Onset of the humoral immune response of layer chicks vaccinated in ovo with strain F *Mycoplasma gallisepticum* vaccine and evidence of male-biased mortality[1,2]

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ABSTRACT Previous trials in which layers were in ovo-vaccinated against strain F *Mycoplasma gallisepticum* (**FMG**) showed that nearly 50% of the birds produced IgM antibody against FMG at 6 wk of age (**WOA**). Standard FMG vaccination application at 9 or 10 woa, result in this percentage at approximately 15 woa. This study investigated when FMG in ovo-vaccinated birds initiate a humoral immune response prior to 6 wk, and if sex influences this response. Hy-Line W-36 embryonated eggs were either not vaccinated (controls) or in-ovo vaccinated with a 50 μL volume of a 10^{-6} dilution of Poulvac MycoF vaccine (Zoetis). For each treatment group, 384 straight-run chicks were reared. At hatch and at 2, 3, 5, 7, 14, 21, and 28 d post-hatch, 54 birds per treatment were individually weighed and a blood sample was collected for *Mycoplasma gallisepticum* (**MG**) IgM antibody detection. ELISA was run on blood samples at 14, 21, and 28 d to distinguish IgG antibody production. At each age, BW was not different between vaccinated and control chicks (all $P > 0.19$). Males, however, outweighed females starting at d 5 ($P = 0.02$). Mortality was 1.0% for the control birds and 12.2% for the FMG birds during the first 2 wk. The majority (72.3%) of the mortalities in the FMG group were male. The percentage of control and FMG in ovo-vaccinated birds with IgM antibody production was 0% and 1.9% on d 7, 0% and 31.5% on d 14, 1.9% and 55.9% on d 21, and 0% and 60.6% on d 28, respectively. IgG antibody production in the FMG in ovo-vaccinated birds was 0.0% at 14 d, 2.9% at 21 d, and 21.2% at 28 d of age. All control birds tested negative for FMG-IgG production. In conclusion, the earliest detection of MG antibodies after in ovo vaccination with live FMG occurred at 7 d. Male layer chickens were more susceptible to the effects of an in ovo FMG vaccine than females.

Key words: *Mycoplasma gallisepticum*, layer, in ovo vaccination, sex, antibody

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INTRODUCTION

To combat Marek's disease in chickens, Sharma and Burmester (1982) administered the herpes virus of turkey (**HVT**) vaccine to chickens in ovo (prior to hatch) or after hatch and found greater protection against an early Marek's disease challenge when the birds were vaccinated before hatching. Since this initial discovery of the success of in ovo vaccination, the technology of administering the vaccine to large numbers of embryonated eggs was developed, and the majority of commercial broilers in the United States were switched from subcutaneous HVT vaccine administration to in ovo vaccination over a span of 20 yr (Gildersleeve et al., 1993 Ricks et al., 1999;). The exact mechanism as to why in ovo vaccination of HVT was and is successful has not been fully confirmed to date (Davison, 2014).

Commercially, in ovo vaccines are employed against Marek's disease, infectious bursal disease, infectious laryngotracheitis, poxvirus, Newcastle disease, avian influenza, and coccidiosis (all viral diseases with the exception of coccidiosis Schijns et al., 2014;). A number of other candidate vaccines, including bacterial vaccines, are under investigation for in ovo vaccination application (Peebles, 2018).

One such bacterial vaccine under investigation for in ovo application is against *Mycoplasma gallisepticum* (**MG**), which causes financial losses for the commercial table egg industry (Elliott et al, 2017, 2018). Standard

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commercial vaccination methods for MG include vaccinating the birds as pullets prior to the initiation of egg production as early as 9 wk of age **(woa)** via spray, eyedrop, wingweb, or intramuscular or subcutaneous routes depending on the vaccine form utilized (Evans et al., 2005 Ferguson-Noel et al. 2012;).

The initial MG in ovo studies evaluated a live strain F MG (**FMG**) vaccine, since post-hatch vaccination with FMG confers protection for the life of the bird (Hy-Line International, 2013). Both IgM and IgG antibody production against FMG has been found in FMG in ovo-vaccinated birds at 6 woa (Elliott et al., 2018). However, evaluations of antibody production in FMG in ovo-vaccinated birds prior to 6 woa has not yet been performed. Thus, the main objective of this study was to determine the timing of the humoral immune response in the chick during the first 4 wk post-hatch following an in ovo vaccination with FMG.

