Factors Influencing Microbial Growth and Viability of Wood Duck Eggs

by

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Abstract

Egg viability in birds declines with increasing length of incubation delay and may be influenced by microbial infection and exposure of eggs to temperatures above physiological zero (>24°C). Reuse of nests is common in cavity-nesting species and may lead to increased microbial levels within the nest. Onset of incubation during egg laying may help to maintain viability of first-laid eggs. We manipulated incubation delay of Wood Duck (*Aix sponsa*) eggs and tested the effects of incubation delay and ambient temperature, as well as the effects of nest reuse and onset of night incubation, on microbial growth and egg viability. Hatching success declined slowly with increasing length of incubation delay, but was not affected by increasing exposure to temperatures >24°C or microbial growth. We found increased levels of heterotrophic bacteria in uncleaned nests, and gram-negative bacteria decreased following onset of night incubation. We suggest early onset of incubation in precocial birds may be more important in reducing incubation period and predation risk than in maintaining viability of first-laid eggs, and that relatively low levels of bacterial infection of eggs at our temperate study site, together with increased antimicrobial properties of Wood Duck eggs and incubation by females before clutches were complete contributed to the negligible effect that bacteria had on egg viability.

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EFFECTS OF INCUBATION DELAY ON VIABILITY AND MICROBIAL GROWTH OF WOOD DUCK EGGS

ABSTRACT.—Egg viability in birds declines with increasing length of incubation delay and may be influenced by microbial infection and exposure of eggs to temperatures above physiological zero (>24°C). Onset of incubation during egg laying results in developmental asynchrony of embryos, but may help to maintain viability of first-laid eggs. We manipulated incubation delay of Wood Duck (*Aix sponsa*) eggs and tested the effects of incubation delay and ambient temperature on egg viability and microbial infection. We also examined onset of incubation by egg-laying females. Hatching success declined slowly with increasing length of incubation delay, but was not affected by increasing exposure to temperatures >24°C. The probability of hatching for an egg delayed 7 days was reduced by 8.6% (95% CI: 4.3 – 15.2). Microbial infection of eggshells was not related to length of incubation delay or mean daily temperature of the exposure period. Egg-laying females began incubating at night approximately 7 days after nest initiation, and 3 days before starting full incubation. Number of days between nest initiation and the start of night incubation declined as clutch size declined. However, number of nights that egg-laying females incubated before starting full incubation did not vary with clutch size. We show that egg viability did not decline with increasing exposure to temperatures >24°C and was weakly affected by incubation delays ≤7 days. We suggest early onset of incubation in precocial birds may be more important in reducing incubation period and predation risk than in maintaining viability of first-laid eggs.

Key Words: *Aix sponsa*, ambient temperature, egg viability, microbial ecology, onset of incubation, Wood Duck.

EGG VIABILITY IN many species of birds declines with increasing length of incubation delay (Arnold et al. 1987, Meijerhof 1992, Stoleson 1999). Studies of both wild and captive birds have shown viability of unincubated eggs begins to decline in as little as 3 days (Veiga 1992, Stoleson and Beissinger 1999). Loss of egg viability is slower in waterfowl and declines when eggs are 5–10 days old (Arnold 1993). Ambient temperature and microbial infection are two important factors that may act independently during incubation delay to affect egg viability (Cook et al. 2005a).

Temperature generally is considered to be the most important factor affecting egg viability (Webb 1987, Stoleson 1999). Optimal incubation temperature for most avian species falls between 36°C and 38°C, but embryo development begins when egg temperature exceeds physiological zero (24°C–27°C; Webb 1987). Prolonged exposure of unincubated eggs to temperatures above physiological zero (>24°C) can result in developmental anomalies and reduced hatchability (Webb 1987, Ewert 1992, Meijerhof 1992). Egg viability of several waterfowl species declined more rapidly with higher ambient temperatures (Arnold 1993).

Microbes grow rapidly on shells of unincubated eggs (Cook et al. 2005b, Shawkey et al. 2008). Trans-shell microbial infection of the embryo can occur in as little as 3 days and greatly reduces egg viability (Cook et al. 2003, 2005a). However, eggs have physical and chemical barriers that help inhibit microbial infection (Board 1966, Board and Fuller 1974). The eggshell cuticle provides a physical defense against microbial contamination by covering eggshell pores and limiting pathways for

microbes to enter the egg (Board and Board 1967). The cuticle also is comprised of antimicrobial proteins that may aid in microbial defense (Wellman-Labadie et al. 2008a). Egg albumen provides not only water and nutrients to the developing embryo, but contains proteins that help to prevent growth of microorganisms (Burley and Vadehra 1989). Lysozyme and ovotransferrin, two proteins typically found in albumen and known for their antimicrobial properties, also have been found within the eggshell matrix of the domestic chicken and some cavity-nesting species, including the Wood Duck (*Aix sponsa*; Wellman-Labadie et al. 2008a, b). These albumen proteins are more effective at impeding microbial infection at temperatures experienced during incubation (Tranter and Board 1984, Wellman-Labadie et al. 2008b).

Starting incubation before clutches are complete may maintain viability of first-laid eggs by initiating embryo development and raising the temperature of eggs to levels that increase the effectiveness of antimicrobial proteins (Hussell 1985, Board and Tranter 1986). Many birds initiate incubation during egg laying, and in passerine species this results in asynchronous egg development and hatching (Stoleson 1999, Hébert 2002). Waterfowl generally start incubation 1–4 days prior to clutch completion (Cargill and Cooke 1981, Loos and Rowher 2004). Early initiation of incubation in waterfowl results in age hierarchies averaging 2–3 days between developing embryos, but young hatch synchronously (Caldwell and Cornwell 1975, Cargill and Cooke 1981, Kennamer et al. 1990).

The Wood Duck is a relatively small-bodied species that nests in natural tree cavities and artificial nest boxes (Bellrose and Holm 1994). Wood Ducks lay one egg per day, and clutch size averages 10–12 eggs (Drobney 1980, Hepp and Bellrose 1995). Wood Ducks are monogamous, and females are solely responsible for incubation and brood rearing (Hepp and Bellrose 1995). Incubation period is 31–32 days (Manlove and Hepp 2000, Hepp et al. 2006), and females begin incubating at night before the clutch is complete (Wilson and Verbeek 1995, Hepp 2004). Full incubation begins after laying of the penultimate egg (Kennamer et al. 1990, Wilson and Verbeek 1995).

In this study of Wood Ducks, we randomly exposed freshly laid eggs to incubation delays of 0, 1, 3, 5, 9, and 13 days and tested the effects of length of incubation delay and exposure to temperatures $>24°C$ on egg viability. We predicted that viability of unincubated eggs would decline with increasing length of incubation delay and increasing exposure to temperatures $>24°C$. If females start incubation during egg-laying to help maintain viability of first laid eggs, we predicted a close relationship between when eggs begin to lose viability and the number of days between nest initiation and the start of incubation by egg-laying females. We also sampled eggshell microbes and examined changes in the incidence of microbial infection with lengthening of incubation delay and changes in ambient temperature.

Methods

Study site.—We conducted this study on the U. S. Department of Energy's Savannah River Site (SRS) in the upper coastal plain of west-central South Carolina

(33·1° N, 81·3° W). SRS encompasses approximately 80,289 ha and borders the Savannah River for 43.5 km. Nest boxes (n = 112) were maintained at Par Pond (~1,000 ha) and L Lake (~400 ha).

Nest checks and onset of incubation.—Nest boxes were checked every four days for nesting activity during the breeding season. Eggs in new nests were marked with a non-toxic waterproof marker (Sharpie®, Sanford). In 2008 and 2010, temperature data-loggers (HOBO® Stowaway XTI and Pro V2, Onset Computer Corp.) were added to nests the day after they were found. Protocol similar to Manlove and Hepp (1998) was used for data-logger installation. We first removed all nesting material and installed a platform containing a wooden egg with an embedded thermistor. The wooden egg was attached to the platform with a deck screw (7.62 cm) to prevent females from moving it. The thermistor tip was exposed on top of the wooden egg to ensure contact with the brood patch of the incubating female. The thermistor was connected to the data-logger by a cable (184 cm). Nesting material was returned to the box after installation and the wooden egg was positioned in the center of the nest. Data-loggers were secured to the underside of the box or buried in nesting material. Data-loggers recorded nest temperature every 6 min, and temperature data were downloaded and plotted for each 24 h period. Examining data from graphs and spreadsheets provides a very accurate method of determining when incubating females were on and off the nest (Manlove and Hepp 2000). Night incubation began on the first day that females spent the night on the nest (Hepp 2004).

For purposes of analysis only data from non-parasitized nests were examined. Identification of non-parasitized nests was based on at least one of the following criteria: (1) egg deposition rate did not exceed one egg per day, (2) viable non-term eggs were not present at hatching, and (3) clutch size was < 16 eggs (Hepp and Kennamer 1993).

