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RESEARCH ARTICLE

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Quantifying the tolerance of chick hip joint development to temporary paralysis and the potential for recovery

Devi L. Bridglal[1] | **Colin J. Boyle**[1] | **Rebecca A. Rolfe**[1,2] | **Niamh C. Nowlan**[1]

[1]Department of Bioengineering, Imperial College London, London, UK

[2]Department of Zoology, School of Natural Sciences, Trinity College Dublin, Dublin, Ireland

Correspondence
Niamh C. Nowlan, Department of Bioengineering, Imperial College London, London SW7 2AZ, UK.
Email: n.nowlan@imperial.ac.uk

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ABSTRACT

Background: Abnormal fetal movements are implicated in joint pathologies such as arthrogryposis and developmental dysplasia of the hip (DDH). Experimentally induced paralysis disrupts joint cavitation and morphogenesis leading to postnatal abnormalities. However, the developmental window(s) most sensitive to immobility—and therefore the best time for intervention—have never been identified. Here, we systematically vary the timing and duration of paralysis during early chick hip joint development. We then test whether external manipulation of immobilized limbs can mitigate the effects of immobility.

Results: Timing of paralysis affected the level of disruption to joints, with paralysis periods between embryonic days 4 and 7 most detrimental. Longer paralysis periods produced greater disruption in terms of failed cavitation and abnormal femoral and acetabular geometry. External manipulation of an immobilized limb led to more normal morphogenesis and cavitation compared to un-manipulated limbs.

Conclusions: Temporary paralysis is detrimental to joint development, particularly during days 4 to 7. Developmental processes in the very early stages of joint development may be critical to DDH, arthrogryposis, and other joint pathologies. The developing limb has the potential to recover from periods of immobility, and external manipulation provides an innovative avenue for prevention and treatment of developmental joint pathologies.

KEYWORDS

arthrogryposis, biomechanics, cartilage, cavitation, fetal movements, hip dysplasia, immobilization, synovial joint development

1 | INTRODUCTION

Fetal movements are critical to healthy synovial joint development. Reduced, restricted, or abnormal fetal movements are implicated in joint pathologies such as arthrogryposis and developmental dysplasia of the hip (DDH), reviewed in Nowlan, 2015.[1] DDH has been estimated to affect 6.6 per 1000 live births,[2] with a late diagnosis (after the age of 1 year) rate of 1.28 per 1000 in the UK.[3] DDH—particularly when diagnosed late—increases the risk of osteoarthritis in later life.[4] Arthrogryposis (joint contractures in two or more body parts) affects 1 per 3000 to 5000 live births[5] and can have severe effects on mobility and lifelong musculoskeletal health.

Synovial joint development occurs as a tightly-regulated cascade of events that are conserved across species.[6] First, the interzone appears as an interruption in the cartilaginous mesenchyme of the presumptive skeletal anlage.[7] Morphogenesis—the emergence of shape—commences prior to physical separation of the rudiments (cavitation) at the interzone,[8] and continues throughout development, including postnatally. The regulation of morphogenesis is critical to the long-term function and maintenance of the joint.

Abnormal fetal movements have been shown to disrupt joint development in multiple animal model systems including avian,[9] rodent,[10-12] and fish[13,14] models. Osborne et al.[15] showed that cavitation of the chick hindlimb is prevented by a three-day paralysis period prior to embryonic day 8 (when cavitation normally occurs), while the joint cavity is resorbed by inducing rigid paralysis after day 8. In addition to preventing cavitation, early, maintained paralysis also affects joint morphology, with joint shape abnormalities present in the immobilized chick[9,16,17] and mouse.[10,11] In the chick, joint shape abnormalities due to sustained paralysis are most pronounced after day 8.[9,16] While these findings demonstrate the necessity of fetal movements to joint development, an open challenge in the field has been to quantify the characteristics of fetal movement that are sufficient for normal joint development.