Additionally, MG infections have been documented as being more severe in male house finches than in female house finches (Nolan et al., 1998), yet to our knowledge, the effect of sex on the severity of an MG infection has not been confirmed in domesticated poultry (Bradbury, 2005). Thus, the further objective in this study was to examine if the introduction of FMG in layer embryos caused a greater impact on males compared with females post-hatch.

MATERIALS AND METHODS

Hy-Line W-36 hatching eggs (12 total 90-egg capacity trays) from breeders tested NPIP (National Poultry Improvement Plan, 2019) *Mycoplasma gallisepticum*-clean were incubated under standard incubation conditions in a NOM 2000 NatureForm incubator. At 18 days of incubation 6 trays of eggs were in ovo-vaccinated with a 10^{-6} dilution (Elliott et al., 2017, 2018, 2020) of Poulvac MycoF vaccine (Zoetis, New York, NY) in Poulvac Marek's diluent (Zoetis, Exton, PA) using an Embrex Inovoject M machine delivering a 50 μL vaccine volume. The vaccine was plated on four replicate plates on Frey's Mycoplasma agar (Frey et al., 1968) and incubated at 37°C for at least 5 d. The plate counts indicated that each embryonated egg received an average of 3.73 CFU of FMG. The other half of the eggs (controls) were subjected to the same handling practices but were not injected. The designation of trays of eggs as being control or MG-vaccinated was made at the time the eggs were initially placed in the incubator, so that a tray of eggs from each treatment were represented on each level within the incubator prior to vaccination. At the time of injection, 2 randomly selected eggs from each of the 12 trays were injected with Coomassie brilliant blue R-250 dye (Genlantis, San Diego, CA) to determine where the injection occurred within the embryonated eggs and to determine the stage of the embryos when injected (Avakian, 2006).

The MG-vaccinated eggs and the intact non-vaccinated control eggs were hatched in separate NOM 2000 NatureForm hatchers with exhausted air from each hatcher being independently filtered and directed outside of the hatchery to prevent MG transmission to non-vaccinated chicks upon hatching. The chicks were removed from the hatchers at 22 d of incubation, allowing for the recommended incubation duration for Hy-Line W-36 eggs to be 21 d and 18 hours (Hy-Line International, 2016). The control chicks were removed first and counted for hatch success. A total of 384 straight-run chicks were placed between 2 rooms consisting of 4 pens per room (1.92 m^2 area per pen): 48 chicks were placed in each of the pens following the space guidelines of the Hy-Line W-36 Commercial Layer Management Guide for 0 to 3 wk-old Hy-Line W-36 chicks (Hy-Line International, 2020). The 48 chicks in each pen consisted of 8 randomly selected chicks from each of the 6 trays. The same placement was followed for the MG-vaccinated chicks in 2 separate rooms with 4 pens per room, totaling 768 birds placed (384 per each treatment). All birds were placed as straight-run (not sexed prior to placement). Within each pen, the birds were placed on fresh, clean pine shavings with ad libitum access to water via a nipple water line and a mashed layer pullet starter diet, fed for the duration of the post-hatch experiment (from hatch date until 4 woa) that was formulated to meet or exceed NRC requirements (NRC, 1994). Brooding temperatures followed the recommendations set by Hy-Line with an initial floor temperature in the range of 33 to 35°C that was decreased by 2 to 3°C per week. Lighting was provided at 21 h of light (at 30 lux) with 3 h of dark during the first wk with decreasing light duration by 1 h each week. Birds were checked once a day, with both control rooms being entered before both MG-positive rooms were entered. Caretakers wore clean protective coverings daily (coveralls, hairnets, gloves, boot covers, and dust masks) and stepped into a quaternary ammonia footbath both before entering and when leaving each of the 4 rooms. Any mortality from each pen was recorded daily and all mortality were removed and necropsied for internal assessment of the cause of death and to determine the sex of each chick. Specifically, all mortalities were necropsied noting any airsacculitis, congested lungs, and any incidence of caseous exudate in the birds. The sex of each bird was determined via the presence of testes or a rudimentary ovary. The sexing of dead birds was not performed in previous trials in which the effects of diluted dosages of in ovo-administered FMG were tested (Elliott et al., 2018, 2020).

