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Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: field and experimental studies

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## Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: field and experimental studies

A. Feberwee, J.J. de Wit & W.J.M. Landman

### Abstract

A novel eggshell pathology, characterized by an altered shell surface, thinning, increased translucency, and cracks and breaks in the eggshell apex, has become increasingly common in layer flocks of various breeds in The Netherlands. Two field studies found an association between the eggshell apex abnormalities (EAA) and infection with *Mycoplasma synoviae*. *M. synoviae* was isolated from the oviduct of birds that produced abnormal eggs, but not from birds in control flocks, although both affected and control birds had agglutinating antibodies against *M. synoviae*. After a single injection with long acting oxytetracycline, the production of abnormal eggs ceased, but then reoccurred about 12 days later. A causal relationship between EAA and *M. synoviae* infection was subsequently demonstrated experimentally. EAA occurred after intratracheal inoculation of birds with *M. synoviae*, and even more frequently in birds infected with infectious bronchitis virus (IBV) five days before inoculation of *M. synoviae*. EAA also occurred, although less frequently, in birds inoculated intravenously with *M. synoviae* and infected with IBV. It did not occur in birds only inoculated intravenously with *M. synoviae*. *M. synoviae* was only isolated from the oviducts of birds producing abnormal eggs. The mean daily egg production was reduced in all groups infected with *M. synoviae*. Examination of the eggshells by scanning electron microscopy revealed that the mammillary layer of the calcified zone was absent and that the inner eggshell membranes were thicker. Isolates of *M. synoviae* from the oviducts of birds from farms producing abnormal eggs were examined by amplified fragment length polymorphism analysis and did not appear to be clonal.

## Introduction

*Mycoplasma synoviae* is considered the second most important mycoplasma affecting chickens (Stipkovits & Kempf, 1996; Kleven & Ley, 2003). It causes respiratory disease and subsequent condemnations due to airsacculitis, although most respiratory tract infections seem to be subclinical. *M. synoviae* also causes synovitis in chickens and turkeys (Landman & Feberwee, 2001; Kleven, 2003; Landman & Feberwee 2004).

The effect of *M. synoviae* infections on egg production by layer chickens remains unclear. In one report a cumulative egg production loss of 10% was seen in flocks infected with *M. synoviae* (Morrow *et al.*, 1990), but in another study no association was seen between *M. synoviae* infection and egg production (Mohammed *et al.*, 1987). However, layer flocks infected with arthropathic strains of *M. synoviae* may suffer severe losses from growth retardation and culling of lame birds (Landman & Feberwee 2001; van Beek *et al.*, 2002).

Since 2000, eggshell apex abnormalities (EAA) have been increasingly seen in layer flocks in The Netherlands. The EAA are characterized by a roughened shell surface, shell thinning, increased translucency, cracks and breaks. The abnormalities are confined to the top cone of the egg, up to approximately 2 cm from the apex, and almost always have a very clear demarcation zone. The proportion of affected eggs varies between flocks, from a few percent up to 25%. After onset, abnormal eggs are produced throughout the remainder of the laying period. The economic impact, which results from eggshell breakage, parallels the occurrence of defective eggshell apices. EAA were first described in white layers housed in cages, but was later also seen in brown layers housed in cages, and in both types of birds kept in other housing systems.

The aim of the work described here was to determine whether there is an association between infection of the oviduct with *M. synoviae* and the production of abnormal eggs. Initially two field studies were conducted to look for this association. Subsequently the causal relationship between infection of oviducts with *M. synoviae* and EAA was examined by experimentally infecting birds with *M. synoviae*.

## Materials and Methods

**Field studies.** *Field study 1.* Three caged white layer flocks on different farms producing eggs with EAA were studied. The proportion of eggs with EAA on each of the three farms was 3, 10 and 25%, respectively. A further three flocks (one of white and two of brown layers, all housed on the floor) that had agglutinating antibodies against *M. synoviae*, but did not produce abnormal eggs, were also included. Between three and six birds were investigated from each flock. In the flocks producing eggs with EAA, only birds producing abnormal eggs, were examined. The chickens were stunned using CO<sub>2</sub> and O<sub>2</sub>, exsanguinated by incision of the jugular vein and examined macroscopically. Serum was collected for *M. synoviae* serology, and swabs taken from the oviducts were used for general bacteriology and mycoplasma culture. Additionally, DNA was extracted from *M. synoviae* cultures for molecular typing.

*Field study 2 (Treatment trial).* A second longitudinal study was conducted over 7 weeks on another affected flock. Forty 70-week-old white layers that were producing eggs with EAA were housed in individual cages at the farm. Three weeks after commencement of the study (W3), 20 of the birds were individually treated once subcutaneously (0.5 ml/chicken) with long acting oxytetracycline (Terramycin/LA, Pfizer, Capelle a/d IJssel, The Netherlands). A layer flock that was serologically positive for infection with *M. synoviae*, but that was not producing abnormal eggs, was included as a control. The control birds were 3 weeks younger than those in the affected flock. Birds on both farms had been vaccinated against Newcastle disease virus (NDV), infectious bronchitis virus (IBV) and avian metapneumovirus (AMPV), and were serologically negative for infection with avian influenza virus, *M. gallisepticum* and the egg drop syndrome (EDS) adenovirus.

Blood samples for *M. synoviae* serology were collected at day 0 (D0), week 3 (W3), week 4 (W4) and week 7 (W7) of the study. At W7 blood samples were also collected from the control farm for *M. synoviae* serology. At W4 ten medicated and 10 unmedicated birds were autopsied. At W7 the remaining birds were autopsied. Ten birds that originated from the affected farm but that were not producing eggs with EAA were used as controls, as well as 10 birds of approximately the same age from the control farm. Chickens were stunned as described above, exsanguinated, subjected to macroscopic examination, serum collected for *M. synoviae* serology, and swabs taken from the oviducts for general bacteriology and mycoplasma culture.

The production of eggs with EAA, shell-less eggs and broken eggs was recorded daily over the 7 weeks by the farmer. Egg quality (egg white thickness, Haugh units and egg shell

strength) was determined at W3 (before treatment), W4 and W7 (after treatment) for up to 19 eggs from the untreated birds and up to 19 eggs from the treated birds. Egg quality was also measured at W3, W4 and W7 for 20 eggs from the control farm.

DNA was extracted from *M. synoviae* cultures for molecular typing. The shells of one normal egg and one egg with EAA were examined by scanning electron microscopy (SEM).

**Experimental infection study.** Seven-week-old commercial white layer pullets that were serologically negative for egg-drop syndrome (EDS) adenovirus, avian influenza virus, *M. gallisepticum* and *M. synoviae* infection were bought from a poultry farm. They had been vaccinated twice against IBV (on day 1 with a Massachusetts-like vaccine strain by spraying, and on day 14 with a 793B-like vaccine by spraying) and NDV (on day 7 and 28 by spraying), and once against infectious bursal disease virus (on day 10). All the birds were kept together in a floor pen until they were 16 weeks of age. They were then weighed, divided into weight classes, allocated into five experimental groups and left to acclimatize for two weeks. Each group was housed in a different negative pressure HEPA-filtered isolator (194 cm wide, 95 cm high and 75 cm deep; Beyer & Eggelaar, Utrecht, The Netherlands) containing four laying nests. The temperature ranged from 18 to 20°C and birds were exposed to fourteen hours of light per day, with food and drinking water provided *ad libitum*.

