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ORIGINAL ARTICLE

An experimental test in Mallards (*Anas platyrhynchos*) of the effect of incubation and maternal preen oil on eggshell microbial load

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Abstract Microbial infection is one of the main factors reducing survival in the first stages of life in oviparous species, and recent studies have shown that the avian eggshell harbors an important variety of microorganisms that can rapidly multiply and penetrate the shell, leading to a decrease in hatchability. Here, we report the results of an experiment in which we examined how incubation and maternal preen oil affect the growth of avian eggshell microbes, using the Mallard (*Anas platyrhynchos*) as a model species. We compared the bacterial and fungal loads on the shell of non-incubated eggs and eggs incubated by females having free or blocked access to their preen gland. An increase of eggshell bacterial loads was observed in all conditions, but bacterial growth was higher on the shell of incubated eggs than on non-incubated eggs. We did not find any significant difference in eggshell bacterial growth for eggs incubated by females with free or blocked access to their preen gland. In addition, fungal growth during our experiment was not affected by incubation or the mother's preen oil. Our findings are in contrast with those of previous studies which showed that incubation limited or had no effect on eggshell bacterial growth. Differences in environmental conditions and/or species ecology may explain the difference between the results of our experiment and those of previous studies. Our study provides the first data on the effect of maternal preen oil on eggshell microorganisms, showing that preen oil does not limit eggshell microbial growth.

Keywords Preen oil · Incubation · Eggshell bacteria · *Anas platyrhynchos*

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Zusammenfassung

Ein experimenteller Test an Stockenten (*Anas platyrhynchos*) zum Effekt von Bebrütung und mütterlichem Bürzeldrüsenfett auf die Mikrobenbelastung von Eierschalen

Mikrobielle Infektionen sind ein Hauptfaktor für reduzierte Überlebensraten der ersten Lebensstadien bei oviparen Arten. Aktuelle Studien belegen, dass bei Vögeln die Eierschale eine erhebliche Vielfalt an Mikroorganismen beherbergt, die sich schnell vermehren und in die Schale eindringen können. Dies führt zu einer verringerten Schlupfrate. Hier stellen wir die Ergebnisse eines Experimentes vor, bei dem wir untersucht haben, wie Bebrütung und mütterliches Bürzeldrüsenfett das Wachstum von Mikroben auf Eierschalen von Vögeln beeinflussen. Als Modellart diente die Stockente (*Anas platyrhynchos*). Wir verglichen den Bakterien- und Pilzbefall der Schalen bebrüteter und nicht-bebrüteter Eier mittels Weibchen, die einen freien oder blockierten Zugriff auf ihre Bürzeldrüse hatten. Bei allen Eierschalen wurde ein Anstieg der Bakterienbelastung beobachtet, wobei das Bakterienwachstum auf Schalen bebrüteter Eier höher war als auf den Schalen unbebrüteter Eier. Hinsichtlich der Weibchen mit freiem oder blockiertem Zugriff auf ihre Bürzeldrüse konnte kein signifikanter Unterschied im Bakterienwachstum auf den Eierschalen festgestellt werden. Darüber hinaus war das Pilzwachstum während des Experimentes nicht beeinflusst durch die Bebrütung oder das Bürzeldrüsensekret des Weibchens. Unsere Ergebnisse stehen im Gegensatz zu früheren Studien, die gezeigt haben, dass die Bebrütung entweder einen limitierten oder gar keinen Effekt auf das Bakterienwachstum auf Eierschalen hatte. Unterschiede in den Umweltbedingungen und/oder der Ökologie der Arten könnten den Unterschied zwischen diesem Experiment und vorherigen Studien erklären. Die vorliegende Studie liefert die ersten Daten zum Einfluss von mütterlichem Bürzeldrüsensekret auf Mikroorganismen von Eierschalen, die zudem zeigen, dass das Bürzeldrüsensekret das mikrobielle Wachstum auf Eierschalen nicht begrenzt.

