Full Title:

Non-Invasive Monitoring Of Chick Development In Ovo Using A 7T MRI System From Day 12 Incubation Through To Hatching

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ABSTRACT

Purpose: Movement of the chick *in ovo* severely degrades MR images to the point where its development can not be monitored. We wished to determine if mild cooling of the egg could reduce such motion to the point where an ultra high field (7 Tesla) MRI system could be used to non-invasively monitor chick growth *in ovo* from 12 days incubation through to hatching.

Materials and Methods: Group A eggs were incubated at 37.5^0C for 21 days; Group B eggs were removed from the incubator on days 12, 15, 17, 18, 19, and 20 of incubation, cooled for 1 hr then returned to the incubator; Group C eggs were cooled as for Group B then individually imaged for 25 minutes using a 7T MRI system before being returned to the incubator. The average size (volume) of the heart, liver and brain at each stage of incubation were estimated from the T_2-weighted images and compared to existing values in the literature.

Results: The combination of cooling and MR imaging significantly reduced chick movement to allow excellent image acquisition at each stage of incubation. Repeated cooling and MR imaging did not significantly slow down or arrest the development of the chicks in either of the experimental groups.

Conclusions: MRI provides a powerful noninvasive tool to study the chick growth and the growth of individual organs including the brain, liver and heart *in ovo* from 12 days incubation.

Key words: 7Tesla -MRI; chick embryo; fertile eggs, development *in ovo.*

INTRODUCTION:

The avian embryo has occupied a unique position among higher vertebrates in that it provides an excellent model of embryology. This is because all of the developing chicks' requirements, with the exception of oxygen and heat, are provided by the egg contents and the surrounding eggshell. The first preserved account of a detailed description of the chick embryo is credited to Aristotle (384-322 B.C) a Greek philosopher. Today developing eggs remain widely used by workers in both the pure and applied sciences.

There are several approaches to studying the dynamics of embryonic development and growth (1) but most require the chick embryos to be sacrificed at different stages of incubation to allow body size and individual organs to be measured. An alterative approach is to make repeated observations on the same embryo using non-invasive imaging techniques such as magnetic resonance imaging (MRI) The advantage of this is that the number of embryos needed to attain statistical significance is significantly reduced, and repeated observations can be made on the same chick throughout the incubation process and subsequently related to the final phenotype at hatching (2). Another advantage of MRI over other non-invasive technologies such as ultrasound is that presence of the eggshell does not present a problem in terms of acquiring images of the interior of the egg. Nevertheless, there are only a few accounts of MRI being used to image fertile eggs and most of these relate to the initial stages of incubation (3-6); chick movement in the later stages of incubation has made imaging much more problematic particularly beyond the 10th day of incubation (2,7).

In recent years MRI techniques have advanced rapidly and as a result the signal to noise ratios have increased, and scanning time has dramatically decreased. The challenge or aim of the current study was to test the feasibility of using a state-of-the-art ultra high field (7 Tesla) MRI system to monitor chick growth *in ovo* from the 12^{th} day of incubation through to hatching. To minimise motion artefacts the eggs used in these experiments were cooled for 1 hour at 4°C in a refrigerator prior to imaging. Besides the problem of chick motion, the bio-effects of repeatedly exposing chick embryos to moderate short-term cooling and then high magnetic fields is also largely unknown. Another aim of this study was therefore to see if repeatedly cooling and or imaging developing chick embryos was detrimental to their survival, growth and hatching success.

MATERIALS AND METHODS:

Animals and treatments

30 broiler breeder eggs weighing 50-55g were obtained from a commercial hatchery and placed in a digital tabletop incubator (Brinsea Products Ltd, Standford, UK). All eggs were laid on the same day and were stored for 3 days prior to the experiment commencing. After the eggs had been incubated at 37.5^0C for 6 days they were 'candled' (i.e. light was shone through them using a hand held light source) to check if they were fertile and developing normally as would be revealed by the shadows of internal structures. Three eggs were removed from the incubator at this time, one of which was cracked, one was infertile and the other showed signs of bacterial contamination. The remaining 27 eggs were then divided into three groups of nine eggs and

treated as follows: Group A eggs were used as a control and left in the incubator for the full duration of the experiment (21 days); Group B eggs were removed from the incubator on days 12, 15, 17, 18, 19, and 20 of incubation, cooled for 1 hr in a refrigerator set at 4°C then returned to the incubator; Group C eggs were cooled as for Group B on days 12, 15, 17, 18, 19, and 20, but were then individually imaged for 25 minutes before being returned to the incubator. Throughout the experiment egg weight loss was monitored and the incubation temperature and relative humidity adjusted to optimal conditions in accordance with the incubator manufacturers recommendations. Candling was repeated on days 12, 15 and 18 to check the viability of the embryos in both experimental and control groups. From day 20 the eggs remained undisturbed in the incubator so that the hatching success rate and average hatching time could be monitored.

