The role of embryo movement in the development of the furcula

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ABSTRACT

The pectoral girdle is a complex structure which varies in its morphology between species. A major component in birds is the furcula, which can be considered equivalent to a fusion of the paired clavicles found in many mammals, and the single interclavicle found in many reptiles. These elements are a remnant of the dermal skeleton and the only intramembranous bones in the trunk. Postnatally, the furcula plays important mechanical roles by stabilising the shoulder joint and acting as a mechanical spring during flight. In line with its mechanical role, previous studies indicate that, unlike a number of other intramembranous bones, furcula growth during development can be influenced by mechanical stimuli. This study therefore investigated the response of individual aspects of furcula growth to both embryo immobilisation and hypermotility in the embryonic chicken. The impact of altered incubation temperature, which influences embryo motility, on crocodilian interclavicle development was also explored. We employed whole-mount bone and cartilage staining and 3D imaging by microCT to quantify the impact of rigid paralysis, flaccid paralysis and hypermobility on furcula growth in the chicken, and 3D microCT imaging to quantify the impact of reduced temperature (32°C to 28°C) and motility on interclavicle growth in the crocodile. This revealed that the growth rates of the clavicular and interclavicular components of the furcula differ during normal development. Total furcula area was reduced by total unloading produced by flaccid paralysis, but not by rigid paralysis which maintains static loading of embryonic bones. This suggests that dynamic loading, which is required for postnatal bone adaptation, is not a requirement for prenatal furcula growth. Embryo hypermotility also had no impact on furcula area or arm length. Furcula 3D shape did however differ between groups; this was marked in the interclavicular component of the furcula, the hypocleideum. Hypocleideum length was reduced by both methods of immobilisation, and interclavicle area was reduced in crocodile embryos incubated at 28°C, which are less motile than embryos incubated at32°C. These data suggest that the clavicular and interclavicle components of the avian furcula respond differently to alterations in embryo movement, with the interclavicle requiring both the static and dynamic components of movement-related loading for normal growth, while static loading preserved most aspects of clavicle growth. Our data suggest that embryo movement, and the mechanical loading this produces, is important in shaping these structures during development to suit their postnatal mechanical roles.

Key words: Furcula, clavicle, chicken embryo, crocodile embryo, intramembranous ossification.

INTRODUCTION

The pectoral girdle is a complex structure which is highly variable between species. In birds, a major component of this structure is the furcula, or 'wishbone', a forked bone which is commonly considered homologous to a fusion of the bilateral clavicles found in many mammals (Vickaryous & Hall, 2010). In many vertebrates the clavicle is the sole intramembranous skeletal element in the trunk; it is a remnant of the ancestral dermal skeleton which has become integrated into the rest of the endochondrally derived pectoral apparatus (McGonnell, 2001). In addition to its role in stabilising and supporting the shoulder joint, particularly in species with laterally positioned limbs like reptiles and amphibians, the furcula is important in acting as a spring during flight in many birds (Jenkins et al. 1988). Accordingly, the proportions of the furcula vary between avian species with different locomotor styles. The furcula's bony fusion of the two lateral clavicular arms, the hypocleideum, is an attachment site for the pectoral muscle, and is considered homologous to the interclavicle, a bone located between the clavicles in many tetrapods (Vickaryous & Hall, 2010). Interclavicles are completely absent in marsupials and placental mammals but present in monotremes and many reptiles such as crocodilians, which retain only a single flat, bar or paddle-like interclavicle with no lateral processes (Vickaryous & Hall, 2010). Such phylogenetic and functional insights clearly support a mechanical role for the furcula, yet the role of movement in the formation of its clavicular and interclavicular components remains incompletely defined.

The developmental origin of these different components of the furcula has been the subject of some debate (Lansdown, 1968; Russell, 1985). The avian furcula and crocodilian interclavicle have been shown to form by intramembranous ossification, during which the cells in mesenchymal condensations differentiate directly into osteoprogenitors without an initial cartilaginous stage; secondary cartilage formation may occur at the lateral extremities of the clavicles after the onset of mineralisation (Vickaryous & Hall, 2010). The paired clavicles of mice, however, have a dual origin: the articular ends of each clavicle form by endochondral ossification, during which mesenchymal condensations differentiate to form cartilage models which are later replaced by bone, whereas the rest of the clavicle forms by intramembranous ossification (Huang et al. 1997; Hall, 2001). Cell labelling studies have shown that, in both the mouse and chick, the cells which form the bony clavicles are of lateral plate mesoderm and somitic mesoderm origin (McGonnell, 2001; Matsuoka et al. 2005).