Introduction

The first stages of life of living organisms are very critical, especially for oviparous species where non-mobile eggs are subject to attacks by predators and parasites (Clutton-Brock 1991). Infection with microbial pathogens might be one of the most important factors decreasing egg survival and affecting embryo development before hatching. Studies on poultry species have shown that eggshell bacteria are able to grow and penetrate into eggs through eggshell pores, leading to decreased hatchability (Baggott and Graeme-Cook 2002). In addition, recent field experiments with domestic and wild bird eggs have demonstrated that microbial infection of egg contents is positively related to shell microbial density and that microbial infection causes a decline in hatching success (Cook et al. 2003, 2005a).

As egg microbial infection strongly decreases individual fitness, selection may have favored the evolution of physiological and behavioral adaptations to limit or control microbial infection of eggs in oviparous vertebrates. One of these mechanisms is the deposition of antimicrobial substances and antibodies (lipophilic components, lysozyme, ovotransferrin, avidin, maternal antibodies) in the albumen and in the eggshell of newly laid eggs to prevent bacteria from altering embryonic development (Kowalczyk et al. 1985; Board and Fuller 1994; Shawkey et al. 2008; Wellman-Labadie et al. 2008, 2010). Cook et al. (2005a) recently proposed that incubation could constitute another mechanism by which birds limit egg microbial infection. In support of this hypothesis, these authors showed that eggshell bacteria and fungal loads decreased on incubated eggs of the Pearly-eyed Thrasher (*Margarops fuscatus*), while these loads increased rapidly on non-incubated eggs exposed to ambient conditions in the tropical climate of Puerto Rico (Cook et al. 2005a). Incubation also inhibits the diversification of bacterial assemblages on the eggshell in this species (Shawkey et al. 2009). In temperate climates, where eggshell bacterial growth should be limited by low temperatures and low humidity levels, the results are more contrasted. In a study on the Tree Swallow (*Tachycineta bicolor*), Western Bluebird (*Sialia mexicana*) and Violet-green Swallow (*Tachycineta thalassina*), Wang et al. (2011) found that eggshell microbial loads did not increase with exposure to ambient conditions and were unaffected by parental incubation. However, Ruiz-De-Castaneda et al. (2011) showed that early incubation in the Pied Flycatcher (*Ficedula hypoleuca*) is associated with an inhibition of eggshell bacterial growth. Given the paucity of studies, these results need to be expanded and confirmed by other studies in temperate climates using different ecological species.

Several mechanisms have been proposed to explain a decrease of eggshell microbial load associated with incubation. First, female presence on eggs during incubation may protect the eggs from environmental humidity and precipitation, leading to a decrease of microbial growth since most pathogenic microorganisms require damp conditions for survival and transport through shell pores into the egg contents (Board and Halls 1973; D'Alba et al. 2010; Ruiz-De-Castaneda et al. 2011). Second, females may spread preen oil with antibacterial activities over the eggshell (Jacob 1978; Menon and Menon 2000; Shawkey et al. 2003) through contact of their oiled feathers with the

eggshell. However, only a few studies have tested these mechanisms experimentally (see D'Alba et al. 2010; Ruiz-De-Castaneda et al. 2011).

Here we describe an experiment designed to test (1) the effect of incubation on bacterial growth in a continental temperate environment using the Mallard *Anas platyrhynchos* as a model species and (2) whether maternal preen oil constitutes one of the mechanisms used by birds to control bacterial growth on eggshell. We thus compared fungal and bacterial growth on the shell of eggs incubated by female Mallards with free or blocked access to preen glands, and on the shell of non-incubated eggs. Based on previous studies (Cook et al. 2005a, b; Shawkey et al. 2009), we expected to find (1) a higher growth of bacteria on eggs incubated by females that had their preen gland access blocked than on eggs incubated by control females and (2) a higher bacterial abundance for non-incubated eggs compared with the incubated ones.