Egg collection and incubation.—In 2009, new nests were visited daily and freshly-laid eggs (i.e. unmarked eggs) were removed on six consecutive days. Individual eggs were placed into labeled sterile bags (Whirl-Pak®, NASCO) and taken to the Savannah River Ecology Laboratory (SREL) in a padded case (model 1600, Pelican Products Inc.) cooled with an ice pack. Sterile techniques were used whenever handling eggs. Fresh eggs were replaced with wooden eggs to prevent nest abandonment by females. Wooden eggs were cleaned with 70% EtOH before being placed in nests.

Eggs were removed from bags at SREL in a sterile laminar-flow hood and labeled on the blunt end with a non-toxic waterproof marker. We randomly assigned fresh eggs collected from nests on each of six consecutive days to one of six incubation delays of 0, 1, 3, 5, 9, and 13 days. Eggs being delayed were placed in newly-constructed cypress nest boxes (30x38x61 cm) and held in ambient conditions. To help standardize the holding environment, nest boxes were disinfected with a weak bleach solution, and autoclaved wood chips were placed in the bottom of nest boxes. Eggs were placed on chips so that they were not touching and were turned 180° twice daily during the exposure period (Mayes and Takeballi 1984). Temperature data-loggers

(HOBO® Pro V2) were placed in each nest box and recorded ambient temperature every 6 min. An onsite weather station recorded relative humidity levels every 30 min (HOBO® U30).

Eggs were removed from nest boxes at the end of exposure periods and artificially incubated (Grumbach model BSS420, Lyon Technologies Inc.) at 37.5°C and 55-65% relative humidity. Control eggs (0 day) were placed in the incubator on the day they were collected. Eggs were turned hourly, and we programmed two cool down periods (60 min each) each day to simulate natural incubation behavior of Wood Ducks (Manlove and Hepp 2000). We candled eggs weekly during incubation to assess egg viability and stage of development (Hanson 1954). Dead eggs were removed from the incubator and opened to determine age of dead embryos. In the last week of incubation, we checked eggs daily for pipping. Pipped eggs were removed from the incubator and placed in a brooder at 37.5°C and 90% relative humidity until ducklings hatched.

Eggshell microbes.–Eggshells were swabbed on two occasions to obtain samples of shell microbiota. Approximately 20% of the shell was swabbed on the day eggs were collected (pre-delay) and again at the end of incubation delay (post-delay). Swabs were placed in 3 ml of sterile physiological saline. After taking pre-delay swabs, we randomly selected half the eggs in each delay group and cleaned them daily with 70% EtOH to kill microbes on the shell surface. Eggshells were cleaned so we could

separate effects of incubation delay, exposure to temperatures >24°C, and microbial infection on egg viability.

We cultured microbes by plating two 0.1 ml replicates of saline supernatant on each of two growth media: MacConkey agar (MAC) and tryptic soy agar (TSA). Media were selected to detect the most common groups of bacteria known to colonize bird eggshells (Cook et al. 2003): MAC will grow Gram-negative enteric bacteria, and TSA will grow heterotrophic bacteria. Fungi will grow on both types of media. Cultures were incubated (Percival model 1-37LLVL, Percival Scientific Inc.) aerobically: MAC was incubated at 35°C, and TSA was incubated at 23°C. Microbial colony forming units (CFUs; CFUs/0.1 ml) were counted following 48 h of incubation (12 h:12 h L:D photoperiod). The culturing procedure was randomly repeated without swabbing eggs to test for contamination. One batch of TSA and MAC showed contamination (>0 colonies) of control plates. We eliminated all eggs associated with the contaminated batches which reduced our sample of eggs from 277 to 117 for both TSA and MAC.

Data analysis.—Spearman's rank correlation (PROC CORR; SAS Institute Inc. 2004) was used to examine the relationship between clutch size and nest initiation date. We also examined the relationship between number of nights egg-laying females incubated nests and nest initiation date using Spearman's rank correlation. We examined the relationship between clutch size and number of days until start of incubation by egg-laying females using linear regression (PROC GLM). We

standardized nest initiation date by subtracting date of first nest each year from nest initiation date of each nest.

We tested for differences in collection date of eggs, loss of egg mass, average daily temperature of nest boxes used to hold eggs, and number of hours eggs were exposed to temperatures >24°C by length of incubation delay using one-way analyses of variance (PROC GLM). We also compared hours eggs were exposed to temperatures >24°C and average daily temperature of nest boxes used to hold eggs by cleaning treatment. We used Tukey's test for means separation.

We developed a suite of *a priori* logistic regression (PROC LOGISTIC) models to evaluate the relationship between egg viability (yes/no) and the explanatory variables of length of incubation delay (DELAY), exposure to temperatures >24°C (TEMP), egg collection date (DATE), and cleaning of eggshells (CLEAN). We treated DELAY, TEMP, and DATE as linear variables, and CLEAN as a class variable (yes/no). In preliminary analyses of egg viability, we included DELAY either as a linear, quadratic or categorical variable. Quadratic and categorical terms did not improve model fit, so we chose to treat DELAY linearly.

Levels of microbial infection (CFUs) were low and variable across all six delay treatments for both pre-delay and post-delay cultures of MAC and TSA; therefore, we chose to treat microbial infection as a binary variable (present/absent) and used logistic regression for analyses (Appendices 1.1, 1.2). Differences in sample sizes caused by the TSA contamination prohibited us from including microbial infection data in our

overall models of egg viability; therefore, we created a separate suite of *a priori* models to examine the effects of DELAY, mean daily temperature of nest box used to hold eggs (HBMEAN), and CLEAN on eggshell microbes following incubation delay. We treated DELAY and HBMEAN as linear variables, and CLEAN as a class variable (yes/no). In preliminary analyses of microbial infection, we included DELAY either as a linear, quadratic or categorical variable. Quadratic and categorical terms did not improve model fit, so we chose to treat DELAY linearly.

We compared models using Akaike's information criterion for small sample size (AIC_c; Burnham and Anderson 2002). We present and rank models ≤ 4 AIC_c units of the best ranking model (ΔAIC_c). Only the top model or models structurally simpler than the top model within this model set were used to draw inference. Akaike weights (w_i) are the relative likelihood of the models given the data. Parameter likelihoods are made by summing Akaike weights across all models that include the variable and are used to judge the relative importance of explanatory variables. Parameters with good support will have values close to 1. We calculated parameter estimates using model-averaging based on AIC_c model weights for all candidate models (Burnham and Anderson 2002). Means and parameter estimates are presented $\pm$ SE.

Results

Onset of incubation.—Date of nest initiation ($n = 25$) was 25 April $\pm$ 5 days (range: 3 March – 5 June), and data loggers were placed in nests 4.1 $\pm$ 0.3 days after nest initiation. Females began incubating at night 7.1 $\pm$ 0.3 days after nest initiation

and 2.9 ± 0.2 days before clutches were completed and full incubation began. Number of days between nest initiation and the beginning of night incubation increased with clutch size (r^2=0.27, β=0.49 ± 0.17, P=0.008, Fig. 1.1). Clutch size decreased as the breeding season progressed (r^2=-0.25, P=0.01); however, there was no relationship between clutch size and number of nights egg-laying females incubated nests (r^2=0.03, P=0.41).

Egg viability.—We exposed 277 eggs from 54 different nests to incubation delays of 0, 1, 3, 5, 9, and 13 days (Table 1.1). Average date of nest initiation was 3 April ± 1 day (range: 12 March – 11 May), and collection date of eggs did not differ by length of incubation delay (F=0.16, df=5, 271, P=0.98; Table 1.1). Loss of egg mass increased with longer incubation delays (F=25.53, df=4, 229, P<0.0001) and was greatest for eggs that were delayed 13 days (1.95%; Table 1.1). Daily temperature of nest boxes used to hold eggs did not differ by length of incubation delay (F=0.54, df=4, 243, P=0.71) and averaged 17.9 ± 0.3°C (Table 1.1). Number of hours eggs were exposed to temperatures above physiological zero (>24°C) increased with length of incubation delay (F=14.18, df=4, 243, P<0.0001; Table 1.1). Overall, 18.4% (51 of 277) of eggs failed to hatch. Embryo mortality was bimodal and was greatest in the first and last weeks of incubation (Fig. 1.2).

We considered two candidate models to make inferences regarding variation in the viability of Wood Duck eggs (Table 1.2). The top model (w_i=0.16) included DELAY and TEMP, and the second model (w_i=0.13) included DELAY. Parameter

likelihood values indicated DELAY (0.95) had greater relative importance than TEMP (0.60). Hatching success declined with increasing incubation delay ($\beta = -0.12 \pm 0.04$; Fig. 1.3), and the odds of an egg hatching decreased by 11% (95% confidence interval [CI]: 3.5 – 18.5) for every additional day of incubation delay. The 95% CI of the model-averaged parameter estimate of TEMP ($\beta = -0.006 \pm 0.004$) included zero.

Microbial growth.—We examined shells of 117 eggs from 41 different nests for microbial infection. Because of low colonization of MAC by gram-negative bacteria [pre-delay (5%: 6 of 117) and post-delay (1%: 1 of 117); Appendix 1.2], we restricted our analyses of microbial infection to TSA. Microbial infection occurred on 43% (50 of 117) of eggs before delaying incubation and cleaning (Table 1.3). Relative humidity at the site averaged 73.9 ± 0.4%. As expected, the percentage of eggs with microbial infection declined for cleaned eggs following incubation delays, but surprisingly, for eggs not cleaned with EtOH, only eggs delayed 13 days showed an increase in microbial infection (Fig. 1.4).

