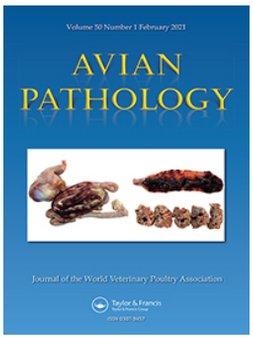

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Success rates of inoculation of the various compartments of embryonated chicken eggs at different incubation days

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ORIGINAL ARTICLE

Success rates of inoculation of the various compartments of embryonated chicken eggs at different incubation days

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ABSTRACT

Inoculation of embryonated chicken eggs has been widely used during the past decades; however, inoculation success rates have not been investigated systematically. In this study named success rates were assessed in brown eggs incubated between 5 and 19 days, which were inoculated with 0.2 ml methylene blue per egg. Inoculations were performed in a simple and fully standardized way. Five embryonic compartments were targeted blindly (amniotic cavity, embryo, allantoic cavity, albumen and yolk) with needles of four different lengths; albumen and yolk were targeted with eggs in upside down position. Three compartments were inoculated within sight (air chamber, chorioallantoic membrane and blood vessel). Twenty embryos were used per incubation day, intended deposition site and needle length. Success rates were assessed by visual inspection after breaking the eggs. The inoculations targeting albumen, yolk, amniotic cavity and embryo yielded low scores. Magnetic resonance imaging was performed to elucidate the reason(s) for these low success rates: needles used were of appropriate length, but embryo and amniotic cavity had variable positions in the eggs, while albumen and yolk rapidly changed position after turning the eggs upside down. The latter led to adjustment of the inoculation method for albumen and yolk. Failures to inoculate compartments within sight were immediately visible; therefore, these eggs could be discarded. Except for the amniotic cavity, full scores (20/20) were obtained for all compartments although not always on every day of incubation. In conclusion, the present study may serve as a guide to more accurately inoculate the various chicken embryo compartments.

RESEARCH HIGHLIGHTS

- Blind inoculation of embryonated egg compartments was successful, except for the amniotic cavity.
- MRI showed rapid position change of albumen and yolk after turning eggs upside down.
- *In ovo* vaccination against Marek's disease might be improved by using 38 mm needles.

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In ovo; inoculation; success rates; embryo; chicken; methylene blue; magnetic resonance imaging

Introduction

The embryonated chicken egg has been used extensively during many decades in different scientific disciplines such as embryology, immunology and virology. In virology, the embryo, and especially its extra-embryonic structures, are used as a medium for virus replication. Based on the former, the inoculation of the embryonated egg serves a wide range of purposes, such as: isolation and propagation of field virus, preparation of attenuated vaccine virus from field virus by successive passages in chicken embryos (Bijlenga *et al.*, 2004; Oade *et al.*, 2019), production of human (Ulmer *et al.*, 2006; Reisinger *et al.*, 2009) and avian vaccine virus (Bijlenga *et al.*, 2004; Coppo *et al.*, 2013), titration of virus stocks (Reed & Muench, 1938), assessment of the virulence of viruses by mean death time (Hanson & Brandly, 1955; Perdue *et al.*, 1990) and induction of immunity by *in ovo* vaccination (Sharma & Burmester, 1982; Ricks *et al.*, 1999; Islam *et al.*, 2001; Wakenell *et al.*, 2002).

The introduction of tissue and cell culture during the second half of the twentieth century reduced the need for embryonated eggs; however, the latter medium remains the method of choice for isolation and replication of the majority of pox- and myxoviruses, and for avian viruses (Horzinek, 1975).

In bacteriology, the embryonated egg is useful, e.g. to perform lethality tests in order to assess the virulence of bacterial strains (Bumgarner & Finkelstein, 1973; Nolan *et al.*, 1992; Blanco *et al.*, 2017). Large scale tylosine tartrate inoculation in the air chamber of hatching eggs was performed in 1960s and 1970s to control *Mycoplasma gallisepticum* infections (Smit & Hoekstra, 1967). Moreover, improvement of the performance of commercial chickens might be achieved by *in ovo* feeding (Zhai *et al.*, 2008; Saeed

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et al., 2019; Zhu *et al.*, 2019). Sexing chickens before hatch using a sample of allantoic fluid is an area of recent research in which the embryonated egg is punctured as well (Weissmann *et al.*, 2013).

Prior to the inoculation of embryonated eggs, a few questions must be addressed: which egg compartment should be inoculated/sampled, at which day of incubation and how should the needle be inserted. The embryonated egg compartments attain a substantial volume from incubation day 6 onwards (Romanoff & Romanoff, 1967a, 1967b; Baggott, 2009), implicating that they can be inoculated from that time. After 19 days of incubation the internal pip of the air chamber, in combination with the size of the embryo, will lead to disappearance of compartments of the embryonated egg (Kuo & Shen, 1937; Van de Ven *et al.*, 2011).

The embryonic compartment to be inoculated depends on the virus species. The allantoic cavity is generally used for isolation and propagation of avian respiratory (vaccine) viruses (Alexander, 2000; Bijlenga *et al.*, 2004; Spackman & Killian, 2020), the chorioallantoic membrane (CAM) especially for poxvirus (Tripathy & Reed, 2013) and infectious laryngotracheitis virus (Hitchner & White, 1958; Noormohammadi & Devlin, 2019) and yolk for reovirus (Guneratne *et al.*, 1982), chicken anaemia virus (Tan & Tannock, 2005) and avian encephalomyelitis virus (Suarez, 2016). As a rule, inoculations are performed between days 5 and 12 of incubation. *In ovo* vaccination and *in ovo* feeding aim at injecting vaccine and feed, respectively, in the amniotic cavity on day 18 of incubation. Inoculation of other compartments of the embryonated egg, e.g. air chamber, albumen and blood vessel, are only used on a small scale in experimental settings (Kelling & Schipper, 1976; Ohta *et al.*, 1999; Wakenell *et al.*, 2002; Tare & Pawar, 2015).

Likely, the publication of Centanni in 1902 was the first in which egg inoculation was reported. Inoculations were performed in the albumen of chicken eggs prior to incubation with blood of diseased chickens from an outbreak of fowl plague in northern Italy (Centanni, 1902).

Review of early literature on inoculation of embryonated eggs was presented by Beaudette *et al.* (1952). From 1935 onwards the same authors developed a technique for the inoculation of embryonated eggs, which may provide a 100% success rate by enabling visualization of all parts of the egg. Briefly, a cap with a diameter of 2 cm of the shell, including the outer shell membrane that covers the air chamber, is removed and the inner shell membrane, as well as the chorioallantoic membrane (CAM), are clarified with a light mineral oil. When the amniotic cavity is targeted, moreover an incision is made in the inner shell membrane and the CAM, and the amnion is lifted with a pipette through the CAM. After inoculation, the egg opening is covered with sterile paper and paraffin (Beaudette *et al.*, 1952). The mentioned technique is very cumbersome, time consuming and poses a significant risk for contamination with microorganisms. Therefore, this technique has not found widespread use. Nowadays, inoculations are performed according to procedures given in laboratory manuals (Guy, 2008; Spackman & Stephens, 2016; OIE, 2019; Spackman & Killian, 2020). Most of these inoculations are done blind, i.e. the targeted compartment is not visualized, and their accuracy has not been assessed, except for a few studies concerning the older embryo only.

The success rates of embryo inoculations have been described to examine the effectiveness of vaccine delivery on days 17, 18 or 19 of incubation (Sharma & Burmester, 1982; Islam *et al.*, 2001; Wakenell *et al.*, 2002; Williams & Hopkins, 2011; Sokale *et al.*, 2019) or on day 17 of incubation to examine *in ovo* feed delivery (Zhai *et al.*, 2008).

As a comprehensive study on the accuracy of inoculation of embryonated chicken egg compartments is lacking, in the present study embryonated chicken eggs were inoculated using needles of different lengths in a standardized way on several days between days 5 and 19 of incubation with a dye (methylene blue). Eight embryonated egg compartments were targeted at each embryonic age. The success rates of inoculations were assessed.

The study consisted of three parts: (1) Research as described before. (2) Magnetic Resonance Imaging (MRI) of embryonated eggs on the different days of incubation to provide an explanation for the low success rates of inoculations in a number of embryonated egg compartments. (3) Adjustment of embryo inoculation procedures of part 1 based on MRI.