A *M. synoviae* isolate (chicken/NL/Dev/SP255/Tom/05) obtained from the oviduct of a bird from Farm 3 of Field study 1 and cultured in Mycoplasma Experience (ME) broth (Mycoplasma Experience, Reigate, Surrey, UK) and IBV strain D1466, grown in the allantoic cavity of embryonated eggs, were used for experimental infections. The five experimental groups included: a negative control group ( $n = 12$ ) inoculated intravenously (i.v.) with 2 ml ME broth; a *M. synoviae* i.v. group inoculated i.v. with 2 ml broth containing  $10^6$  colony forming units (cfu) *M. synoviae*/ml ( $n = 18$ ); a *M. synoviae* i.v./IBV group inoculated intratracheally (i.t.) with 1 ml and intramuscularly with 0.5 ml allantoic fluid containing  $10^{6.6}$  median egg infectious doses (EID<sub>50</sub>) IBV/ml, then 5 days later with 2 ml broth containing  $10^6$  cfu *M. synoviae*/ml ( $n = 17$ ); a *M. synoviae* group inoculated i.t. with 1 ml broth containing  $10^6$  cfu *M. synoviae* ( $n = 18$ ); and a *M. synoviae* i.t./IBV group inoculated i.t. with 1 ml and intramuscularly with 0.5 ml allantoic fluid containing  $10^{6.6}$  EID<sub>50</sub> IBV/ml, then 5 days later inoculated i.t. with 1 ml broth containing  $10^6$  cfu *M. synoviae*/ml ( $n = 17$ ).

Blood samples for *M. synoviae* and IBV serology were collected from individual chickens at D0 (IBV inoculated groups only), W4, W8 and W11. Chickens were also tested

for the presence of antibodies against EDS adenovirus and *M. gallisepticum*. At D0 and W11 the birds were weighed individually.

At W11 all birds were stunned and exsanguinated as described above at the time that calcified eggs were expected to be present in the uterus (between 10 a.m. and 12 noon). Serum was collected for *M. synoviae* serology, and swabs taken from the oviducts were used for general bacteriology and mycoplasma culture. Histology was performed on sections of the oviduct of three hens producing eggs with EAA and 3 hens from the negative control group.

From week 4 to 6 and week 9 to 11 the eggshell strength was measured using an eggshell compression device. At week 4, 60 normal eggs collected from the control group, 60 normal eggs collected from the infected groups producing eggs with EAA and 60 eggs with EAA from the infected groups were tested. Between week 9 and 11, 30 normal eggs from the control group and from each infected group ( $n = 150$ ) and 30 eggs with EAA from the infected groups were also tested.

DNA extracted from the *M. synoviae* cultures was used for molecular typing and scanning electron microscopy (SEM) was performed on the shell of one normal egg and one egg with EAA.

**Serology.** Serum antibodies against *M. synoviae* were detected using the rapid plate agglutination (RPA) test. Sera were tested within 24 hours of collection of blood samples. Two-fold dilutions of sera were tested with the RPA antigen (Nobilis MS antigen batch numbers 74074A and A012A01, Intervet International, Boxmeer, The Netherlands) as described by Feberwee *et al.* (2005). The sensitivity and specificity of both batches were assessed before use with a standardised panel of sera, following the international standard ISO 17025 (2005). Positive sera were then serially diluted from 1:4 to 1:32 in phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 0.27 g  $\text{KH}_2\text{PO}_4$  and 1.16 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  per litre, pH 7.2) and re-tested. Agglutination titres of 8 or above were considered to be specific for *M. synoviae*. Serum antibodies against *M. gallisepticum* were detected using a RPA test (Nobilis MG antigen, Intervet International) and a haemagglutination inhibition (HI) test as described by Feberwee *et al.* (2005). Agglutination and HI titres of 2 or less were regarded negative. Antibodies against IBV and the EDS adenovirus were detected using HI tests, and those against avian influenza virus by ELISA (FlockChek AIV Antibody Test Kit, IDEXX Corporation, Westbrook, Maine, USA) as described by De Wit *et al.* (1997, 2004a, 2004b).



**Bacterial culture of the reproductive tract.** The outer surface of the oviduct was seared with a hot scalpel blade, an incision then made with a sterile scalpel and two sterile cotton swabs used to swab both the isthmus and the uterus. One swab was used to inoculate a 5% sheep blood agar (SBA) plate and the other a ME mycoplasma agar plate (Mycoplasma Experience). The ME agar plates were incubated at 37°C in a humid environment and examined for colonies every 2-3 days for up to 28 days. One isolated colony was subcultured onto a fresh ME agar and a 2 x 0.5 cm<sup>2</sup> piece of agar containing mycoplasma colonies transferred into 5 ml ME broth and incubated at 37°C.

Positive mycoplasma cultures were identified as *M. synoviae* by polymerase chain reaction (PCR). In Field study 1, one culture per farm was examined to confirm that it was *M. synoviae*, in Field study 2, five cultures from birds producing abnormal eggs that were treated with oxytetracycline, four cultures from birds producing abnormal eggs that were not treated with oxytetracycline and two cultures from control birds that were not producing abnormal eggs were examined to confirm that they were *M. synoviae* and in the experimental infection study one or two positive oviduct cultures were examined per experimental group.

**Molecular identification.** Positive broth cultures were pelleted for 10 min at 16,000 x g and the pellets were resuspended in 200 µl of sterile PBS. DNA was extracted with QiaAmp DNA mini kits (Qiagen Benelux B.V., Venlo, The Netherlands) using the protocol for cultured animal cells provided with the kits (Mekkes & Feberwee, 2005). The forward primer for identification of *M. gallisepticum* by quantitative PCR (Q-PCR) was 5'-GAG CTA ATC TGT AAA GTT GGT C-3' and the reverse primer was 5'-GCT TCC TTG CGG TTA GCA AC-3' (Lauerman, 1998). These primers amplify a 186 bp fragment of the 16S ribosomal RNA gene of *M. gallisepticum*. For the *M. synoviae* Q-PCR the forward primer was 5' GAG AAG CAA AAT AGT GAT ATC A-3' and the reverse primer was 5' CAG TCG TCTCCG AAG TTA ACA A-3'. These primers amplify a 211 bp fragment of the 16S ribosomal RNA gene of *M. synoviae*.

**Molecular epidemiology.** The DNA extracted from two isolates from Field study 1 (SP2005-00257 A (Farm 1) and SP2005-00255 B (Farm 3)), from five isolates from Field study 2 (two isolates from untreated birds necropsied at W7 (SP2007-00333-3 and SP2007-00333-4), two isolates from treated birds necropsied at W7 (SP2007-00333-14 and SP2007-00333-15) and one isolate from one control bird that was not producing abnormal eggs (SP2007-00426-1), and from two isolates from the experimental infection study (SP2007-00597-15 and SP2007-

00601-24), were used for molecular typing by amplified fragment length polymorphism (AFLP) (Feberwee *et al.*, 2006). In addition, DNA from isolates of *M. synoviae* from two other farms that were not included in the other studies but that had hens producing eggs with EAA (SP2002-00804 and SP2007-01054) and four reference strains were analyzed. The *M. synoviae* reference strains were ATCC 25204 (M1996.01), K870 (M2001.29), K1968 (M2001.27) and Chicken/NL/Dec/801979Rob/00 ((M2000.05) (Feberwee *et al.*, 2004; 2006). The DNA concentrations for AFLP analysis were between 4 and 15 ng/ $\mu$ l. Digestion with *Hind*III and *Hha*I, ligation of AFLP adaptors and amplification of the modified fragments were carried out as previously described by Feberwee *et al.* (2006). Site-specific adaptors were ligated to the digestion products and a pre-selective PCR amplification was followed by a selective PCR using one fluorescently labeled primer containing a selective sequence. Final products were analyzed on the Applied Biosystems Avant 310 DNA sequencer. The AFLP banding patterns were imported into Bionumerics 4.61 and comparisons were made using the unweighted paired group method with arithmetic averages (UPGMA).