We considered two candidate models to make inferences regarding microbial infection of Wood Duck eggs following incubation delay (Table 1.4). The top model ($w_i = 0.21$) included DELAY and CLEAN, and the second candidate model ($w_i = 0.11$) included CLEAN. Parameter likelihood values indicated CLEAN (0.53) had a greater relative importance than DELAY (0.42). However, the model-averaged parameter estimates for CLEAN ($\beta = 0.32 \pm 0.32$) and DELAY ($\beta = 0.04 \pm 0.04$) both included zero in the 95% CI.

Discussion

Female Wood Ducks began incubating eggs at night an average of 7 days after nest initiation. These results are similar to patterns reported by Wilson and Verbeek (1995) and Hepp (2004) where onset of night incubation in Wood Ducks occurred after laying 7 eggs. Studies of other species of waterfowl have shown that females increasingly spent time on the nest during the day as laying progressed (Afton and Paulus 1992, Loos and Rohwer 2004). Onset of incubation by Mallards (*Anas platyrhynchos*) began as early as after laying the sixth egg in a clutch of 10–12 eggs, but night incubation began only after the last egg was laid (Caldwell and Cornwell 1975). Nest attentiveness by American Coots (*Fulica americana*) gradually increased as laying progressed from the first to the sixth egg (Arnold 2011). Flint et al. (1994) reported Black Brant (*Branta bernicla nigricans*) incubated first-laid eggs as much as 48 h before clutch completion.

Early onset of incubation has been reported for many species of birds and has been suggested as a way to maintain viability of first-laid eggs (Stoleson and Beissinger 1995, Beissenger et al. 2005). Viability of unincubated eggs has been shown to decline in several species of birds with increasing ambient temperatures and number of days eggs are exposed before incubation begins (Arnold et al. 1987, Arnold 1993, Stoleson and Beissinger 1999); however, Hepp (2004) did not find evidence to support the egg viability hypothesis in Wood Ducks. Several studies have found that early onset of incubation can protect nests from both conspecific and heterospecific brood parasitism

(Neudorf and Sealy 1994, Stoleson and Beissinger 1995, Clotfelter and Yasukawa 1999). However, Hepp (2004) found no evidence that night incubation by Wood Ducks reduced conspecific brood parasitism. Early incubation also has been suggested to produce shortened incubation periods (Flint et al. 1994, Persson and Anderson 1999). Hepp (2004) found some indication that more time spent incubating by egg-laying Wood Ducks resulted in shortened incubation periods.

If early incubation is used to maintain viability of first-laid eggs, we predicted that onset of incubation by egg-laying females should be closely related to the timing of loss of egg viability. We found that egg viability decreased slowly with increasing incubation delay. Females, on average, began night incubation 7 days after nest initiation, but egg viability had only declined 8.6% (95% CI: 4.3 – 15.2) by day 7. Further, there was no evidence that increased exposure to temperatures $>24°C$ reduced hatchability of Wood Duck eggs. Clutch size declined as the breeding season progressed, and length of incubation delay decreased with clutch size. If early onset of incubation is used to maintain viability of first-laid eggs, and exposure to high ambient temperatures does not reduce viability, then we would expect onset of night incubation to be fairly consistent among females and not vary seasonally or with clutch size. We found little support that loss of egg viability explains early onset of incubation in Wood Ducks.

Because early onset of incubation in precocial birds results in asynchronous development of embryos, late-laid eggs must accelerate development to achieve

synchronous hatching (Davies and Cooke 1983, MacCluskie et al. 1997, Persson and Andersson 1999, Boonstra et al. 2010). There is a limit to the amount of asynchrony that late-laid eggs can overcome. In Lesser Snow Geese (*Chen caerulescens caerulescens*) for example, eggs delayed more than 4 days did not hatch (Davies and Cooke 1983), and Wood Duck clutches with more than 3 days of developmental asynchrony experienced reduced hatching success (Kennamer et al. 1990). Similar to Hepp (2004), we found that incubating females spent approximately 3 nights incubating eggs before beginning full incubation and this did not vary with clutch size. Accelerated development is achieved through increased metabolism and use of egg lipids (MacCluskie et al. 1997, Boonstra et al. 2010). Therefore, there may be costs associated with accelerated development and increased metabolic rates. Embryos from late-laid eggs that accelerate development may use more nutrients and exhibit reduced maturity at hatching which may affect post-hatching survival (Ricklefs and Starck 1998, Hepp et al. 2006).

Early incubation also may help to reduce microbial growth on eggs. Several studies have shown rapid increases of eggshell microbes within the time needed to complete clutches, but incubation of eggs reduced levels of pathogenic bacteria (Shawkey et al. 2009, Cook et al. 2005b). Two separate studies found that unincubated eggs exposed to moisture had higher levels of microbial growth (Godard et al. 2007, D'Alba et al. 2010). In contrast, our results did not show increases in microbial growth on eggshells until day 13 of exposure. Antimicrobial attributes of Wood Duck eggs

seem to work well to protect unincubated eggs from increased microbial growth during the delay period. Antimicrobial proteins (lysozyme and ovotransferrin) of the eggshell cuticle and albumen have been found in higher concentrations in eggs of the Wood Duck than in other species (Wellman-Labadie et al. 2008b). These proteins are known to prevent the growth of bacteria, and are more effective at impeding microbial infection at temperatures experienced during incubation (Wellman-Labadie et al. 2008a).

In this study we found little evidence that early onset of incubation is important for maintaining viability of first-laid eggs. We also found colonization of eggshells by bacteria was not related to increased exposure of eggs and found no evidence to support the negative effects of bacteria on egg viability. In birds, predation risk is a major factor influencing reproductive success and shortening the incubation period would decrease predation risk (Martin 1995). For Wood Ducks and other precocial species of birds, shortening the incubation period may be the most important factor influencing early onset of incubation (Hepp 2004).

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TABLE 1.1. Summary statistics of Wood Duck eggs by incubation delay. Means (±SE) within rows followed by different letters are significantly different (P<0.05).

	Incubation delay (days)					
	0	1	3	5	9	13
n	29	52	48	54	45	49
Hatching success (%)	82.8	90.4	87.5	88.9	73.3	65.3
Collection date	6 April ± 4	7 April ± 3	8 April ± 3	7 April ± 3	10 April ± 3	9 April ± 3
Mass loss (%)	–	0.15 ± 0.02 a	0.37 ± 0.02 ab	0.78 ± 0.05 bc	0.98 ± 0.04 c	1.95 ± 0.29 d
Nest box temperature (°C)	–	17.6 ± 0.7	17.3 ± 0.7	18.0 ± 0.4	18.3 ± 0.5	18.3 ± 0.5
Exposure to temperatures >24°C (hr)	–	3.4 ± 0.7 a (0 – 11.7)[a]	11.4 ± 2.4 b (0 – 41.4)	12.6 ± 2.4 bc (0 – 45.4)	28.5 ± 5.1 cd (0 – 77.3)	43.5 ± 7.6 d (0 – 129.7)

[a] Range (min – max)

TABLE 1.2. Top ranked models (ΔAIC_c<4.0) used to evaluate the influence of incubation delay and exposure of eggs to ambient temperatures >24°C on viability of Wood Duck eggs on the Savannah River Site, SC. Only the top model (ΔAIC_c=0) or models structurally simpler than the top model were considered for interpretation (in bold).

Model[a]	K[b]	AIC_c[c]	ΔAIC_c[d]	w_i[e]
Intercept	1	239.90	13.01	0.00
DELAY + TEMP	**3**	**226.89**	**0.00**	**0.16**
DELAY	**2**	**227.21**	**0.32**	**0.13**
DELAY + TEMP + DATE	4	227.95	1.06	0.09
DELAY + TEMP + CLEAN + DELAY*CLEAN	5	228.03	1.14	0.09
DELAY + CLEAN + DELAY*CLEAN	4	228.08	1.19	0.09
DELAY + TEMP + CLEAN	4	228.75	1.86	0.06
DELAY + DATE	3	228.99	2.10	0.06
DELAY + CLEAN	3	229.01	2.12	0.05
DELAY + TEMP + CLEAN + DATE + DELAY*CLEAN	6	229.22	2.33	0.05
DELAY + TEMP + CLEAN + DATE	5	229.88	2.99	0.04
DELAY + CLEAN + DATE + DELAY*CLEAN	5	229.91	3.02	0.03
DELAY + TEMP + CLEAN + TEMP*CLEAN	5	230.17	3.28	0.03
DELAY + CLEAN + DATE	4	230.79	3.90	0.02
TEMP + DATE	3	230.89	4.00	0.02

[a] DELAY = length of incubation delay (1, 3, 5, 9, or 13 days); TEMP = hours of exposure to ambient temperatures >24°C; DATE = egg collection date; CLEAN = cleaned (yes/no)
[b] Number of parameters in each model.
[c] Akaike's information criterion for small sample size.
[d] Difference between each model and the best-fitting model.
[e] Akaike weights.