At 8 sampling dates, a subset of birds from each treatment (control and MG-vaccinated) were randomly selected, individually weighed, euthanized via decapitation, and a blood sample was collected immediately for antibody detection. For the first sampling date at hatch, a total of 54 chicks from each treatment were selected and sampled (9 randomly selected chicks from each of the 12 total incubation trays). These initial chicks were selected as hatch was pulled for each treatment. After the 384 chicks from each treatment were placed in the house, 54 birds from each treatment were sampled at 2,

3, 5, 7, 14, 21, and 28 d of age. Six or 7 birds were randomly selected from each of the 8 total pens from each treatment to total the 54 birds sampled on each date. Blood collection via euthanasia reduced the stocking density and the number of birds per feeder space and per each nipple waterer in each pen after each sampling. Sex was noted for all the sample birds via necropsy following blood collection by evaluating the presence of testes or a rudimentary ovary. Any observable evidence of disease (congested lungs, airsacculitis, and any incidence of caseous exudate) within the sample birds during necropsy were also noted.

Bird incubation, management, and handling practices were reviewed and approved by a USDA-ARS Animal Care and Use Committee (Mississippi State, MS) prior to the initiation of the research. The health and welfare of the birds were additionally monitored by a Veterinary Medical Officer during the study. All mortality necropsies were carried out by the Veterinary Medical Officer.

Laboratory testing (Serum Plate Agglutination [**SPA**] and ELISA) were performed as designated by Elliott et al. (2018). All blood samples were evaluated for IgM antibody production via SPA testing. All birds with a SPA score of 1 and above were considered positive. Since class switching to IgG occurs at a later time point than initial IgM antibody production (approximately 2 wk later), ELISA tests for IgG antibody production were performed on the later age samples at 14, 21, and 28 d of age for all control and MG birds sampled.

The hatch results, body weight data, and ELISA titer data were analyzed as an ANOVA using the proc mixed procedure of SAS (Version 9.4; SAS Institute, 2020) with either the incubation tray or the pen treated as a random effect. The data were tested for the main effects of injection treatment, sex, and their interaction.

RESULTS AND DISCUSSION

Incubation and Hatch

As has been found in previous trials (Elliott et al., 2017, 2018, 2020), there was no difference in the hatch success (percentage hatch of live embryonated eggs) between the control non-injected eggs and the MG-injected eggs ($P = 0.3157$). Additionally, as in previous trials when layer embryos were injected at 18 d of incubation, and when incubated at the same facility and under the same incubation conditions, most of them were injected in the amnion (Elliott et al., 2017, 2018, 2020). Embryos (12 total) selected from the control treatment trays had an average development stage score of 1.7, and 2 were injected in the allantois and 10 in the amnion. Embryos (12 total) selected from the FMG treatment incubation trays had an average development stage score of 1.6, and similarly, 2 embryos were injected in the allantois and 10 embryos were injected in the amnion. This information is merely important to note that the attenuated bacteria (FMG vaccine) was deposited within the amnion of the embryo near the time of hatch in the majority of the eggs. The location of vaccine deposition is important to note and can have potential implications on the immune response of the hatched chick (Wakenell et al., 2002 Avakian, 2006;).

Mortality

The birds that were reared experienced mortality during the first 2 weeks after hatch as in previous trials (Elliott et al., 2018, 2020). The first mortalities (aside from sampled birds) occurred at d 3 of age and continued until d 14 of age. Only one mortality occurred after 14 d of age: a female from the control group died on d 19 due to intestinal obstruction/volvulus (Fulton, 2017). Additionally, one control male chick escaped from its pen during catching on d 3 and was removed from the study due to biosecurity concerns. Thus, percentage mortality during the first 2 wk, calculated as a percentage of 383 chicks placed in the control group and the 384 chicks placed in the FMG treatment group, was 1.0% for the control birds and 12.2% for the FMG birds (Table 1).

