Ontogenetic development of the uncinate processes in the domestic turkey (*Meleagris gallopavo*)

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ABSTRACT Uncinate processes extend off the vertebral ribs in most species of bird. The processes are a crucial component of ventilatory mechanics, being involved in inspiration and expiration. Here we examine the pattern of ossification of the uncinate processes using histochemistry and biomechanical testing in developing domestic turkeys (*Meleagris gallopavo*). Ossification begins just before hatching, and the processes are fully ossified in the adult bird. We suggest that the development of these processes is linked to the onset of air breathing and the increase in sternal mass that occurs after hatching.

Key words: uncinate process, galliform, ontogeny, ossification

INTRODUCTION

Birds and mammals are the only vertebrates capable of very high rates of oxygen consumption relative to body mass. However, whereas the mechanics of breathing are well-documented for mammals, our understanding of how the avian lung is ventilated is comparatively poor. The avian respiratory system is composed of a set of rigidly fixed lungs and series of approximately 9 air sacs. The bellows-like action of the air sacs and the unidirectional passage of air through the lungs are facilitated by movements of the ribs and sternum during ventilation (Claessens, 2004). Developmental studies of the avian respiratory system are often focused on the structure and function of the parabronchial lung and air sac system (Duncker, 1978; Maina 2003a,b, 2006). The main site of gas exchange for the avian embryo is the chorioallantois, a membrane that adheres to the inner membrane of the shell and permits diffusion of oxygen and carbon dioxide between the blood and environment (Wangensteen and Rahn, 1970/71; Tullett and Deeming, 1982). As incubation progresses, an air cell forms in the blunted end of the egg through water loss across the membranes. During the period around 24 to 48 h before hatching, the embryo pips internally and begins to use pulmonary ventilation in addition to the chorioallantoic membrane for gas exchange. The relative contribution of the chorioallantoic membrane to respiration decreases rapidly after external pipping. Upon hatching, movements of the skeleton fulfill respiratory requirements by pumping air around the air sacs (Menna and Mortola, 2002).

Uncinate processes (UP) are bony projections that extend off the posterior edge of the vertebral ribs in most species of extant birds. Although UP were previously thought to be adaptations for flight (Welty, 1988) or to strengthen the ribs and rib cage (Kardong, 1988), these processes have recently been demonstrated to be integral components of the ventilatory mechanics of birds. The UP are involved in both ventilation and running locomotion in the giant Canada goose (Codd et al., 2005). The appendicocostales muscle originates from the caudal surface of the processes and facilitates cranial and ventral movements of the ribs and sternum, respectively, during inspiration. The UP also act as a brace for the externus obliquus abdominus that inserts onto the base of the processes and pulls the sternum dorsally during expiration (Codd et al., 2005). Geometric modeling of the rib cage demonstrated that the UP function as levers for the forward movement of the ribs and ventral rotation of the sternum during respiration (Tickle et al., 2007). Given the lever action of the processes on the ribs and sternum, changes in length will have a significant functional impact. For example, a longer process provides a greater surface area for muscle attachment and a greater mechanical advantage for movements of the ribs and sternum (Tickle et al., 2007).

The avian pattern of skeletal development is typical of amniotes. A cartilaginous skeleton is subsequently replaced by bone mineralisation. The majority of developmental studies have focused on the sequence of ossification in galliform birds, such as the domestic chick-

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en (Gallus gallus) and the Japanese quail (Coturnix coturnix). Fell (1925) undertook the first detailed investigation of histogenesis of bone and cartilage in the developing fowl, and subsequent research has tended to focus on the long bones (e.g., Simmons and Pankovich, 1963; Blom and Lilja, 2004). An understanding of the skeletal changes during ontogeny has been used to model avian growth (Starck, 1994) and to examine the significance of developmental patterns in phylogenetics (Burke and Feduccia, 1997). However, existing research into the ontogeny of skeletal development in birds provides sparse information on the chondrification and ossification of UP. Recent comparative analyses of the embryonic skeleton in galliform birds suggest that although ossification of the UP occurs in the quail (Nakane and Tsudzuki, 1999) and the chicken (Hogg, 1980), it is absent in stage 40+ turkeys (Maxwell, 2008). Hogg (1980) highlighted the uncertainty surrounding the sequence of UP ossification in the chicken embryo; onset of bone growth has been reported from as early as d 17 (Fujioka, 1952) to as late as posthatch (Hamilton, 1952). Here we examine the pattern of ossification of the UP in the developing domestic turkey using histochemical and biomechanical techniques.