TABLE 1.3. Percentage of Wood Duck eggshells infected with microbes before and after incubation delay. Eggshells were either cleaned or not cleaned daily with 70% EtOH. Cultures were grown using tryptic soy agar (TSA). Nest box temperature (mean ±SE) followed by different letters are significantly different (P<0.05).

Cleaning treatment		Incubation delay (days)				
		1	3	5	9	13
Clean	*n*	9	19	16	10	6
	Nest box temperature (°C)	22.5 ± 0.7 a	18.2 ± 1.2 b	19.0 ± 0.8 b	20.6 ± 0.8 ab	17.6 ± 1.0 b
	Microbial infection (%)					
	Pre-delay[a]	55.6	26.3	37.5	40.0	66.7
	Post-delay[b]	22.2	5.3	12.5	20.0	33.3
Not Clean	*n*	13	12	10	13	9
	Nest box temperature (°C)	20.9 ± 1.3 a	17.5 ± 1.5 b	20.9 ± 0.7 a	20.4 ± 0.7 ab	18.8 ± 1.0 ab
	Microbial infection (%)					
	Pre-delay	30.8	66.7	40.0	46.2	44.4
	Post-delay	23.1	33.3	40.0	38.5	55.6

[a] Eggshells were swabbed on the day they were collected.
[b] Eggshells were swabbed after incubation delay.

TABLE 1.4. Top ranked models (ΔAIC_c<4.0) used to evaluate the influence of incubation delay, daily cleaning, and exposure of eggs to increasing ambient temperatures on microbial infection of Wood Duck eggshells on the Savannah River Site, SC. Only the top model (ΔAIC_c=0) or models structurally simpler than the top model were considered for interpretation (in bold).

Model[a]	K^b	AIC_c[c]	ΔAIC_c[d]	w_i[e]
Intercept	1	135.24	6.76	0.01
DELAY + CLEAN	**3**	**128.48**	**0.00**	**0.21**
CLEAN	**2**	**129.86**	**1.37**	**0.11**
DELAY + CLEAN + HBMEAN	4	130.58	2.10	0.07
DELAY + CLEAN + DELAY*CLEAN	4	130.61	2.13	0.07
CLEAN + HBMEAN	3	131.85	3.37	0.04

[a] DELAY = length of incubation delay (1, 3, 5, 9, or 13 days); HBMEAN = mean temperature of nest box used to hold eggs; CLEAN = cleaning treatment (yes/no)
[b] Number of parameters in each model.
[c] Akaike's information criterion for small sample size.
[d] Difference between each model and the best-fitting model.
[e] Akaike weights.

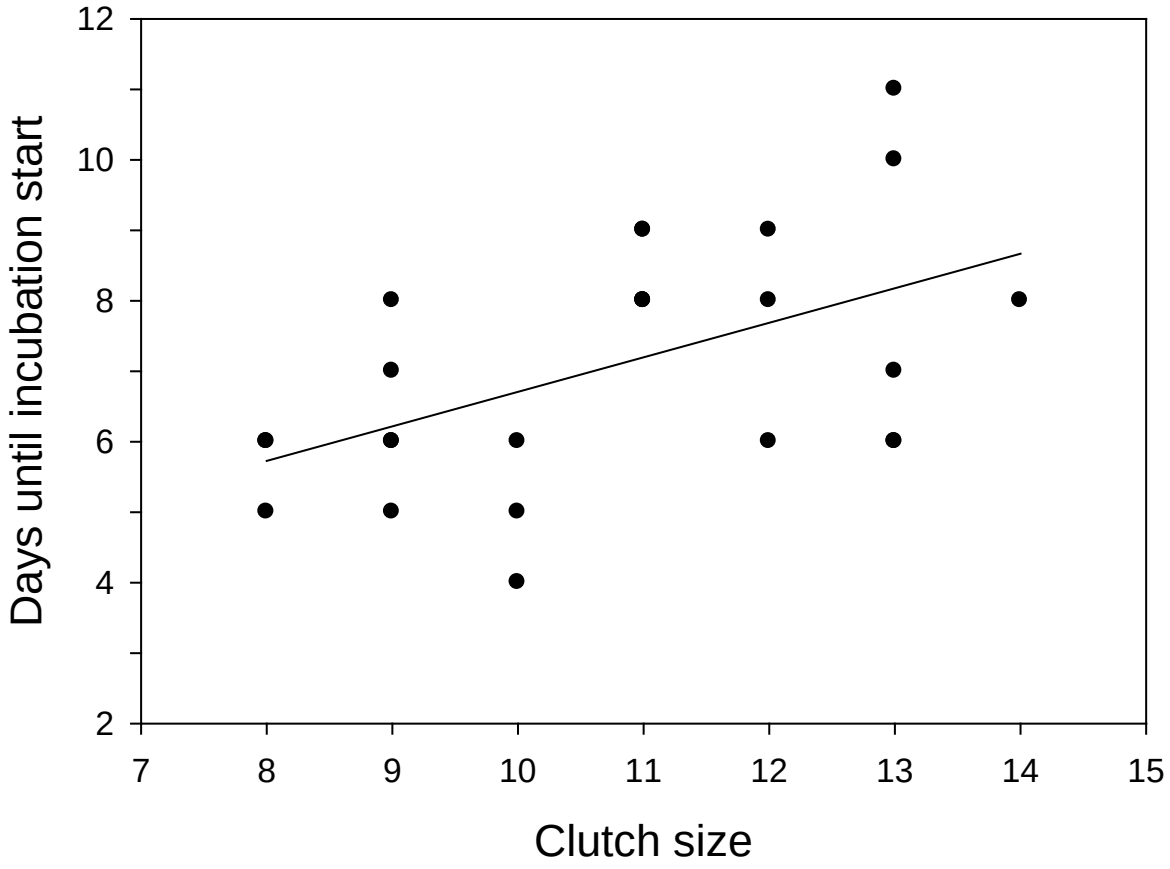

FIG. 1.1. Relationship between clutch size and the number of days between nest initiation and the start of nocturnal incubation by Wood Ducks (*n*=25) on the Savannah River Site, SC.

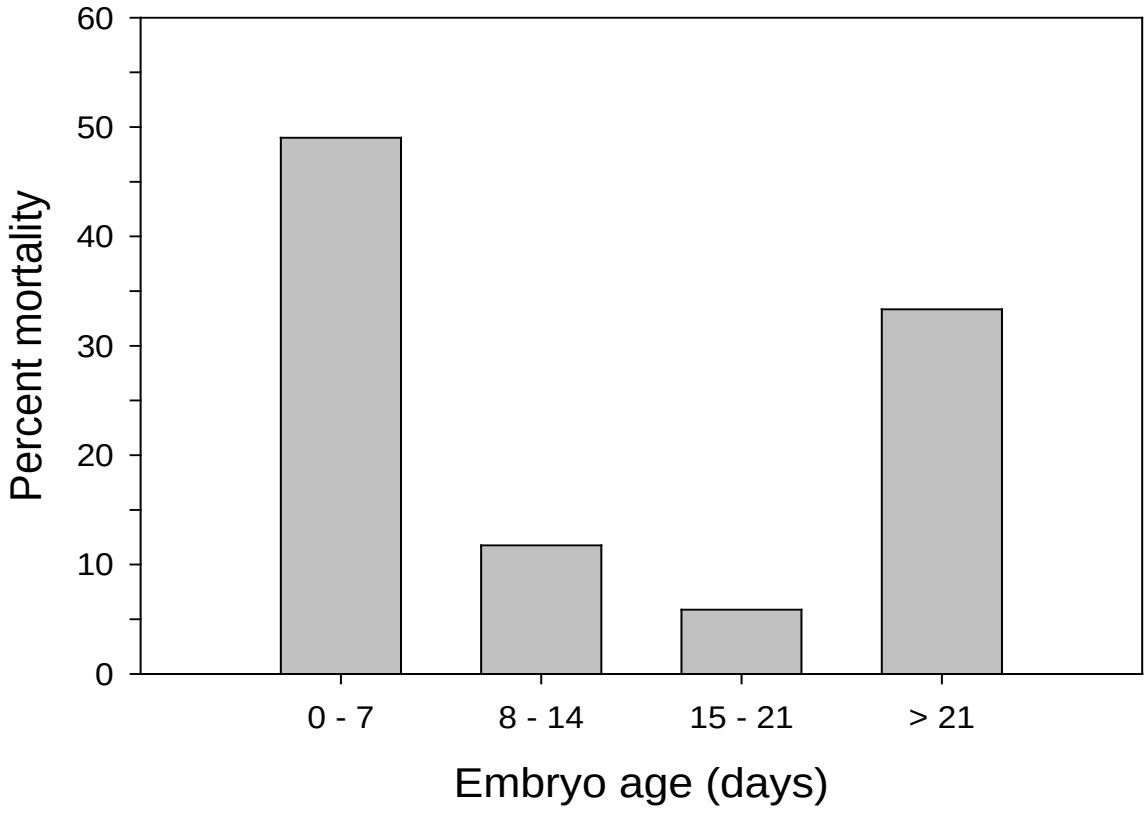

FIG. 1.2. Percent mortality of artificially-incubated Wood Duck eggs (*n*=51) in relation to age that embryos died.