The daily mortality rate for each treatment and sex are presented in Figure 1. The control bird mortalities occurred on d 3, 6, and 7 and consisted of 2 males and 2 females for a 50:50 sex ratio (Table 1). Upon necropsy, the deceased control birds showed symptoms associated with omphalitis.

Percentage mortality for the FMG birds by 7 d of age was 7.8% with the greatest numbers of birds found dead on d 5, 6, and 7. The 2 wk mortality of the FMG group is one of the higher levels recorded in which this dilution of the Poulvac Myco F vaccine was tested for in ovo use. Previous studies have recorded mortality levels ranging from 1.1% to 11.7% at this dosage level during the first 2 wk of rearing for straight-run Hy-Line W-36 birds (Elliott et al., 2018, 2020). In the FMG group, however, 72.3% of the mortalities were male during the first 2 wk (Table 1). During the first week mortalities (d 3−7),

Table 1. Total percentage mortality of control (non-vaccinated) and FMG *in ovo*-vaccinated[1] Hy-Line W-36 layer chickens within the first 14 d post-hatch[2].

Control	1.0%
FMG in ovo	12.2%

Percentage of the 14 d mortality of control (nonvaccinated) and FMG in ovo-vaccinated layer chickens by sex[3]

	Male	Female
Control (of the 1.0%)	50%	50%
FMG in ovo (of the 12.2%)	72.3%	27.7%

[1]Chickens were vaccinated with Poulvac Myco F (Zoetis) live attenuated strain F *Mycoplasma gallisepticum* (FMG) vaccine at 18 d of incubation with a 10^{-6} dilution of the vaccine in a 50 μL volume. The vaccine was resuspended and diluted in Poulvac Marek's diluent (Zoetis, Exton, PA).

[2]Percentage mortality calculated as the total number of dead birds out of 383 total birds in the control group and 384 total birds in the FMG in ovo vaccinated group

[3]Sex was determined by necropsy of the deceased birds and evaluation of the internal reproductive organs.