Materials and methods

Part I: success rates of inoculation (I)

Experimental design

Embryonated chicken eggs were inoculated with methylene blue targeting the air chamber, the amniotic cavity, the embryo, the allantoic cavity, the albumen, the yolk, the CAM or a blood vessel. Inoculations were made on days 6, 8, 10, 12, 14, 16 and 18 of incubation. Every time, 20 groups each consisting of 20 embryonated eggs were used. The rationale behind the number of groups is as follows. Attempts to inoculate the amniotic cavity/embryo, allantoic cavity and albumen/yolk were made blindly as these embryonated egg compartments are not visible during inoculation. Therefore, to increase the chance of success, needles of four different lengths were used for these inoculations. Targeting of the amniotic cavity and embryo was performed by the

same inoculation method; this was also the case for the inoculation of albumen and yolk. Here, only the needle length determines which compartment is reached. Allantoic cavity inoculation attempts were made by two (slightly) different routes. The foregoing resulted in 16 groups: four (inoculation routes) × four (needle lengths). Air chamber, CAM and blood vessel were made visible, and therefore were inoculated with needles of one length only. However, blood vessels were injected with needles of two different diameters. These inoculations required another four groups, yielding a total of 20 groups.

Immediately after inoculations, the eggs were opened and the deposition site(s) of the methylene blue was (were) assessed by visual inspection.

Embryonated chicken eggs

A total of 3500 brown-shelled eggs, from a Ross 308 broiler breeder flock of 39 weeks of age, were incubated at a commercial hatchery following standard procedures. The average egg weight according to the Ross 308 performance objectives was 63.3 g (Aviagen®, 2016). Every 2 days, from incubation day 6 onwards, 500 eggs were collected and transported to the animal research facilities of Utrecht University, where all inoculations and examinations were performed. Eggs were candled with a LED flashlight (MAG-TAC® LED rechargeable flashlight system, model number TRM4TRE4L, Mag Instruments Inc., Ontario, CA, USA) with a candling rubber (RCom digital incubators, RCom Egg Cool Candler, model no. CANDLER 200, Wichita, KS, USA) and only eggs with viable embryos and the air chamber present at the blunt end of the egg were used. Embryos from surplus eggs were immediately killed by decapitation, and discarded.

Inoculations

Common procedures. Preceding inoculations, except for CAM and blood vessel inoculations, a small hole (approximate diameter 1 mm) was made in the shell leaving the underlying shell membrane(s) intact, with an electric engraver (Hugo Brennenstuhl GmbH & Co Kommanditgesellschaft, Electric Engraver Signograph 25 Set, article number 1500740, Tübingen, Germany). The injection needles used to inoculate embryonated egg compartments blindly (amniotic cavity/embryo, allantoic cavity and albumen/yolk) had the following sizes: 0.5 × 6 mm (25G × 2/8″), 0.5 × 16 mm (25G × 5/8″), 0.5 × 25 mm (25G × 1″) and 0.5 × 38 mm (25G × 1.5″) (B. Braun Melsungen AG, Sterican®, REF 4657853 (16 mm), REF 9186158 (25 mm), REF 9186166 (38 mm), Melsungen, Germany). Since needles of 0.5 × 6 mm were not available, they were made by inserting 0.5 × 16 mm needles in a 10 mm long rubber tube with a diameter of 2.5 mm (Terumo, Surflo® winged infusion set, REF SV*19NL30, Tokyo, Japan).

Needles were inserted completely following the long central axis of the egg to inoculate the air chamber (Figure 1(A)), the amnion/embryo (Figure 1(B)) and the albumen/yolk (Figure 1(D1)), and parallel to the long central axis to target the allantoic cavity (Figure 1(C1,C2)).

In all cases, inoculations were performed with 0.2 ml methylene blue per egg (10 mg methylthionine chloride /ml, batch number 08E19-002; Pharmacy Faculty of Veterinary Medicine, Utrecht, the Netherlands).

Specific procedures for each egg compartment. The air chamber was injected from the centre, which was marked during candling, with a needle of 0.5 × 6 mm (Figure 1(A)). The amniotic cavity/embryo were targeted from the centre of the blunt side of the egg using needles of the four different sizes (Figure 1 (B)). Attempts to inoculate the allantoic cavity were made using two methods. In both methods, the limit of the air chamber was marked and the needle was inserted either 2-3 mm under the mark (Figure 1 (C1)) or 2–3 mm above the mark (Figure 1(C2)). Note that in the latter method (Figure 1(C2)) the needle penetrates the air chamber prior to inoculation in the allantoic cavity. The downside of the method in which the needle does not penetrate the air chamber (Figure 1(C1)), is the spill of a drop of inoculum and/or allantoic fluid. On the other hand, when the needle penetrates the air chamber (Figure 1(C2)) the named compartment may be contaminated by the inoculum. The allantoic cavity was targeted using needles of four different sizes.

Prior to inoculations of albumen/yolk, the eggs were turned upside down (apex up) for reasons of convenience. Needles of the four sizes were inserted from the centre of the apex (Figure 1(D1)).

Injection of a blood vessel was done after candling the egg and marking with a pencil a relative big vessel of the CAM with a triangle with sides of approximately 4 mm. The eggshell was thereafter carved following the triangle's shape by using the electric engraver and was subsequently carefully removed with a blunt needle, while leaving the shell membranes intact. The injection of the vessel was favoured by holding the candling lamp against the eggshell during the procedure (Figure 1(E)). The inoculation was considered successful if the methylene blue was visible in the blood vessel and no leakage of blood or dye could be seen on the CAM or in the allantoic cavity. Needles of two sizes were used: 0.4 × 13 mm (27G × 1/2″) (BD Microlane™ 3, REF 300635, Drogheda, Ireland) and 0.3 × 13 mm (30G × 1/2″) (B. Braun Melsungen AG, Omnican F, REF 9161502S).

Prior to inoculation of the CAM, a small hole was made in the eggshell and the outer shell membrane at the centre of the air chamber. Thereafter, at halfway along the egg length, a site which lacked major blood vessels on the CAM by candling was marked