Methods

The experiments were carried out from February to April 2008 at the Centre d'Etudes Biologiques de Chizé (Western France) using 60 adult Mallards (2 or 3 years old) descended from individuals caught in the wild. The birds were kept in semi-captive conditions for at least 2 years before the experiments and were therefore accustomed to their aviary environment. Birds were fed with an ad libitum mixture of crushed corn, wheat and commercial duck food. Housing conditions and the experiments were carried out in compliance with European legal recruitment guidelines and national permission (European convention ETS123).

Experimental design

To test the effect of maternal preen oil on eggshell bacterial growth, we used a previously described device that we designed to prevent bill–uropygial gland contact and the spreading of preen gland secretions on the feathers (Giraudeau et al. 2010a, b). Briefly, the device consists of a rubber tube (diameter 1 cm, height 2.5 cm) that is glued to the feathers and skin around the small feathered nipple of the uropygial gland. This structure is reinforced with a flexible plastic square (pierced in the middle at the ring level) glued to the plastic ring and set around the uropygial gland (Giraudeau et al. 2010a, b). A first group of females was equipped with this anti-preening mechanism (APM group, $N = 14$). The control group of females ($N = 16$) was handled in the same way but had no device attached. Ducks were observed with binoculars at least twice a week to check that the APM remained attached. At the end of the experiment the devices were removed by gently pulling on the plastic square. We detected no obvious signs of stress or unusual behavior after fitting the birds with the system (Giraudeau et al. 2010b).

Females were housed individually in a 6-m^2 pen with an artificial nest box and a small pool, and each female was randomly assigned one male for the entire clutch. Nest boxes were monitored daily (between 0800 and 1000 hours) to determine laying dates. The surface of the eggshell of the second and third eggs of each clutch was swabbed (2 cm^2 at the center of the egg) within 2 h of laying to collect microbiota. The swabs were then kept in individual sterile 1.5-ml Eppendorf tubes containing 0.8 ml of sterile physiological (0.90 % w/v) saline solution. The eggs were then marked and returned to natal nest boxes. To test the effect of incubation, we returned the second egg laid by each female to the clutch, while the third egg was placed on some nest materials but in a separate section of the nestbox behind a separation barrier and therefore inaccessible to females. To record eggshell bacterial growth, we swabbed the second and third eggs of each clutch a second time 5 days after laying. Samples were stored at −20 °C until microbial analyses.

Microbial analyses

Microbiological analyses were performed under sterile conditions following the methods of Møller et al. (2009), Czirják et al. (2010) and Giraudeau et al. (2010c). Briefly, samples were vortexed for 20 s and the bacterial suspensions transferred to a sterile 1.5-ml Eppendorf tube. Swabs were re-suspended in 0.5 ml of sterile physiological saline and vortexed again for 20 s. The supernatant was transferred in a sterile tube, obtaining approximately 1.3 ml of solution.

Two different growth media were used to quantify cultivable microorganism and bacterial loads. First, Tryptic Soy Agar (TSA, #22091; Fluka, Sigma-Aldrich, St. Louis, MO), a rich medium on which heterotrophic bacteria and fungi grow, was used to assess total cultivable microorganism load of the eggshells. Second, the same media (TSA), but with 100 mg mL^{-1} of cycloheximide, which inhibits fungal growth, was used to quantify bacterial load (TSA_{cy}; Smit et al. 2001).

To measure microbial counts, we spread 100 µL of the microbial solution on the different growth media and incubated the plates at 25 °C, for 3 days (Shawkey et al. 2003; Møller et al. 2009). After incubation, the numbers of visible colony-forming units (CFU) on each plate were counted, and the number of CFU/2 cm^2 of eggshell was calculated. We used only one dilution because preliminary tests showed that the counts were always between 0 and 150 CFU per plate (Giraudeau, unpublished data). All counts were performed by an investigator blind to the

Table 1 Results of statistical tests examining the effects of incubation and mother's preen oil (anti-preening mechanism vs. control) on eggshell bacterial and fungal loads