**Assessment of egg production and egg quality, and estimation of economic impact.** Lay was synchronized to 10 a.m. and total egg and abnormal egg production (including broken eggs) were assessed daily between 1 and 2 p.m.

Eggshell strength and the egg quality measurements (Haugh units and egg white thickness) were determined using the Futura egg quality measuring system version 3/A (Futura-Werner Fürste Gbr, Lohne, Germany). The eggshell strength was measured using an eggshell compression device (Futura 3/A, OQT-II) and a height gauge was used to measure the Haugh units, egg white and the albumen (Futura 3/A, FIE-A).

The economic impact of EAA was estimated based on the average number of eggs with EAA, the loss in production and the increased costs related to breakage of soft shelled eggs, the increased percentage of downgraded eggs and the increased labour costs resulting from the need to identify abnormal eggs and from the need to clean equipment after egg breakage.

**Histological examination of the oviduct.** The whole oviducts of hens producing eggs with EAA ( $n = 3$ ) and of those producing normal eggs ( $n = 3$ ) were collected for histopathological examination. Oviducts were fixed in 4% buffered formalin and paraffin embedded. Sections of the infundibulum, the magnum, the transition zone between the magnum and isthmus, the isthmus and the uterus were stained with haematoxylin/eosin.

**Scanning electron microscopy (SEM) of the eggshell.** The structure of the eggshell was studied in two eggs with EAA and two normal eggs using SEM. Samples were submerged in Karnovsky fixative for more than a week. After washing with 0.1 M cacodylate buffer pH 7.4, they were post-fixed in 2% osmium tetroxide, buffered with 0.1 M cacodylate pH 7.4, for 2 hours. The samples were then washed in 0.1 M cacodylate for 10 min and then 6 times in distilled water over 30 min. Thereafter, they were incubated for 1 h in 2% tannin and again washed 6 times in distilled water. Eggshell samples were then fixed in 2% OsO<sub>4</sub> in 0.1 M cacodylate buffer pH 7.4 for 1 h and washed in distilled water 3 times over 20 min. The samples were dehydrated in graded concentrations of acetone (50, 70, 80, 96 and 100%), spending at least at 30 min in each acetone concentration. Finally, the samples were critical-point dried in carbon dioxide (CPD 030 GRE, Bal-Tec AG, Balzers, Liechtenstein) according the manufacturer's manual and coated with platinum to a thickness of about 10 nm before SEM (XL30 SFEG, Fei Company, Eindhoven, The Netherlands).

The thickness of the calcified layer was measured using a universal SEM imaging platform based on Analysis 5 from Olympus Soft Imaging Systems ([www.soft-imaging.net](http://www.soft-imaging.net) Soft Imaging System, Münster, Germany). The thicknesses of the calcified eggshell layer of the apex of two eggs with EAA (one from Field study 2 and one from the experimental infection study) and two normal eggs (one from Field study 2 and one from the experimental infection study) were measured at 6-7 locations in 6 different fields for each egg.

**Statistical analysis.** The proportions of sera that had detectable antibodies against *M. synoviae* and the proportions of cultures of oviducts that yielded mycoplasmas were compared using the Two-Sample Proportion Test (Statistix<sup>®</sup>, 2005). IBV antibody titres, egg quality measurements, egg weights, egg production, production of eggs with EAA and the thicknesses of the calcified eggshell layer were analyzed using Kruskal-Wallis One Way analysis of variance. The Kruskal-Wallis all pairwise comparisons test was performed as a *post hoc* analysis in order to compare all possible pairwise differences between the means of the different treatment groups (Statistix<sup>®</sup>, 2005).

## Results

**Field study 1.** *M. synoviae* was only isolated from the oviducts of birds that produced eggs with EAA or that came from a flock that produced eggs with EAA, not from control flocks, although all groups contained birds with detectable antibodies against *M. synoviae* (Table 1). At postmortem no macroscopic abnormalities were seen other than cystic degeneration of the right oviduct in some birds.

**Field study 2 (Treatment trial).** Both the groups that produced eggs with EAA and the groups that only produced normal eggs contained hens with serum antibodies against *M. synoviae*. At postmortem at week 4 large numbers of mycoplasmas were isolated from the oviduct of 7/10 medicated and 8/10 unmedicated birds producing eggs with EAA. At the end of the experiment mycoplasmas were isolated from 9/10 medicated birds and 9/10 unmedicated birds (Table 2). Mycoplasmas isolated from the oviduct were identified as *M. synoviae* by PCR. *M. synoviae* was isolated from the oviducts of 2/10 birds producing normal eggs within a flock producing eggs with EAA but not from the oviducts of 9 hens from a farm that was not producing eggs with EAA. No other bacteria were isolated from the oviducts.

All birds in the longitudinal study produced eggs with EAA most of the time. In some cases soft-shelled eggs were also produced. A few days after antibiotic treatment the production of eggs with EAA ceased, but it reoccurred about 12 days later (Figure 1). Egg compression tests revealed a significant decrease in mean eggshell strength ( $P < 0.05$ ) in affected eggs ( $15.9 \pm 2.2$  N,  $n = 15$ ) compared to normal eggs ( $34.1 \pm 6.9$  N,  $n = 20$ ). After antibiotic treatment eggshell strength improved temporarily. One week after treatment the mean eggshell strength was  $30.4 \pm 10.8$  N ( $n = 13$ ). No significant difference was seen in the mean Haugh units or egg white thicknesses of affected and normal eggs (Table 2).

At postmortem no macroscopic abnormalities were detected in birds from the farm producing eggs with EAA or in those from the control farm.

**Characteristics of eggshell apex abnormalities and estimation of economic impact.** EAA was characterized by an altered shell surface, shell thinning, increased translucency and the occurrence of cracks and breaks (Figure 2). The eggshell abnormalities were confined to a region extending approximately 2 cm from the apex of the egg and in most cases there was a very clear demarcation zone (Figure 2). The estimated average economic loss of a flock in which 5% of the eggs had EAA between 30 weeks and 75 weeks of age was about 3% of the gross return on the egg price.

**Experimental infection study.** No serum antibodies against avian influenza virus, EDS adenovirus, *M. gallisepticum* or *M. synoviae* were detected in the flock of origin at week 7. No clinical signs of disease were observed in any experimental group throughout the study.

Antibodies against *M. synoviae* were only detected in the birds inoculated with *M. synoviae* (Table 3). Both groups that were inoculated with IBV developed HI antibodies against IBV, while the other groups did not.

*M. synoviae* could not be isolated from the oviducts of birds in the control group or from those of birds in the *M. synoviae* i.v. group. However, mycoplasmas were isolated from the oviducts of one layer in the *M. synoviae* i.v./IBV group, six in the *M. synoviae* i.t. group and seven in the *M. synoviae* i.t./IBV group. These were also the groups that produced eggs with EAA. The rate of isolation of mycoplasmas from oviducts of the *M. synoviae* i.t./IBV group (7/17) was significantly higher ( $P < 0.05$ ) than in the control group (0/12), the *M. synoviae* i.v. group (0/17) and the *M. synoviae* i.v./IBV group (1/16), but was not significantly different from the rate of isolation in the *M. synoviae* i.t. group (6/17). The rate of isolation of mycoplasmas from oviducts of the *M. synoviae* i.t. group was not significantly different ( $P = 0.0563$ ) from the rate of isolation in the control group, but was significantly different ( $P < 0.05$ ) from the rate of isolation from the *M. synoviae* i.v. group (Table 3).