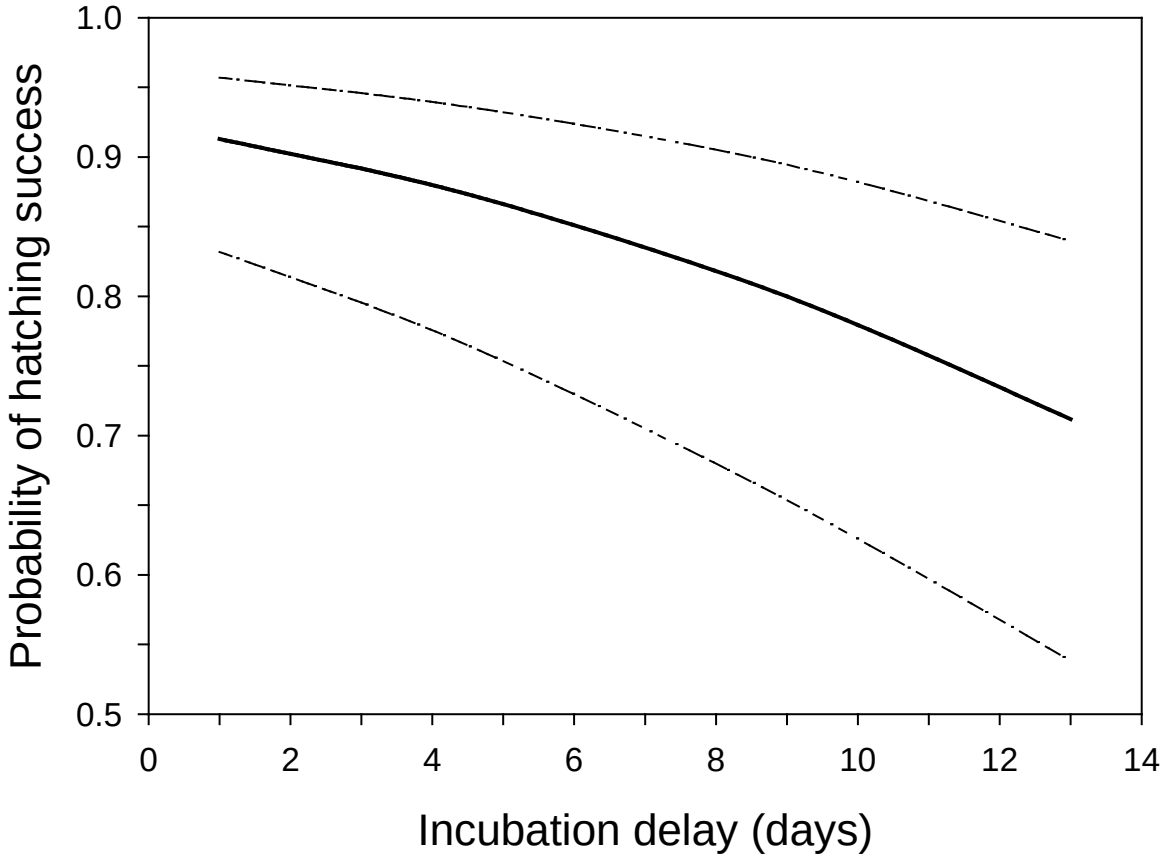

FIG. 1.3. Model predicted probability of hatching success for Wood Duck eggs in relation to length of incubation delay.

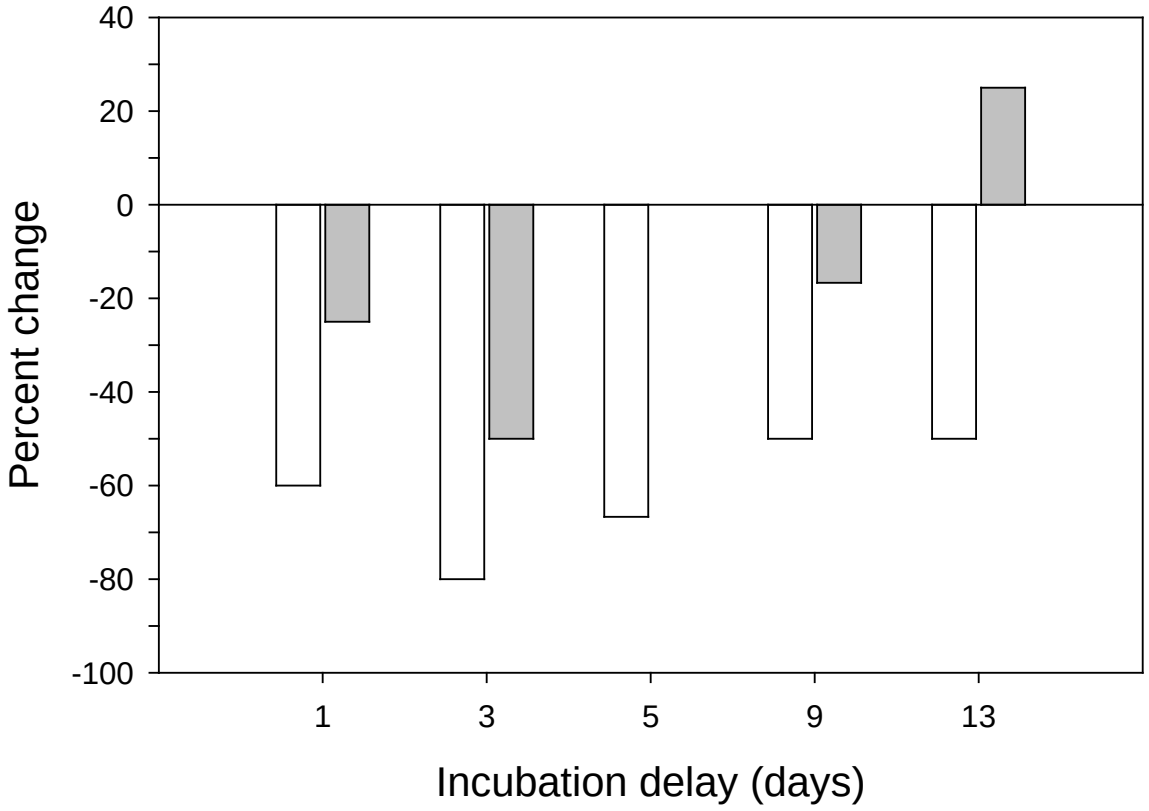

FIG. 1.4. Percent change in microbial infection of Wood Duck eggshells after incubation delay. Eggshells were cleaned (white bars) or not cleaned (gray bars) daily with 70% EtOH.

ONSET OF INCUBATION AND NEST REUSE: EFFECTS ON MICROBIAL GROWTH AND VIABILITY OF WOOD DUCK EGGS

ABSTRACT.—Egg viability in birds declines with increasing length of incubation delay and may be influenced by microbial infection. Reuse of nests is common in cavity-nesting species and may result in increased exposure to microbes. We examined the effects of nest cleaning and onset of night incubation on microbial growth and egg viability in Wood Ducks (*Aix sponsa*). We detected levels of eggshell microbes that were on the order of 10^1 CFUs/egg. Levels of heterotrophic bacteria were 2.2 times greater in uncleaned nests than in clean nests that had previous nesting materials removed. Gram-negative bacteria on eggshells declined by 87% (3.10 to 0.41 CFUs) after onset of night incubation and before full incubation began. We show that nest reuse can lead to higher levels of bacterial growth on shells of unincubated eggs, and that onset of night incubation reduced levels of gram-negative bacteria. Further, we found no relationship between levels of bacteria and hatching success. We suggest relatively low levels of bacterial infection of eggs at our temperate study site, together with increased antimicrobial properties of Wood Duck eggs and incubation by females at night before clutches were complete contributed to the negligible effect that bacteria had on egg viability.

Key Words: *Aix sponsa*, egg viability, microbial ecology, nest reuse, onset of incubation, Wood Duck.

EGG VIABILITY IN many species of birds declines with increasing length of incubation delay (Arnold et al. 1987, Meijerhof 1992, Stoleson 1999, Walls et al. in review). Studies of both wild and captive birds have shown viability of unincubated eggs begins to decline in as little as 3 days (Veiga 1992, Stoleson and Beissinger 1999). Ambient temperature and microbial infection are two important factors that may act independently during incubation delay to affect egg viability (Cook et al. 2005a).

Reuse of nests is common in cavity-nesting species. Benefits of nest reuse include knowledge of previous nest success, earlier nest initiation, reduced energy expenditure for nest creation, and increased reproductive success (Greenwood and Harvey 1982, Hepp and Kennamer 1992, Wiebe et al. 2007). Costs of nest reuse also exist and may include increased levels of competition, nest predation, and ectoparasites (Hepp and Kennamer 1992, Wiebe et al. 2007).