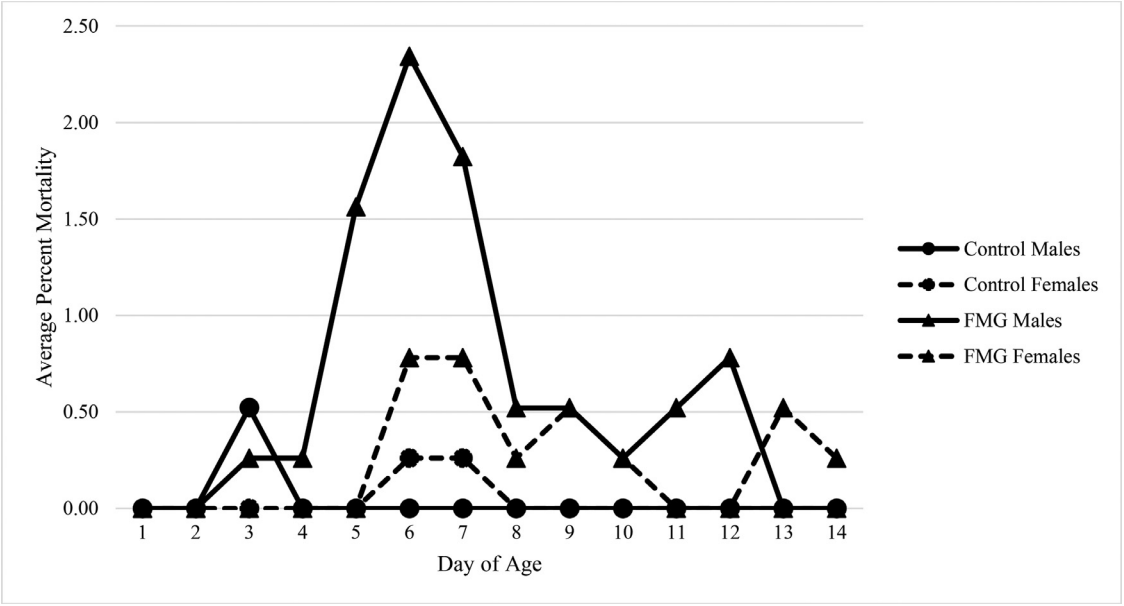

Figure 1. Percentage mortality by day, treatment, and sex of Hy-Line W-36 layer chickens from a control (nonvaccinated) group and a strain F *Mycoplasma gallisepticum* (FMG) in ovo-vaccinated group. The control birds were hatched without receiving any in ovo vaccination and were hatched and reared separately from any FMG-vaccinated birds. The FMG birds were administered a 50 μL volume injection volume of a 10^{-6} dilution of the Poulvac Myco F vaccine (Zoetis, New York, NY) diluted in Poulvac Marek's diluent (Zoetis, Exton, Pennsylvania) at 18 d of incubation. Daily percentages are taken out of 383 birds for the control group and out of 384 birds for the FMG group.

80% of the 7.8% mortality were males. Thus, in total, the 2 wk mortality rate for pullets in the FMG treatment group totaled 3.4%. Male mortality within the FMG treatment group totaled 8.8%. To our knowledge, this is the first confirmation of male-biased mortality due to *Mycoplasma gallisepticum* in a domestic chicken.

The earliest mortality in the FMG-treated birds on d 3 and 4 exhibited only dehydration. Birds found dead on d 5 from the FMG group showed the first incidences of airsacculitis in 5 out of the 6 birds. Caseous exudate was first found in the day 6 mortality. Caseous exudate that predominantly occurred in the cranial airsacs was found in the majority of the total number of deceased FMG birds (35/47 or 74.5%). Nearly all FMG birds that deceased showed signs of dehydration (43/47 or 91.5%). The findings of airsacculitis and caseous exudate hint that mortality was in-part due to effects of the FMG in the FMG-in ovo vaccinated birds.

Male house finches have been known to have a greater susceptibility to MG infection during an MG pandemic (Nolan et al., 1998). In mice infected with *Mycoplasma pulmonis*, males had more severe respiratory disease symptoms than females, and neutered male mice showed a reduction in disease symptoms (Yancey et al., 2001). The effects of testosterone on the adaptive immune system of chickens has also been shown. It was found that an adequate method to reduce or inhibit the development of the Bursa of Fabricius was to place embryonated chicken eggs in testosterone propionate at 3 d of incubation (Glick and Sadler, 1961). Thus, Bradbury (2005) posed the question, "Does gender influence the severity of poultry mycoplasma respiratory disease?" Based upon the mortality findings of this study, young Hy-Line W-36 males up to 2 woa are more susceptible to a vaccine containing attenuated strain F of *Mycoplasma gallsepticum* bacteria.

High levels of estradiol within the developing chicken embryo can also have negative effects on the development of the Bursa of Fabricius (Norton and Wira, 1977). A recent paper has highlighted the changes in the immune cells present within the blood and spleen of laying hens and has demonstrated that more dramatic changes correspond with the onset of lay (Schmucker et al., 2021). Susceptibility of chickens to FMG at an older age was not tested in this current study but would comprise an interesting future study to evaluate the susceptibility of females once they are producing more steroid hormones closer to the onset of lay. However, *Mycoplasma gallisepticum* is a concern of the commercial layer industry, which does not retain commercial males for table egg production. Determination of the sex of the birds that died in this study indicates that the higher mortalities seen in previous studies that also tested this in ovo vaccine dose of FMG (Elliott et al., 2018, 2020) would more likely have observed a lower mortality if only pullets had been reared.

Body Weights

Body weights of the birds selected for sampling on each day during the study are presented in Table 2. Due to mortality, less than 54 birds per treatment were sampled in the FMG treatment on d 21 and 28, and for the control group on d 28. Starting from hatch through 4 woa, there was no effect due to the in ovo vaccination of FMG on the BW of the birds. Males, however, as expected, obtained a greater BW on d 5, 7, 21, and 28. Birds that had been in ovo-vaccinated with the current dose did have BW at 6 woa that was decreased by 13.8 g

Table 2. Average BW (grams) of control (non-vaccinated) and Strain F *Mycoplasma gallisepticum* (FMG) in ovo-vaccinated[1] Hy-Line W-36 layer chickens from 0 to 28 d of age by sex[2].