The mean weights did not differ between the groups at D0. At W11 the mean increases in body weight in the *M. synoviae* i.v./IBV ( $368 \pm 18$  g) and *M. synoviae* i.t./IBV ( $372 \pm 20$  g) groups were significantly lower than in the control group ( $473 \pm 20$  g) and the *M. synoviae* i.t. group ( $486 \pm 28$  g) (Table 3). However, there was no significant difference between the weight increases of the *M. synoviae* i.v. group ( $384 \pm 21$  g) and those of the other groups.

The mean daily egg production per chicken was significantly lower in the *M. synoviae* i.t. group ( $0.49 \pm 0.02$  eggs) than in most of the other groups:  $0.75 \pm 0.02$  eggs in the controls,  $0.61 \pm 0.03$  eggs in the *M. synoviae* i.v. group and  $0.62 \pm 0.03$  eggs in the *M. synoviae* i.v./IBV group. The exception was the *M. synoviae* i.t./IBV group ( $0.54 \pm 0.03$  eggs) (Table 3).

Eggs with EAA were produced by the *M. synoviae* i.v./IBV group (15/778, 2%), the *M. synoviae* i.t. group (49/685, 7%) and the *M. synoviae* i.t./IBV group (96/673, 14%). The production of eggs with EAA started at day 37, 26 and 23 after *M. synoviae* inoculation, respectively, in these groups (Figure 3).

The mean rate of production of eggs with EAA was significantly higher in the *M. synoviae* i.t./IBV group ( $0.10 \pm 0.01$  eggs/chicken/day) than in the *M. synoviae* i.t. group

( $0.06 \pm 0.01$  eggs/chicken/day) and the *M. synoviae* i.v./IBV group ( $0.02 \pm 0.00$  eggs/chicken/day).

At weeks 4-5 egg compression tests revealed a significant ( $P < 0.05$ ) decrease in the average eggshell strength in affected eggs ( $16.8 \pm 0.9$  N ( $n = 60$ )) compared to that of unaffected eggs of all groups taken together (*M. synoviae* i.v., *M. synoviae* i.t. and *M. synoviae* i.t./IBV, ( $37.7 \pm 1.4$  N ( $n = 60$ )). At weeks 9-11 a significant difference was also found in the eggshell strength of affected eggs ( $12.7 \pm 1.0$  N ( $n = 30$ )) compared to that of eggs from the control group ( $31.7 \pm 1.8$  N) and unaffected eggs from the infected groups (*M. synoviae* i.v.,  $35.1 \pm 1.8$ , *M. synoviae* i.v./IBV,  $35.2 \pm 2.0$  N; *M. synoviae* i.t.,  $35.6 \pm 1.6$  N and *M. synoviae* i.t./IBV,  $37.2 \pm 1.6$  N).

At postmortem, which was performed between 9 and 10 a.m., no gross macroscopic abnormalities were detected in the oviduct. In 83% to 94% of the birds in each group an egg was found in the uterus. Eggs with EAA were found in one bird in the *M. synoviae* i.t. group and in three birds in the *M. synoviae* i.t./IBV group. The oviducts of these three birds in the *M. synoviae* i.t./IBV group also yielded positive mycoplasma cultures.

**Molecular epidemiology.** Three different clusters of *M. synoviae* strains could be identified by AFLP (Figure 4). Cluster 1 contained an isolate from Farm 1 (SP2005-00257 A), the isolate from joint lesions (M2000.5) and the reference strain K1968 (M2001.27). The patterns yielded by these organisms had more than 89% similarity with each other. Cluster 2 contained the reference strain K870 (M2001.29), and the pattern had 75% similarity with those in clusters 1 and 3. Cluster 3 contained two distinct groups of isolates, one containing the inoculum strain (SP2005-00255 B), two field isolates (IP2007-01054 and IP2002-00804), the isolates made from the oviducts during the experimental infection study (SP2007-00597-15 and SP2007-00601-24) and the isolates from both treated and untreated birds in Field study 2 (SP2007-00333-3, SP2007-00333-4, SP2007-00333-14 and SP2007-00333-15). The second group within cluster 3 contained the isolate from a hen producing normal eggs in Field study 2 (SP2007-00426-1) and the ATCC strain (M1996.01). The pattern produced by the inoculum strain had 88% similarity to the isolates from the oviducts of birds in the experimental infection study. The patterns yielded by all the reisolates from the oviducts had  $\geq 93\%$  similarity with each other. The patterns produced by the two isolates in cluster 3 from oviducts of birds from the field (SP2007-01054 and SP2002-00804) that came from farms producing eggs with EAA had 88% similarity with each other. The pattern produced by the isolate from the oviduct of a hen producing normal eggs in Field study 2 (SP2007-00426-1)

had 97% similarity to that produced by the ATCC strain (M1996.01) and 86% similarity to those produced by organisms in the cluster containing the other isolates from oviducts in this field study.

**Histological examination oviduct.** Histopathological lesions were not detected in the oviducts of any bird examined in the experimental infection study.

**Scanning electron microscopic examination of egg shells.** SEM analysis of eggshell samples demonstrated a change in the calcified layer and the inner membranes of the eggs with EAA. The mammillary knob layer (Figure 5) was absent in the apical shell and the palisade layer was reduced in width. The inner membranes were thicker, apparently as a result of an increase in the density of the meshwork of proteinaceous fibres (Figure 5).

The mean thickness of the calcified layer of the apex of the egg with EAA from Field study 2 ( $212 \pm 1.5 \mu\text{m}$ ) was significantly ( $P < 0.05$ ) less than that of the normal egg ( $311 \pm 1.7 \mu\text{m}$ ). The overall thickness of the shell of the egg with EAA from the experimental infection study was also significantly less than that of the normal egg ( $246.9 \pm 1.7 \mu\text{m}$  compared to  $286.7 \pm 1.7 \mu\text{m}$ ).

## Discussion

Eggshell apex abnormalities in table eggs, leading to a significant decrease in eggshell strength, are described here for the first time. The egg shell pathology was associated with an increased incidence of soft-shelled eggs and egg breakage, as well as a general decrease in egg production.

Our egg quality studies showed that eggshell strength was severely diminished (up to half of that of normal control eggs), which was in agreement with the translucency detectable macroscopically, particularly at candling. SEM provided a further explanation for the diminished eggshell strength and translucency: the apical shell of the eggs with EAA lacked the mammillary knob layer and part of the palisade layer. Whether the ultrastructural changes found in the apical eggshell are also present in other parts of the shell remains to be investigated. However, this is not likely as affected eggs almost invariably tended to break at the altered apex and macroscopically the region of abnormal eggshell appeared to be confined to the apex.

Both field studies demonstrated an association between the presence of *M. synoviae* in the oviduct and the production of eggs with EAA. This association was further supported by the fact that normal egg shell strength was temporarily regained after treatment of layers with long acting oxytetracycline.

A causal relation between EAA and *M. synoviae* infection was established by experimental infection. Eggs with EAA were produced by all the groups inoculated with *M. synoviae*, with the exception of the group only inoculated intravenously with *M. synoviae*. The number of eggs with EAA was highest in the *M. synoviae* i.t./IBV group. The birds in this group and the *M. synoviae* i.v./IBV group also had the slowest growth rate. The egg production per chicken was lowest in the *M. synoviae* i.t. and *M. synoviae* i.t./IBV groups. Total egg production and production of eggs with EAA may have been underestimated by the greater susceptibility of affected eggs to breakage, resulting in some losses before daily egg collection.

The synergism seen between *M. synoviae* and IBV was not unexpected, as it has been demonstrated previously in the pathogenesis of arthritis (Landman & Feberwee, 2004) and respiratory disease caused by *M. synoviae* (Kleven *et al.*, 1972; Springer *et al.*, 1974; Hopkins & Yoder, 1982). The IBV strain used in this study has been administered to layers in previous studies, but this has never resulted in the production of eggs with EAA (J.J. de Wit, unpublished data).