Microbes can grow rapidly on shells of unincubated eggs (Cook et al. 2005b, Shawkey et al. 2008). Trans-shell microbial infection of the embryo can occur in as little as 3 days and greatly reduces egg viability (Cook et al. 2003, 2005a). However, eggs have physical and chemical barriers that help inhibit microbial infection (Board 1966, Board and Fuller 1974). The eggshell cuticle provides a physical defense against microbial contamination and is comprised of antimicrobial proteins that also may aid in microbial defense (Board and Board 1967, Wellman-Labadie et al. 2008a). Egg albumen provides not only water and nutrients to the developing embryo, but contains proteins that help to prevent growth of microorganisms (Burley and Vadehra

1989). Lysozyme and ovotransferrin, two proteins typically found in albumen and known for their antimicrobial properties, also have been found within the eggshell matrix of some cavity-nesting species, including the Wood Duck (*Aix sponsa*; Wellman-Labadie et al. 2008a, b). These albumen proteins are more effective at impeding microbial infection at temperatures experienced during incubation (Tranter and Board 1984, Wellman-Labadie et al. 2008b).

Cavity-nesting birds have developed several adaptations to combat nest microbes. Many species add green vegetation to nests during egg laying, and cavity-nesting birds that reuse nests are six times more likely to use green nest material as cavity-nesters that do not reuse nests (Clark and Mason 1985). Several studies have shown reduced bacteria and ectoparasite levels in the presence of green nest material and have hypothesized that green nest material likely protects nestlings from pathogens (Clark and Mason 1985, Gwinner and Berger 2005). Further, eggs of many cavity-nesting species have higher levels of antimicrobial proteins, thicker eggshells, and improved eggshell cuticles than open-nesting species (Board 1966; Board and Fuller 1974; Cook et al. 2005b; Wellman-Labadie et al. 2008a, b). Incubation also may reduce levels of microbes on eggshells by reducing water on the eggshell, thereby lessening the conditions for microbial growth (D'Alba et al. 2010). Starting incubation before clutches are completed may maintain viability of first laid eggs by reducing eggshell moisture, initiating embryo development, and raising the temperature of eggs to levels

that increase the effectiveness of antimicrobial proteins (Hussell 1985, Board and Tranter 1986, D'Alba et al. 2010).

The Wood Duck is a secondary cavity-nesting species that uses natural tree cavities and artificial nest boxes (Bellrose and Holm 1994). Females are highly philopatric, and 79% of females annually return to the same wetland for nesting and 42% return to the same nest box (Hepp et al. 1989, Hepp and Kennamer 1992). Wood Ducks are monogamous, and females are solely responsible for incubation and brood rearing (Hepp and Bellrose 1995). Incubation period is 31–32 days (Manlove and Hepp 2000, Hepp et al. 2006), and females begin incubating at night approximately 3 days before the clutch is complete (Wilson and Verbeek 1995, Hepp 2004, Walls et al. in review). Full incubation begins after laying of the penultimate egg (Kennamer et al. 1990, Wilson and Verbeek 1995).

In this study of Wood Ducks, we used CLEAN and DIRTY nest boxes to investigate effects of reusing nests on microbial infection of eggs. CLEAN nest boxes were cleaned of nesting materials from the previous breeding season and DIRTY nest boxes were those that were not cleaned of old nesting materials. We sampled eggshell microbes during early and late stages of egg laying and again after the start of night incubation. We predicted that microbe levels on eggshells would increase from early to late egg laying and that microbial infection of eggshells would be greater in DIRTY nests than in CLEAN nests. We also predicted that microbial infection would decline after start of night incubation in both nest types. If microbial infection reduces egg

viability in Wood Ducks, we predicted a negative relationship between the level of microbial infection and hatching success.

METHODS

Study site.–We conducted this study on the U. S. Department of Energy's Savannah River Site (SRS) in the upper coastal plain of west-central South Carolina (33·1° N, 81·3° W). SRS encompasses approximately 80,289 ha and borders the Savannah River for 43.5 km. Nest boxes (n = 82) were maintained at Par pond (~1,000 ha).

Nest treatment and monitoring.–Before the breeding season of 2010, intact eggs and egg membranes from 2009 nests were removed from DIRTY nests, but all other nesting material including eggshell fragments, feather down, and used wood chips were left in the nest box. Old nesting material was completely removed from CLEAN nests and replaced with new wood chips. Sterile techniques were used whenever working in nest boxes or handling eggs.

Nest boxes were checked every four days for nesting activity during the breeding season of 2010. Eggs in new nests were numbered with a non-toxic waterproof marker (Sharpie®, Sanford). These nests were visited again the next day to check and number any new eggs, to take the first swab of eggs (see below), and to install a temperature data-logger (HOBO® Pro V2, Onset Computer Corp.). Protocol similar to Manlove and Hepp (1998) was used for data-logger installation. We first

removed all nesting material and installed a platform containing a wooden egg with an embedded thermistor. The wooden egg was attached to the platform with a deck screw (7.62 cm) to prevent females from moving it. The thermistor tip was exposed on top of the wooden egg to ensure contact with the brood patch of the incubating female. After installing the data-logger, nesting material and eggs were returned to the nest box. The wooden egg was positioned in the center of the nest, and the data-logger was secured to the underside of the box. Data-loggers recorded nest temperature every 6 min, and temperature data were downloaded and plotted for each 24 h period. Examining data from graphs and spreadsheets provides a very accurate method of determining when incubating females were on and off the nest (Manlove and Hepp 2000). Night incubation began on the first day that females spent the night on the nest (Hepp 2004).

Nest initiation date was estimated by subtracting the number of eggs in the nest when it was first found from the Julian date that the nest box was checked. We assumed a laying rate of one egg per day (Hepp and Bellrose 1995). Incubating females were captured in nest boxes during the first and last week of incubation. Unmarked females were banded with U.S. Fish and Wildlife Service leg bands, and all females were weighed to the nearest 5 g with a spring scale (Pesola®, Pesola AG). For nests with more than twelve eggs clutch size was reduced to twelve eggs during the first week of incubation by removing the newest eggs in the clutch. Clutch reduction was used to normalize clutch size for incubating females, and twelve eggs is the average size of a Wood Duck clutch (Hepp and Bellrose 1995). Hatching success was determined by

visiting the nest within one week of hatching and recording the numbers of any unhatched eggs.

Eggshell microbes.–Eggshells were swabbed on three occasions to obtain samples of shell microbiota. Approximately 20% of each eggshell was swabbed on the day after the nest was found (first swab), these eggs were swabbed again three days later (second swab) and after initiation of night incubation (night swab). Data-loggers were checked frequently, so we knew when night incubation began. Swabs were placed in 3 ml of sterile physiological saline and sample tubes were transported back to the Savannah River Ecology Laboratory (SREL) in a padded case (model 1600, Pelican Products Inc.) cooled with an ice pack.

We cultured microbes by plating two 0.1 ml replicates of saline supernatant on each of two growth media: MacConkey agar (MAC) and tryptic soy agar (TSA). Media were selected to detect the most common groups of bacteria known to colonize bird eggshells (Cook et al. 2003): MAC will grow Gram-negative enteric bacteria, and TSA will grow heterotrophic bacteria. Fungi will grow on both types of media. Cultures were incubated (Percival model 1-37LLVL, Percival Scientific Inc.) aerobically: MAC was incubated at 35°C, and TSA was incubated at 23°C. Microbial colony forming units (CFUs; CFUs/0.1 ml) were counted following 48 h of incubation (12 h:12 h L:D photoperiod). The culturing procedure was randomly repeated without swabbing eggs to test for contamination. No contamination was found.

Data analysis.–One-way analyses of variance (ANOVA; PROC GLM; SAS Institute Inc. 2004) were used to test for differences in nest initiation date and hatching success between CLEAN and DIRTY nests. We arcsin transformed the square root of the percent hatch for each nest prior to ANOVA. We used Tukey's test for means separation. Spearman's rank correlation (PROC CORR; SAS Institute Inc. 2004) was used to examine the relationship between MAC CFUs and TSA CFUs.

We used average CFUs from replicate plates in generalized linear mixed models (PROC GLIMMIX) to evaluate the relationship between CFUs and the explanatory variables of nest type (CLEAN), swab timing (SWAB), the first order interaction of CLEAN and SWAB, and nest initiation date (DATE). We separately tested MAC and TSA. Non-significant ($P > 0.05$) interaction terms were removed from the models. We treated the fixed effects CLEAN (yes/no) and SWAB (first, second, and night) as categorical variables, and DATE as a linear variable. Eggs within nests were treated as random events and SWAB was treated as a repeated measure. We used an unstructured covariance matrix, and denominator degrees of freedom were calculated using the Kenward Rogers methods. We calculated least squares means and used Tukey's test for means separation.

We tested the relationship between egg viability (yes/no) and microbial growth using generalized linear mixed models using the logit link. Nests were treated as random effects. Denominator degrees of freedom were calculated using the Kenward

Rogers methods. The explanatory variables, MAC CFUs and TSA CFUs, were from cultures of the second swab.

Results

Nest monitoring and swabbing.–Date of nest initiation was 19 March ± 4 days (range: 14 February – 19 May) and did not differ ($F=3.48$, df = 1, 28, $P=0.07$) between CLEAN and DIRTY nests. Data-loggers were installed in nests and first swabs of eggshells were taken 2.3 ± 0.2 days after nest initiation. Second swabs were taken 3 days after first swabs. Night swabs were taken 2.3 ± 0.2 days after night incubation began and 11.1 ± 0.6 days after nest initiation. Hatching success of eggs examined for microbial infection was 82.5 ± 5.8% and did not differ ($F=1.58$, df = 1, 25, $P=0.22$) between CLEAN and DIRTY nests.

