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EFFECTS OF *MYCOPLASMA ANATIS* AND COLD STRESS ON HATCHING SUCCESS AND GROWTH OF MALLARD DUCKLINGS

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ABSTRACT: We inoculated game-farm mallard (*Anas platyrhynchos*) eggs and 1-day-old birds with *Mycoplasma anatis* to determine its effect on hatching success and growth rates of ducklings. Inoculations of eggs reduced hatching success, hatchling size, and duckling growth rates, compared to controls. Intratracheal inoculations of 1-day-old birds did not affect growth rates. Hatchlings and 1-day-old ducklings grew much slower for the first 7 to 10 days when raised at 17 to 19 C, compared to controls raised at 30 to 35 C. The effect of cold stress on growth was greater than the effect of *M. anatis* infection; we found no synergistic effects between cold stress and *M. anatis* infection.

Key words: *Anas platyrhynchos*, cold stress, ducklings, growth, hatch success, mallards, mycoplasmas, *Mycoplasma anatis*.

INTRODUCTION

Mycoplasmas (*Mycoplasma* spp.) frequently have been isolated from domestic chickens (*Gallus gallus*) and turkeys (*Meleagris gallapavo*). Recently, mycoplasmas have been recovered from domestic and semi-domestic ducks throughout the world (Bradbury et al., 1987; El-Ebeedy et al., 1987; Ivanics et al., 1988) and from wild ducks in Spain (Poveda et al., 1990) and the United States (Goldberg et al., 1995). Many of these mycoplasmas cause clinical disease of the respiratory system and joint synovia (Jordan, 1975). In addition, avian mycoplasmas cause decreased productivity in captive-reared wild turkeys (Rocke et al., 1988) and domestic poultry, and suppress growth rates in young birds (Stipkovits, 1979). *Mycoplasma anatis* can be pathogenic to domestic ducklings and eggs (Stipkovits, 1979), and mycoplasmas can be transmitted vertically from infected females (Yoder, 1991). However, little information is available about the effects of *M. anatis* on hatching success or on duckling growth.

In wild birds, about half of the ducklings that hatch die before fledging, with most losses occurring during the first 2 wk of life (Sargeant and Raveling, 1992). The importance of predation on ducklings has been documented by Sargeant and Raveling (1992), but less is known about the effects of weather or disease agents on survival (Johnson et al., 1992) and growth of wild ducklings. Cold, rainy, or windy weather conditions can increase mortality of ducklings, especially the very young (Makepeace and Patterson, 1980; Johnson et al., 1992). Exposure of canvasback (*Aythya valisineria*) ducklings to cold and wet weather during their first 7 to 10 days is an important cause of mortality (M. D. Samuel, unpubl.). Adverse weather conditions also may affect duckling physiology (Koskimies and Lahti, 1964) and survival (Makepeace and Patterson, 1980; Mendenhall and Milne, 1985) or reduce their growth.

Our objective was to determine the effects of *M. anatis* on hatching success and growth of ducklings. In the first experiment we inoculated game-farm mallard (*Anas platyrhynchos*) eggs with *M. anatis* and a control group with media only. In the second experiment we assessed the effects of *M. anatis* and cold stress on growth rates of mallard ducklings; we used successfully hatched birds from the first experiment and an additional group of 1-day-old ducklings intratracheally inoculated with *M. anatis* or media only. Both ex-

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periments were conducted between July and August 1991.

MATERIALS AND METHODS

The *M. anatis* culture used for all inoculations was isolated from the trachea of a wild adult mallard carcass recovered in Kulm, North Dakota (USA) (46°25'N, 98°55'W) and was identified using a Western blot procedure (Thomas et al., 1991). Mycoplasma culture media were prepared as described by Jordan (1983) and Goldberg et al. (1995). *Mycoplasma anatis* cultures were grown in mycoplasma culture media for 24 hr before inoculation.