MATERIALS AND METHODS

Eggs of the domestic turkey (Meleagris gallopavo) were purchased from a licensed breeder and placed in an incubator (Ova-Easy 190; Brinsea Products Ltd., Sandford, UK) within 1 wk of laying. Eggs were incubated for 28 d. Temperature and humidity during the initial 25 d of incubation were maintained at 37.5°C and 50%, respectively. For the remainder of incubation humidity was increased to 65%. Eggs were turned every hour until d 25 of incubation, whereupon eggs were left undisturbed to hatch. Before hatching, embryos were culled by rapid freezing to −40°C daily from d 14 of incubation to d 28. After hatching, chicks were transferred to a brooder and given access to food (chick crumbs) and water ad libitum. Single samples for histochemical analysis were taken daily from d 29 to 35. Birds were also culled on d 42, 49, 56, 63, and 94. Posthatch birds were culled by dislocation of the cervical vertebrae. Single samples for mechanical testing were taken on d 25 and 94. Birds were frozen immediately after death for future processing.

Dissection and Histochemistry Protocol

At d 25 and 94, two specimens were taken, one for histochemical analysis and the other for biomechanical testing. At all remaining time points, one sample was collected for histochemistry. Birds were skinned and eviscerated, and the superficial thoracic musculature was carefully removed to expose the ribcage. Prepared specimens were stained for bone and cartilage according to a protocol adapted from the mouse method of Miller and Tarpley (1996). Samples were fixed in 90% ethanol for 24 h before immersion in Alcian blue solution (Acros Organics, Geel, Belgium; uptake corresponds to the presence of cartilage) for 72 h. Skeletons were then rehydrated in a series of ethanol solutions (70, 40, and 15%, respectively) for 2 h and finally rinsed in distilled water. Remaining muscle tissue was then macerated by exposure to 1% KOH solution for 24 to 48 h. Skeletons were then transferred to a solution of Alizarin red (Sigma-Aldrich, St. Louis, MO; to indicate the presence of bone) for 72 h followed by repeat exposure to 1% KOH as required. Stained skeletons were passed through a series of glycerol solutions (20, 50, and 80%, respectively) and stored in 100% glycerol. All stages of the staining protocol were conducted at room temperature (22°C). The UP are numbered from the anterior end of the skeleton, according to the vertebral rib from which they project (i.e., the most cranial rib is rib 1, UP 1). Stained skeletons were then examined for the presence of bone and cartilage using a Leica MZ9s light microscope (Leica Microsystems, Milton Keynes, UK). Digital photographs of the ribcage and UP were then taken and analyzed using the Leica Application Suite Software. The most anterior and posterior UP are typically reduced in morphology, whereas the UP on the remaining ribs are uniform in size (Tickle et al., 2007). For comparative analyses, the relative area of bone and cartilage was calculated for the UP that extends from the fourth vertebral rib in all specimens. Areas of blue and red stain were measured and then calculated as a percentage of the total process area.