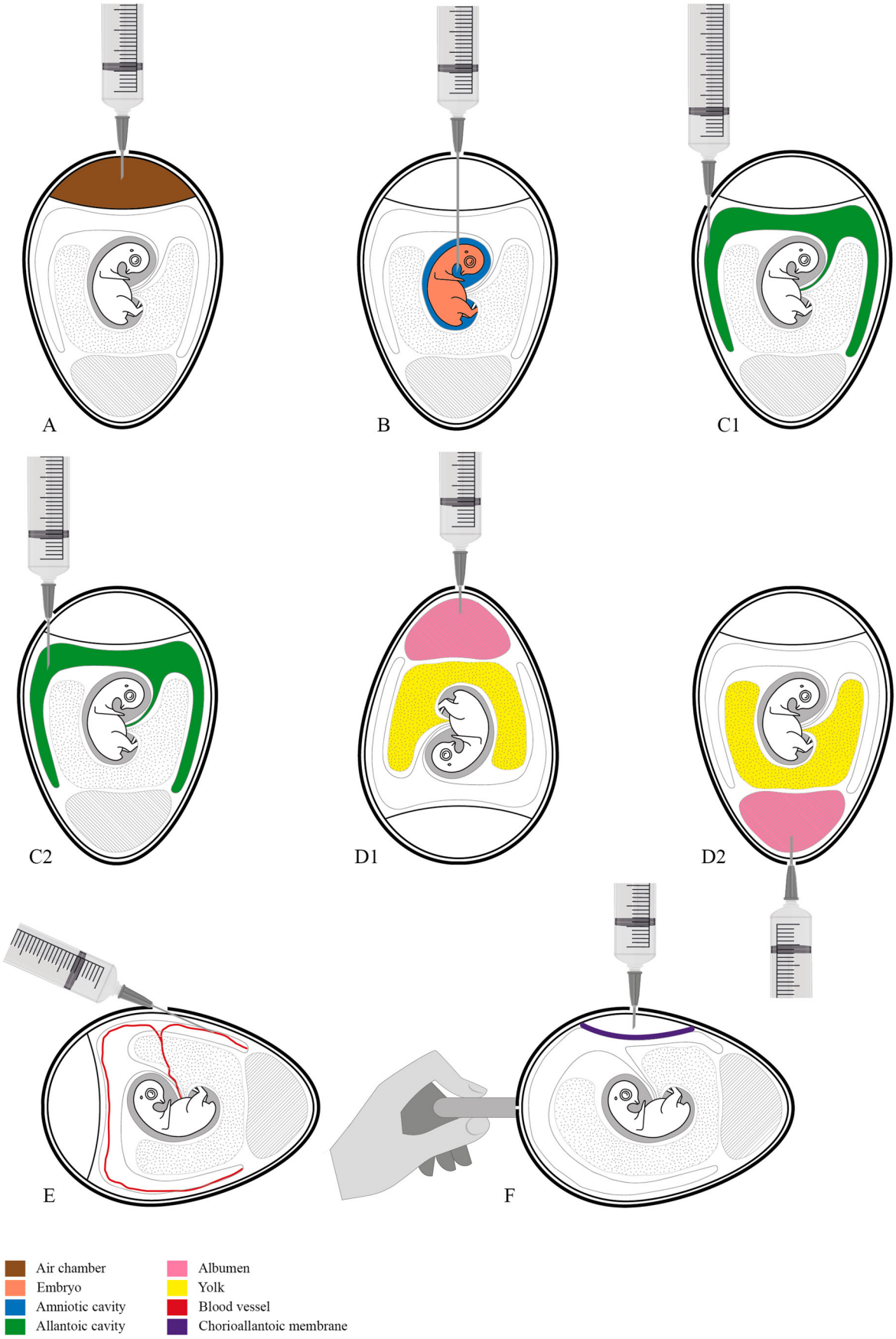

Figure 1. Schematic illustration of the inoculation of the compartments of approximately 8–12 days incubated embryonated chicken eggs. Note that, except for illustrations E and F, the needles were completely inserted following the longitudinal axis or parallel to the longitudinal axis of the egg. Also note, that in C1 the needle does not penetrate the air chamber, while it does in C2, and that in D1 the egg is positioned upside down (apex up). In F an artificial air chamber is created in order to access the chorioallantoic membrane.

with a pencil. At this mark a small hole was made in the eggshell, while leaving the underlying shell membranes intact. The intact shell membranes were perforated with a blunt needle applying gentle pressure and taking care not to damage the underlying CAM. Subsequently, a squeezed rubber pipette bulb (volume 3 ml) was placed on top of the small eggshell hole at the blunt end of the egg in order to create negative pressure inside the egg, resulting in an artificial air chamber on top of the CAM. Then, methylene blue was dropped on it (Figure 1(F)).

Assessment of deposition site

Within 45 s after inoculation, eggs were opened as follows: at half-way along the length of the egg a crack in the eggshell was made by gently hitting it with forceps, after which the eggshell was broken into two halves. Then, the egg content was carefully poured in a Petri dish and the deposition of methylene blue was assessed by visual inspection. When methylene blue was only observed in the targeted compartment, the inoculation was considered successful. Whenever inoculations targeting a particular compartment were successful in all embryonated eggs ($n = 20$), a 100% success rate (= full score) was obtained. Embryos were killed immediately by decapitation.

Part II: magnetic resonance imaging study

Rationale

Success rates of inoculations of the amniotic cavity, embryo, albumen and yolk performed in part I of the study were poor. Two possible explanations for these failures exist: the targeted compartment was not laying in the needle's path or the series of needles was inadequately chosen, i.e. a particular needle was too short to reach the targeted compartment, while the next size up was too long. These possibilities were explored in two trials applying MRI.

Experimental design

In trial I, eggs were scanned in the normal position (apex down), in trial II, in both normal and upside down position, while keeping the time between turning of the eggs and scanning to a minimum. In both trials, four embryonated eggs were scanned on each of incubation days 6, 8, 10, 12, 14, 16 and 18. In trial I, directly after scanning, the eggs were frozen at −20°C and subsequently cut midsagittal with a band saw. Egg cuts were used as a reference to identify the chicken embryo compartments on magnetic resonance images (MRIs).

Embryonated chicken eggs

Brown-shelled eggs originating from Ross 308 broiler breeder flocks of 36 (first trial) and 28 (second trial) weeks of age, were used. For each trial, 56 eggs were obtained. The selection of eggs for MRI was performed as presented in Materials and Methods part I. Moreover, the length of the eggs was measured with a caliper and only eggs of 55 or 56 mm long were used. MRI scans were made of four embryonated eggs simultaneously per incubation day studied and, as these eggs had the same length, transversal MRI views could also be selected for these four eggs simultaneously. The mean weight ± standard deviation of the selected eggs from both trials was 60.8 ± 1.9 g. The eggs were incubated at 37.5°C and 53% relative humidity at Utrecht University. A blue line was drawn on each eggshell to mark a midsagittal plane before MRI scanning. Thereafter, the embryonated eggs were cooled for 1 h at 4°C prior to MRI scanning to minimize embryo movement, which can lead to artefacts on the scans (Bain *et al.*, 2007).

In the first trial, eggs were placed in a custom made plastic box (191 × 55 × 75 mm) and aligned in such a way that the blue line identifying the midsagittal plane of the eggs paralleled the length of the box. Eggs were submersed in cold water and restrained with tape to prevent floating and subsequent misalignment (Figure 2(A)). Submersion in water is necessary to visualize the outer limits of the eggshell on the MRI scans. After the scanning procedure, eggs were frozen and cut following the blue line on the midsagittal plane.

In the second trial, eggs were placed in the normal position on a cardboard egg tray (Figure 2(B)) instead of in the custom made plastic box to minimize the time between turning the eggs and the MRI scans. T1 and T2 weighed images were taken within 44 s and within 5 min respectively, after turning the eggs.

MRI scanning

MRI scans (1.5 T MRI system; Philips Ingenia, Eindhoven, the Netherlands) were performed at the division of Diagnostic Imaging, Department of Clinical Sciences of the Veterinary Faculty, Utrecht University.

The MRI scans included T1 weighted turbo-spin echo (TR = 450, TE = 20, flip-angle = 90 degrees, field-of-view = 210, matrix size = 524 × 405, slice thickness = 1.5 mm, interslice gap including the slice thickness = 1.65 mm) and T2 weighted turbo-spin echo (TR = 7410, TE = 100, flip-angle = 90 degrees, field-of-view=210, matrix size = 420 × 316, slice thickness = 1.5 mm, interslice gap including the slice thickness = 1.65 mm).

Software (Medixant, 2020) was used to evaluate the MRI scans, and to select and export images. The exported images were processed using InDesign (Adobe, 2017) and incorporated in figures 3–5.

In both trials, 27 sagittal views were made per egg; of these only the midsagittal image was exported. Moreover, in the first trial, four transversal images at different distances from the top of the egg, (i.e. 6, 16, 25, and 38 mm), were exported. The images were

