxies of probability of trans-shell bacterial infection of embryos. It should be noted here that our predictions deal with eggshell bacterial density, and that it can be assumed that estimates from culture and more direct methods (i.e. molecular analyses) should be closely positively related (Stolp, 1988).

Uropygial gland, diversity and density of feather mites and hatching success

We tested for reliability of size estimates for uropygial glands using information on the mass of the uropygial gland from the study by Jacob & Ziswiler (1982) and Møller *et al.* (2010a). As explained in the study by Møller *et al.* (2010a), the glands of Danish birds were carefully dissected and weighed to the nearest 0.001 g on a precision balance. Moreover, estimates of the mass and size (i.e. external volume) of the uropygial gland were highly repeatable when comparing ten common species from the data set in the study by Møller *et al.* (2010a) and that of Jacob & Ziswiler (1982) [$F_{9,10} = 10.95$, $r^2 = 0.91$, $P = 0.0004$, repeatability R (SE) = 0.83 (0.14)] (Møller *et al.*, 2010a). This provides evidence of consistency in estimates among different sources. Furthermore, we know that uropygial gland mass at the interspecific level is strongly positively related to uropygial gland volume as estimated from external measures (A.P. Møller, unpublished data: log-transformed values, $r_{27} = 0.76$, $P < 0.0001$), and both gland mass ($r_{25} = 0.44$, $P = 0.023$) and volume ($r_{44} = 0.67$, $P < 0.0001$) are positively related to volume of uropygial secretions by analysing unpublished data collected by APM in Ukraine. At the intraspecific level, it is also known that volume of the uropygial gland predicted the volume of secretions (Martín-Vivaldi *et al.*, 2009; Møller *et al.*, 2009; Pap *et al.*, 2010). Thus, the use of size of the uropygial gland as a

proxy of volume of secretion of different bird species is well founded.

The mean numbers of feather mites (based on total number of mites on the right wing) were obtained from the study by Galván *et al.* (2008). Diversity of feather mites found in different hosts was estimated as the number of different species reported in the literature survey posted at http://www.biology.ualberta.ca/faculty/heather_proctor/?Page=5626. However, diversity of feather mites found for a target species of bird was closely related to the number of performed studies ($R = 0.96$, $N = 22$, $P < 0.00001$). Thus, we divided the number of detected feather mite species by the number of reported studies per bird species and used these average values of diversity of feather mites in subsequent analyses.

We used hatching success of eggs that had been incubated for the normal incubation period, thus excluding depredated or deserted eggs, as reported by Spottiswoode & Møller (2004) combined with data from Cramp (1998) as reported by Møller *et al.* (2010a). Finally, we also used information of water habitat [i.e. terrestrial (0; not commonly encountering water), partly aquatic (1; spending at least part of the time in water) or completely aquatic (2; spending most or all of the time in water)] based on habitat descriptions in Cramp (1998) as described in the study by Møller *et al.* (2010a), because this variable would affect bacterial and fungal growth (Shawkey *et al.*, 2003; Burtt & Ichida, 2004), uropygial gland size and probability of successful hatching (Møller *et al.*, 2010a).

Sample sizes and statistical analyses

We successfully collected information on eggshell bacterial loads for 954 nests belonging to 22 species of birds (21 from Denmark and 15 from Spain) (see Appendix 1). We know from previous analyses that interspecific variation in eggshell bacterial load is significantly larger than intraspecific and interyear variation (Peralta-Sánchez, 2011). Thus, for each species and country, we estimated geometric means of $\log_{10}$-transformed eggshell bacterial load. Frequency distributions of $\log_{10}$ bacterial density did not differ from normality for mesophilic bacteria or *Enterobacteriaceae* (Kolmogorov–Smirnov tests for continuous variables, $P > 0.2$), but did so for *Enterococcus* and *Staphylococcus* (Kolmogorov–Smirnov tests for continuous variables, $P < 0.05$). Thus, because we were interested in considering all estimations of eggshell bacterial load or growth in the same analyses, we conservatively ranked values and used these in the subsequent analyses. Moreover, because we were not interested in differences among locations (J.M. Peralta-Sánchez, A.P. Møller, M. Martín-Vivaldi & J.J. Soler, in prep.), but in maximizing the number of species with information on eggshell bacterial loads, we used residuals of such values after controlling for the effect of country (GLM weighted by number of sampled nests per species, effect of country: mesophilic bacteria, $F_{1,89} = 2.79$, $P = 0.098$; *Enterococcus*, $F_{1,89} = 3.83$, $P = 0.053$; *Staphylococcus*, $F_{1,89} = 10.39$, $P = 0.0018$; *Enterobacteriaceae*, $F_{1,89} = 2.79$, $P = 0.107$). For species sampled in both countries, we used mean values of these residuals. Note that the estimation of residuals from ranked values is well founded because nonparametric tests work with these values and estimate parametric statistics (e.g. Sprent, 1993). Thus, the use of parametric tests in subsequent analyses is appropriate.