Bone growth during development, like postnatal skeletal mass and architecture, is influenced by mechanical loading to match a bone's structural parameters to facilitate locomotor performance. A number of studies have established that embryonic skeletal growth is subject to regulation by the mechanical stimuli resulting from embryonic muscle contraction, by focusing primarily on the role of embryo movement in regulating the endochondral ossification process by which the long bones of the limbs grow (Hall & Herring, 1990; Hosseini & Hogg, 1991a,b; Germiller & Goldstein, 1997; Osborne et al. 2002; Lamb et al. 2003; Nowlan et al. 2010b; Pollard et al. 2014). The role of muscle contraction in the development of intramembranous bones has thus far received much less attention. Studies in embryonic mice and chickens have shown that the growth of intramembranous neurocranium skull bones is largely unaffected by embryo paralysis, and the intramembranous calvaria bones of postnatal mice do not respond in the same way as limb bones to microgravity (Zhang et al. 2013). In vitro studies using cultured murine calvaria and long bones, and primary osteoblasts isolated from murine intramembranous calvaria elements and endochondrally derived ulnae, demonstrate that calvaria cells are less responsive than those from limb bones to dynamic strain (Rawlinson et al. 1995). The growth of the zebrafish skull, on the other hand, is severely affected by microgravity, although many craniofacial bones in this species are endochondral in origin, which may explain this difference (Cubbage & Mabee, 1996; Edsall & Franz-Odendaal, 2014). The mandible, which is primarily intramembranous in origin, is reported to be reduced in growth by paralysis in both mouse and chick embryos, but is typically considered the least severely changed of the affected skeletal elements (Hall & Herring, 1990). Whether the regulation of growth in bones derived by endochondral or intramembranous ossification has similar requirements for mechanical stimuli is therefore still somewhat unclear.

There is evidence that the growth of the interclavicle/furcula, another intramembranous bone, is indeed severely affected by altered movement. Clavicle shape and size have been reported to be altered in muscle-less mice and in mice with muscular dysgenesis, which results in flaccid paralysis and unloading of the skeleton throughout prenatal development (Nowlan et al. 2010a). These changes include a reduction in secondary cartilage formation at the articular ends of the clavicle, at the acromioclavicular joint (Rot-Nikcevic et al. 2007). Induction of rigid paralysis with the neuromuscular blocking agent decamethonium bromide (DMB) has also been shown to result in reductions in clavicle mass, fusion of the sternal rudiments and collapse of the thorax, leading to altered furcular shape in the chick (Hall & Herring, 1990; Rot-Nikcevic et al. 2006). In these experiments, the growth of the furcula/clavicle structure was more severely affected at relatively early developmental stages than were endochondrally formed limb bones, indicating that the

growth of at least some intramembranous bones is dependent upon embryonic muscle contraction. The impact of altered embryo movement on the development of the reptilian interclavicle is, however, unknown.

In this study we aimed to identify more fully the contribution of mechanical loading to the development of the clavicular and interclavicular furcula components in the chick and interclavicle in crocodilians. We investigated the impact of flaccid paralysis, which results in complete unloading of the skeleton, rigid paralysis, which maintains only the static component of loading resulting from embryonic muscle contraction, and stimulating skeletal muscle contraction with 4-aminopyridine (AP) on chick furcula growth. In addition to this pharmacological modulation of chick embryo movement, we also investigated the impact of altered incubation temperature and embryo motility, which could occur naturally, on interclavicle growth in the West African Dwarf crocodile, to test whether the growth of this intramembranous skeletal element is dependent on muscle contraction.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by local ethics committee.

Fertilised white leghorn chicken eggs (*Gallus gallus*) were obtained from Henry Stewart & Co. (Norfolk, UK) and incubated at 37°C with 45% relative humidity. The first day of incubation was designated E0. The eggs were 'windowed' according to standard protocols (Fisher and Schoenwolf, 1983) on E4. Chicks were treated with decamethonium bromide, pancuronium bromide or 4-aminopyridine to alter embryo motility between stage Hamburger and Hamilton stage 36-37, 36-40 and 36-44 (Osborne et al., 2002{Heywood, 2005 #26)}. Another three groups of chicks received the vehicle, Tyrode's salt solution, at the same times to act as a control. On the final day of treatment, chicks were killed by Schedule 1 decapitation in accordance with the Animals (Scientific Procedures) Act 1986.

Crocodile (*Osteolaemus tetraspis*) eggs from a single clutch were obtained from Crocodiles of the World conservation and education centre (Witney, UK) at E10. They were randomly assigned into groups and incubated at 28°C or 32°C (n=3 per group) with 90% relative humidity. Embryos incubated at 28°C exhibit significantly reduced embryo motility during mid-gestation relative to

those incubated at 32°C (Pollard et al., manuscript submitted). Crocodile embryos were euthanized by a Schedule 1 method at E70, of an approximately 75 day incubation period and staged according to Ferguson's staging series for *Alligator mississippiensis* (Ferguson, 1985).

Manipulation of embryo movement

Embryonic chicks were treated with pharmacological agents which induced flaccid paralysis, rigid paralysis or skeletal muscle hyperactivity at stage 36 (equivalent to E10). Chicks were treated with 100 µL of a sterile-filtered solution of 8 mg mL−1 pancuronium bromide (PB), 5 mg mL−1 decamethonium bromide (DMB) or 2 mg mL−1 4-aminopyridine (AP) in Tyrode's salt solution (TS: 0.8% NaCl, 0.02% KCl, 0.005% NaH2 PO4.H2O, 0.1% glucose, 0.1% NaHCO3 at pH 7.4). These methods for pharmacological manipulation of movement in embryonic chickens have been validated by a number of previous studies (Hall & Herring, 1990; Hosseini & Hogg, 1991a,b; Osborne et al. 2002; Lamb et al. 2003; Heywood et al. 2005). Each dose was injected through the egg window onto the chorioallantoic membrane. On each subsequent day, embryos received a 100-µL top-up dose of 1 mg mL−1 PB, DMB or AP at 24-h intervals to maintain paralysis or hyperactivity. We examined the impact of 1, 4 or 8 days of altered embryo movement on clavicle growth.