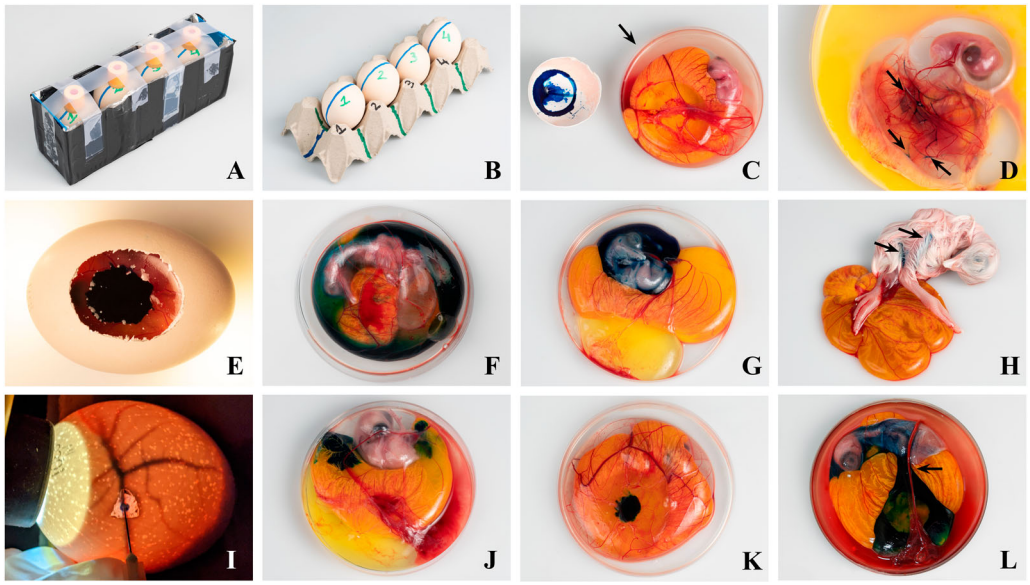

Figure 2. A and B: egg containing boxes to perform magnetic resonance imaging (MRI). The blue lines on the eggs mark a midsagittal plane of the eggs and were used to align the eggs in the boxes and to mark the plane along which eggs were cut when frozen (see Materials and Methods part II: MRI study). A: custom made plastic box (191 × 55 × 75 mm) in which eggs were restrained with tape, after which the box was filled with cold tap water. B: cardboard egg tray with marked eggs used for MRI of eggs in normal (apex down) and upside down (apex up) positions. C-L: methylene blue in various embryonated egg compartments. All inoculations were performed on day 12 of incubation unless otherwise stated. C: the air chamber and no methylene blue in other compartments (arrow), D: slight diffusion of methylene blue to the chorioallantoic membrane, indicated by arrows, after inoculation into the air chamber of an 8-day incubated embryonated egg. E: chorioallantoic membrane, F: allantoic cavity in a 14-day incubated embryonated egg, G: amniotic cavity, H: embryo with methylene blue deposition indicated by arrows in an 18-day incubated embryonated egg, I: blood vessel, J: yolk, K: albumen and L: sero-amniotic connection (arrow) and amniotic fluid stained with methylene blue after inoculation into the albumen of a 14-day incubated embryonated egg.

selected by using the measurement tool of the software program (Medixant, 2020). The distances from the top of the egg corresponded with the length of the needles used in part I of the study. The midsagittal and transversal images were inserted in Figures 3 and 4 together with the pictures of the sagittal cuts of the frozen eggs. Yellow horizontal lines were drawn on the midsagittal images and on the photo of the sagittal cut of the frozen egg at the four different distances from the top of the egg (Figure 4).

Part III: success rates of inoculation (II)

Rationale

MRI showed that, except for the air chamber, the compartments of embryonated eggs changed position rapidly following turning of the eggs upside down. Therefore, the inoculation method to target the albumen and yolk was adjusted: instead of inoculation via the apex with the egg in upside position as performed in part I of the study (Figure 1(D1)), inoculations were now made via the apex with the egg in normal position (Figure 1(D2)).

Experimental design

The experimental design was equivalent to that given in Materials and Methods part I, i.e. on incubation days 6, 8, 10, 12, 14, 16 and 18, embryonated eggs were inoculated with methylene blue using needles of four different lengths (6, 16, 25 and 38 mm). Per incubation day and needle length, 20 embryonated eggs were used. Within 45 s after inoculations the eggs were opened and the inoculation site(s) was (were) assessed by visual inspection.

Embryonated chicken eggs and inoculations

Seven hundred brown-shelled eggs from a Ross 308 broiler breeder flock of 40 weeks of age and with a mean weight of 63.7 g (Aviagen®, 2016) were incubated at 37.5°C and 53% relative humidity at Utrecht University. Selection of eggs for inoculation and inoculations with methylene blue was performed as presented in Materials and Methods part I.

Ethics

The use of embryonated eggs in experimental studies is not classified as an animal trial under Dutch law (Dutch Animal Procedure Act (Wet op de dierproeven)).

Results

Part I: success rates of inoculation (I)

Embryonated egg compartments inoculated blindly

The highest success rates of inoculation of embryonated egg compartments with the corresponding

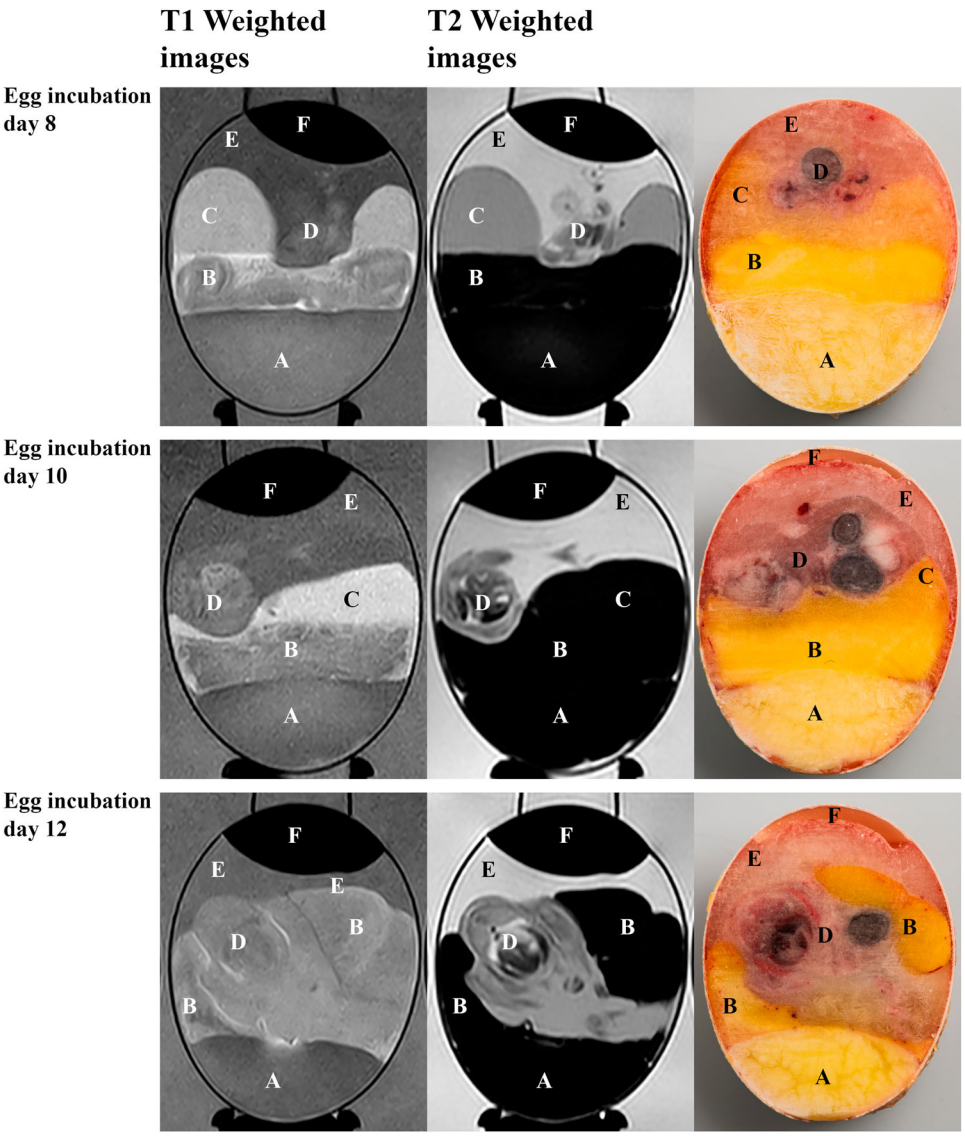

Figure 3. Sagittal magnetic resonance images of embryonated chicken eggs using T1 and T2 weighing made on incubation days 8, 10 and 12. Sagittal cuts of the same eggs in frozen condition were used as reference to identify the various embryonated chicken egg compartments. A, albumen, B, yolk, C, sub-embryonic fluid, D, embryo + amniotic cavity, E, allantoic cavity and F, air chamber.

needle length on each of the egg incubation days are presented in Table 1. Allantoic cavity inoculations were highly successful, in contrast to those of the other compartments. By either one or both methods targeting the allantoic cavity (Figure 1(C1,C2)) scores of 100% were achieved using 6 mm needles at all embryo ages, except for the age of 18 days. At certain ages, 16 mm long needles also yielded good results. Spilling of a drop of inoculum and/or allantoic fluid over the eggshell was observed in approximately 4% of the eggs if the needle was inserted outside the air chamber (Figure 1(C1)). This phenomenon was hardly observed in the air chamber in cases where the needle penetrated it (Figure 1(C2)). Only in one egg on incubation day 8, was a small amount of methylene blue observed in the air chamber.