Imaging

The MR imaging were carried out using a 7 Tesla Bruker BioSpec 70/30 system (Bruker, Germany), together with a 120 mm inner-diameter actively shielded gradient set (400 mT/m maximum gradient) and a 72 mm birdcage volume resonator. The eggs were placed into a custom-built polystyrene holder, which was suspended within the resonator/magnet in order to minimize vibrations arising in the gradient coils. For the purpose of this investigation, where high contrast / high-resolution images were a prerequisite, T_2-weighted imaging was carried out using a multi-spin multi-echo pulse sequence (TR/TE 6424/56msec, 195 μm in-plane resolution, 0.5 mm slice thickness, 0.2 mm inter-slice distance). 60 slices were required

to cover the entire egg, with a total acquisition time of 25 minutes per egg. T_2-weighted images were chosen because they produced high signal contrast between the organs of interest, making them readily identifiable. T_1-weighted images acquired in 3D mode provided higher resolution images, particularly through plane, but produced a much reduced signal contrast thus making reliable organ delineation in the subsequent analysis very difficult.

Image Analysis

Different tissues and organ systems were identified on the basis of their anatomical position and signal contrast with other structures. For demonstration purposes, measurements of the volume of the heart, liver and brain were subsequently carried out using the Bruker ParaVision software by the same observer using an electronic cursor to manually delineate each organ in each slice of the dataset. The software then automatically integrated these areas over the slices to produce an estimate of the heart, liver and brain volume for each egg (n=9) at 12, 15, 17, 18, 19, and 20 days incubation. These values were then averaged to give an indication of incubation time-dependent changes in the volume of each of these organs.

RESULTS

T_2 imaging of chick embryos

Examples from T_2-weighted MRI multi-slice scans obtained at days 12, 15, 17, 18, 19 and 20 of incubation are shown in Fig.1 (i-vi). Examples of the complete 60 slice acquisitions for each of these time points can be viewed at www.gla.ac.uk/7tmr/development.htm

At 12 days incubation the chick is very small and most of the available space is taken up by the yolk and the extra-embryonic membranes and their associated fluid compartments (Fig.1 (i)). The air sac is also very prominent. The most distinguishing features of the embryo are the eyes, the brain and the heart which serve as useful points of reference. By day 15 the chick has grown considerably and feathers can now be visualized (Fig.1 (ii)). Many of the other organ systems are now also discernable including the liver, the gizzard, intestinal loops and the kidneys. The dorsal border of the lung tissue can also be seen embedded in the vertebral ribs. From 17 days incubation the chick's orientation changes in such a way that the head progressively moves down towards the air sac (Fig.1 (iii-vi)) The depressed state of the air sac in the images obtained from the later stages of incubation confirms that there must be considerable pressure exerted on this compartment as the chick continues to grow. The albumen mass is also no longer visible and the yolk sac surrounding the rapidly growing chick decreases in size. In the image corresponding to 20 days incubation (Fig.1 (vi)) the chick has just 'pipped', that is its head has penetrated through into the air sac at the blunt end of the shell. At this stage the chick actively begins to breath and the yolk sac becomes reabsorbed into the abdominal cavity via the naval in readiness for hatching.

MRI estimates of organ sizes and differential growth profiles

The MRI volume estimates (mean +/-SD) of the heart, liver and brain at 12, 15, 17, 18, 19, and 20 days of incubation are shown in Fig. 2. The volume of the liver is proportionally greater than both that of the brain and the heart at

each time point. As these data come from the same nine individuals it is possible to gain an appreciation of how these organs change in size over time; all three organ systems undergo the greatest increase in size between days 12 and 17 of incubation. The low variance at each time point suggests that the MRI volume estimates are reproducible between individuals.