The production of eggs with EAA started three weeks after inoculation with *M. synoviae*, a similar incubation period to that seen in experimental induction of *M. synoviae* arthritis (Landman & Feberwee, 2001; 2004). It is notable that the induction of EAA by *M. synoviae* was dependent on the route of inoculation, with the i.t. route being most efficient. Mycoplasmas may be able to translocate from a colonized airsac to the adjacent ovary (Roberts & McDaniel, 1967). The results of the study reported here suggest that the translocation of *M. synoviae* from the air sacs to the oviduct is more efficient than colonization of the oviduct via the circulation. A predisposing factor affecting the oviduct, such as IBV infection, may be necessary to induce colonization of the oviduct via the i.v. route (Cavanagh & Naqi, 2003).

Other researchers have shown that the route of infection with *M. synoviae* affects pathogenicity. Inoculation via the food pad or the intravenous route are more likely to cause synovitis, while these lesions occur less frequently after respiratory exposure (Lockaby *et al.*, 1998; Landman & Feberwee, 2004). IBV infection also enhances the prevalence of joint pathology after aerosol exposure to *M. synoviae* (Landman & Feberwee, 2004).



The comparison of isolates of *M. synoviae* by AFLP showed that isolates from the oviducts could be assigned to two different clusters of strains, suggesting that they were not clonal. The similarity between the strain used for experimental infection and reisolates from the oviducts was 88%. Generally isolates with similarities of 90% or more are considered identical. However the inoculated strain could still be considered the same as the oviduct reisolates as Feberwee *et al.* (2005) have shown that intra-flock variation can occur (similarities of 85% have been seen between different isolates within one flock), indicating that genetic variation can occur over the course of infection. There is considerable capacity for generation of variation within the *vIhA* locus by site-specific recombination (Noormohammadi *et al.*, 2000) and it is possible that this variation results in some heterogeneity within AFLP patterns within a single clone. The lack of clonality suggests that a common virulence factor or a common predisposing factor might explain the novel tissue tropism involved in EAA.

Although *M. synoviae* was isolated in large numbers from the oviduct, this was not associated with histologically detectable oviduct abnormalities. Nevertheless, the eggshell defect, which was mainly located in the mammillary layer of the calcified zone, may have been caused by functional and/or ultrastructural defects in the oviduct.

It is not clear how *M. synoviae* affects the normal eggshell calcification process or why the defect is confined to a distinct zone at the apex of the egg. *M. synoviae* may affect the composition and concentration of eggshell matrix proteins in the uterine fluid, which are needed for the regulation of the growth of calcite during eggshell calcification (Gautron *et al.*, 1997; Hincke *et al.*, 1999, 2003). *M. synoviae* may also affect ciliary motility in the oviduct, which could lead changes in the uterine fluid content affecting the deposition of calcium carbonate crystals (Dominiquez-Vera *et al.*, 2000). Preferential colonization of a specific area of the uterus might also offer an explanation for the localisation of the eggshell defects.

Treatment with oxytetracycline improved eggshell strength significantly, but the effect was temporary, an observation in agreement with the other studies showing that antimicrobial therapy is unable to eliminate *M. synoviae* from infected flocks (Kleven & Ley, 2003). The limited benefit from antimicrobial therapy and the risk of deposition of residues in eggs indicate that alternative strategies, such as eradication or vaccination, should be evaluated to control the induction of EAA by *M. synoviae*.

Although respiratory infections with *M. synoviae* are generally considered to be subclinical (Van Eck *et al.*, 1980), an increasing number of reports have documented economic losses attributable to respiratory infections (Morrow *et al.*, 1990; Lockaby *et al.*,

1998; Kang *et al.*, 2002) and arthropathic strains (Landman & Feberwee, 2001; van Beek *et al.*, 2002; Kleven, 2003). The eggshell pathology described here and the concomitant egg production losses that result from it further highlight the economic significance of *M. synoviae* in commercial poultry.

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## Legends to figures

**Figure 1.** *Total egg production and production of eggs with EAA in Field study 2. Boxed numbers (21-40) indicate the chickens treated with long acting oxytetracycline at day 25. Note the temporary production of normal eggs for 12 days (day 28 until day 40) after administration of antibiotic.*

**Figure 2.** *(I). Eggshell apex abnormality characterized by an altered shell surface, shell thinning, increased translucency, and the occurrence of cracks and breaks. Eggshell pathology is confined to a region approximately 2 cm from the apex. The picture shows eggs originating from brown and white layer chickens. (II). The clear demarcation zone separating the affected eggshell from the remainder of the shell is more apparent at candling. The abnormal eggshell has increased translucency.*

**Figure 3.** *EAA egg production after experimental infection. Eggs with EAA were not produced in the control and group inoculated intravenously *M. synoviae* but not infected with IBV.*

**Figure 4.** *Dendrogram constructed from UPGMA analysis of HindIII/HhaI AFLP patterns. The strains and isolates included are: from Field study 1, SP2005-00257 A (farm 1) and SP2005-00255 B (farm 3, also inoculum used for experimental infection); from Field study 2, SP2007-00333-3 (untreated bird), 00333-4 (untreated bird), 00333-14 (treated bird), 00333-15 (treated bird) and SP2007-00426-1 (bird producing normal eggs); from the experimental infection study, SP2007-00597-15 and SP2007 00601-24; and two additional field isolates, SP2002-00804 and SP2007-01054. Three different clusters of *M. synoviae* strains could be identified. *M. synoviae* isolates from oviducts of hens producing eggs with EAA were not clonal. The inoculum strain had 88% similarity with the oviduct reisolates from the animal experiment. Isolate SP2007-00426-1 from Field study 2 had 85% similarity with the other oviduct isolates from this study.*

**Figure 5.** *(I). Scanning electron microscopy of a normal eggshell showing the inner membranes (A), the mammillary knob layer (B) and part of the palisade layer (C). Bar = 50  $\mu$ m. (II). Scanning electron microscopy of an abnormal apical eggshell. The mammillary knob layer is absent and only part of the palisade layer is present: inner membranes (A), palisade*

*layer (B), vertical layer (arrow) and cuticule (arrow head). Note also the increased density of the inner membranes. Bar = 100  $\mu$ m.*

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**Table 1.** Serology and mycoplasma culture of oviducts of hens from farms producing eggs with EAA and farms producing normal eggs

	Farm Number	Number of birds investigated	Serum antibodies against <i>M. synoviae</i> <sup>a</sup>	Oviduct positive by mycoplasma culture <sup>b</sup>
Producing eggs with EAA	1	3	1	1
	2	6	6	1
	3	3	1	2
Producing normal eggs	4	5	5	0
	5	5	3	0
	6	5	5	0

<sup>a</sup>Agglutination titre  $\geq 8$

<sup>b</sup>Mycoplasma cultures identified as *M. synoviae* by PCR

**Table 2.** Egg quality measurements, mycoplasma serology and mycoplasma culture of oviducts in Field study 2 (treatment trial)