Not a single embryo was successfully inoculated prior to 14 days of age (Table 1); thereafter increased success rates occurred. One hundred per cent success was achieved at 18 days of age with 38 mm needles. Methylene blue was found in the abdominal cavity of 19/20 embryos and 1/20 embryos in the yolk present in the abdominal cavity. The inoculator (T. T. M. Manders) seemed to sense embryonic retraction upon inoculation attempts at young ages.

Full scores (20/20) targeting the amniotic cavity were not obtained at any age. The highest score was 19/20 on day 12 of incubation using 38 mm needles. Adding the number of eggs inoculated in the amniotic cavity to the number inoculated in the embryo gave a full score at incubation days 16 and 18. This score was achieved with 38 mm needles on day 16 and with 25 mm (methylene blue was also observed in the allantoic cavity in 3/20 eggs) and 38 mm needles on day 18 (data not shown in Table 1).

Yolk and albumen inoculations with the egg in the upside down position (Figure 1(D1)) were 100% successful on incubation day 6 using 25 and 38 mm

needles to target the yolk and 6 mm needles to target the albumen. Moreover, on incubation day 8 a full yolk score was obtained via an allantoic cavity route (Figure 1(C1)) with 38 mm needles. Yolk absorbed by the abdomen of the embryo at 18 days of age was successfully inoculated with 25 mm needles.

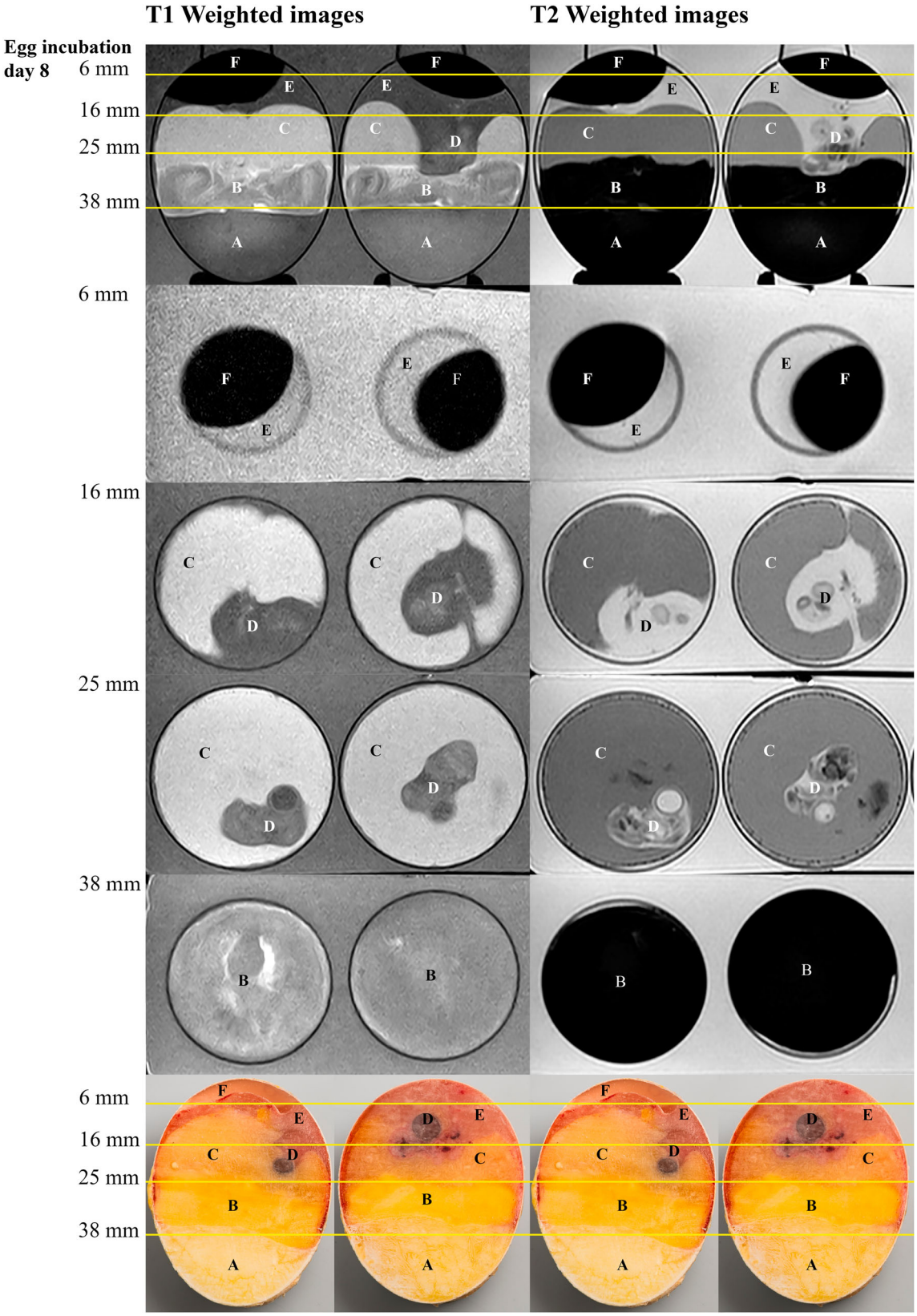

Figure 4. (A+B+C). Sagittal and transverse magnetic resonance images (MRIs) of two embryonated chicken eggs using T1 and T2 weighing made on incubation days 8 (A), 10 (B) and 12 (C). Transverse images were selected that matched the applied needle lengths as indicated in the sagittal images (yellow horizontal lines). Sagittal cuts of the same eggs in frozen condition were used as a reference to identify the various embryonated chicken egg compartments. Note that the position of the embryo is variable. Based on the transverse MRIs all targeted embryonated chicken egg compartments could theoretically be reached. A, albumen, B, yolk, C, sub-embryonic fluid, D, embryo + amniotic cavity, E, allantoic cavity and F, air chamber.