The second data set shown on Fig. 2 corresponds to published weights (g) of the same organs obtained by sacrificing individuals at each respective time point (8). These data serve as a useful bench mark on which to compare our MRI volume estimates. For the liver and the brain there is a good correlation between the latter and the published data, but this is not the case for the heart. The most likely explanation for this effect probably relates to the fact that the volume of the blood in each of the heart chambers will have been taken into account in the MRI volume estimate. The post-mortem measurements in contrast are more likely to only include the weight of the cardiac muscle *per se.*

MRI and chick survival

The survivability and hatching success of the chick embryos was not adversely affected by the cooling treatment (Group B) or by the combination of cooling and imaging (Group C). With just one exception (Group B) all the experimental eggs hatched within 4-6 hours of the controls (Group A) on day 21. Hatchability, (number of hatched eggs/ total number of eggs incubated) was calculated to be 86.7%, which is typical of the expected hatchability values for the Ross 308 broiler breeder eggs (www.aviagen.com) which were used in this study.

DISCUSSION

A pilot study carried out using the 7T MRI system prior to this experiment confirmed that if fertile eggs, which have been incubated for 12 days or more, are not cooled prior to imaging then the image quality is poor due to excessive chick movement. In the current study we found that cooling the eggs for 1 hour in a refrigerator set at 4^0C prior to scanning significantly reduced chick movement and as a result the MR image acquisitions were with few exceptions, excellent. In addition we were able to show that repeated moderate cooling alone or in combination with MR imaging did not significantly slow down or arrest the development of the chicks (Groups B and C). With only one exception (in Group B), all of our treated eggs hatched on day 21 within a few hours of one another and with the untreated controls (Group A). Moreover there was no obvious pattern to the timing at which hatching occurred between the groups. We therefore feel confident that survivability and hatching success were not affected by either the cooling treatment and or the MR imaging used in this study.

The change in postural twist of the chick over time meant that it was not possible to image the chicks or their organs in the same orientation in a single scan on consecutive days. Nevertheless the yolk, air cell, limbs, spinal column and structures of the head including the brain, eye and beak could all clearly be defined in the T_2-weighted images associated with each 60-slice acquisition. The heart, liver, gizzard, intestine, lungs, kidneys and pectoral muscles plus many of the major blood vessels were also easily identifiable (Fig. 1(i-vi)).

For illustrative purposes the average volume or size (mean +/-SD) of the heart, liver and brain at 12, 15, 17, 18, 19, and 20 days of incubation were estimated using the Bruker paravison software program (Fig. 2). According to this data the heart, liver and brain grow most rapidly between days 12 and 17 of incubation. These findings are consistent with the changes in overall chick size, as illustrated in Fig.1 (i-vi). As the MR images obtained in this study did not 'cut' these organs in the same orientation each time, it could be argued that there will be errors in these measurements introduced by the so-called 'partial volume' effect (individual image pixels containing contributions from more than one tissue can reduce the clarity of the organ boundary). Errors introduced by the 'partial volume' effect can be reduced by acquiring images at a higher resolution, or by acquiring an image-set oriented individually for each organ. However the low variances observed in our measurements (Fig. 2) suggest that the partial volume effect was of little consequence in our study. The MRI estimated volumes for the brain and liver were also comparable with published weights for each stage of incubation (8). The former were obtained by continuously monitoring the same animals, as opposed to sacrificing multiple animals at each time point, and so probably give a more realistic impression of the differential growth of each of these organs over time. The MRI volume estimates for the heart were considerably different than those obtained by directly sacrificing chicks and weighing the heart but this is probably due to the fact that blood within the heart chambers is inclusive in the MRI volume estimate of this organ.

As previously stated, MRI has significant advantages over other less invasive systems as the number of embryos needed to attain statistical

significance is significantly reduced. The fact as shown here that repeated observations can also be made on the same chick throughout the incubation process and then related to the phenotypic characteristics of the emerging chick is also highly beneficial compared to more invasive investigative methods.

Today the developing avian embryo continues to be routinely used as an experimental model in a wide variety of science based disciplines concerned with such diverse fundamental questions as the effect of nutritional or endocrine deficiencies on embryonic development and growth, disease transmission, and the development of vaccines. This study has shown that a 7T MRI system can be used as a powerful investigative tool for non-invasively studying the development and growth of chicks and individual organ systems *in ovo* from 12 days incubation. Moreover, the image quality is exceptional allowing sequential quantifiable measurements to be made on the same chick throughout the incubation process. In order to image the chicks successfully it is however necessary to mildly cool the eggs for 1 hour prior to imaging. The fact that 95% of the experimental eggs in groups B and C went on to hatch successfully suggests that treating the eggs in this way does not adversely affect the prenatal growth and development of the chick embryos. Additional studies are therefore currently being conducted to determine if cooling is necessary before day 12 of incubation and thus determine if MRI can be used to continuously monitor chick development and growth from day 0 through to hatching. Studies of this type are pivotal to improving our understanding of early developmental processes, and will help to answer key questions such as how do the extra-embryonic membranes and egg contents become partitioned

during the early stages of embryonic development *in vivo* (9). Further experiments are also being planned to ensure that the postnatal chick growth is also unaffected by the exposure of chick embryos to these treatments.