	Week	Farm producing eggs with EAA			Control farm
		Untreated hens producing eggs with EAA	Treated hens producing eggs with EAA <sup>a</sup>	Hens producing normal eggs	Hens producing normal eggs
<i>M. synoviae</i> RPA test <sup>b,d</sup>	0	20/20 <sup>A</sup>	20/20 <sup>A</sup>	-	-
	3	-	-	-	-
	4	10/10 <sup>A</sup>	8/10 <sup>A</sup>	-	-
	7	10/10 <sup>A</sup>	10/10 <sup>A</sup>	9/10 <sup>A</sup>	9/9 <sup>A</sup>
Mycoplasma culture oviduct <sup>c, e</sup>	4	8/10 <sup>A</sup>	7/10 <sup>A</sup>	-	-
	7	9/10 <sup>A</sup>	9/10 <sup>A</sup>	2/10 <sup>B</sup>	0/9 <sup>B</sup>
Eggshell strength (N) (n = 10-20) <sup>c</sup>	3	15.9 (2.2) <sup>A</sup>	-	-	34.1 (1.7) <sup>B</sup>
	4	14.4 (3.2) <sup>A</sup>	30.4 (3.0) <sup>B</sup>	-	32.4 (1.3) <sup>B</sup>
	7	16.2 (1.8) <sup>A</sup>	13.9 (1.8) <sup>A</sup>	-	31.1 (1.8) <sup>B</sup>
Haugh units (n = 12-20) <sup>c</sup>	3	90.1 (1.5) <sup>A</sup>	-	-	87.2 (1.5) <sup>A</sup>
	4	85.1 (2.3) <sup>A</sup>	82.5 (1.2) <sup>A</sup>	-	80.1 (1.2) <sup>A</sup>
	7	74.2 (5.3) <sup>A</sup>	81.2 (2.0) <sup>A</sup>	-	80.1 (1.6) <sup>A</sup>
Egg white thickness (mm) (n = 10-20) <sup>c</sup>	3	8.4 (0.3) <sup>A</sup>	-	-	7.8 (0.3) <sup>A</sup>
	4	7.4 (0.4) <sup>A</sup>	6.9 (0.2) <sup>A</sup>	-	6.6 (0.2) <sup>A</sup>
	7	6.4 (0.4) <sup>A</sup>	6.9 (0.3) <sup>A</sup>	-	6.8 (0.3) <sup>A</sup>

<sup>a</sup>Treated birds were given a single dose of long-acting oxytetracycline at 3 weeks.

<sup>b</sup>Two-Sample Proportion Test. Data within the same row with the same superscript are not significantly different ( $P > 0.05$ ).

<sup>c</sup>Mean ( $\pm$  SEM) and statistical analysis with Kruskal-Wallis One Way ANOVA.

<sup>A, B</sup>Means within the same row with the same superscript are not significantly different ( $P > 0.05$ ).

<sup>d</sup>Agglutination titres  $\geq 8$  were considered positive.

<sup>e</sup>Between two and five mycoplasma positive cultures were tested by PCR and identified as *M. synoviae*.



**Table 3.** Serology, mycoplasma culture of the oviduct, growth, eggshell strength, egg production and production of eggs with EAA in the experimental infection study

	Week	Control	<i>M. synoviae</i> (i.v.)	<i>M. synoviae</i> (i.v.) +IBV	<i>M. synoviae</i> (i.t.)	<i>M. synov.</i> +IBV
<i>N</i>		12	18	17	18	17
Mean IBV HI titre <sup>a</sup>	0	-	-	4.4 (0.2) <sup>A</sup>	-	4.4 (0.2) <sup>A</sup>
	4	4.5 (0.2) <sup>A</sup>	3.6 (0.3) <sup>A</sup>	7.1 (0.3) <sup>B</sup>	4.9 (0.3) <sup>A</sup>	7.1 (0.3) <sup>A</sup>
	8	4.5 (0.3) <sup>A</sup>	4.8 (0.2) <sup>A</sup>	6.9 (0.3) <sup>B</sup>	3.9 (0.1) <sup>A</sup>	7.8 (0.3) <sup>A</sup>
	11	4.4 (0.1) <sup>A</sup>	4.1 (0.1) <sup>A</sup>	6.8 (0.2) <sup>B</sup>	4.2 (0.3) <sup>A</sup>	7.4 (0.3) <sup>A</sup>
<i>M. synoviae</i> RPA test positive <sup>b, e</sup>	0	0/12 <sup>A</sup>	0/18 <sup>A</sup>	0/17 <sup>A</sup>	0/18 <sup>A</sup>	0/17 <sup>A</sup>
	4	0/12 <sup>A</sup>	15/18 <sup>B, d</sup>	12/17 <sup>B</sup>	14/18 <sup>B</sup>	12/17 <sup>B</sup>
	8	0/12 <sup>A</sup>	17/17 <sup>B</sup>	17/17 <sup>B</sup>	17/17 <sup>B, c</sup>	13/17 <sup>B</sup>
	11	0/12 <sup>A</sup>	13/17 <sup>B</sup>	13/16 <sup>B, d</sup>	15/17 <sup>B</sup>	11/17 <sup>B</sup>
Oviduct Mycoplasma culture positive <sup>b, f</sup>	11	0/12 <sup>CD</sup>	0/17 <sup>C</sup>	1/16 <sup>AC</sup>	6/17 <sup>ABD</sup>	7/17 <sup>B</sup>
Weight (g) <sup>a</sup>	0	1230 (19) <sup>A</sup>	1248 (17) <sup>A</sup>	1261 (14) <sup>A</sup>	1261 (18) <sup>A</sup>	1258 (14) <sup>A</sup>
Increase in body weight (g) <sup>a</sup>	0-11	474 (20) <sup>A</sup>	384 (21) <sup>AB</sup>	368 (18) <sup>B</sup>	486 (28) <sup>A</sup>	372 (20) <sup>A</sup>
Mean daily egg production/chicken <sup>a</sup>	1-11	0.75 (0.02) <sup>A</sup>	0.61 (0.03) <sup>B</sup>	0.62 (0.03) <sup>B</sup>	0.49 (0.02) <sup>C</sup>	0.54 (0.02) <sup>B</sup>
Eggs with EAA	4-11	0	0	15	49	90
Mean daily EAA eggs/chicken <sup>a</sup>	4-11	0.0 (0.00)	0.0 (0.00)	0.02 (0.00) <sup>A</sup>	0.06 (0.01) <sup>B</sup>	0.1 (0.01) <sup>B</sup>
<i>Eggshell strength (N)</i>						
Unaffected eggs ( <i>n</i> = 60 per group) <sup>c</sup>	4-5	37.7 (1.4) <sup>A</sup>	-	38.2 (1.5) <sup>A</sup>	37.7 (1.4) <sup>A</sup>	-
EAA eggs of 3 groups ( <i>n</i> = 60) <sup>c</sup>	4-5	-	-	16.8 (0.9) <sup>B</sup>	-	-
Unaffected eggs ( <i>n</i> = 30 per group) <sup>d</sup>	9-11	31.7 (1.8) <sup>A</sup>	35.1 (1.8) <sup>A</sup>	35.2 (2.0) <sup>A</sup>	31.7 (1.8) <sup>A</sup>	35.1 (1.8) <sup>A</sup>
EAA eggs of 3 groups ( <i>n</i> = 30) <sup>d</sup>	9-11W	-	-	12.7 (1.0) <sup>B</sup>	-	-

<sup>a</sup>Mean ( $\pm$  SEM) and statistical analysis with Kruskal-Wallis One Way ANOVA.,

<sup>A, B</sup> means with the same superscript within the same row are not significantly different ( $P > 0.05$ ).