APM, Anti-preening mechanism

* Value is statistically significant at $P < 0.05$

Factor	Start of experiment		Growth rate	
	Bacterial load	Fungal load	Bacterial load	Fungal load
Female treatment (APM vs. control)	$F_{1,28} = 0.72$ $P = 0.4$	$F_{1,28} = 2.14$ $P = 0.15$	$F_{1,28} = 0.001$ $P = 0.97$	$F_{1,28} = 2.47$ $P = 0.12$
Egg incubation (incubated vs. non-incubated)	$F_{1,28} = 0.02$ $P = 0.9$	$F_{1,28} = 0.02$ $P = 0.9$	$F_{1,28} = 6.63$ $P = 0.01$*	$F_{1,28} = 0.05$ $P = 0.8$
Interaction Female treatment × egg incubation	$F_{1,28} = 0.05$ $P = 0.82$	$F_{1,28} = 0.03$ $P = 0.86$	$F_{1,28} = 0.01$ $P = 0.92$	$F_{1,28} = 0.09$ $P = 0.77$

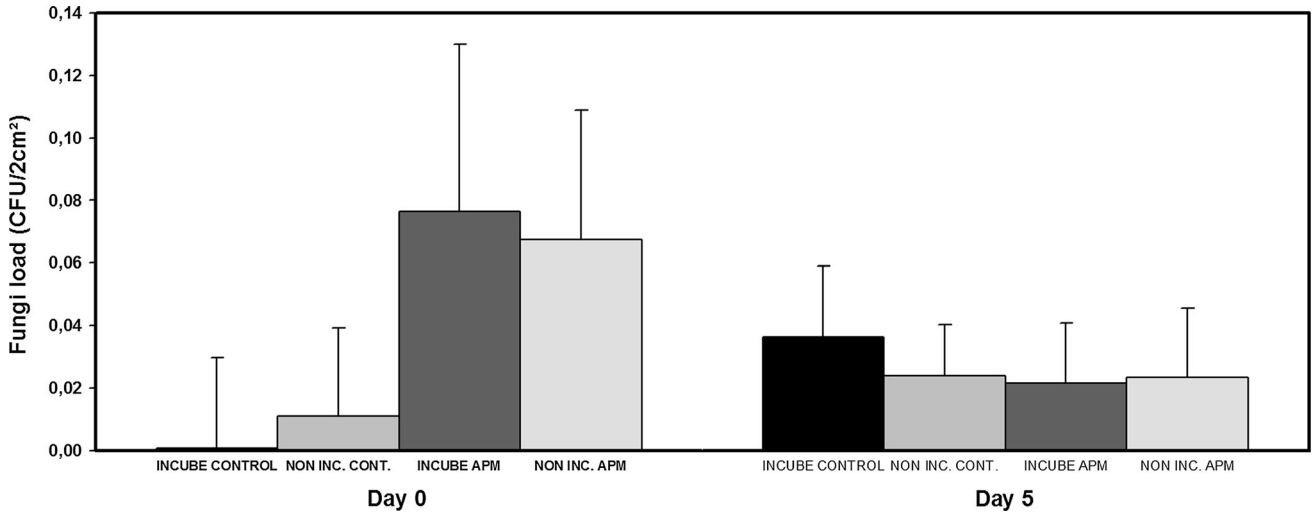

Fig. 1 Eggshell fungal load at the start (day 0; *left*) and end of the experiment (day 5; *right*) for non-incubated eggs and eggs incubated by females having free (control) or blocked access (APM) to their preen gland

treatments (incubated or not, APM or control) applied on the egg from which the samples originated. The fungal load of eggshell was estimated indirectly by calculating the difference between total microorganism counts and total bacterial counts ($TSA - TSA_{cy}$) (Møller et al. 2009). Three plates that were not inoculated with microbial solution were incubated to detect any contamination of media. We measured the bacterial growth rate by calculating the difference in bacterial load between day 5 and day 0.