Fertile game-farm mallard eggs (Whistling Wings, Hanover, Illinois, USA) were held in a Jamesway incubator-hatcher (Model 252B, Butler Manufacturing Company, Fort Atkinson, Wisconsin, USA) for 10 days. All eggs were externally disinfected with an iodine alcohol solution (3% potassium iodine in 95% ethyl alcohol). Eighty-three eggs were inoculated via the yolk sac with 0.1 ml of *M. anatis* culture ($>10^{10}$ color changing units) following the methods of Rovozzo and Burke (1973). A control group of 83 eggs was inoculated with 0.1 ml culture medium only. Mycoplasma-inoculated and control eggs were incubated separately, at 37.3 to 37.7 C and relative humidities of 30 to 32%, until hatch or mortality occurred. All eggs were candled daily; eggs containing dead embryos were removed and stored at 4 C overnight to constrict the blood vessels before isolation of mycoplasma was attempted. At the onset of pipping, the eggs were moved to hatching trays and misted and checked every 6 hr. Once hatched, the ducklings were moved to experimental isolation rooms and held under brooder lamps for 6 to 12 hr until dry. Survival to pipping and hatch were recorded. Hatching success was measured as the percentage of birds surviving to 24 hr post-hatch.

Because mortality was higher than expected in the egg inoculation experiment, we supplemented the groups of surviving ducklings with 1-day-old game-farm mallards (Whistling Wings) in the second experiment. These ducklings were inoculated intratracheally with 0.2 ml of *M. anatis* culture ($>10^{10}$ color changing units) in broth (63 birds) or mycoplasma medium alone (20 birds). A butterfly infusion set (Abbott Hospitals, Inc., North Chicago, Illinois), with butterfly and needle removed, attached to a 1-cc syringe, was inserted into the trachea until it reached just above the syrinx; the fluid then was slowly delivered. All air bubbles were removed before inoculation. Birds were restrained briefly to ensure retention of the inoculum.

Approximately half of the birds in each experimentally infected and control group were maintained at normal brooding temperature (30 to 35 C); the other half were subjected to mild cold stress (17 to 19 C) for the first 7 to 10 days, creating four experimental groups (control-cold, control-normal, mycoplasma-cold, mycoplasma-normal). Birds in the 30 to 35 C groups were housed at seven to 11 per cage in Horsfall Bauer units (Drury et al., 1969), five cages per isolation room. Birds maintained at 17 to 19 C were housed in rabbit cages (Lab Products, Inc., Aberdeen, Maryland, USA) for 7 to 10 days at seven to 11 per cage; then they were transferred to Horsfall units and kept at 30 to 35 C. Hatched ducklings were kept in separate cages from tracheally-inoculated ducklings. After approximately 2 wk, the temperature gradually was decreased to 26 to 29 C in all cages for the duration of the experiment to compensate for increased duckling metabolic activity. Cage temperature was regulated by a combination of room thermostats, portable space heaters, and cage lights. A size-3 metal wing tag (National Band and Tag Co., Newport, Kentucky, USA) was attached to the patagium of each bird. Duck starter feed number 8855 (Purina Mills, Inc., St. Louis, Missouri, USA) and water were provided ad libitum. Waste food and feces were removed daily.

To prevent cross-contamination, each of the four experimental groups were housed in a separate isolation room with negative air pressure (0.253 g/cm^2 relative to hallway access) and air-locked anteroom systems. Personnel in contact with the ducklings wore protective coveralls, face masks, bonnets, rubber boots, and gloves. Control rooms always were visited before the mycoplasma-infected rooms. Showers were taken by all personnel following daily activities with the animals.

Body weights, and culmen and tarsus lengths were measured on all birds every 2 days (half of the birds each day) until the birds were 21 days old. Weights were recorded to the nearest 1 g for birds $\leq$500 g using a 100 or 500-g spring balance or to the nearest 5 g for birds >500 g using a 1 kg spring balance. Culmen and tarsus lengths were determined to the nearest 0.1 mm using a dial caliper, according to the methods described by Lightbody and Ankney (1984).

Following storage at 4 C, all eggs that died before hatch were disinfected with an iodine alcohol solution and opened, as described by Rovozzo and Burke (1973). Using aseptic techniques, two Dacron swabs were inserted into the yolk sac. To check for bacterial contamination, one swab was swirled in a tube of brain heart infusion broth (Difco Laboratories, Detroit, Michigan, USA); the swab was removed and the tube was incubated for 24 hr at 37 C. The other swab, used for mycoplasma isolation, was swirled

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in a tube of broth; the swab was discarded and the tube was incubated as described by Goldberg et al. (1995).