Interspecific variation in both eggshell bacterial loads (Peralta-Sánchez, 2011) and diversity of feather mites from different sources at http://www.biology.ualberta.ca/faculty/heather_proctor/?Page=5626) ($F_{21,285} = 2.30$, $P = 0.0013$) were larger than the within-species variation, which justifies the use of species-specific values in the comparative analyses. Interspecific statistical relationships could be affected by phylogenetic nonindependence (Harvey & Pagel, 1991). Therefore, we tested residuals of the regressions between estimated eggshell bacterial loads, mass of the uropygial gland, abundance and diversity of feather mites and hatching success, using the lambda statistic of Pagel (1999; Freckleton *et al.*, 2002) and a molecular phylogeny of European birds recently published (Thuiller *et al.*, 2011) (Appendix 2). To control for possible effects of a common ancestor, we performed phylogenetic generalized least-squares regression (PGLS) analyses (Pagel, 1997, 1999) as implemented in *R* with the appropriate libraries ('ape', 'MASS' and 'mvtnorm') and additional functions by R. Freckleton (University of Sheffield) as implemented in the package 'caic'. The PGLS approach characterizes evolutionary changes along each branch of a phylogeny through the variance components of traits, and it controls for the nonindependence among species by incorporating a matrix of the covariances among species based on their phylogenetic relationships (Martins & Hansen, 1997; Pagel, 1997, 1999). Thus, phylogenetic information is incorporated to the error term, thus controlling for the shared evolutionary history among species (Harvey & Pagel, 1991; Martins & Hansen, 1997). The method applies likelihood ratio statistics to test evolutionary hypotheses and also to estimate the importance of phylogenetic corrections in the models (λ) (Freckleton *et al.*, 2002), which vary between 0 (phylogenetic independence) and 1 (species' traits covary in direct proportion to their shared evolutionary history) (Pagel, 1997, 1999). Then, we conducted all analyses setting the degree of phylogenetic dependence (λ) to the most appropriate degree evaluated for each model. This was carried out by using the *pglmEstLambda* function, which automatically estimates the λ parameter simultaneously with other parameters of the model (Freckleton *et al.*, 2002).

In addition, we corrected our comparative analyses for heterogeneity in data quality due to the large variation in sample sizes among species by using weights in the comparative analyses (Garamszegi & Møller, 2010).

Briefly, following Garamszegi & Møller (2007), we combined variance factors due to phylogenetic and weight effects as error terms in the form of a matrix using the $\boldsymbol{Q} = \boldsymbol{V} + c\boldsymbol{W}$ equation, where $\boldsymbol{V}$ is the phylogeny matrix; $\boldsymbol{W}$ is the diagonal matrix of 1/weights; and c is a constant (Martins & Hansen, 1997). By varying the c constant, we calculated the maximum likelihood of different combinations of the phylogeny and weight matrices. At the combination which resulted in the highest maximum likelihood, we determined the slope of the effect in focus. This additional PGLS exercise was also performed in the *R* statistical computing environment and by setting the degree of phylogenetic dependence (λ) to the most appropriate degree evaluated for each unweighted model.

Mass of the uropygial gland is strongly related to body mass at the interspecific level (our data: PGLS, $R^2 = 0.76$, Beta (SE) = 0.88 (0.11), $F_{1,21} = 64.3$, $P < 0.0001$), and thus, we used residuals of mass of the uropygial gland in our analyses. Variation in uropygial gland mass can be affected by bacterial environment (i.e. eggshell bacterial load) and vice versa. Thus, for simplicity, we used residuals of mass of the uropygial gland after controlling for the effect of body mass as dependent variable, and bacterial loads or growth estimated for each of the four media as independent variables in our models.