Statistical analyses

Since two eggs per female were used, we used a mixed procedure (PROC MIXED) with female identity considered as a random factor. The two factors (experimental female or not; incubated egg or not) were statistically tested using a full model (single factors and their interaction) to test for the effect of maternal preen oil and incubation on eggshell microorganism loads. Statistical tests were carried out on SAS ver. 9.1 (SAS Institute, Cary, NC).

Results

In a preliminary study, we found that incubation behavior (time spent on eggs and mean temperature/day in the nest) of female Mallards (measured with temperature recorders in the nest) did not differ between females with or without access to their preen gland (Legagneux and Giraudeau, unpublished data).

At the beginning of the experiment, neither bacteria nor fungal loads differed on the shell of eggs according to female or egg status and their interaction (Table 1). After 5 days, the fungal load on eggshells was not statistically different from that at day 0, and there was no effect of female and egg treatments on fungal growth (Table 1; Fig. 1). In contrast, the bacterial load on eggshells increased in all groups, but the level of increase differed significantly according to egg treatment (Table 1; Fig. 2). The increase of bacterial load was higher on the shells of incubated eggs than on those of non-incubated eggs. Female treatment did not affect bacterial growth during the experiment.

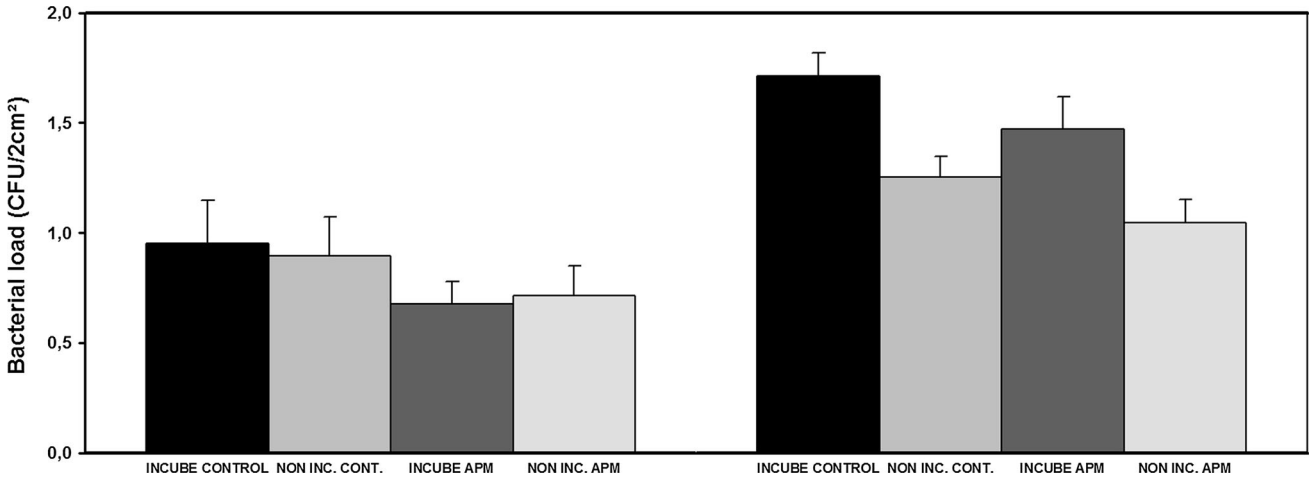

Fig. 2 Eggshell bacterial load (mean, +SE) at the start (day 0, *left*) and end of the experiment (day 5, *right*) for non-incubated eggs or eggs incubated by females having free (control) or blocked access (APM) to their preen gland