	Hatch	D 2	D 3	D 5	D 7	D 14	D 21	D 28
Control								
Male	40.5	41.4	46.4	56.8	70.6	122.7	200.1	303.7
Female	40.5	41.5	44.6	55.0	67.9	118.5	183.3	260.6
FMG in ovo								
Male	41.0	42.4	46.4	57.6	68.2	122.3	205.4	305.5
Female	40.0	41.1	46.3	54.3	65.8	121.7	176.4	263.5
Pooled SEM	0.3	0.2	0.4	0.5	0.6	1.3	3.2	4.5
Probability								
Trt	0.9212	0.5077	0.3233	0.9480	0.1912	0.5775	0.8919	0.7688
Sex	0.4579	0.2257	0.2306	0.0164	0.0248	0.3487	0.0004	<0.0001
Trt × Sex	0.4940	0.1424	0.2516	0.4794	0.8536	0.4774	0.3599	0.9439
Numbers of birds weighed								
Control	54	54	54	54	54	54	54	53
FMG in ovo	54	54	54	54	54	54	34	33

[1]Chickens were vaccinated with Poulvac Myco F (Zoetis) live attenuated strain F *Mycoplasma gallisepticum* (FMG) vaccine at 18 d of incubation with a 10^{-6} dilution of the vaccine in a 50 μL volume. The vaccine was resuspended and diluted in Poulvac Marek's diluent (Zoetis, Exton, PA).
[2]Sex was determined by necropsy of the deceased birds and evaluation of the internal reproductive organs.

in comparison to non-injected controls in one study (Elliott et al., 2018), but exhibited no significant difference at 6 woa in another study (Elliott et al., 2020). Further tests utilizing only pullets in larger sample sizes would help elucidate any vaccine effect on BW for commercial application.

Sample Bird Necropsy

All sampled birds were additionally evaluated for any airsacculitis, congested lungs, and any amount/incident of caseous exudate present within the body upon necropsy. Within the control group, one bird (interestingly all females) on d 0 (hatch), 2, 5, and 21 were found to have some incidence of caseous exudate present in the cranial aspect at the base of the heart. On d 5, 5 female control birds were noted to have congested lungs. No other incident of congested lungs was found in any control birds. No incidence of airsacculitis was noted in any control birds during the duration of the study. All control birds on d 7, 14, and 28 appeared to be normal with no health concerns upon necropsy.

Within the FMG in ovo treatment, none of the birds that were sampled exhibited airsacculitis. One female on d 3 and one male on d 5 were noted to have congested lungs, but no other incident of congested lungs was found within any of the sampled birds in the FMG treatment throughout the duration of the study. A speck of caseous exudate was frequently present within the bodies of the birds. The percentage incidence of casesous exudate within each sex belonging to the FMG treatment is presented within Table 3. The percentages of the incidence of caseous exudate within the males is numerically higher than that of the females on sample d 3, 5, 14, 21, and 28. In comparison to sampled males, the percentage of birds with some caseous exudate was numerically higher within the sampled females on d 7. However, there was only a 1% difference between the sexes on d 28. It is important to note that while some incidence of caseous exudate was found within birds sampled, no birds exhibited extensive incidences of caseous exudate during the study.

Thus, FMG-treated birds were noted to have very mild internal reactions comparable to the control group, with the exception of the incidences of caseous exudate that persisted in some sampled birds throughout the duration of the study. When compared to nonvaccinated and nonchallenged/infected birds, the FMG vaccine is known to have a negative effect on birds during lay (Carpenter et al., 1981). How these FMG in ovo-vaccinated pullets compare in performance to standard post-hatch vaccinated pullets still needs to be determined.

Antibody Detections

Detection of IgM antibody production by SPA analysis was conducted on all samples collected. The numbers of birds sampled and the percentage of birds showing IgM antibody production on each collection date are presented in Table 4. The percentage of FMG in ovo-vaccinated birds showing IgM antibody production was 1.9% on d 7, 31.5% on d 14, 55.9% on d 21, and 60.6% on d 28. A sample from one control bird tested positive

Table 3. Percentages of any incidence of caseous exudate within each sex[1] on 8 sample days in Hy-Line W-36 layer chickens that had been in ovo vaccinated with a strain F *Mycoplasma gallisepticum* vaccine[2].