Monitoring of the frequency of embryo movement by candling crocodile eggs revealed that embryos incubated at 28 °C were significantly less motile than those incubated at 32 °C (Pollard et al. private communication).

Assessment of furcula growth

The skin and viscera were removed from embryonic chicks, which were then fixed in 96% ethanol for 3 days. Embryos were immersed in acetone for 48 h to remove fat. Embryos were then washed in 96% ethanol for 2 h prior to staining in a solution of 0.015% Alcian blue, 0.005% Alizarin red in 70% ethanol, 20% acetic acid and 10% dH2O at 37 °C for 4 h. Embryos were washed in 96% ethanol for 1 h followed by running tap water for 1 h. Muscle was cleared in an aqueous solution of 1% potassium hydroxide (KOH) until the skeletons were visible (approximately 24 h). The embryos were then de-stained in a graded series of glycerol/potassium hydroxide concentrations: 20% glycerol/0.8% aqueous KOH, 50% glycerol/0.5% aqueous KOH, 80% glycerol/0.2% KOH. Stained embryos were stored in 100% glycerol.

Images of each embryonic furcula, oriented in the coronal plane, were acquired using a KS300 Imaging System with JVC 3CCD colour camera. Total furcula area (the area of tissue stained with Alizarin red), the length of the left and right clavicular arms of the furcula, the length of the

hypocleideum and the angle between the two arms were measured using image j. The furculae from a minimum of five chicks from each treatment group were assessed at each time-point. Additionally, 3D images of the furcula from PB-treated and TS-treated control chicks (n = 3) were obtained by microCT imaging. Limbs were scanned with a SkyScan 1172 microCT system (Bruker, Belgium) with a resolution of 5 µm, voltage of 40 kv, current of 250 µA, exposure time of 1600 ms and a 0.5 mm aluminium filter. Images were reconstructed using NRecon.

The interclavicles from embryonic crocodiles were fixed with 4% paraformaldehyde for 24 h. 3D images were obtained by microCT imaging. Limbs were scanned with a SkyScan 1172 microCT system with a resolution of 5 µm, voltage of 40 kv, current of 250 µA, exposure time of 1600 ms and a 0.5 mm aluminium filter. Images were reconstructed using NRecon. The volume, length and width of the interclavicle were measured using mimics research 18.0 (Materialise NV, Leuven, Belgium).

Statistics

Measurements of furcula area, arm length and angle, and hypocleideum length from embryonic chicks were compared using the Kruskal-Wallis one-way analysis of variance test. Mean interclavicle volume, length and width values from crocodile embryos incubated at 28°C and 32°C were compared using Welch's *t*-test.

RESULTS

Furcula growth is influenced by embryonic muscle contraction

We investigated the impact of both rigid and flaccid embryo paralysis and skeletal muscle hyperactivity on furcula growth in the embryonic chicken. Whole-mount Alizarin red staining of embryonic furculae revealed that the angle between the two arms of the furcula increases during normal development, from 60° ± 4.3 at E11, to 70° ± 3.6 at E14, and 101° ± 4.1 at E18 (Fig. 2). This was largely unaffected by the two modes of embryo paralysis used in this study. The angle between the two arms was marginally increased in embryos treated between E10 and 18 with AP (to induce hyperactivity) relative to rigidly and flaccidly paralysed embryos when treated from E10 to 18, but not relative to controls (Fig. 2i), which may result from an acceleration in forelimb skeletal muscle and long bone growth (Heywood et al. 2005). In contrast, whole mount Alizarin red staining of embryonic furculae and 3D imaging by microCT revealed that the shape of the hypocleideum was indeed modified by pharmacological treatment which induces alterations in embryonic muscle

contraction (Fig. 2. a–f). Flaccid immobilisation in particular produced a significantly shorter, more rounded hypocleideum relative to that observed in control embryos (Fig. 2 e–f). The articular ends of the furcula arms also varied in shape between groups.

Unsurprisingly, total furcula area increased throughout normal development (Fig. 3 a–c). However, the growth rate of each component of the furcula was not equal. The longitudinal growth of the two arms of the furcula was significantly decreased in rate after E14 (Fig. 3 d–i), suggesting that rapid growth of these elements occurs relatively earlier during development. The hypocleideum, in contrast, undergoes a period of rapid growth between E11 and 14 (Fig. 3. j–l). Our investigation revealed that the growth of all furcula components is not equally influenced by embryonic muscle contraction, perhaps related to these differences in growth rate.