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REFERENCES

1. Bellairs R, Osmond M. The atlas of chick development, 1st edition. London: Academic Press Ltd; 1998. 323p.
2. Effmann EL, Johnson GA, Smith BR, Talbott GA, Cofer G. Magnetic-resonance microscopy of chick embryos in ovo. Teratology 1988:38: 59-65.
3. Falen S, Szeverenyyi NM, Packard DS, Rucocco MJ. Magnetic resonance imaging study of the structure of the yolk in the developing avian egg. J Morphol 1991:209: 331-342.
4. Hutchison MJ, Lirette A, Etches RJ, Towner RA, Janzen EG. An assessment of egg yolk structure using magnetic resonance imaging. Poultry Sci 1991:71: 2117-2121.
5. Klein S, Rokitta M, Baulain U, Thielebein J, Hasse A, Ellendorff F. Localization of the fertilized germinal disc in the chicken egg before incubation. Poultry Sci 2002: 81:529-536.
6. Klein S, Baulain U, Rokitta M, Marx G, Thielebein J, Ellendorff F. Sexing the freshly laid egg- development of embryos after manipulation; analytical

approach and localization of the blastoderm in the intact egg. World Poultry Sci J 2003:59:39-45.

7. Moseley ME, Wendland MF, Darnell DK, Gooding CA. Metabolic and anatomic development of the chick embryo as studied by phosphorous-31 magnetic resonance spectroscopy and proton MRI. Pediatr Radiol 1989:19:400-405.
8. Romanoff AL. The avian embryo. New York: McMillan Company; 1960. p1305.
9. Baggot GK. Development of extra-embryonic membranes and fluid compartments. In: Baggot GK, Bakst MK, Bellaire R, Christensen VL, Fasenko GK, Starck JM, editors. Perspectives in fertilization and embryonic development in poultry, Lincs, UK: Ratite Conference Books; 2001. p23-29.