Discussion

To the best of our knowledge, this study is the first one to investigate the effect of incubation on eggshell microbial load in a waterbird species. Wang et al. (2011) found that incubation did not affect bacterial growth on the eggshell of three species of passerines in northern California. Cook et al. (2005a, b) found that incubation decreased bacterial growth on the eggshell of the Wild Pearly-eyed Thrasher. In contrast to these published studies on passerines (Cook et al. 2005a, b; Ruiz-De-Castaneda et al. 2011; Wang et al. 2011), we found a greater increase in bacterial abundance on incubated eggs compared to non-incubated eggs. This discrepancy between our experimental results and those of previous studies could be explained by at least three non-exclusive hypotheses. First, many bacterial strains grow well in warm and damp conditions, but our experiment was performed in the dry and temperate climate of central France where during the 3 months of the experiment the maximum and minimum temperature was 15 °C and −5 °C, respectively, the average temperature was 4 °C and total rainfall was 221 mm. Thus, non-incubated eggs may have experienced poor conditions for bacterial growth, which was not the case during the experiment of Cook et al. (2005a, b), which was performed in the tropical climate of Puerto Rico. The results obtained by Wang et al. (2011), who reported that eggshell microbial loads did not increase with exposure to ambient conditions in the temperate climate of northern California, provides support for this hypothesis.

Second, incubating females may contaminate their eggs with numerous bacterial strains due to contact between their non-sterile plumage, brood patch (Burtt and Ichida 1999) and eggshell. Mallards are ground birds that live on in mudflats known to present high microbial densities (Burtt and Ichida 1999; Giraudeau et al. 2010c). Thus, it is highly probable that the transfer of bacterial cells from the female to eggs leads to a higher contamination in Mallards, which do show a higher abundance of bacteria on their plumage (Giraudeau et al. 2010c) than the passerine aerial species studied in previous studies.

Third, incubation has been shown to shift microbial communities from more pathogenic to less pathogenic members (Cook et al. 2005b). Females may increase eggshell bacterial load with benign microbes during incubation since Gram-positive (less pathogenic) microbes dominate avian feathers. Thus, while in our experiment microbial loads were higher on incubated eggs than on non-incubated, it does seem possible that the microbes growing on the incubated eggs may have differed from those that grew on non-incubated eggs. Future studies should examine how the bacterial community is affected during incubation in Mallards.

In this study, we also tested for the first time the antimicrobial activity of mother's preen oil on eggshell microorganisms by comparing bacterial growth on the shell of eggs incubated by female Mallards with or without access to their preen gland; no effect of female treatment on eggshell bacteria and fungal growth was found. Future studies should examine if the preen oil of the female Mallard has antibacterial properties against some specific bacterial species or whether it has the ability to change or control the eggshell bacterial community. However, if the preen oil does have such inhibitory effects on specific harmful bacterial strains, this should be coupled with the enhancement of the growth of other harmless bacteria strains, and we did not find any effect of our treatment on total bacterial growth. Shawkey et al. (2003) found in vitro evidence that the preen oil of the House Finch (*Carpodacus mexicanus*) inhibits the growth of several strongly feather-

degrading bacterial strains but enhances the growth of one weakly feather-degrading isolate. These results on plumage bacteria suggest that birds may defend themselves against some feather-degrading bacteria using uropygial oil. It could be interesting to test if preen oil could have similar effects on eggshell bacteria, especially on pathogens. An alternative hypothesis would be that preen oil might form a physical barrier to the passage of microbes from the shell surface into the egg and the embryo (Reneerkens et al. 2008). Such a barrier would not affect the abundance of the surface microbiota, but would protect the embryo from eggshell pathogenic bacteria. A detailed examination of the role of preen oil on the eggshell bacteria community using newly available molecular techniques (Bisson et al. 2007, 2009) would be particularly interesting.

In conclusion, the way by which avian incubation may limit—or not—microbial infection remains unclear. However, our study provides the first information on the interaction between maternal preen oil and eggshell microbial growth. In addition, we showed that the bacterial load increased significantly during egg incubation in Mallards. A fruitful direction for future research would be to understand how avian species that do not inhibit microbial growth during incubation use other mechanisms to prevent infection, such as thicker eggshells, more cuticle on the shell and/or higher concentrations of antimicrobial compounds in the shell or in the albumen. Shawkey et al. (2008) found, for example, that the Blue-winged Teal (*Anas discors*), a waterfowl species, has higher concentrations of antimicrobial proteins in the egg albumen relative to several species of passerines.

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