	Percentage incidence out of males or females							
	Hatch	2	3	5	7	14	21	28
Male	0.0%	0.0%	6.5%	17.4%	22.7%	36.0%	42.9%	27.8%
Female	3.7%	5.9%	0.0%	12.9%	25.0%	10.3%	15.4%	26.7%
	Total numbers sampled of each sex							
Male	27	20	31	23	22	25	21	18
Female	27	34	23	31	32	29	13	15

[1]Sex was determined by necropsy of the deceased birds and evaluation of the internal reproductive organs.
[2]Chickens were vaccinated with Poulvac Myco F (Zoetis) live attenuated strain F *Mycoplasma gallisepticum* (FMG) vaccine at 18 d of incubation with a 10^{-6} dilution of the vaccine in a 50 μL volume. The vaccine was resuspended and diluted in Poulvac Marek's diluent (Zoetis, Exton, PA).

Table 4. Percentage of control (non-vaccinated) and Strain F *Mycoplasma gallisepticum* (FMG) in ovo-vaccinated[1] Hy-Line W-36 layer chickens that tested positive[2] for IgM and IgG antibody production against MG (*Mycoplasma gallisepticum*) from 0 to 28 d of age.

	IgM antibody production (% Serum plate agglutination positive)							
	Hatch	D 2	D 3	D 5	D 7	D 14	D 21	D 28
Control	0	0	0	0	0	0	1.9	0
FMG in ovo	0	0	0	0	1.9	31.5	55.9	60.6
	IgG antibody production (% ELISA positive)							
Control	NA	NA	NA	NA	NA	0	0	0
FMG in ovo	NA	NA	NA	NA	NA	0	2.9	21.2
	Numbers of birds tested							
Control	54	54	54	54	54	54	54	53
FMG in ovo	54	54	54	54	54	54	34	33

[1]Chickens were vaccinated with Poulvac Myco F (Zoetis) live attenuated strain F *Mycoplasma gallisepticum* (FMG) vaccine at 18 d of incubation with a 10^{-6} dilution of the vaccine in a 50 μL volume. The vaccine was resuspended and diluted in Poulvac Marek's diluent (Zoetis, Exton, PA).

[2]Blood samples collected from the birds were tested for IgM antibodies against MG by serum plate agglutination (SPA) testing and were tested for IgG antibodies against MG by ELISA testing.

Table 5. The percentage of each sex[1] that tested positive[2] for IgM and IgG antibody production in the FMG in ovo vaccination treatment[3] at 14, 21, and 28 d of age.

	IgM antibody production (% Serum plate agglutination positive)		
Age	Total # positive	% Male positive	% Female positive
14	17	58.8	41.2
21	19	73.7	26.3
28	20	50.0	50.0
	IgG antibody production (% ELISA positive)		
Age	Total # positive	% Male positive	% Female positive
14	0	0.0	0
21	1	0	100
28	7	42.9	57.1

[1]Sex was determined by necropsy of the deceased birds and evaluation of the internal reproductive organs.

[2]Blood samples collected from the birds were tested for IgM antibodies against MG by serum plate agglutination (SPA) testing and were tested for IgG antibodies against MG by ELISA testing.

[3]Chickens were vaccinated with Poulvac Myco F (Zoetis) live attenuated strain F *Mycoplasma gallisepticum* (FMG) vaccine at 18 d of incubation with a 10^{-6} dilution of the vaccine in a 50 μL volume. The vaccine was resuspended and diluted in Poulvac Marek's diluent (Zoetis, Exton, PA).

for IgM antibody production on d 21, however this sample tested negative for IgG antibody production.

Assessments of IgG antibody production were performed using ELISA on all samples collected on d 14, 21, and 28. The ELISA results were negative for all the control samples tested. At 14 d of age, all FMG-treated birds tested negative for IgG antibody production. On d 21 of age, one female out of 34 total birds sampled in the FMG treatment group bird tested positive for IgG antibody production. This bird had also tested SPA positive. At 28 d of age, out of 33 sampled birds from the FMG group, 7 birds (4 females and 3 males) tested positive for IgM and IgG antibody production. Thus, in the birds vaccinated in ovo with live FMG at 18 d of incubation, the switch from IgM to IgG antibody production occurred in 0.0% of the birds at 2 woa, in 2.9% of the birds at 3 woa and in 21.2% of the birds at 4 woa (4 weeks plus 4 days since the initial antigen introduction to the immune system of the bird).