Total furcula area was largely unaffected by muscle contraction but was found to be reduced in embryos treated with PB between E10 and 18 (Fig. 3c). Total unloading of the pectoral girdle in flaccidly immobilised embryos therefore negatively influenced total furcula area, but static muscle contraction induced by DMB treatment appeared sufficient to maintain furcula area. Measurements obtained from whole mount Alizarin red-stained furculae showed that longitudinal growth of the two arms is not altered by either the flaccid or rigid elimination of embryo motility or by the imposition of increased embryo motility by AP treatment (Fig. 3 d–i). In contrast, hypocleideum length was very markedly reduced in embryos treated with PB and DMB between E10–14 and E10–18 relative to both control and AP-treated embryos (Fig. 3 j–l). 3D imaging of this region revealed that both reduced growth and altered shape accounted for the measured reduction in hypocleideum length (Fig. 2 e–g). The role of embryonic muscle contraction in influencing bone shape and promoting longitudinal growth is well established (Hall & Herring, 1990; Pitsillides, 2006; Nowlan et al. 2010b; Sharir et al. 2011), but it primarily has been studied in bones that form and expand by an endochondral ossification process. The chicken hypocleideum is in contrast known to form by intramembranous ossification and our findings demonstrate that the expansion of bones by this process is also subject to influence by the elimination of muscle contraction. The impact we observe on hypocleideum but not ramus growth also indicates that, in addition to some intramembranous bones displaying a greater sensitivity to mechanical input than others, some regions of individual bones may be more sensitive than others.

Interestingly, increasing the frequency of embryonic muscle contraction by treatment with AP did not significantly alter furcula growth of either the clavicles or the hypocleideum relative to controls. This suggests that the relationship between muscle contraction and growth of the furcula by

intramembranous ossification is not as simple as a reduction in or removal of muscle contraction resulting in reduced growth, and an increase in the frequency and/or magnitude of force produced by muscle contraction resulting in increased growth. Rather, our data suggest that there may be a threshold of mechanical loading below which furcula growth is influenced negatively, but above which there is no additional impact.

The impact of temperature and increased motility on interclavicle growth in the embryonic crocodile

The crocodilian interclavicle is thought to be homologous to the avian hypocleideum (Vickaryous & Hall, 2010). We therefore investigated the impact of incubation temperature, which is a major influence on embryo motility, on interclavicle growth in West African Dwarf crocodile embryos (Pollard et al. private communication). Incubation of crocodile embryos at 28 °C, which is considered sub-optimal for this species and results in lower levels of embryo motility, produced dramatic differences in interclavicle growth relative to those incubated at 32 °C. The interclavicle of embryos incubated at 32 °C was well-formed, large and paddle-like. In contrast, the interclavicle of embryos incubated at 28 °C was only ossified cranially and its volume, length and width were significantly reduced compared with the interclavicle in chicks incubated at 32 °C (Fig. 4).

Staging of embryos according to Ferguson's alligator embryo staging series (Ferguson, 1985), which is based on withdrawal of the yolk sac into the abdominal cavity at very late stages of development, determined that embryos incubated at 28 °C were mildly developmentally delayed relative to those incubated at 32 °C. Embryos incubated at about 28 °C corresponded to FS stage 26 (60–63 days into an approximately 70-day incubation period), whereas those incubated at 32 °C corresponded to FS stage 27 (equivalent to 64–70 days). We therefore cannot rule out that the differences in hypocleideum growth we observed are not stage-related or due to a direct impact of temperature on intramembranous bone growth. However, the impact of immobilisation we observed on the chicken hypocleideum suggests that reduced crocodile embryo movement in response to incubation temperature may contribute to the measured reduction in interclavicle growth (Fig. 4).

DISCUSSION

Our data demonstrate that the growth of the clavicular and interclavicular components of the furcula may be regulated separately during normal development. In control embryos we observed that growth rate of the two arms of the furcula and the hypocleideum were not synchronised. It is likely that rapid longitudinal growth of the two arms occurs relatively early in development; they grow relatively little between E14 and E18. In contrast, a period of rapid growth of the hypocleideum

region occurred earlier between E11-14. The underlying reason for this divergence in growth in these two components of the same skeletal element is unclear. It is unlikely that the explanation will emerge from divergence in the origins of these two components as, in contrast to some mammals, the clavicular and interclavicular components of the avian furcula are entirely comprised of intramembranous bone and are of lateral plate mesoderm origin. Changes in the angle between the two arms of the furcula, and hence its overall shape, were also observed during normal development in the chicken. The angle between the two arms of the furcula increased during development in control embryos, which is perhaps related to outgrowth of the long bones and muscle growth in the pectoral limb. Long bone outgrowth and muscle growth are known to be increased following treatment with AP, and a small increase in mean inter-arm angle was observed in the furculae of AP-treated relative to DMB and PB-treated chicks, providing further evidence that these are linked, but the increase in angle was modest and not significantly altered relative to controls.