At 21 days of age, tracheal swab samples were taken from all ducklings using a Type 1 Calgiswab® (Baxter Scientific Products, McGaw Park, Illinois). Swabs were cultured in mycoplasma broth, overlaid on an agar slant (mycoplasma broth containing 1% molten agarose; Difco) designed to provide a structure for mycoplasma adherence and growth. Two tracheal swab samples were taken from each egg-inoculated bird; one swab was cultured immediately and a duplicate was frozen at −20 C to assess recovery of mycoplasmas under similar conditions used in our field studies (Goldberg et al., 1995). Tracheal swabs from 1-day-old birds were immediatly stored at −20 C and cultured 1 mo later, along with the frozen swabs (duplicates) from the egg-inoculated group. Birds were euthanized by cervical dislocation and frozen at −20 C for 3 mo until necropsied. The sex of each bird was determined at necropsy, and trachea and lungs were cultured for *M. anatis* (Goldberg et al., 1995).

We compared pipping and hatching success between mycoplasma inoculated and control eggs using a chi-square (χ^2) statistic (Daniel, 1978). McNemar's test (Daniel, 1978) was used to compare the frequency of *M. anatis* recoveries from duplicate swab samples obtained from ducklings.

We used a repeated measures multivariate analysis of variance (ANOVA) (Milliken and Johnson, 1984) to evaluate the effects of mycoplasma and cold stress on duckling growth. Response variables included body weight, culmen length, and tarsus length. Separate analyses were conducted for birds from the egg experiment and from the 1-day-old duckling experiment, because these two experiments represented differences in route of *M. anatis* inoculation and duration of infection. Due to the lower hatch success for mycoplasma inoculated eggs, we randomly assigned more 1-day-old ducklings to the mycoplasma inoculation group. These distributions resulted in unbalanced cells within the ANOVA. Therefore, we used type III sums-of-squares in our analyses (SAS Institute Inc., 1987). To standardize our results for different hatch dates, the repeated measures portion of our analyses were based on the age of each bird. The repeated measures analyses were further complicated because we measured growth on half of the birds each day (each bird was measured every 2 days). To accommodate this sampling scheme, we fit the weight, culmen, and tarsus data from each bird to a separate exponential curve and produced predicted values for each growth response on odd days of duckling age. The log-transformed predicted growth values were then used in our repeated measures ANOVA. Finally, four apparently healthy ducklings (two hatchlings and two 1-day-olds) with unusually slow growth were removed from our reported analyses. We believe most of these undersized ducklings were harassed by other birds and therefore grew poorly.

Multivariate ANOVA also was used to compare the weights of 1-day-old birds that successfully hatched from the egg-inoculation experiment. We evaluated the effect of disease treatment, sex, and the interaction using the predicted weights determined from the exponential growth model.

RESULTS

Pipping success in the mycoplasma-inoculated eggs was 78%, significantly ($P = 0.057$) lower than the 89% observed among the control eggs (Table 1). The difference in hatching success between the two groups was much more pronounced ($P < 0.001$); only 50% of the eggs inoculated with *M. anatis* survived to 24 hr post-hatch, compared with 86% of the control group. *Mycoplasma anatis* was cultured from all 36 mycoplasma-inoculated eggs that died before hatch. *Mycoplasma anatis* also was recovered from one control egg that died following pipping and was subsequently removed from all tallies. We also excluded 10 eggs that were broken accidentally or cracked during incubation, and 22 eggs that died in the first 24 hr after hatch due to poor brooding conditions.

In the egg-inoculation experiment, birds inoculated with *M. anatis* were smaller ($P = 0.04$) at 1 day of age than control birds. Mycoplasma-inoculated hatchlings had lower mass [mean (95% confidence interval), 48.3 (46.1 to 50.5) vs. 49.6 (48.1 to 51.1) g], smaller culmens [15.3 (14.9 to 15.6) vs. 15.7 (15.5 to 16.0) mm], and smaller tarsi [26.6 (26.1 to 27.1) vs. 27.3 (27.0 to 27.7) mm] than control birds. There were no differences in initial measurements for males and females ($P = 0.88$) or for the interaction of sex and disease treatment ($P = 0.59$). No significant ($P > 0.05$) interactions were found in duckling growth rates among the ANOVA main effects of

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TABLE 1. Pipping and hatching success of game-farm mallard eggs inoculated with *Mycoplasma anatis*.