The relationship between eggshell bacterial loads and hatching success was analysed by means of PGLS models with the latter as dependent variable and the former as independent factors. Because eggshell bacterial loads were predicted to depend on abundance and diversity of feather mites, the former were in this case used as dependent variables.

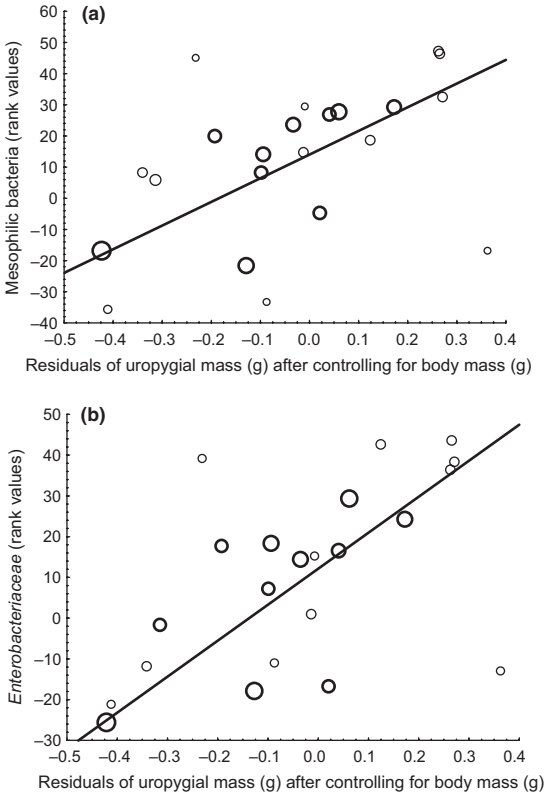

Fig. 2 Relationship between eggshell bacterial loads estimated for mesophilic bacteria, and *Enterobacteriaceae*, and residual of uropygial gland size after correcting for the allometric effect of body mass for different species of birds. Lines are weighted regression lines. Circle areas are proportional to log-transformed number of nests for each species sampled for eggshell bacterial loads.

Results

Eggshell bacterial load and uropygial gland size

We found that the species with larger uropygial glands had higher eggshell bacterial density, especially for mesophilic bacteria and *Enterobacteriaceae* (Fig. 2), even after correcting for the nonsignificant effects of water habitat (Table 1). These bacteria include pathogens that are known to infect developing embryos in avian eggs, and thus, these results suggest that size of the uropygial gland is at least partially adjusted to the bacterial environments of nests, as reflected by eggshell bacterial loads.

Eggshell bacterial load and hatching success

Hatching success was negatively associated with eggshell bacterial loads, especially for estimates of mesophilic bacteria, *Enterococcus* and *Enterobacteriaceae*, but not for *Staphylococcus* (Fig. 3) even after controlling for the effect of water habitat. Interestingly, when including in the models information on uropygial gland size, none of the estimations of eggshell bacterial loads or gland size explained a significant proportion of variance in hatching success (PGLS, $P > 0.23$), which suggests that shared variance between eggshell bacterial loads and uropygial gland size was responsible for the detected association between eggshell bacterial loads and hatching success. In these models, uropygial gland size was negatively related to hatching success [Beta (SE) = −0.20 (0.05), $t_{20} = 2.39$, $P = 0.03$] after controlling for the nonsignificant effect of *Staphylococcus* (Beta (SE) = 0.001 (0.001), $t_{20} = 1.29$, $P = 0.22$), but not after controlling for the effect of other bacterial groups.

Eggshell bacterial load and diversity and abundance of feather mites

In agreement with the hypothesis that feather mites affect the bacterial environment of eggs, we found that species with a more diverse fauna of feather mites harboured lower bacterial density on eggshells, especially for *Enterococcus* and *Staphylococcus* (Table 2; Fig. 4). Moreover, species with more abundant feather mites were

Table 1 Relationship between eggshell bacterial loads, as reflected by aerobic cultures of mesophilic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*, and size of the uropygial glands (residuals values after controlling for the effect of body mass) and hatching success of different species of birds after controlling for the nonsignificant effects of water habitat. Analyses are phylogenetic generalized linear models (PGLS) weighted by sample size. Phylogenetic signals (λ) of statistical models are also shown.