Mechanical Testing

Nanoindentation was used to calculate the elastic modulus at the base of the UP. Using the data collected from the rib histochemistry, vertebral rib 4 was dissected from the right-hand side of representative specimens obtained on d 25 and 94. Specimens were dehydrated in 95% ethanol for 24 h before embedding in a noninfiltrating polyester resin (Kleer set; Metprep Ltd., Coventry, UK). To ensure accurate calculation of mechanical properties, surface topography of the samples was imaged by atomic force microscopy, enabling the selection of relatively smooth areas for indentation. A TriboScope (Hysitron Inc., Minneapolis, MN) nanomechanical system was then used for material testing. Nanoindents were made using a maximal loading force of 5,000 μN applied via a tetrahedral diamond Berkovich indenter tip. The indenter detects force and displacement to form a nanoindentation curve. This curve consists of a loading phase (the tip is pressed into the material up to a maximal force), holding period (the tip creeps into the material), and an unloading phase (force on the sample is released; Hengsberger et al., 2001). The unloading force-displacement curve was then used to calculate reduced modulus using the equations of Oliver and Pharr (1992, 2004). Calculation of the elastic moduli assumed the Berkovich tip with an...
 elastic modulus of 1,140 GPa and Poisson ratio of 0.07. Given the different distribution of cartilage and bone in the UP of different ages, elastic moduli were calculated using contrasting Poisson ratios. In accordance with published values we used a value of 0.3 for bone (Rho et al., 1997; Zysset et al., 1999) and 0.5 for cartilage (Mak et al., 1987; Wong et al., 2000). Mean elastic moduli values were compared using an independent sample t-test (SPSS 15.0).

RESULTS AND DISCUSSION

Turkey embryos and chicks had 7 vertebral ribs of which 5 were paired with a sternal rib, whereas vertebral ribs 1 and 2 were unpaired. The UP occurred on ribs 2 to 6 in all specimens. A very short UP occurred on vertebral rib 1 in specimens taken on d 15, 19, 20, 25, and 31. The UP were absent on rib 7 in all specimens. The reduced size and morphology of UP 2 and 6 indicate that their role in ventilation is limited (Tickle et al., 2007). For ease of comparison (and because the timing of ossification and morphology were similar in UP 3, 4, and 5), uncinate process 4 was considered to be representative.

Days 14 to 21

The rib cage appeared cartilaginous until d 18 of incubation when all vertebral ribs began to ossify. By d 21 the vertebral ribs were ossified except for the ventral tip, capitulum, and tuberculum. The sternal ribs that pair with the vertebral ribs 5 and 6, displayed bone growth on d 19. Ossification began on d 20 for the sternal ribs paired with vertebral ribs 4 and 7, whereas the remaining sternal rib, paired with vertebral rib 3, began bone proliferation on d 21. The sternum remained entirely cartilaginous during this period. By d 17 the characteristic adult morphology of the UP was fixed, although they remained cartilaginous. The UP 2 and 6 appeared curved and were relatively short compared with those on ribs 3, 4, and 5. Uncinate process 3 was straight with a flared base, whereas UP 4 and 5 were L-shaped with a distended tip (Figure 1A, 2). Uncinate process 4 was entirely cartilaginous during these stages (Figure 1A, 2A, 2D).

Days 22 to 28

Ossification of the UP began on d 22 with process 5. By d 23 UP 2 to 5 underwent bone growth. The timing

Figure 1. Pattern of ossification of uncinate process 4 from 19 to 94 d. (A) d 19 embryo, stained Alcian blue, ×25, (B) d 25 embryo, Alizarin red staining locates the ossification center, ×20 (C) d 28 chick, further bone growth apparent, ×20, (D) d 36 chick, ×20, (E) d 49 bird, ×12.5, (F) d 94, ossification of the uncinate process base to the vertebral rib, ×12.5. Scale bars represent 1 mm.
of ossification of process 2 varied between specimens, such that bone growth was not detected until hatching in some birds. Bone proliferation extended from the center of the process toward the cartilaginous tip and base. Vertebral and sternal ribs exhibit widespread ossification (Figure 2B), whereas sternal bone growth begins in the cranial aspect of the keel on d 24. During this period rapid ossification increased the bone area of process 4 from 13 to 37% (Figure 1B, 1C, 2D).

**Days 29 to 35**

Progressive ossification of the UP continued, including process 6 by d 31. The base and tip of each process remained cartilaginous. The ventral and dorsal tips of the vertebral and sternal ribs remained cartilaginous, whereas sternal ossification continued in the craniodorsal keel. Rapid mineralization increased the proportion of bone from 37 to 59% in process 4 (Figure 1D, 2D).