Our measurements of furcula growth with and without manipulation of embryo movement reveal that embryonic muscle contraction is required for the development of normal furcula morphology. However, the growth of the clavicular and interclavicular regions of the furcula was not equally dependent upon muscle contraction at the time-points considered in this study. Static loading resulting from treatment with DMB, which induces a rigid form of paralysis, preserved most furcula growth parameters including total furcula area, while induction of flaccid paralysis with PB, which results in unloading of the skeleton, produced a significant reduction in total furcula area. This contrasts with the findings of previous studies, which compared the impact of these two forms of paralysis on the growth of endochondrally-derived bones, and found that they produced a similar impact on longitudinal growth. This suggests that intramembranous and endochondral bones may exhibit differential sensitivity to mechanical stimuli. It is possible that either intramembranous bones have less of a requirement for mechanical stimuli in regulating their growth, and that intrinsic growth factors may promote growth even in the absence of dynamic embryo movement, or that intramembranous bones exhibit a reduced threshold for sensitivity to mechanical stimuli, and even limited mechanical stimuli are sufficient to promote growth. There is evidence for the latter explanation in the findings of Hall and Herring (1990) which conclude, on the basis of preserved secondary cartilage formation in the furculae of pharmacologically immobilised chicken embryos, which otherwise do not experience dynamic loading, that even contractions of the amnion are sufficient stimulus to promote this aspect of furcular development. This should however be viewed a perhaps a special case, since both endochondral ossification in long bones and intramembranous

ossification of the furcular in chicks have previously been shown to be affected by such immobilisation (Hall, 1986).

The longitudinal growth of the two arms of the furcula was unaffected by all methods used to alter embryo motility at the time-points considered in this study. It is possible that this is related to the relatively reduced growth rate of the arms at time-points later than E14. Previous studies have reported a more severe impact of embryo immobilisation on total furcula growth at earlier developmental stages, although it is unclear which aspects of furcula growth were affected, as only the impact on total furcula mass or size are reported (Hall & Herring, 1990; Hall, 2001). It is possible that immobilisation prior to the onset of mineralisation of the furcula arms results in a more severe impact on their growth. The growth of the hypocleideum, on the other hand, was more severely influenced by muscle contraction; its length and shape were altered by both DMB and PB treatment between E10–14 and 10–18, which encompass periods of relatively rapid growth of this element. Intrinsic differences in the growth trajectories of the clavicular and interclavicular components of the furcula may therefore explain the differential impact of embryo paralysis that we observe on their growth. Our observations on the impact of altered incubation temperature and hence motility on interclavicle growth in embryonic crocodiles provide further evidence for a role of embryo movement in regulating the growth of the interclavicular component of the pectoral girdle. Interclavicle growth was severely impacted in less motile embryos incubated at 28 °C. However, further work is required to separate the contribution of temperature and embryo movement to regulating interclavicle growth in this crocodilian model, and some of this growth difference may be attributed to a mild developmental delay in embryos incubated at 28 °C vs. 32 °C. Staging of *Osteolaemus* embryos according to existing staging series for Alligator mississippiensis (Ferguson, 1985) revealed a delay equivalent to 1–10 days (FS 26 relative to FS27, out of approximately 70 total) resulting from incubation at the cooler temperature. Data from Alligator missippiensis indicate that the interclavicle is present from FS 19 onwards, is mineralised cranially, as observed in our *Osteolaemus* embryos incubated at 28 °C, by FS 21, and is spade-like and fully mineralised as in *Osteolaemus* embryos incubated at 32 °C by FS 24 (equivalent to 46–50/70 days; Vickaryous & Hall, 2010). This is a larger difference in mineralisation of the interclavicle than would be expected to be produced by a developmental delay of 1–10 days. This suggests that the differences we observed are unlikely to be due solely to a uniform developmental delay in embryos incubated at the cooler temperature, and that altered embryo movement is likely to contribute to interclavicle growth in crocodilians as in chickens. Our estimates of *Osteolaemus* developmental stages are based on those from a relatively closely related crocodilian species which shares a similar incubation period. However, our data should be interpreted with the caveat that a comprehensive staging series for

Osteolaemus would need to be developed to be more certain of this conclusion. Together, the data gathered from embryonic chicks and crocodiles suggest that this interclavicular component is particularly sensitive to the absence of or a reduction in embryonic muscle contraction.

Interestingly, we observed little impact of AP treatment on furcula growth. Treatment with AP induces skeletal muscle hyperactivity to increase the frequency of muscle contraction, and has also been shown to promote the growth of a number of skeletal muscles including the pectoral muscle (Heywood et al., 2005). As muscle force production is proportional to its physiological cross sectional area (Allen et al., 2010), AP treatment therefore likely increases the magnitude of loading produced by each muscle contraction. However, this had no significant impact upon any aspect of furcula growth measured in this study. Previous studies into the impact of embryo movement on endochondral ossification of limb bones indicate that the removal of embryo movement has a considerably larger impact on limb growth than increasing embryo motility. Together with the data gathered from pharmacologically immobilised embryos, this suggests that there may be a threshold, or an optimum level of embryo motility, above which additional movement does not promote increased growth but below which growth is impaired.