Size of the uropygial gland					
Models		λ	Beta (SE)	t_{20}	P
1	Mesophilic bacteria	0.55	-0.53×10^{-2} (0.17×10^{-2})	3.05	0.007
	Water habitat		0.124 (0.099)	1.25	0.23
2	*Enterococcus*	7.48×10^{-05}	-0.01×10^{-2} (0.28×10^{-2})	0.04	0.97
	Water habitat		0.191(0.093)	2.04	0.055
3	*Staphylococcus*	6.61×10^{-05}	-0.08×10^{-2} (0.28×10^{-2})	0.27	0.79
	Water habitat		0.186 (0.091)	2.06	0.056
4	*Enterobacteriaceae*	6.50×10^{-05}	-0.60×10^{-2} (0.17×10^{-2})	3.61	0.0019
	Water habitat		0.083 (0.086)	0.96	0.347
Hatching success					
Models		λ	Beta (SE)	t_{16}	P
1	Mesophilic bacteria	0.22	-0.98×10^{-3} (0.40×10^{-3})	2.36	0.032
	Water habitat		0.029 (0.055)	0.54	0.60
2	*Enterococcus*	0.27	-0.14×10^{-2} (0.06×10^{-2})	2.33	0.039
	Water habitat		0.054 (0.057)	0.95	0.36
3	*Staphylococcus*	0.68	0.13×10^{-2} (0.08×10^{-2})	1.57	0.14
	Water habitat		0.058 (0.040)	1.45	0.17
4	*Enterobacteriaceae*	0.34	-0.92×10^{-3} (0.40×10^{-3})	2.30	0.036
	Water habitat		0.047 (0.057)	0.83	0.42

those with lower density of *Enterococcus* and *Enterobacteriaceae* on their eggshells (Table 2). Other bacterial counts were not significantly related to density of feather mites.

Discussion

The main findings of this study are that (i) eggshell bacterial load at the beginning of incubation is positively related to size of the uropygial gland of different species of birds; (ii) interspecific variation in eggshell bacterial loads explained significant interspecific variation in hatching success; and (iii) host species with more diverse and abundant feather mites are those with lower eggshell bacterial loads. These results suggest, first, that the previously detected association between size of the uropygial gland and hatching success of birds was mediated by the effect of antimicrobial properties of uropygial secretion on eggshell bacterial loads. Second, in accordance with the hypothetical beneficial effects of feather mites of their avian hosts, we show for the first time a negative relationship between feather mites and bacteria on eggshells. These novel findings constitute the first evidence of a mutualistic relationship between feather mites and their avian hosts because feather mites benefit from consumption of secretions from the uropygial gland and the microorganisms found on feathers, whereas bird hosts benefit from reduced eggshell bacterial load, a proxy of hatching failure. Below, we discuss these interpretations centring on the role of bacteria as important agents of selection driving the evolution of size of the uropygial gland.

Møller *et al.* (2010a) detected a positive association between hatching success and size of the uropygial gland of different bird species interpreted to be a consequence of the antimicrobials of abundant uropygial secretion acting against pathogenic microorganisms of avian embryos. In accordance with this interpretation, we found that bird species with lower eggshell bacterial loads enjoyed higher hatching success. Thus, it is likely that variation in selection pressures due to pathogenic microorganisms explains at least partially the variation in size of the uropygial gland of birds. In accordance with this interpretation, we have found that bird species with higher eggshell loads of mesophilic bacteria and *Enterobacteriaceae* were those with larger uropygial glands. However, given the positive association between eggshell bacterial load and gland size reported here, the association between eggshell bacterial load and hatching success would predict a negative (See Fig. 1) rather than the positive association between uropygial gland size and hatching success reported by Møller *et al.* (2010a). For the group of species studied here, we in fact found a negative association between hatching success and gland size, suggesting that birds under higher selection presures due to causes of hatching failures had larger uropygial glands. Interestingly, the detected relationships between eggshell bacterial loads and hatching success disappeared when including information on the size of