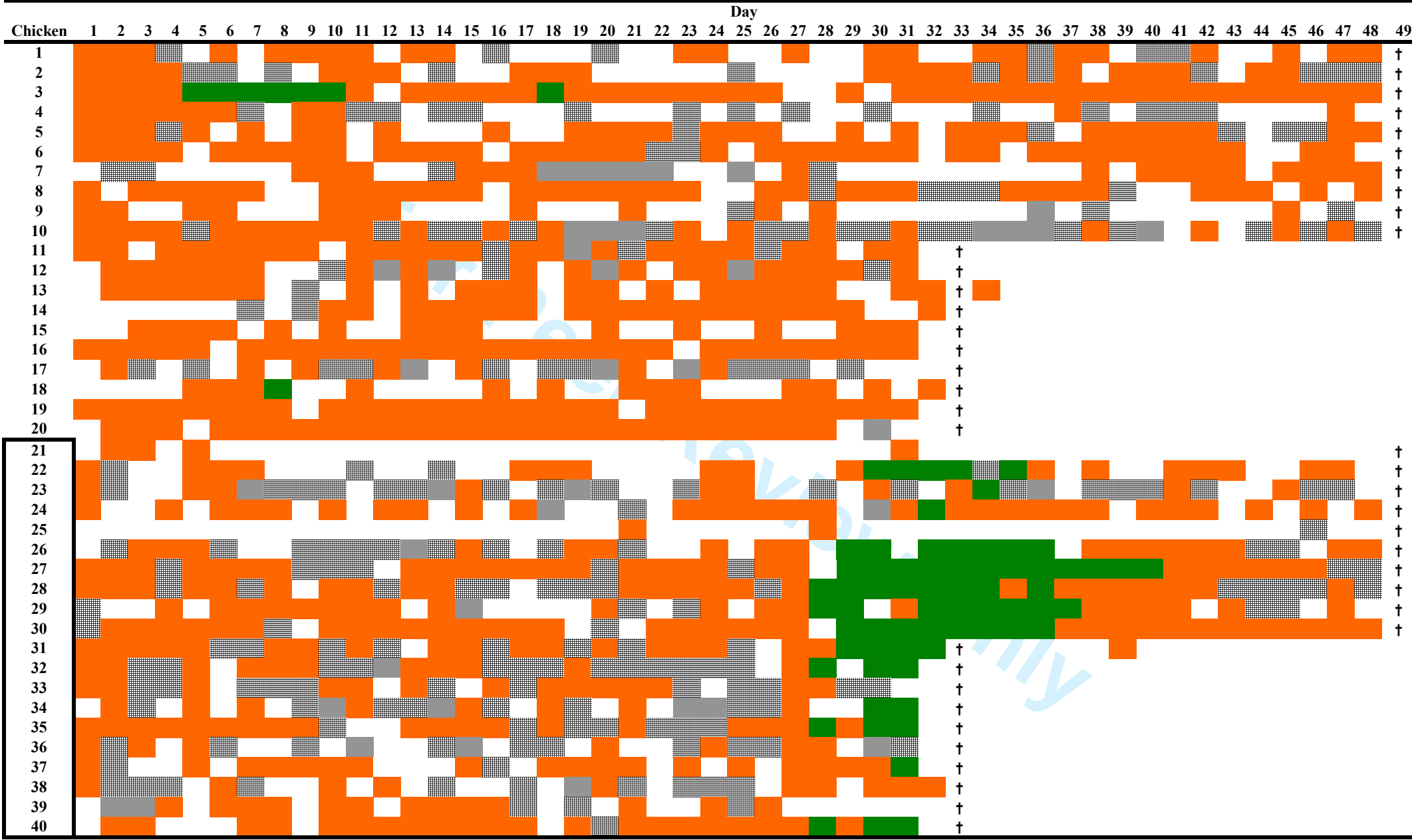
<sup>b</sup>Two-Sample Proportion Test. Data with the same superscript within the same row are not significantly different ( $P > 0.05$ ).

<sup>c</sup>Mean ( $\pm$  SEM) and statistical analysis with Kruskal-Wallis One Way ANOVA, means within the same row (no EAA eggs and EAA eggs) with the same superscript are not significantly different ( $P > 0.05$ ).

<sup>d</sup>One bird died.

<sup>e</sup>Agglutination titres  $\geq 8$  were considered positive.

<sup>f</sup>Between one and two mycoplasma positive cultures were tested by PCR and identified as *M. synoviae*



no egg     
  normal egg     
  EAA egg     
  crushed eggs     
  shell-less eggs     
 † postmortem



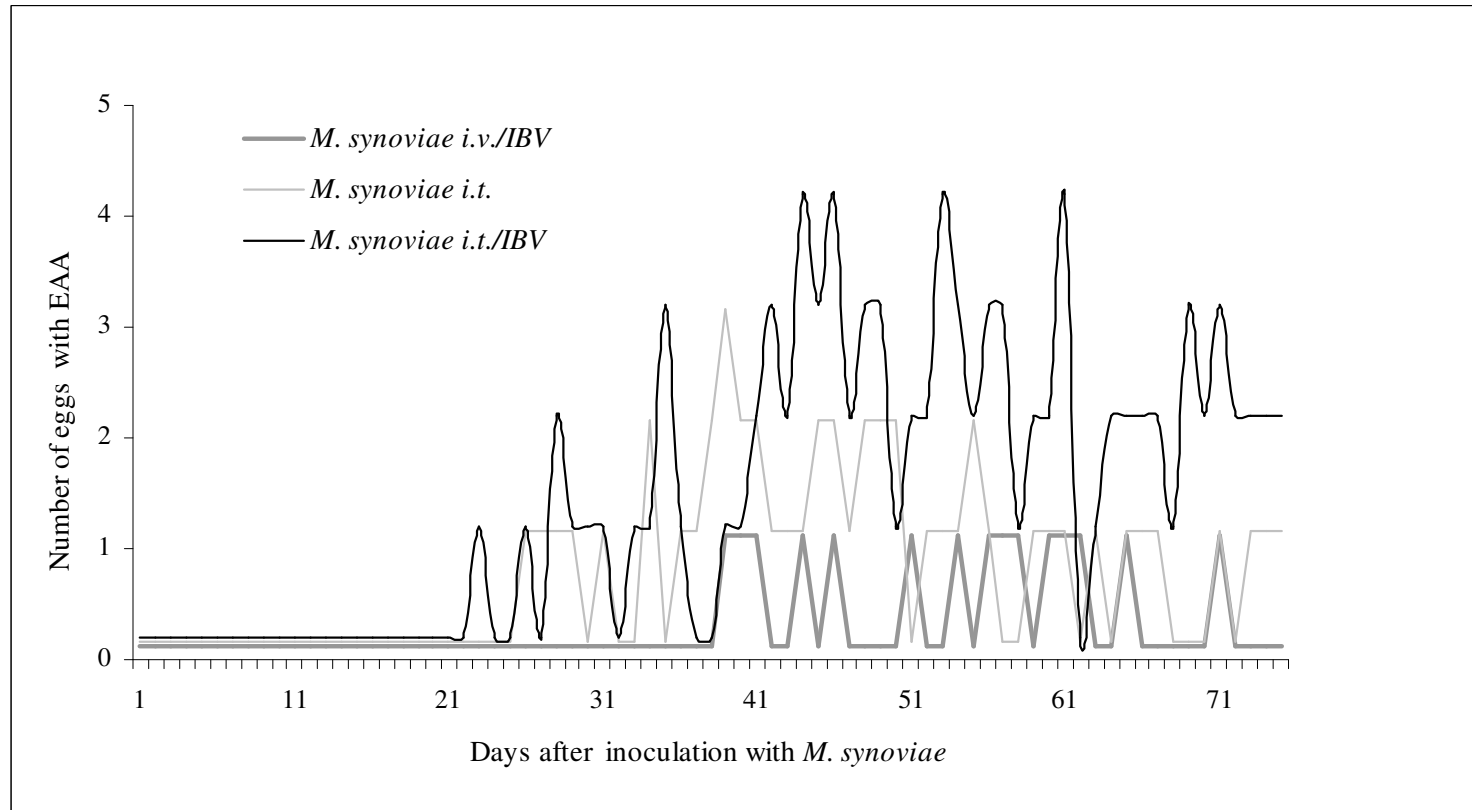
Figure 2.I  
1236x824mm (72 x 72 DPI)