The results of previous studies indicate that different skeletal elements exhibit differential sensitivity to altered mechanical stimuli. Our study now indicates that this can occur even in distinct components of a single skeletal element. Our findings also suggest that the mechanical requirements of intramembranous and endochondral bones differ. However, it is incorrect to assume that all intramembranous bones, like the neurocranial bones, are insensitive to mechanical stimuli. Additionally, the clavicular and interclavicle components of the avian furcula respond differently to alterations in embryo movement, with the interclavicle requiring both the static and dynamic components of movement-related loading for normal growth, while static loading preserved most aspects of clavicle growth. Additionally, environmental factors which influence embryo motility, such as temperature, have the scope to alter the growth and morphology of the interclavicle prior to birth/hatching (although incubation temperature is likely to only vary significantly with environment in ectothermic species such as reptiles). Both the length of the clavicular components of the furcula and the shape/size of the interclavicle vary widely between both extant and extinct avian species with divergent locomotor styles. Our data suggest that embryo movement, and the mechanical loading this produces, is important in shaping these structures during development to suit their postnatal mechanical roles.

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Author Contributions

Designed study: ASP, AAP. Acquired data: SB, ASP. Analysed and interpreted data: SB, ASP, AAP. Wrote and revised manuscript: ASP, IMM, AAP.

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FIGURE LEGENDS

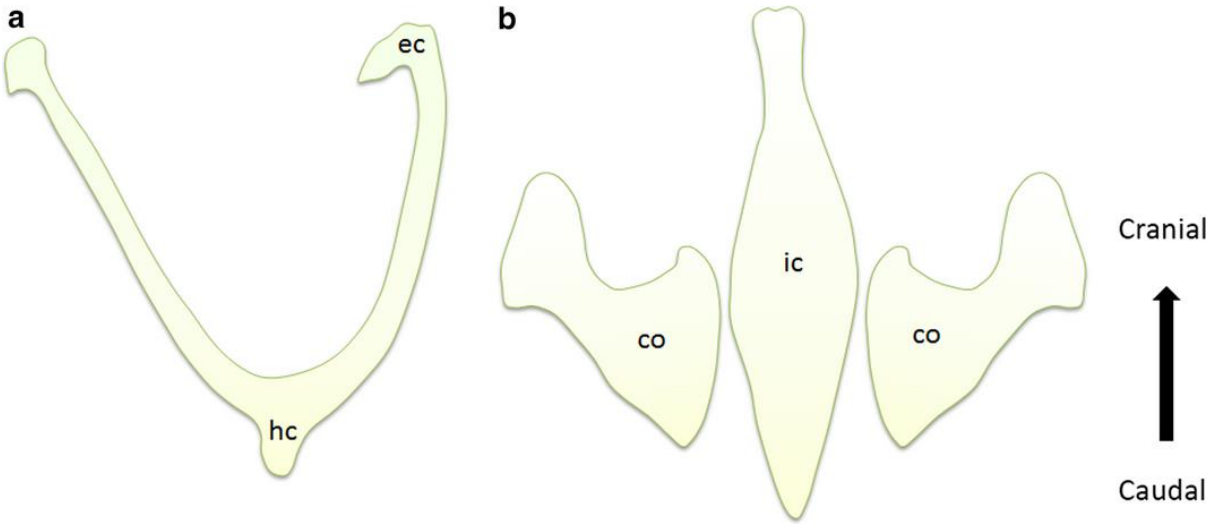

Fig. 1. The skeletally mature avian furcula (a) and crocodilian interclavicle (b). The chevron-shaped furcula is composed of two arms, or rami, which are fused at the midline. A projection at this midline fusion, the hypocleideum (hc), is present in some species, including chickens. The ends of the furcula arms which articulate with the acromion process are referred to as the epicleideum (ec). The crocodilian interclavicle (ic) is a single, paddle-like skeletal element without lateral processes which can be considered equivalent to the avian hypocleideum. Co indicates the coracoid.