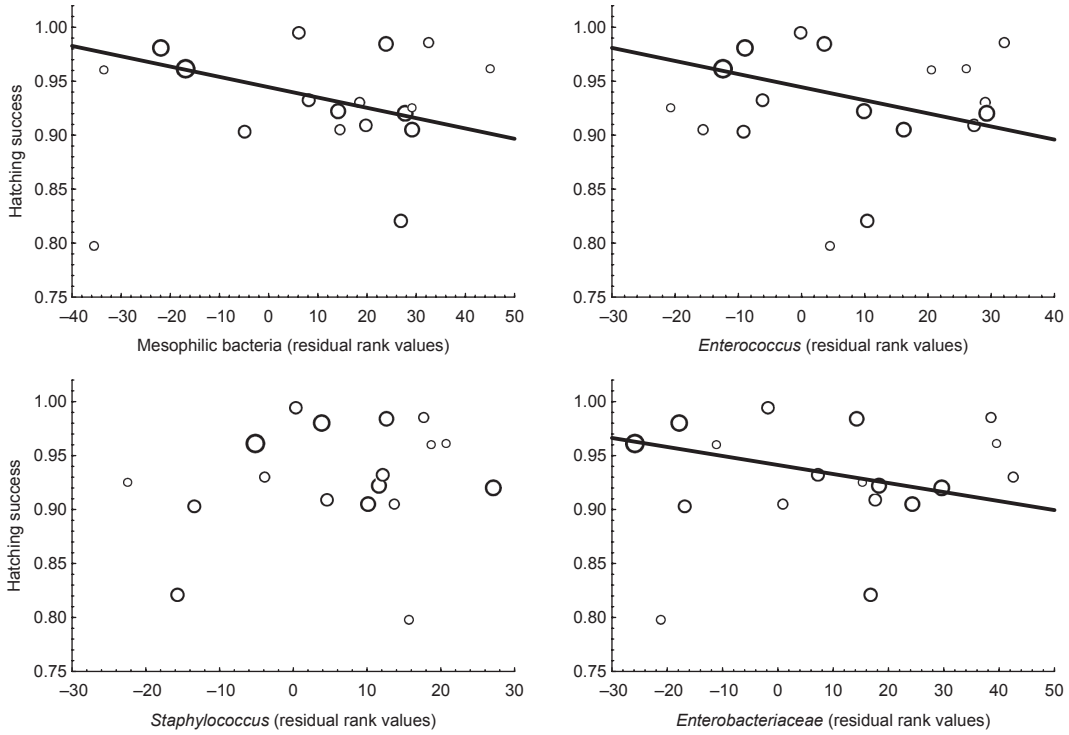

Fig. 3 Relationship between eggshell bacterial loads estimated for mesophilic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*, and hatching success of different species of birds. Lines are weighted regression lines. Circle areas are proportional to log-transformed number of nests of each species sampled for eggshell bacterial loads.

Table 2 Relationship between eggshell bacterial loads, as reflected by aerobic cultures of mesophilic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*, and abundance (as reported by Galván *et al.*, 2008) and average number of feather mite species detected in different species of birds per published study. Feather mite taxa known from different hosts are taken from a literature survey posted at http://www.biology.ualberta.ca/faculty/heather_proctor/?Page=5626. Analyses are phylogenetic generalized linear models (PGLS) weighted by sample size. Phylogenetic signals (λ) of statistical models are also shown.

Log–transformed number of feather mite taxa					
	λ	R^2	Beta (SE)	F_{20}	P
Mesophilic bacteria	0.91	0.08	–25.83 (19.43)	1.77	0.20
Enterococcus	4.9×10^{-5}	0.22	–26.81 (11.43)	5.50	0.029
Staphylococcus	4.8×10^{-5}	0.32	–25.55 (8.38)	9.39	0.006
Enterobacteriaceae	0.91	0.03	–16.06 (19.93)	0.65	0.43
Abundance of feather mites					
	λ	R^2	Beta (SE)	$F_{1,6}$	P
Mesophilic bacteria	0.00	0.06	–0.239 (0.375)	0.41	0.540
Enterococcus	0.00	0.70	–0.418 (0.111)	14.26	0.009
Staphylococcus	0.00	0.68	–0.076 (0.115)	0.44	0.530
Enterobacteriaceae	0.00	0.50	–0.660 (0.267)	6.11	0.048

uropygial glands in the statistical models. Statistically, these results should be explained as covariation between size of the uropygial gland and eggshell bacterial loads being responsible for the separate associations between bacteria on the eggshell (see Results) and size of the uropygial gland with hatching success (Møller *et al.*, 2010a). All these results together suggest that bacteria on eggshells of birds are important agents determining the probability of hatching failure and that the effects of size of the uropygial gland on hatching success are