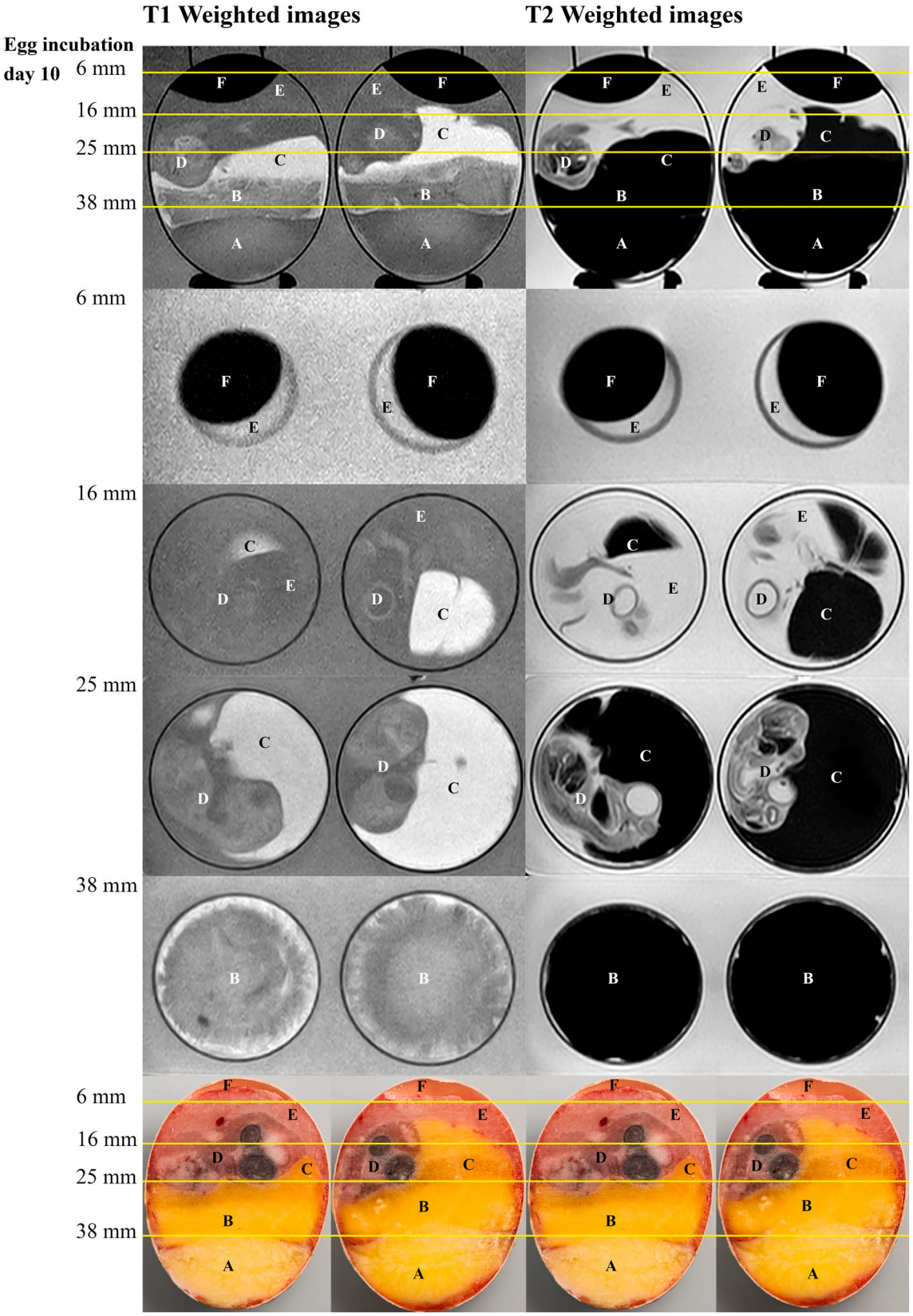

Figure 4 *Continued*

Inoculations resulting in full scores were always clean, i.e. deposition of methylene blue was only found in the targeted egg compartment and not in others.

Pictures of successfully blind inoculated embryonated egg compartments are presented in Figure 2(F–H,J,K).

Embryonated egg compartments inoculated within sight

The air chamber was always successfully inoculated on all incubation days using needles of 6 mm. Especially at young embryo ages, rapid diffusion of slight amounts of methylene blue

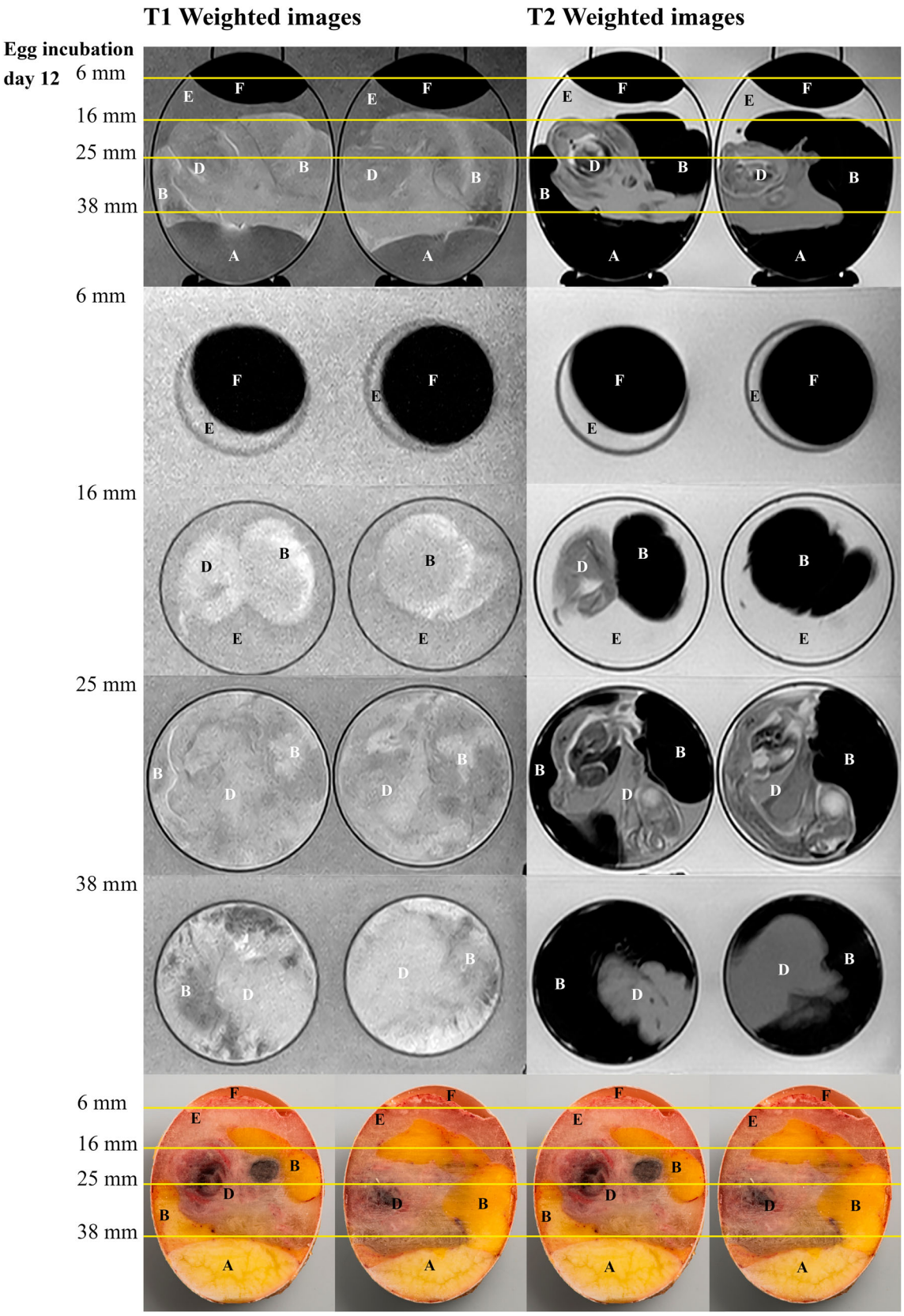

Figure 4 *Continued*

from the air chamber in to the CAM was noticed (Figure 2(D)).

In our hands (T. T. M. Manders), success rates of CAM and blood vessel inoculations varied considerably depending on the age of the embryo. Targeting the CAM, success rates of approximately 90–100% were achieved up to day 14 of incubation, and thereafter decreased to 20% on day 18 of incubation. Blood vessel injections were (almost) fully successful on 14 and 16 days of incubation and ranged from approximately 20–60% on other incubation days. In young embryos, success rates depended predominantly on the diameter