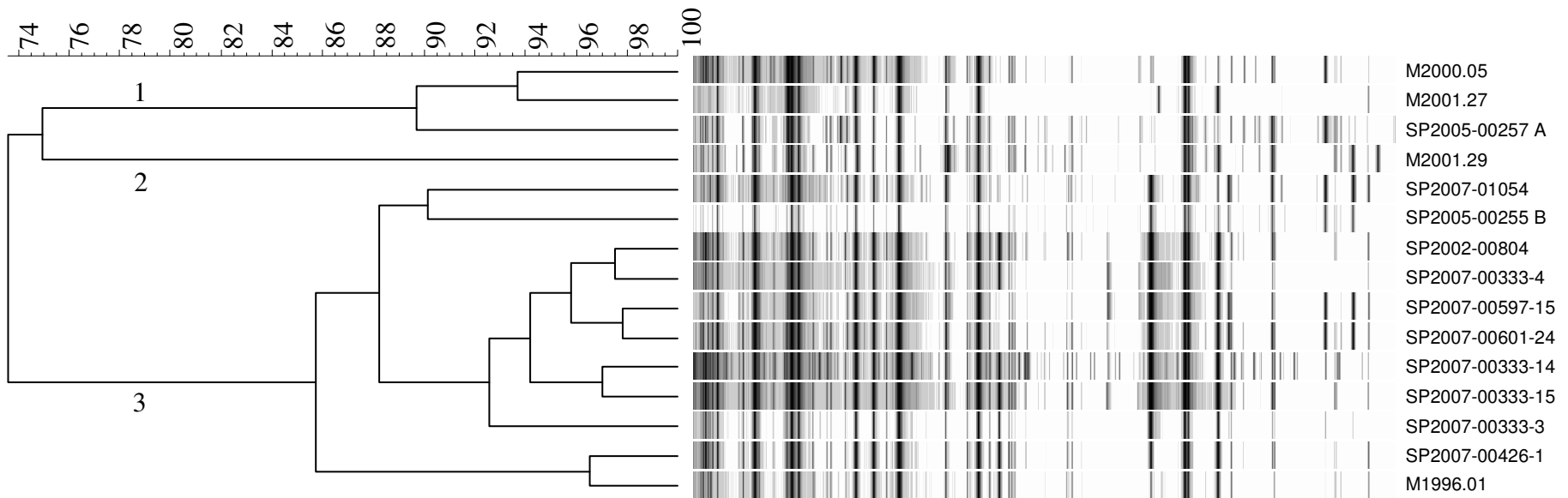
Review Only



Figure 2.II  
99x107mm (300 x 300 DPI)







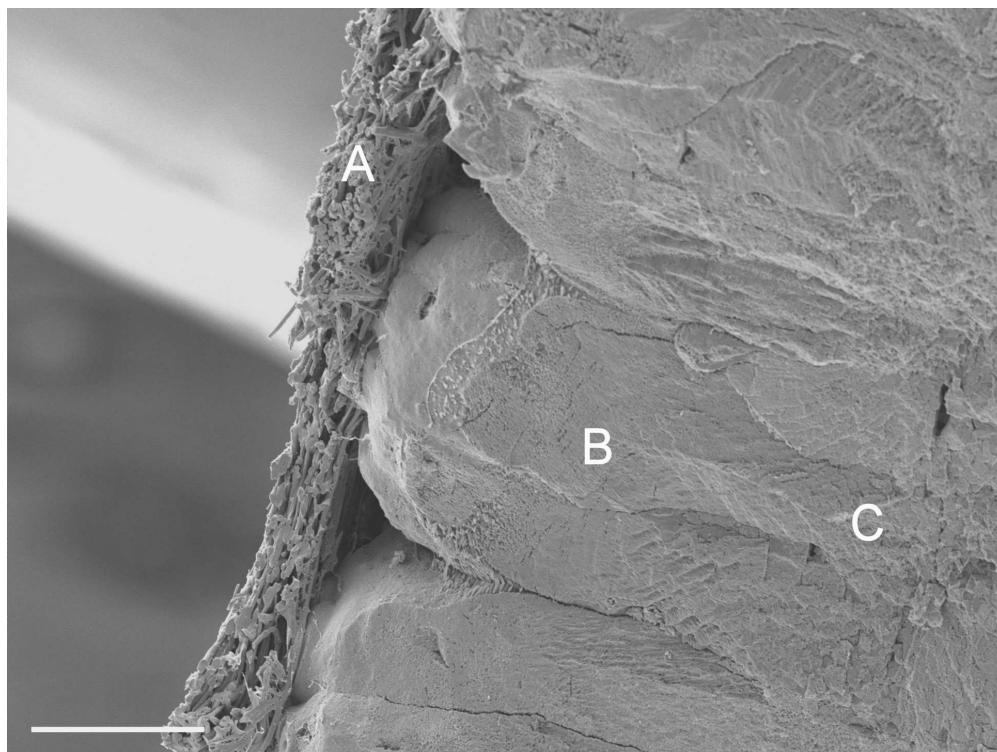


Figure 5.I  
682x512mm (72 x 72 DPI)

View Only



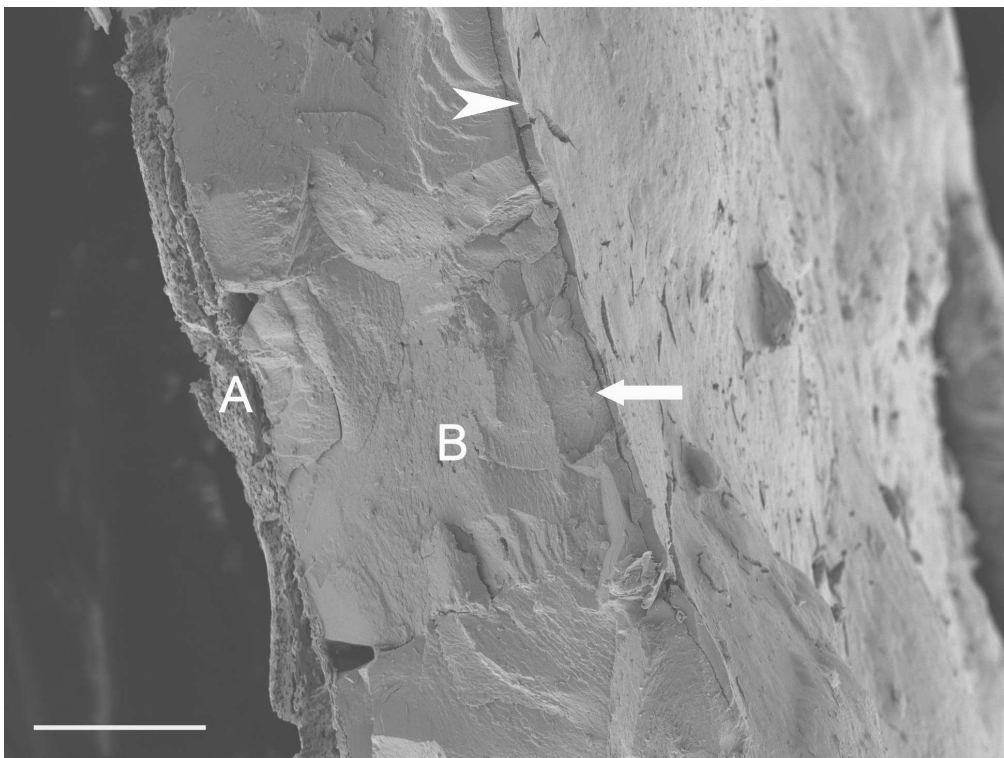


Figure 5.II  
682x512mm (72 x 72 DPI)

Preview Only