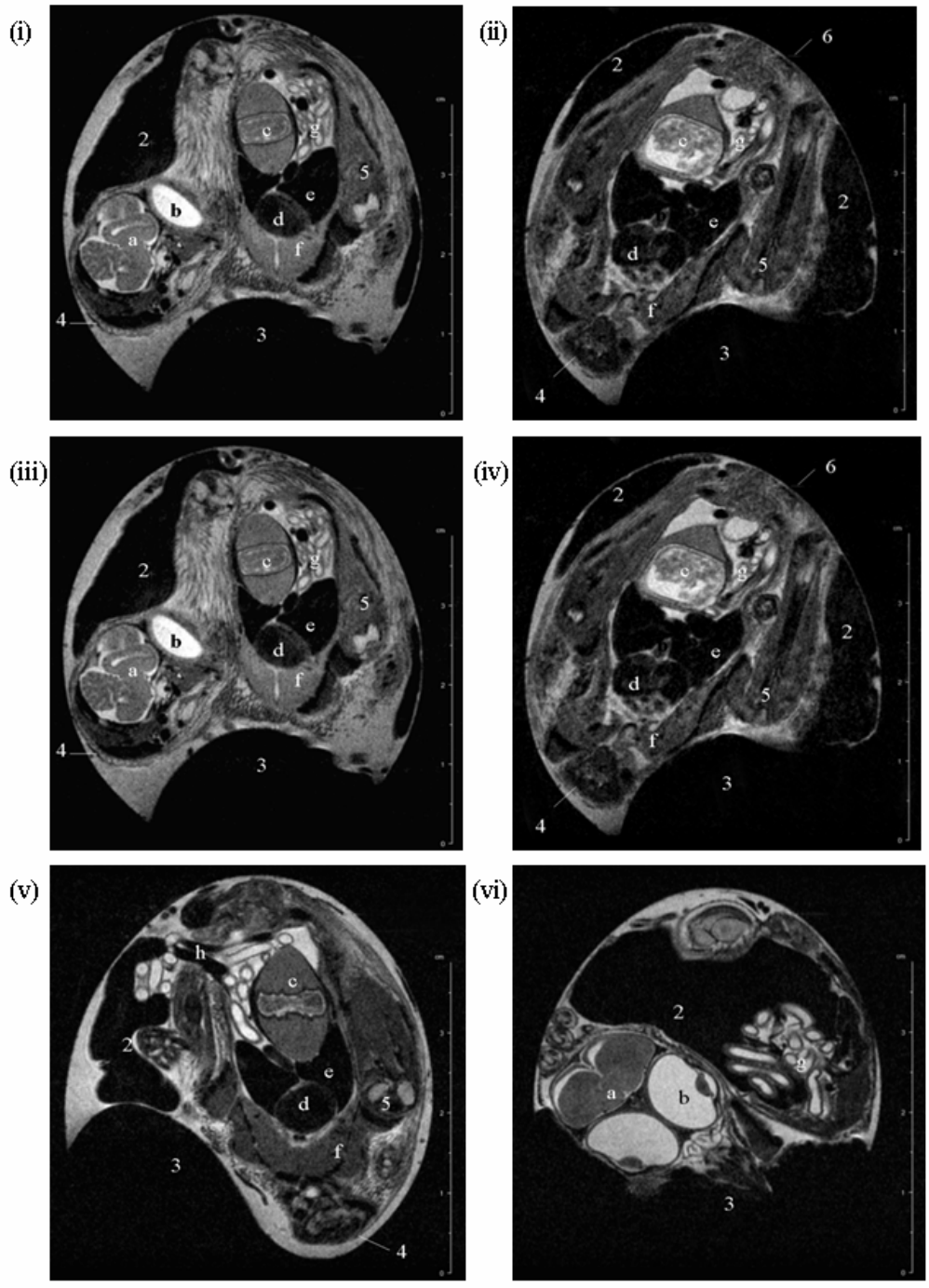

Figure 1: Representative examples of T_2-weighted multi-slice scans of chick embryos *in ovo* at **(i)** 12, **(ii)**15, **(iii)**17, **(iv)** 18**, (v)**19 and **(vi)** 20 days of incubation. 1 = albumen; 2 = yolk; 3 = airsac; 4 = head; 5 = limb; 6 = rump; a = brain; b = eye; c = gizzard; d = heart; e = liver; f = pectoral muscles; g = intestine; h= umbilical vessels. The complete scans can be viewed at www.gla.ac.uk/7tmr/development.htm

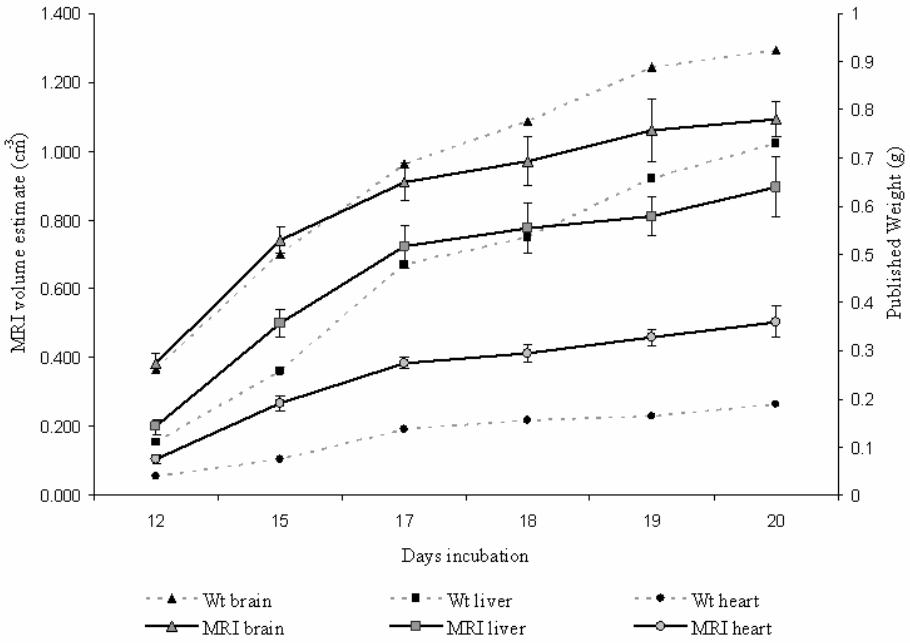

Figure 2: MRI volume estimate of the heart, the liver and the brain size at days 12, 15, 17, 18, 19 and 20 of incubation (mean +/- standard for n=5). Published weights for these organs derived by invasive techniques are also presented to allow comparisons to be made with the non-invasive estimates.