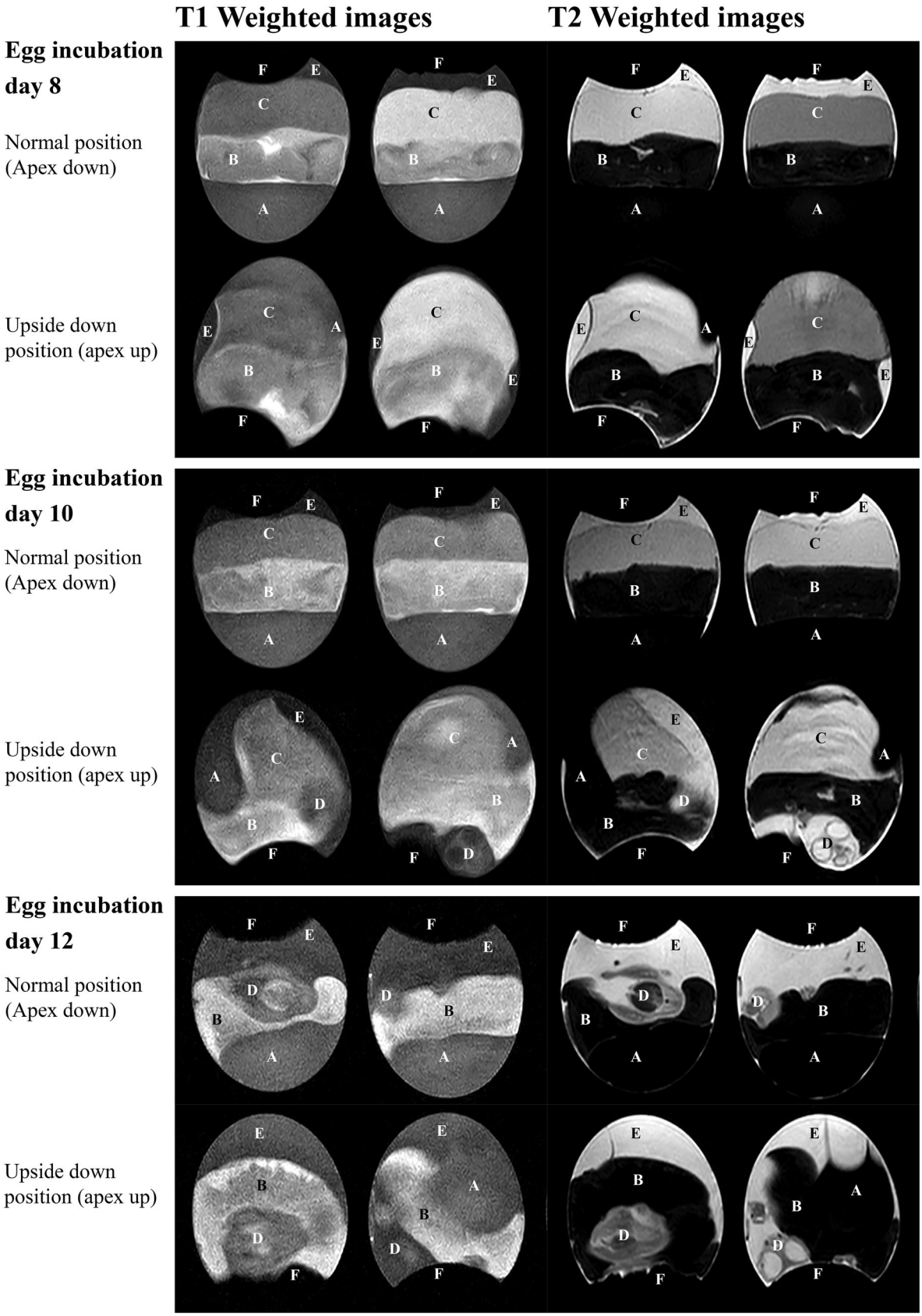

Figure 5. Sagittal magnetic resonance images of two embryonated chicken eggs using T1 and T2 weighing made on incubation days 8, 10 and 12; per incubation day different eggs were used. The eggs were placed in either the normal position (apex down) or the upside down (apex up). T1 and T2 weighted images of eggs in upside down position were made within 44 s and 5 min, respectively, after placing eggs upside down. Note that all of the embryonated chicken egg compartments, except the air chamber, switch position after placing the eggs upside down. Also note that on incubation day 8 the embryo was not visible and on incubation day 10 the embryo only became visible after turning the egg upside down. A, albumen, B, yolk, C, sub-embryonic fluid, D, embryo + amniotic cavity, E, allantoic cavity and F, air chamber.

Table 1. Success rates of the inoculation of compartments of embryonated chicken eggs with methylene blue using needles of various lengths (6, 16, 25, 38 mm). Twenty embryonated eggs were used per incubation day, needle length and intended deposition site. Only the highest success rates achieved with the corresponding needle length are given.

	Intended deposition site											
	Amniotic cavity[a]		Embryo[a]		Allantoic cavity[b]		Allantoic cavity[c]		Yolk[d]		Albumen[d]	
Egg incubation day	Needle length[e] leading to highest score	Highest score	Needle length leading to highest score	Highest score	Needle length leading to highest score	Highest score	Needle length leading to highest score	Highest score	Needle length leading to highest score	Highest score	Needle length leading to highest score	Highest score
6	25	4	All needles	0	6	20	6	12	25	20	6	20
									38	20		
8	38	1	All needles	0	6	20	6	20	38	19[f]	6	13
					16	20	16	20				
10	38	14	All needles	0	6	20	6	20	38	13	25	12
					16	20	16	20				
12	38	19	All needles	0	6	20	6	20	25	10	6	8
					16	20					16	8
											25	8
14	38	16	38	6	6	20	6	20	16	8	6	5
16	25	14	38	13	6	19	6	20	16	12	All needles	0
18	16	18[g]	38	20	6	19[g]	6	19[g]	25	20[h]	All needles	0

Note that the yolk/albumen route, in contrast to other routes, was performed with the egg upside down (apex up) (Figure 1(D1)).

Inoculations on the CAM and in a blood vessel can be assessed by visual inspection and failures discarded, which leads to a 100% success rate. Inoculations into the air chamber, as outlined in Figure 1(A), using a needle with a length of 6 mm were always successful.

[a]Inoculations were performed as outlined in Figure 1(B).

[b]Inoculations were performed as outlined in Figure 1(C1).

[c]Inoculations were performed as outlined in Figure 1(C2). Note that during inoculation the needle penetrates the air chamber prior to inoculation in the allantoic cavity.

[d]Inoculations were performed as outlined in Figure 1(D1).

[e]All needles used had a diameter of 0.5 mm.

[f]A yolk score of 20 in 8-day incubated embryos was achieved using the allantoic cavity route (Figure 1(C1)) with a needle of 38 mm length.

[g]No difference could be observed between the allantoic cavity and amniotic cavity in a number of embryonated eggs with internal pipping.

[h]Methylene blue was found in yolk absorbed in the abdomen of the embryo.

of the blood vessel; in 18-day-old embryos on blood vessel visibility. Results obtained with needles with a diameter of 0.3 or 0.4 mm were similar, except for incubation day 6, at which time 0.3 mm needles proved superior. Inoculations on the CAM and in the blood vessel can be assessed immediately by visual inspection and failures can be discarded, resulting in a 100% success rate.

Pictures with successfully inoculated embryonated egg compartments within sight are presented in Figure 2(C,E,I).

Part II: magnetic resonance imaging study

Figure 3 shows that all compartments of embryonated eggs could be identified on 8, 10 and 12 days of incubation based on T1 and T2 weighed MRIs and cuts of the same eggs in frozen condition, except for the amniotic cavity. Moreover, the sub-embryonic fluid was no longer identified on day 12 of incubation.

In Figures 4(A–C) and 5, MRIs made on 8, 10 and 12 days of incubation are presented, each time showing two eggs representative for the four eggs examined. In the mentioned figures, the following observations were made: (1) The position of the embryo is variable. On egg incubation day 8 in 50% (Figure 4(A)) and on day 10 in 100% (Figure 4(B)) of the eggs the embryo is not positioned in the central long axis of the egg, but off-centre. Only on incubation day 12, the embryo is positioned in the central long axis in all eggs (Figure 4(C)). (2) All embryonated egg compartments, which were targeted blind, could be reached with one or two needle types belonging to the set used in this study, providing that the targeted compartment is located in the needle's path (Figure 4(A–C)). (3) After turning the egg upside down, all embryonated egg compartments, except the air chamber, rapidly changed position. Amongst others, the albumen and yolk moved towards the blunt end of the egg, while the allantoic fluid moved in the opposite direction (Figure 5).

The afore-mentioned three observations were not fully applicable to embryonated eggs on incubation days 6, 14, 16 and 18: the embryos were not positioned off-centre and yolk did not change position on day 18 of incubation after turning the eggs upside down. Displacement of albumen could not be assessed on incubation days 16 and 18 as it was no longer visible on the MRIs.

Part III: success rates of inoculation (II)

Success rates of inoculations of albumen and yolk via the apex with the egg in normal position (Figure 1(D2)) are presented in Table 2. Leakage of fluid (methylene blue and clear fluid) was observed in approximately 3% of the eggs, but only when 6 mm long needles were used. Full albumen scores were obtained on 6, 8, 10, 12 and 14 days of incubation with 6 and 16 mm needles. Albumen inoculations on 12 and 14 days of incubation also resulted in the presence of the dye in the amniotic cavity: in 13 and 14 eggs, respectively, when 6 mm needles were used, and in two and five eggs, respectively, if inoculations were performed with 16 mm needles.

Full yolk scores were achieved on 6 and 8 days of incubation with 38 mm needles.

Table 2. Success rates of the inoculation of yolk/albumen of embryonated chicken eggs with methylene blue using needles of various lengths (6, 16, 25, 38 mm). Twenty embryonated eggs were used per incubation time and needle length. Only the highest success rates achieved with the corresponding needle length are given.