Microbial growth.–We examined shells of 86 eggs from 30 different nests for microbial infection (Table 1). MacConkey cultures of first swabs detected gram-negative bacteria on 41% of eggs and in 63% of nests. Microbial infection detected with MAC did not differ between CLEAN and DIRTY nests ($F=0.59$, df = 1, 157.1, $P=0.44$), but bacterial infection differed by SWAB ($F=15.11$, df = 2, 138.8, $P<0.0001$) and nest initiation date ($F=8.65$, df = 1, 163.9, $P=0.004$). Bacteria levels did not differ between first and second swabs, but declined by 87% once night incubation began (Fig. 1). Bacteria levels detected with MAC also declined seasonally ($\beta = -0.02 \pm 0.007$).

Tryptic soy cultures of first swabs detected heterotrophic bacteria on 92% of eggs and in 97% of nests. Levels of microbial growth detected with first swabsusing MAC and TSA were positively correlated ($r = 0.44$, df = 84, $P < 0.0001$). We found no difference in bacteria levels on TSA by SWAB ($F = 0.19$, df = 2, 98.55, $P = 0.82$), but bacteria levels differed between CLEAN and DIRTY nests ($F = 7.69$, df = 1, 82.07, $P = 0.007$) and nest initiation date ($F = 20.35$, df = 1, 81.98, $P < 0.0001$). Bacteria levels on eggshells were 2.2 times greater in DIRTY nests than in CLEAN nests (Fig. 2). Bacteria levels detected with TSA also declined seasonally ($\beta = -0.03 \pm 0.006$).

Three nests were abandoned or destroyed by predators during incubation. Hatching success was 83% (64 of 77 eggs; $n = 27$). Egg viability was not related to levels of microbial infection detected from second swabs for either MAC ($F = 0.02$, df = 1, 75, $P = 0.88$) or TSA ($F = 0.17$, df = 1, 75, $P = 0.68$).

Discussion

Levels of microbial growth from first swabs of eggs in our study of Wood Ducks were on the order of 10^1 CFUs/egg. In tropical climates microbial growth on shells of unincubated eggs were reported to be on the order of 10^3–10^4 CFUs/egg (Cook et al. 2005a, b). In studies of Pearl-eyed Thrashers (*Margarops fuscatus*), Green-rumped Parrotlets (*Forpus passerinus*), and Hoopoes (*Upupa epops*) using nest boxes in tropical climates, length of exposure, cleaning of eggshells, and incubation all had significant effects on microbial levels of eggshells (Stoleson and Beissinger 1999; Cook et al. 2005a, b; Shawkey et al. 2009). In contrast, studies conducted in more temperate

climates reported that unincubated eggs in nest boxes had microbial levels $< 10^1$ CFUs/egg (Godard et al. 2007, Wang et al. 2011). Wang et al. (2011) found no evidence that length of exposure, eggshell cleaning, or incubation affected microbial growth of unincubated Western Bluebird (*Sialia mexicana*), Tree Swallow (*Tachycineta bicolor*), and Violet-green Swallow (*Tachycineta thalassina*) eggs. Similarly, Godard et al. (2007) found no evidence that length of exposure influenced microbial growth of unincubated eggs in nest boxes. Microbial infection may lead to loss of egg viability in wild birds (Cook et al. 2003, 2005a), but may have a greater impact on egg viability in tropical (i.e., moist) environments.

Wood Ducks do not excavate cavities for nesting, but rely on either natural cavities or nest boxes. Nest reuse has been shown to be related to previous nest success in waterfowl. A study of Common Goldeneyes (*Bucephala clangula*) reported preferences for nest boxes that had been previously used, especially if the box had been used successfully in the previous year (Dow and Fredga 1985). Female Wood Ducks are philopatric and are more likely to select nests that they used successfully in the past (Hepp et al. 1989). Pathogens may build up in nests that are reused both within and across years and may reduce hatching success of unincubated eggs (Cook et al. 2005a, Wiebe et al. 2007). In a study evaluating effects of nest box cleaning on Wood Duck productivity, Utsey and Hepp (1997) reported nest boxes that were never cleaned of nesting materials had fewer nests and lower hatching success than nest boxes that were cleaned at least once during the breeding season.

Changes in bacteria levels on shells of unincubated Wood Duck eggs were influenced by incubation and whether nest boxes were CLEAN or DIRTY. Eggshells from DIRTY nests had heterotrophic bacteria levels that were 2.2 times greater than those found on eggshells from CLEAN nests. Similar findings were reported for several passerine species where eggshell bacteria levels increased with age of nest boxes (Wang et al. 2011). We predicted that bacteria levels on eggs of Wood Ducks would increase during egg laying, but that onset of night incubation would reduce bacteria levels on eggshells. Previous studies in tropical climates have reported increased levels of microbes following exposure (Cook et al. 2005a, b; Shawkey et al. 2009). However, recent studies in temperate climates showed no relationship between exposure length and bacteria levels on shells of unincubated eggs (Godard et al. 2007, Wang et al. 2011). Studies from tropical climates also reported reduced levels of microbes on incubated eggshells (Cook et al. 2005b, Shawkey et al. 2009). We did not find any evidence of increased bacteria levels on eggshells from early to late egg laying. However, we did find that incubation reduced levels of gram-negative bacteria on eggshells by 87% (3.10 to 0.41 CFUs) after the onset of night incubation and before full incubation began.

We also predicted that if bacteria influences viability of Wood Duck eggs, then there should be a negative relationship between bacteria levels and hatching success of eggs. Studies in the tropics having high levels of microbes on eggshells reported reduced hatching success of eggs following incubation delay and suggested that microbial infection was the likely cause of the loss of viability (Stoleson and Beissinger

1999; Cook et al. 2003, 2005b). We found no relationship between bacteria levels and hatching success of Wood Duck eggs. Similarly, Wang et al. (2001) found no reduction in hatching success following exposure where low microbe levels were involved.

In this study we provide evidence that nest reuse can lead to higher levels of bacterial growth on shells of unincubated eggs; however onset of night incubation reduced levels of gram-negative bacteria, but not heterotrophic bacteria. We found no relationship between levels of bacteria and hatching success. We suggest relatively low levels of bacterial infection of eggs at our temperate study site, together with increased antimicrobial properties of Wood Duck eggs (Wellman-Labadie et al. 2008a, b) and incubation by females before clutches were complete contributed to the negligible effect that bacteria had on egg viability.

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TABLE 2.1. Mean (±SE) colony forming units (CFUs/0.1 ml) by media type and nest type for Wood Duck eggs swabbed on three occasions before the start of full incubation.

		Nest Type	
		Clean	Dirty
Media	Swab	(*n*=43)	(*n*=43)
MacConkey	First[a]	1.83 ± 0.86	3.18 ± 0.86
	Second[b]	3.39 ± 1.60	2.55 ± 0.51
	Night[c]	0.46 ± 0.30	0.46 ± 0.13
Tryptic soy	First	33.00 ± 12.22	55.53 ± 14.14
	Second	32.16 ± 12.62	64.11 ± 14.78
	Night	38.06 ± 17.93	32.16 ± 17.43

[a] Eggs swabbed during early egg laying; 2.3 days after nest initiation.
[b] Eggs swabbed during late egg laying; 3 days after first swab.
[c] Eggs swabbed after onset of night incubation; 2.3 days after start of night incubation