	Control	Mycoplasma
Pipping success	67/75 (89)[a]	64/82 (78)[b]
Hatching success	59/69 (86)	33/66 (50)[c]

[a] Number of eggs successfully pipped/number of eggs inoculated (% positive).
[b] Significantly ($P = 0.057$) lower than control group.
[c] Significantly ($P < 0.001$) lower than control group.

temperature, disease treatment, or sex for either hatchlings or 1-day-old ducklings. Ducklings raised at 17 to 19 C had significantly lower growth than ducklings at 30 to 35 C, regardless of disease treatment or sex (Table 2). These differences were present for the birds hatched from eggs ($P < 0.001$) and 1-day-old birds ($P < 0.001$). In the experimental group inoculated as 1-day-olds, male ducklings grew faster ($P < 0.01$) than females, regardless of temperature or disease treatment (Table 3). However, males and females had similar growth ($P = 0.18$) in the egg-inoculated birds. Growth was lower ($P < 0.001$) for the ducklings inoculated with mycoplasma in the egg than for their corresponding uninfected controls (Table 4). These mycoplasma-infected birds had similar body mass, but a smaller culmen and tarsus than controls. However, ducklings inoculated with *M. anatis* at 1 day of age did not show a significant difference ($P = 0.23$) in growth from their corresponding controls, although control birds were consistently larger than mycoplasma-inoculated birds (Table 4).

Mycoplasmas were not isolated from swabs or tissues of any of the 93 control birds used in the growth experiment. *Mycoplasma anatis* was cultured (combined swabs and tissue samples collected on day 21) from 21 (60%) of 35 egg-inoculated mycoplasma ducklings and 41 (68%) of 60 ducklings infected as 1-day-olds. Isolations from 30 to 35 C and 17 to 19 C groups were similar, 28 (62%) of 45 and 34 (68%) of 50 ducklings, respectively. We isolated *M. anatis* from three (8.6%) of 35 lung tissue samples from egg-inoculated birds and three (5.0%) of 60 lung tissue samples from 1-day-old ducklings. *Mycoplasma anatis* was recovered from five (14%) and 17 (28%) of the tracheal tissue samples from those respective groups. From the swab samples, we recovered *M. anatis* from 33 (52%) of 63 frozen swab samples obtained from ducklings inoculated as 1-day-old birds. For the duplicate swab samples taken from egg-inoculated birds, *M. anatis* was isolated infrequently from both swabs (six of 21 recoveries), and recovery frequencies differed by McNemar's test ($P < 0.001$) between frozen swabs (17%) and fresh swabs (58%) from the 36 samples. It appeared that fresh swabs were most effective in isolating mycoplasmas from ducklings in comparison to tissues from frozen carcasses; however, in seven (7.4%) of 95 birds we only recovered mycoplasma from the trachea or lung tissues.

TABLE 2. Least-squares means and 95% confidence intervals for body weights and culmen and tarsus lengths for all ducklings raised at 17 to 19 C and 30 to 35 C. Means approximate the measurements obtained at the midpoint (age 10 to 11 days) of the experiment.

Inoculation group	Temperature treatment	Body weight (g)	Culmen length (mm)	Tarsus length (mm)
Eggs	30 to 35 C	183 (177–189)[a]	26.4 (26.1–26.8)	42.7 (42.2–43.1)
	17 to 19 C	154[b] (150–159)	24.5[b] (24.1–24.8)	39.2[b] (38.7–39.6)
1-day-old	30 to 35 C	173 (167–180)	26.9 (26.4–27.3)	43.1 (42.6–43.7)
	17 to 19 C	159[b] (154–164)	25.9[b] (25.5–26.3)	40.8[b] (40.3–41.2)

[a] Least-squares means (95% confidence intervals).
[b] Significantly ($P < 0.05$) lower than 30 to 35 C treatment.