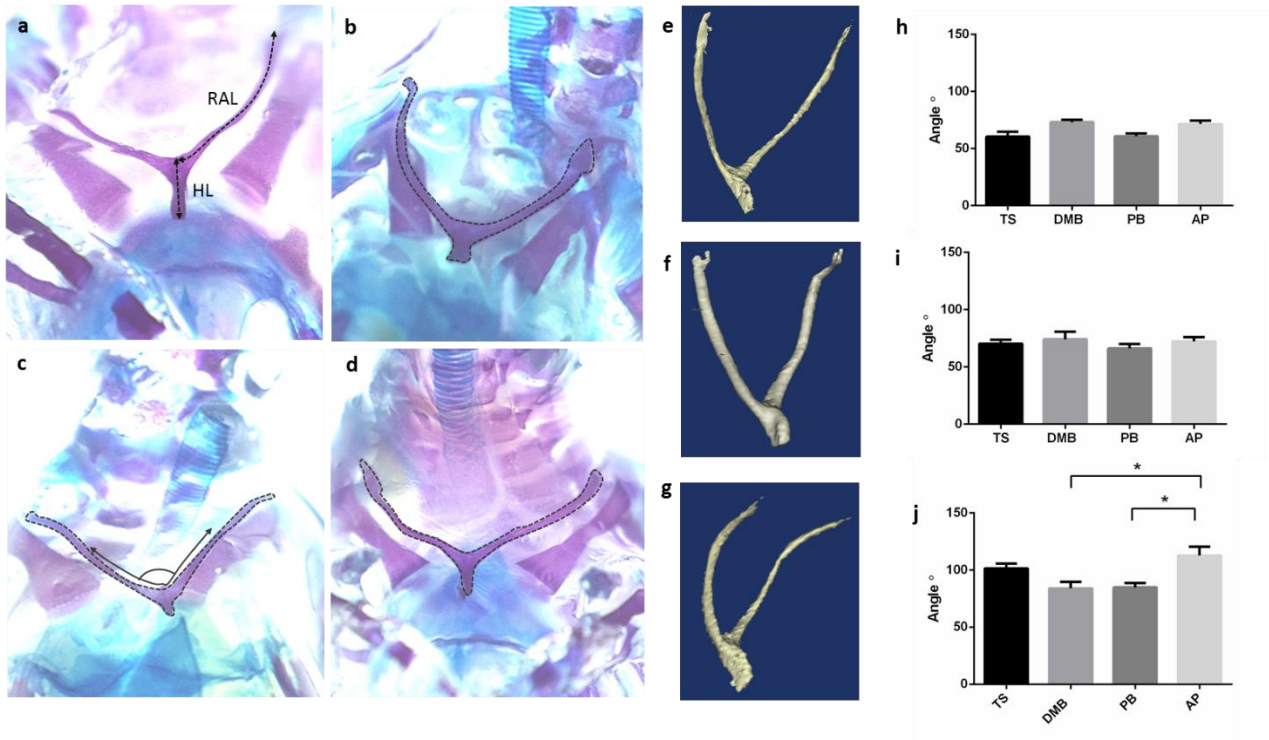

Fig. 2. The impact of altered embryo movement on furcula shape and angle. Whole mount embryonic furculae stained with alizarin red and alcian blue from embryos treated from E10-18 with **A)** control (TS) **B)** DMB **C)** PB and **D)** AP. The furcula is outlined with a dotted line in these images. The three-dimensional shape of the furcula varied between **E)** TS **F)** PB and **G)** DMB-treated limbs. The mean ±SEM angle between the two arms of the furcula was unchanged by pharmacological manipulation of embryo movement between **H)** E10-11 and **I)** 10-14. Furcula angle was also unchanged by both rigid (DMB) and flaccid (PB) paralysis between **J)** E10-18. AP treatment increased the furcula angle significantly relative to DMB and PB-treated embryos at this stage, but not relative to controls. * indicates $P<0.05$.