**Days 36 to 63**

Ossification of vertebral and sternal ribs appeared complete. Ossification of the UP appeared to slow as bone growth continued at the base; however, the tip remained predominantly cartilaginous. The ventral aspect of the tip appeared proportionally more ossified than the dorsal edge (Figure 1E and 1F). The symphysis between the UP and vertebral rib remained cartilaginous, despite extensive ossification at the process base. The relative area of bone in process 4 increased from 59 to 77% (Figure 2D).

**Days 64 to 94**

Ossification of the UP continued, primarily at the base. Staining of the tip remained blue in all processes, indicating that cartilage was the predominant tissue, although the relative size of this region was smaller in processes 2 and 6 (Figure 2C). The process base was seen to ossify to the bony vertebral rib by 94 d (Figure 1F). Uncinate process 4 exhibited a slight increase in bone from 77 to 79% of total area (Figure 2D).

The UP are clearly present in the developing turkey embryo and are initially cartilaginous but undergo ossification from d 22, being completely ossified to the vertebral rib from which they extend by d 94. Cartilage has a lower structural density than bone (Lyman, 1994), meaning it has different biomechanical properties. Two key changes that occur during ontogeny in birds are the switch to pulmonary ventilation and the exponential increase in the pectoralis muscle mass that occur after

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**Figure 2.** Representative skeletons showing the progress of ossification during ontogeny. (A) d 19 embryo, the uncinate processes (UP) are cartilaginous, whereas ossification has begun in the vertebral ribs (rib 1 is missing), ×8, (B) d 28 chick, extensive bone growth in the ribs, whereas ossification is apparent in UP, ×6.3, (C) d 90 bird, ossification is advanced in the ribs and UP. Anterior is to the right. Ribs are numbered from the anterior end. Scale bar 5 mm. (D) Percentage of cartilage (dashed line) and bone (solid line) for uncinate process 4 from d 14 to 94.
hatching (Ricklefs et al., 1994). The morphology of the UP in adult birds is linked to adaptations of the sternum to different forms of locomotion. The UP are short in walking or running birds, intermediate in nonspecialists, and long in diving species; the longer the sternum the longer the UP (Tickle et al., 2007). There may be fundamental differences in the ventilatory mechanics of different species of bird linked to morphological specializations to different forms of locomotion (Tickle et al., 2007). For example, the mass of the flight muscles accounts for up to 35% of the total BW in some species of flying birds (Dial et al., 1988), which may affect the timing of UP ossification. However, it remains to be determined if the pattern of ossification also varies during ontogeny in species adapted to diving, flying, or running.

Maxwell (2008) did not report ossification of the UP for stage 40+ (before hatch) M. gallopavo but did report that accelerated ossification of the UP is characteristic of galliforms. Endochondral bone formation is associated with mechanical stimulation (Carter et al., 1996). For the turkeys used in this study, ossification began just before hatching; therefore, we suggest that the onset of respiratory movements, around the time of internal pipping, may be the trigger for the initialization of bone formation in the UP. Similarly, ossification of the UP has been shown to occur at the time of internal pipping in the Japanese quail (Nakane and Tsudzuki, 1999) and at hatch in the domestic chicken (Hogg, 1980). Therefore, the timing of ossification in the UP may be conserved within the galliformes.

Biomechanical Testing

A section toward the base of the UP on d 25 and 94 specimens (Figure 3A) was exposed by grinding and polishing the resin. Representative force-displacement curves from which the elastic moduli were calculated for each sample are shown in Figure 3B. The disparity between the curves is indicative of a difference among the samples in response to loading. The relatively steep slope in the d 94 specimen indicates a higher elastic modulus when compared with d 25. The mean elastic modulus of the uncinate process base from d 25 (n = 7; 1.20 GPa ± 0.07) was significantly reduced compared with d 94 (n = 8, 6.72 GPa ± 0.31; P < 0.001). Mechanical testing indicated that the stiffness increased as the process ossified, meaning the UP in older birds would function as a more effective lever and brace during respiration. The UP with a cartilaginous attachment to the vertebral rib would also be more likely to flex during muscle shortening, therefore reducing its effectiveness. Given the key role UP have during avian ventilation, an examination of the developmental changes in UP ossification across a wider range of species during ontogeny may shed new light on the mechanics of avian ventilation.

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REFERENCES