The percentage of each sex that tested positive for IgM and IgG antibody production within the FMG in ovo-vaccinated treatment are presented in Table 5. Of the positive birds, more males than females tested positive for IgM antibody production at 14 and 21 d of age. However, at 28 d of age, the percentage of positive tests were the same for both sexes. Again, the one bird that received FMG by in ovo injection, and tested positive for IgG antibody production at 21 d of age was a female. The sex ratio of the birds that tested positive for IgG antibody production at 28 d in the FMG treatment group was nearly equal (3 positive males and 4 positive females).

The average ELISA titers at 14, 21, and 28 d for all birds tested are presented in Table 6. The birds in the FMG in ovo treatment had greater overall titers than the control birds on each tested date. The analysis of the interaction of treatment and sex was not significant but approached significance on d 14 and 21. No significant main effect of sex was noted on the average ELISA titers.

In previous studies at 6 woa following in ovo FMG vaccination, tested birds were 58.2 or 62.2% SPA positive and 42.0 or 30.3% ELISA positive (Elliott et al., 2018, 2020). According to the results of the present study, the birds reached the previously seen 6 wk SPA positive percentage at approximately 3 to 4 woa. The percentage ELISA positive birds in the present study had not yet reached a range of 30 to 40% that has been observed in the previous 6 wk old bird studies. In one study, no sex effect on ELISA titers was found at 6 woa (Elliott et al., 2018). In another study, male Hy-Line W-36 birds had a significantly higher antibody titer than females (Elliott et al., 2020). From this current study, no strong evidence of a greater immune response in males as compared with females was found, even though there was a clear difference in mortality between the sexes. However, only antibody production and class switching were evaluated. There are many facets of the total immune response that have not been evaluated and should be examined in future research concerning the effects of FMG in ovo vaccination.

Table 6. Average ELISA titers of control (non-vaccinated) and FMG in ovo vaccinated[1] Hy-Line W-36 layer chickens at 14, 21, and 28 d of age.

	D 14	D 21	D 28
Control	147.2	218.1	155.1
FMG in ovo	288.1	416.1	660.6
Pooled SEM	18.8	23.7	45.7
Probability			
Trt	0.0062	0.0095	<0.0001
Sex[2]	0.4870	0.2729	0.1306
Trt × Sex	0.0695	0.0607	0.2660

[1]Chickens were vaccinated with Poulvac Myco F (Zoetis) live attenuated strain F *Mycoplasma gallisepticum* (FMG) vaccine at 18 days of incubation with a 10^{-6} dilution of the vaccine in a 50 μL volume. The vaccine was resuspended and diluted in Poulvac Marek's diluent (Zoetis, Exton, PA).

[2]Sex was determined by necropsy of the deceased birds and evaluation of the internal reproductive organs.

SUMMARY

The earliest antibody production (IgM antibody production) in response to in ovo FMG vaccination was found in one bird at 7 d of age. Percentages of IgM positive birds continued to increase particularly in the 2nd and 3rd wk post-hatch. Previous trials (Elliott et al., 2018, 2020) showed that 58.2 and 62.2% of FMG in ovo-vaccinated birds tested positive for IgM antibody production at 6 woa. The current study demonstrated a similar percentage 3 wk earlier at 3 woa.

The earliest class switching to IgG antibody production started at 3 woa in 2.9% of the FMG in ovo-vaccinated birds and increased to 21.2% of the birds at 4 woa but had not yet reached the percentages of birds that exhibited IgG antibody production observed in birds at 6 wk post-hatch in previous studies (Elliott et al., 2018, 2020).

There was no effect of the in ovo vaccination of FMG on BW through 4 woa. More male chicks than female chicks died early due to the in ovo vaccination of FMG. No evidence was found to indicate that females were more likely to produce antibodies or have greater antibody titers in response to the vaccination. As only female layer chickens are reared for commercial egg production, a lower initial mortality due to the in ovo vaccination of FMG is expected (at approximately 3.4%) when only female rather than straight-run birds are reared.

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DISLOSURES

The authors have no conflicts of interest.

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