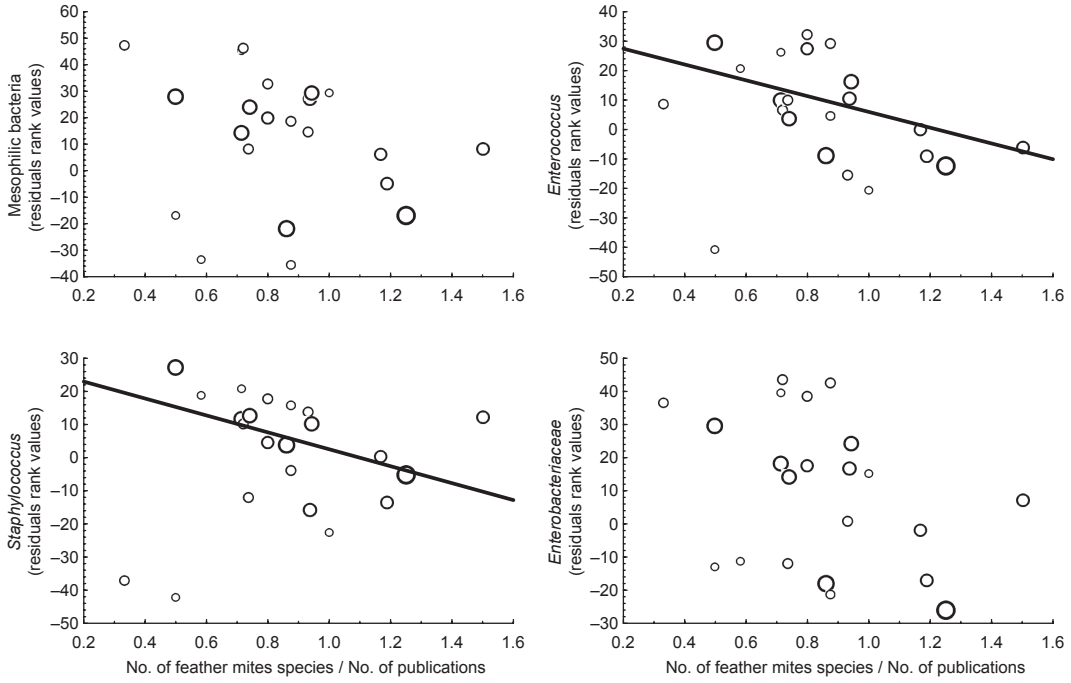

Fig. 4 Relationship between eggshell bacterial loads estimated for *Enterococcus*, and *Staphylococcus*, and number of species of feather mites found in feathers of different species of birds (as reported in the literature survey posted at http://www.biology.ualberta.ca/faculty/heather_proctor/?Page=5626) divided by the number of papers listed for each species. Lines are weighted regression lines. Circle areas are proportional to log-transformed number of nests of each species sampled for eggshell bacterial loads.

determined by the antimicrobial effects of uropygial secretion on eggshell bacterial loads and proliferation. At the intraspecific level, similar to the relationship between uropygial gland size of barn swallows (*H. rustica*) and abundance of feather-degrading bacteria (Møller *et al.*, 2009), individuals with smaller uropygial gland may also be those with higher eggshell bacterial loads.