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TABLE 3. Least-squares means and 95% confidence intervals for body weights and culmen and tarsus lengths for all male and female game-farm mallard ducklings. Means approximate the measurements obtained at the midpoint (age 10 to 11 days) of the experiment.

Inoculation group	Sex	Body weight (g)	Culmen length (mm)	Tarsus length (mm)
Eggs	Male	172 (166–177)[a]	25.7 (25.3–26.1)	41.2 (40.8–41.7)
	Female	165 (160–170)	25.2 (24.8–25.5)	40.6 (40.1–41.0)
1-day-old	Male	173 (168–178)	26.6 (26.3–27.0)	42.4 (42.0–42.9)
	Female	159[b] (153–165)	26.1 (25.7–26.6)	41.4[b] (40.9–42.0)

[a] Least-squares means (95% confidence intervals).
[b] Significantly ($P < 0.05$) lower than males.

DISCUSSION

Mycoplasmas often are vertically transmitted, and have caused considerable reduction in productivity in poultry operations (Yoder, 1991). We have isolated mycoplasmas from 1-day-old wild ducklings (Goldberg et al., 1995), providing evidence that vertical transmission occurs in wild birds. There also is evidence that *Mycoplasma anatis* may be pathogenic to ducklings and eggs (Stipkovits, 1979), and causes reduced growth rates in young birds (Amin and Jordan, 1978). Based on our results, it appears that vertical transmission of *M. anatis* has the potential to reduce mallard hatching success, hatch size, and the growth of mallard ducklings. Inoculation of 1-day-old ducklings with *M. anatis* did not significantly reduce growth. It appears that vertical transmission of *M. anatis* may have a more pronounced effect on hatch size and subsequent growth than post-hatch infection. The effect of mycoplasmas on the productivity of wild duck populations is uncertain; however, further research is needed to determine the extent of mycoplasma infections in wild mallard populations, the degree of vertical transmission during nesting, the importance of sexual transmission during breeding, and the effects of mycoplasmas on wild ducklings.

In young birds, mycoplasmas have synergistic effects with other agents, such as goose parvovirus in goslings (Kisary et al., 1976), and avian influenza virus in ducklings (Roberts, 1964). Thus, birds infected with mycoplasmas may be at higher risk from secondary infections from other pathogens in their environment (Jordan, 1975; Tiong, 1990). Based on our findings, mild cold stress did not appear to have a synergistic effect on the growth of ducklings infected with *Mycoplasma anatis*.

Adverse weather during the first week after hatch can cause substantial mortality in wild ducklings (Johnson et al., 1992; Sargeant and Raveling, 1992), but we know little about the influence of weather on

TABLE 4. Least-squares means and 95% confidence intervals for body weights and culmen and tarsus lengths for all ducklings inoculated with *Mycoplasma anatis* and controls. Means approximate the measurements obtained at the midpoint (age 10 to 11 days) of the experiment.

Inoculation group	Disease treatment	Body weight (g)	Culmen length (mm)	Tarsus length (mm)
Eggs	Control	167 (163–172)[a]	25.7 (25.5–26.0)	41.1 (40.7–41.4)
	Mycoplasma	169 (163–176)	25.1[b] (24.7–25.6)	40.7 (40.2–41.2)
1-day-old	Control	168 (161–175)	26.7 (26.1–27.2)	42.2 (41.5–42.8)
	Mycoplasma	164 (161–168)	26.1[b] (25.8–26.4)	41.7 (41.1–42.0)

[a] Least-squares means (95% confidence intervals).
[b] Significantly ($P < 0.05$) different from control group.

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duckling growth. In our laboratory experiment, mild cold stress reduced the growth of ducklings even when ad libitum food and water were supplied; it also had a more pronounced effect on growth than mycoplasma infection. Thus, severe or prolonged exposure to cold and wet weather may cause mortality of wild ducklings and also may reduce their growth. In agreement with previous studies (Prince et al., 1970; Greenwood, 1974; Bruggers and Jackson, 1977), we also found that males grew at a higher rate than females in the group inoculated as 1-day-old birds. This sex difference was independent of cold stress and mycoplasma effects on growth rates.

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