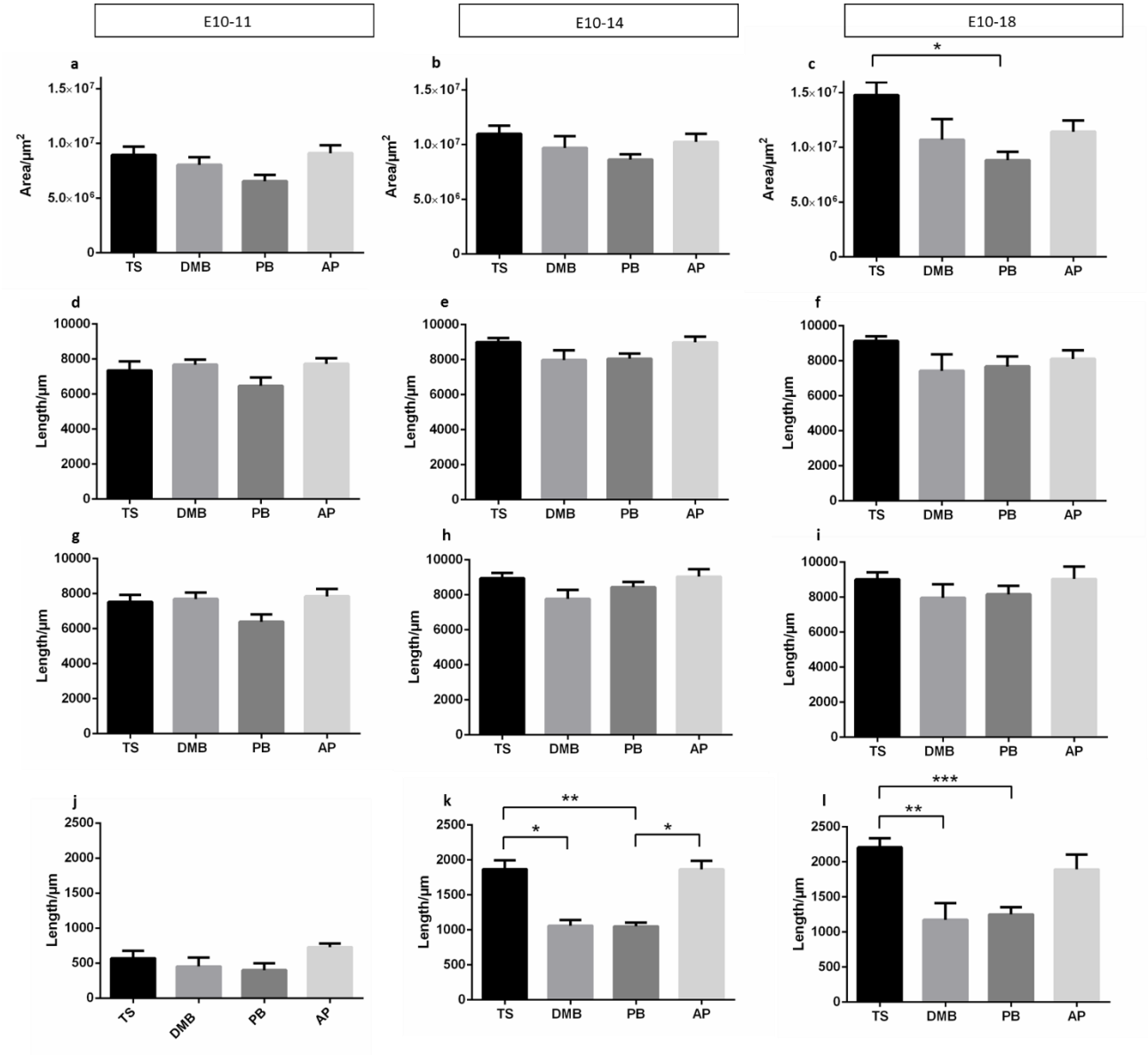

Fig. 3. The impact of embryo movement on furcula growth. Furcula area was unchanged by pharmacological manipulation of embryo movement between **A)** E10-11 and **B)** 10-14, but was reduced by **C)** PB-induced flaccid immobilisation between E10-18. The length of the left arm of the furcula was unchanged by manipulation of embryo movement between **D)** E10-11 **E)** 10-14 and **F)** 10-18. The length of the right arm of the furcular was unchanged by manipulation of embryo movement between **G)** E10-11 **H)** 10-14 and **I)** 10-18. The length of the hypocleideum was unchanged by manipulation of embryo movement between **J)** E10-11. Growth was altered by movement at **K)** 10-14; both rigid paralysis induced by DMB and flaccid paralysis induced by PB treatment significantly reduced hypocleideum length relative to control embryos. AP treatment did not alter hypocleideum length relative to controls. Growth was also altered by manipulation of movement between **L)** E10-18; both rigid paralysis induced by DMB and flaccid paralysis induced by DMB treatment reduced hypocleideum length, which was unaffected by AP treatment. Error bars indicate SEM. * indicates P<0.05. ** indicates P<0.01. *** indicates P<0.001.

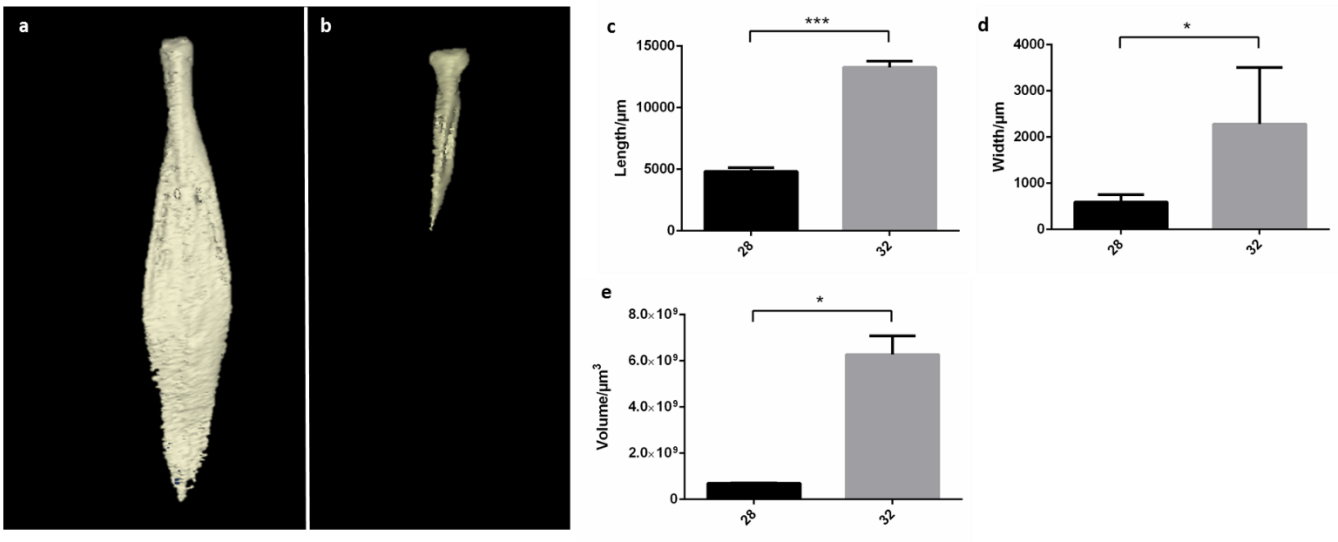

Fig. 4. The impact of incubation temperature and increased motility on crocodile embryo interclavicle growth. Growth of the interclavicle was altered significantly by incubation at **A)** 32°C and **B)** 28°C. Incubation at 28°C (lower than 'optimal' 30°C for this species) resulted in a significant reduction in mean ±SEM interclavicle **C)** length **D)** width and **E)** volume. * indicates $P<0.05$, *** indicates $P<0.001$.