A negative relationship between uropygial gland size and eggshell bacterial loads can also be predicted through indirect effects (see Fig. 1), which would predict a positive association between hatching success and size of the uropygial gland. The function of the uropygial secretions has mainly been framed as preventing feather degradation due not only to microorganisms (see Introduction), but also to environmental conditions and physical abrasion (Moreno-Rueda, 2011). It has also been suggested that uropygial secretions may attract blood-suckling insects (Martinez-de La Puente *et al.*, 2011) and/or favour symbiotic mites that feed on uropygial secretions smeared on feathers (Galván *et al.*, 2008), resulting in beneficial effect for birds if organisms feeding on secretions removed old inactive secretions together with microorganisms living from secretions (Blanco *et al.*, 1997). Both antimicrobial chemicals of uropygial secretions and microorganisms living on feathers of incubating individuals may reach eggshells, the latter increasing the probability of trans-shell embryo infection (Cook *et al.*, 2005a). Thus, because the abundance of feather mites and the size of the uropygial gland are positively related at the interspecific level (Galván *et al.*, 2008), and because feather mites may feed on microorganisms, the amount of bacteria that would reach eggshells would partially depend on the diversity and the abundance of feather mites. We found support consistent with this scenario because both abundance and diversity of feather mites were negatively related to eggshell bacterial loads (see Results). Estimates of feather mite abundance were from wing feathers (Galván *et al.*, 2008), and estimates of feather mite diversity were from feathers of different body parts. Mites are known to specialize on different kind of feathers (Dabert & Mironov, 1999), and thus, it can be argued that our estimates do not closely reflect the abundance and diversity of feather mites that are in contact with incubated eggs. However, the abundance and the diversity of mites at different kinds of feathers are likely positively correlated, and possible variation would in any case only add noise to the model testing our predictions. Therefore, our results constitute the first evidence of beneficial effects of feather mites through their influence on bacteria. Feather mites might indirectly reduce the probability of hatching failure because we found a negative relationship between eggshell bacterial loads and hatching success.

To summarize, we found support consistent with a relationship between size of the uropygial gland and bacterial density of eggshells, a variable that predicted interspecific variation in hatching success. Furthermore, we showed negative relationships between abundance and diversity of feather mites, respectively, and eggshell bacterial loads, which provides the first evidence supporting the hypothesis of a beneficial role of feather mites in reducing the fitness costs of bacteria on birds' eggs.

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Appendix 1

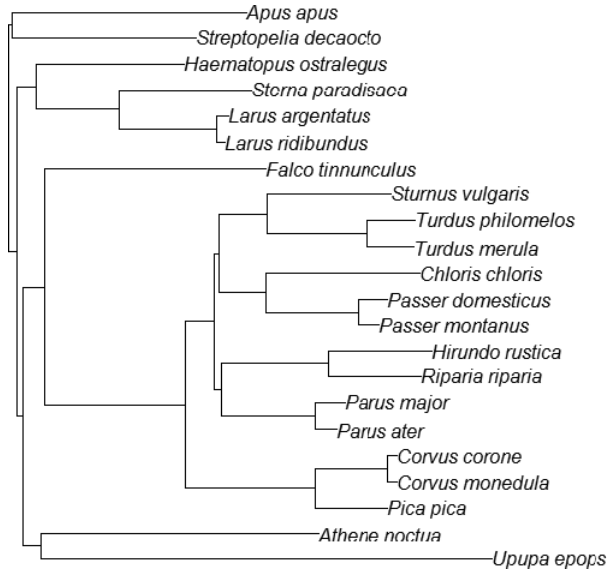
Log-transformed values of body mass, water habitat [i.e. terrestrial (0; not commonly encountering water), partly aquatic (1; spending at least part of the time in water) or completely aquatic (2; spending most or all of the time in water)], residual mass of the uropygial gland after controlling for the effects of body mass (ResUG), log-transformed numbers of species of feather mites together with number of studies per species enclosed in parentheses (log mite diversity), mean values of mite abundance (mean mite abundance), hatching success and residual eggshell bacterial loads in ranks after controlling for the effect of country shown for mesophilic bacteria (TSA), *Enterococcus* (KF), *Staphylococcus* (VJ), Gram-negative *Enterobacteriaceae* (HK). *N* refers to the number of nests sampled for estimates of eggshell bacterial load.