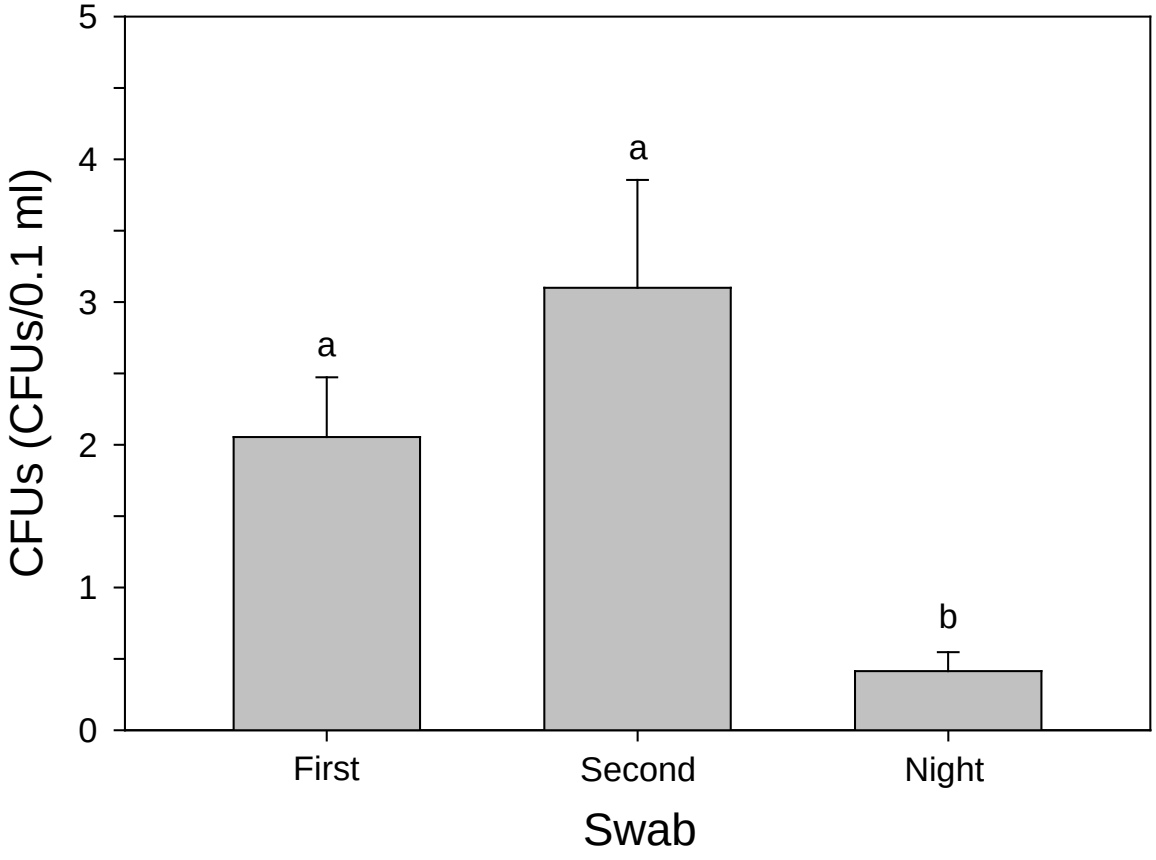

FIG. 2.1. Microbe levels detected on Wood Duck eggshells (*n*=86) using MacConkey agar at the Savannah River Site, SC. First swab was taken 2.3 days after nest initiation; second swab was taken 3 days after first swab; and night swab was taken 2.3 days after start of night incubation. Least squares means (±SE) with different letters are significantly different ($P<0.05$).

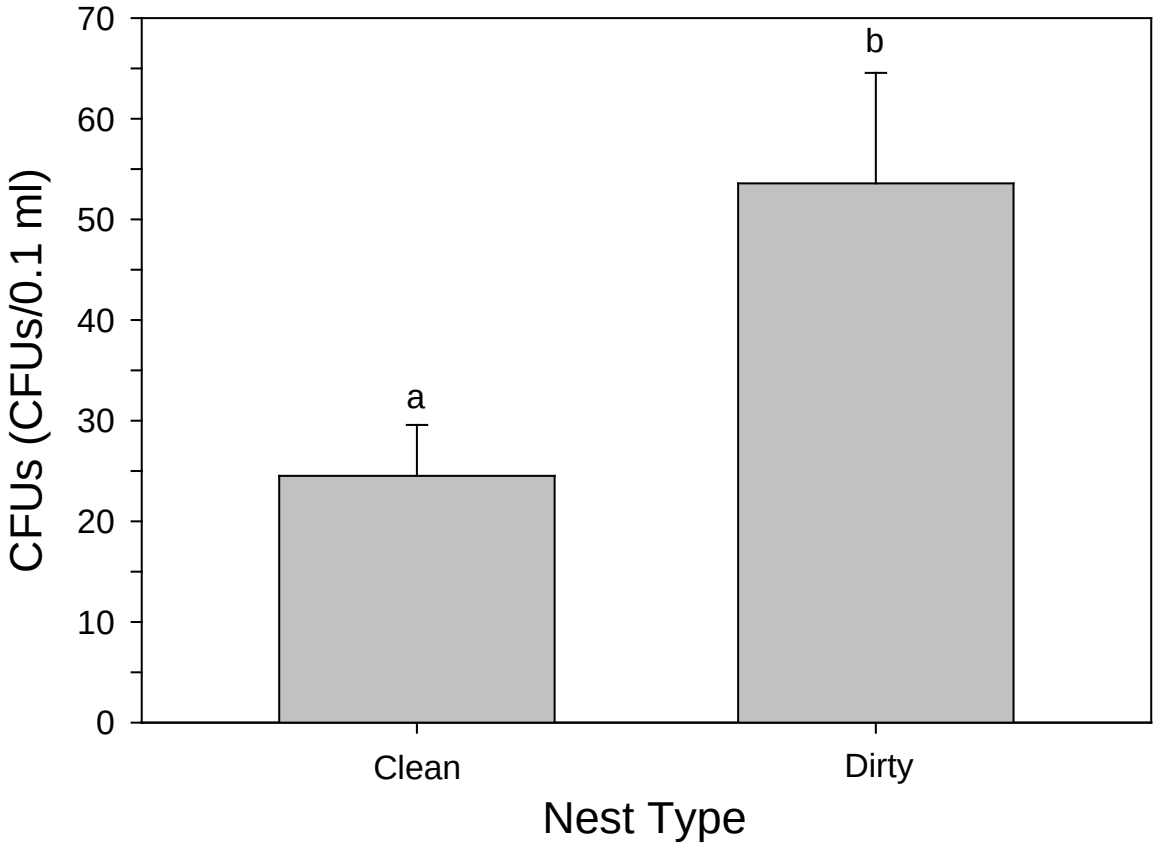

FIG. 2.2. Effects of nest type on microbe levels detected on Wood Duck eggshells (*n*=86) using tryptic soy agar at the Savannah River Site, SC. CLEAN nests had old nesting material removed and replaced with new wood chips; DIRTY nests had intact eggs and egg membranes removed, but all other nesting material was left. Least squares means (±SE) with different letters are significantly different (*P*<0.05).

APPENDIX 1.1. Summary statistics of microbial growth on Wood Duck eggshells by incubation delay. Eggshells were either cleaned or not cleaned daily with 70% EtOH. Cultures were grown using tryptic soy agar (TSA). Colony forming units (CFUs/0.1 ml; Mean ± SE) followed by different letters are significantly different (P<0.05).

			Incubation delay (days)				
Cleaning treatment	Swab		1	3	5	9	13
Clean		n	9	19	16	10	6
	Pre-delay	CFUs	3.11 ± 1.9 (0 – 13.0)[a]	2.68 ± 1.6 (0 – 25.0)	10.25 ± 8.5 (0 – 137.0)	1.40 ± 1.2 (0 – 12.0)	7.58 ± 3.5 (0 – 19.0)
		Microbial infection (%)	55.6	26.3	37.5	40.0	66.7
	Post-delay	CFUs	1.22 ± 1.1 (0 – 10.0)	10.58 ± 10.58 (0 – 201.0)	0.09 ± 0.07 (0 – 1.0)	0.15 ± 0.1 (0 – 1.0)	4.42 ± 4.3 (0 – 26.0)
		Microbial infection (%)	22.2	5.3	12.5	20.0	33.3
Not Clean		n	13	12	10	13	9
	Pre-delay	CFUs	5.73 ± 4.1 (0 – 51.0)	5.33 ± 3.7 (0 – 43.5)	0.65 ± 0.4 (0 – 4.0)	5.23 ± 2.8 (0 – 30.5)	3.22 ± 2.1 (0 – 18.0)

	Microbial infection (%)	30.8	66.7	40.0	46.2	44.4
Post-delay	CFUs	0.31 ± 0.2 (0 – 2.5)	0.50 ± 0.4 (0 – 4.5)	0.35 ± 0.2 (0 – 2.0)	0.81 ± 0.4 (0 – 4.0)	0.50 ± 0.2 (0 – 2.0)
	Microbial infection (%)	23.1	33.3	40.0	38.5	55.6

[a] Range (min – max

APPENDIX 1.2. Summary statistics of microbial growth on Wood Duck eggshells by incubation delay. Eggshells were either cleaned or not cleaned daily with 70% EtOH. Cultures were grown using MacConkey agar (MAC). Colony forming units (CFUs/0.1 ml; Mean ± SE) followed by different letters are significantly different (P<0.05).

Cleaning treatment	Swab		Incubation delay (days)				
			1	3	5	9	13
Clean		n	9	19	16	10	6
	Pre-delay	CFUs	0.00 ± 0.0 (0 – 0.0)[a]	0.05 ± 0.04 (0 – 0.5)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)
		Microbial infection (%)	0.0	10.5	0.0	0.0	0.0
	Post-delay	CFUs	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.17 ± 0.17 (0 – 1.0)
		Microbial infection (%)	0.0	0.0	0.0	0.0	16.7
Not Clean		n	13	12	10	13	9
	Pre-delay	CFUs	1.62 ± 1.62 (0 – 21.0)	7.17 ± 6.4 (0 – 77.0)	0.00 ± 0.0 (0 – 0.0)	0.04 ± 0.04 (0 – 0.5)	0.00 ± 0.0 (0 – 0.0)
		Microbial infection (%)	7.7	16.7	0.0	7.7	0.0

Post-delay	CFUs	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)	0.00 ± 0.0 (0 – 0.0)
	Microbial infection (%)	0.0	0.0	0.0	0.0	0.0

[a] Range (min – max)