	Intended deposition site			
	Yolk		Albumen	
Egg incubation time (days)	Needle length[a] leading to the highest score	Highest score	Needle length leading to the highest score	Highest score
6	38	20	6	20
			16	20
8	38	20	6	20
			16	20
10	25	19	6	20
			16	20
12	38	14	6	20[b]
			16	20[c]
14	25	6	6	20[d]
			16	20[e]
16	6	12	All needles	0
18	6	10	All needles	0

Note that all inoculations were performed with the egg in normal position (apex down) (Figure 1(D2)).
[a]All needles used had a diameter of 0.5 mm.
[b]Thirteen eggs also showed methylene blue in the amniotic cavity.
[c]Two eggs also showed methylene blue in the amniotic cavity.
[d]Fourteen eggs also showed methylene blue in the amniotic cavity.
[e]Five eggs also showed methylene blue in the amniotic cavity.

Overview of procedures resulting in 100% success rates

This overview is given in Table 3. Full air chamber scores were obtained on any day of incubation with 6 mm needles, while full embryo scores were only achieved on day 18 of incubation with 38 mm needles. Full scores for the allantoic cavity can be obtained up to and including day 16 of incubation, for the albumen up to and including day 14 of incubation and for the yolk on days 6 and 8 of incubation.

Frequently, 100% success rates were obtained via different inoculation routes using needles of different lengths (Table 3). Full CAM and blood vessel scores were achieved by discarding eggs that were inoculated unsuccessfully.

Discussion

Inoculations of various compartments of embryonated chicken eggs serve a wide range of purposes

Table 3. Overview of procedures to inoculate compartments of embryonated chicken eggs with methylene blue (MB) at various days of incubation, which resulted in 100% success rates.

Egg compartment	Day of incubation							Remarks
	6	8	10	12	14	16	18	
Air chamber	A[a]-6[b]	A-6	A-6	A-6	A-6	A-6	A-6	Rapid diffusion of MB to CAM[c], especially at young embryo ages
Embryo							B-38	
Allantoic cavity	C1-6	C1-6; C1-16; C2-6; C2-16	C1-6; C1-16; C2-6; C2-16	C1-6; C1-16; C2-6	C1-6; C2-6	C2-6		
Yolk	B-38; D1-25; D1-38; D2-38	C1-38; D2-38					D1-25	Yolk of 18-day incubated eggs was absorbed in the abdomen of the embryo
Albumen	D1-6; D2-6; D2-16	D2-6; D2-16	D2-6; D2-16	D2-6; D2-16	D2-6; D2-16			In a number of eggs of incubation days 12 and 14, MB was also observed in the amniotic cavity (Table 2)

Empty cells: 100% success rate was not obtained.
[a]Inoculation route as shown in Figure 1.
[b]Needle length in mm.
[c]Chorioallantoic membrane.

and have been performed extensively for many decades up to date. However, a comprehensive study outlining the accuracy of these inoculations is lacking. Therefore, in the present study, each of eight compartments of the embryonated chicken egg was targeted with methylene blue on several days between days 5 and 19 of incubation using simple and fully standardized procedures. Five embryonated egg compartments were targeted blindly (amniotic cavity, embryo, allantoic cavity, albumen and yolk) with needles of four different lengths. Three compartments were inoculated within sight (air chamber, CAM and blood vessel).

Chicken egg inoculations are most often performed in the period between days 5 and 14 of incubation and on day 18. Full, clean scores were obtained for all embryonated egg compartments on several days in mentioned time period (Table 3), except for the amniotic cavity and the embryo. Up to day 14 of incubation, embryos were never reached (Table 1). MRIs showed that these failures were not due to a mismatch between the targeted compartments and the lengths of the needles used, but at least in part to the variable position of the embryo in the egg. Moreover, the embryo seemed to retract upon puncture with the needle. Guy (2008) and Spackman & Stephens (2016) described amniotic cavity inoculations in 9–16 day incubated chicken eggs after visualization of the embryo by candling under darkness. Nevertheless, Guy (2008) stated that the principal disadvantage of this method is the difficulty of visualizing the embryo, particularly in dark coloured eggs, which were used in our study. Up to 18 days of incubation a full score for either the amniotic cavity or the embryo can only be obtained when these compartments are within sight, which can be achieved by the technique described by Beaudette *et al.* (1952). However, the mentioned technique is, as already stated in the introduction, very cumbersome, time consuming and poses a significant risk for contamination with microorganisms.

The amniotic cavity can be reached in an indirect way. Injection of methylene blue in the albumen on days 12 and 14 of incubation also resulted in the presence of the dye in the amniotic cavity (Table 2). Two theories exist regarding the mechanism of albumen absorption into the amniotic cavity: (1) via a direct connection between the albumen sac and the amniotic cavity, the so-called sero-amniotic connection, which is opened from day 12 of incubation onwards (Deeming, 1991), and (2) via endocytosis through the albumen sac and extra-embryonic cavity into the amniotic cavity (Yoshizaki *et al.*, 2002). We observed a physical connection between the "neck" of the albumen and the amniotic cavity (Figure 2(L)), which supports the first mentioned theory.

Albumen injections made with 6 mm needles resulted in a greater number of eggs with methylene blue in the amniotic cavity (27/40) compared to injections performed with 16 mm needles (7/40) (Table 2). This might be explained as follows: fresh egg albumen consists of two parts – a thick, jelly-like fraction and a thin, comparatively mobile fraction. The two may be separated by a sieve (Miller, 1941). Methylene blue injected with 6 mm needles reached the thin fraction, which is possibly absorbed faster into the amniotic cavity than the thick fraction.

The indirect approach of the amniotic cavity is not without risks: the albumen might form a hostile environment for microorganisms due to the presence of antimicrobial proteins and a high pH (Willems *et al.*, 2014). Furthermore, the absorption of the albumen is not completed before day 18 of incubation (Romanoff & Romanoff, 1967b), which means that inocula deposited in the albumen will take several days before fully reaching the amniotic cavity.

Success rates of targeting albumen and yolk were poor if injections were made with eggs in the upside down position (Table 1; Figure 1(D1)). This procedure was initially chosen for reasons of convenience. MRIs revealed the cause of these poor results: the albumen and yolk rapidly changed position after turning the eggs upside down. Injecting eggs in the normal position (Figure 1(D2)) significantly improved the results, especially regarding the albumen (Table 2).

Allantoic cavity inoculations were 100% successful on all incubation days examined, except for incubation day 18 (Table 3). The two (slightly) different inoculation methods applied (Figure 1(C1,C2)) gave similar success scores (Table 1). However, spilling of inoculum and/or allantoic fluid on the eggshell was observed in approximately 4% of the eggs in cases when the needle did not penetrate the air chamber (Figure 1(C1)), while spilling in the air chamber when it was penetrated (Figure 1(C2)) occurred only incidentally. The latter method is preferable considering this point of view.

In ovo vaccinations are performed on incubation day 18 via the blunt end of the eggs using 25 mm needles and mainly concerns vaccination against Marek's disease. These vaccinations are intended to deposit the vaccine in the amniotic cavity and/or the embryo. Similar protection against Marek's disease was obtained by vaccination into each of these two deposition sites when chickens were challenged at 5 days of age (Wakenell *et al.*, 2002). Protection against Marek's disease as early as possible is of great importance, as field challenge can occur immediately after placement of day-old chickens at the farm. Vaccine deposition in the embryo might result in earlier protection compared to deposition in the amniotic cavity. Moreover, in the present study it was shown that part of the inoculum was deposited in the allantoic cavity of 3/20 eggs using 25 mm needles, while Wakenell and others (Wakenell *et al.*, 2002) observed that Marek's disease vaccination in this egg compartment induced poor protection. Here we showed that 100% successful inoculation of embryos was obtained with 38 mm needles. However, further research is needed to ascertain whether protection against Marek's disease can be improved by using 38 mm needles.

Successful embryonated egg inoculations have to fulfil two criteria: (1) The inoculum is only injected in the intended compartment, and (2) The inoculation procedure itself should not harm the embryonated egg. The latter aspect was not assessed in the present study as success rates were scored immediately after injection of methylene blue by visual inspection of the opened eggs.

In conclusion, the present study may serve as a guide to more accurately inoculate the various embryonated chicken egg compartments.

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