	log Mass	Water habitat	ResUG	Mean mite diversity	Mean mite abundance	Hatching success	*N*	TSA	KF	VJ	HK
Apus apus	1.5982	0	−0.4112	2.7081 (16)		0.798	6	−35.500	4.492	15.628	−21.131
Athene noctua	2.2253	0	−0.1924	1.6094 (5)		0.909	26	19.724	27.241	4.521	17.500
Chloris chloris	1.4417	0	−0.3395	2.7081 (19)	9.10		10	8.112	9.866	−11.925	−11.816
Corvus corone	2.7360	0	0.0217	2.9957 (16)		0.903	29	−4.888	−9.134	−13.425	−16.816
Corvus monedula	2.3962	0	−0.3135	2.0794 (6)		0.994	24	6.112	−0.134	0.325	−1.816
Falco tinnunculus	2.2418	0	−0.0984	1.9459 (4)		0.932	29	8.112	−6.134	12.075	7.185
Haematopus ostralegus	2.7251	1	−0.0866	2.0794 (12)		0.960	4	−33.500	20.492	18.628	−11.131
Hirundo rustica	1.2810	0	−0.1269	3.4657 (36)	48.34	0.980	132	−21.888	−8.884	3.825	−17.816
Larus argentatus	2.9518	1	0.1259	2.0794 (8)		0.930	10	18.500	28.992	−3.872	42.369
Larus ridibundus	2.4479	1	0.2720	2.1972 (10)		0.985	10	32.500	31.992	17.628	38.369
Parus ater	0.9661	0	−0.0073	2.0794 (7)	0.00	0.925	4	29.112	−20.634	−22.425	15.185
Parus major	1.2672	0	−0.0935	2.3979 (14)	4.09	0.922	73	14.112	9.866	11.575	18.185
Passer domesticus	1.4822	0	0.1724	3.5264 (35)	4.04	0.905	65	29.112	16.116	10.075	24.185
Passer montanus	1.3365	0	0.0416	2.7726 (16)		0.821	38	26.862	10.366	−15.675	16.685
Pica pica	2.3579	0	−0.4210	3.0445 (16)		0.961	295	−16.888	−12.384	−5.175	−25.816
Riparia riparia	1.1189	0	0.2668	2.9444 (25)	50.0		10	46.000	6.492	10.128	43.369
Sterna paradisaea	2.0394	2	0.2632	0.6931 (3)			8	47.000	8.492	−36.872	36.369
Streptopelia decaocto	2.3043	0	0.3628	1.3863 (6)			4	−16.888	−40.634	−41.925	−12.816
Sturnus vulgaris	1.9058	0	−0.0118	3.3322 (29)		0.905	10	14.500	−15.508	13.628	0.869
Turdus merula	1.9816	0	−0.0345	3.0445 (27)	37.83	0.984	62	23.862	3.616	12.575	14.185
Turdus philomelos	1.8482	0	−0.2304	1.7918 (7)	0.75	0.961	4	45.000	25.992	20.628	39.369
Upupa epops	1.8264	0	0.0624	0.6931 (2)		0.920	101	27.724	29.241	27.021	29.500

Appendix 2

Phylogenetic tree in PHYLIP format used for the analyses.

(((((Upupa_epops:0.46842580359,Athene_noctua:0.2881034494):0.018252974437,(((Pica_pica:0.07488956568,
(Corvus_monedula:0.01030648059,Corvus_corone:0.009445764074000001):0.07537231181699999):0.13432180965,
(((Parus_ater:0.022456241102,Parus_major:0.030504919357):0.09750645983,(Riparia_riparia:0.0968341599,
Hirundo_rustica:0.10845752644000001):0.11115278135500001):0.00751467203,
(((Passer_montanus:0.02215067647,Passer_domesticus:0.031500583417):0.09587091783,
(Fringilla_coelebs:0.10514552145,(Chloris_chloris:0.06946376369,Carduelis_carduelis:0.040545961132):
0.05916051848):0.032424203478):0.048447675350000004,((Turdus_merula:0.04964404928,
Turdus_philomelos:0.0505294965):0.103346216358,Sturnus_vulgaris:0.1293460245271):0.05000397905):
0.005038614934):0.02902980694):0.14645662366,Falco_tinnunculus:0.2295270005999):0.02257657166):
0.006400257462,(((Larus_ridibundus:0.009274300906,Larus_argentatus:0.011978476264221):
0.10139970757200001,Sterna_paradisaea:0.137404857547):0.085931385692,
Haematopus_ostralegus:0.15430641736099998):0.02015341826):0.004510076473,(Fulica_atra:0.26752087562,
Cuculus_canorus:0.22755102674529998):0.011098744575707):0.004589088048,
(Streptopelia_decaocto:0.19140464406500002,Apus_apus:0.24433296803341398):0.003641215018):0.3657713382.



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