Both the magnitude and temporal profile of fetal movements may be crucial to joint development. The magnitude of movements required for healthy joint morphogenesis has been investigated using *in vitro* culture of developing limbs in a mechano-stimulation bioreactor,[18,19] while less is known about the temporal profile of movements needed to promote normal joint development. Drachman and Coulombre[20] found that later periods of paralysis (initiated between 13 and 15 days) had more severe effects on joint angulation than earlier periods of paralysis (initiated between 7 and 12 days), and that a two-day period of paralysis had effects on joint mobility which persisted after hatching. Drachman and Sokoloff[16] varied the duration of paralysis (ranging from 1 to 12 days) and found failed or abnormal cavitation and abnormal flattening of the articular surfaces in all those paralysis groups assessed after embryonic day (e8).[16] However, no detailed comparisons were made between immobilization groups, disallowing conclusions on which timing or duration of immobility led to the most severe effects. From these studies, we have learned that the effects of a period of paralysis can vary depending on when that period occurs, and that even a short period of paralysis can have sustained effects. There is also evidence to suggest an intrinsic link between movement-induced cavitation and later progression of morphogenesis.[9] However, we do not know when, or for how long, the embryo must be active for normal cavitation or morphogenesis to proceed. Furthermore, no previous studies have attempted to "rescue" joint development after a period of immobility by externally manipulating the limb.

In this study, we identify the developmental windows in early development that are most sensitive to abnormal fetal movements. We perform a systematic investigation of the effects on joint cavitation and morphogenesis of periods of fetal immobility induced at varying timepoints (between e3, when the very first spontaneous movements commence,[21] and e8) and for varying durations of time (between 1 and 6 days). Hip joints were characterized at e9, immediately following the point at which cavitation normally takes place (at around e8.5[8]). We also show that external manipulation, applied at e8, can induce cavitation in the hip joint of an immobilized chick, and promote more normal morphogenesis by e9. The aims of the study were to identify a critical time—and intervention window—for movement for normal prenatal development of the hip joint. Such an approach, if translated to humans, could enable targeted *in utero* screening in the future, and open avenues to potential treatments to reduce the severity of the effects of reduced fetal mobility on joint shape and health, such as in the case of arthrogryposis.

2 | RESULTS

2.1 | Joint abnormalities are more pronounced with earlier onset of sustained paralysis

A common experimental model to study fetal movements is sustained paralysis, whereby movement is restricted from a set time-point until harvest—that is, movements are not allowed to resume. However, the timing of paralysis onset varies between studies, and the impact of this timing has never been studied.

To quantify the effect of paralysis onset timing on joint development, we induced paralysis in a chick *in ovo* model at six different timepoints from embryonic days 3 to 8 (Figure 1) by administering 0.5% decamethonium bromide daily. All chicks were harvested at day 9. We extracted the surfaces defining the femur and acetabulum at each time point using three dimensional (3D) image segmentation and used outlines to measure the dimensions of the acetabulum and the proximal femur (Figure 2). Cavitation was assessed in sections through the hip joint stained with Toluidine blue. Cavitation was deemed failed if- throughout all sections through the joint- there was obvious fusion (joint line between rudiments indistinguishable), or if there was both decreased joint spacing (relative to age matched controls) and unclear separation ("bleeding" or

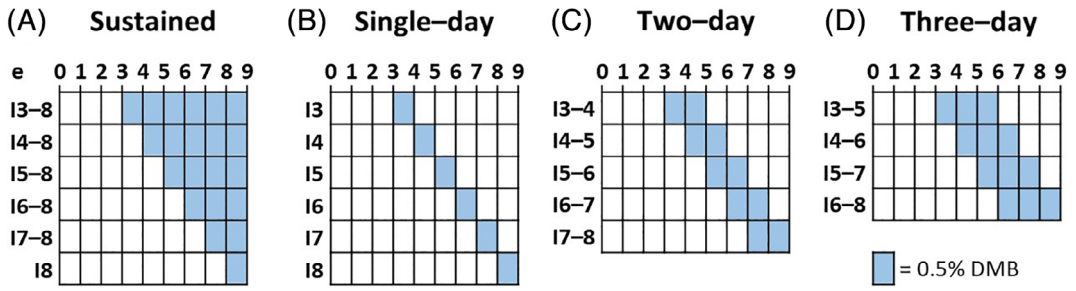


FIGURE 1 Timed immobilization regimes, all of which had variable starting timepoints: A, sustained, B, single-day, C, two-day, and D, three-day. Groups within immobilization regimes are named to the left of the graphs (eg, I3–8 denotes immobilization from embryonic day [e] 3–8). Blue shading denotes a 24-hour period of immobilization with 0.5% (DMB). All embryos were harvested at e9

fuzziness of joint line between rudiments), as shown in Figure 3.

Sustained paralysis initiated at day 3 (I3–8; preventing any spontaneous movements) led to a smaller, dramatically stunted femoral head (Figure 2A) and a smaller, shallower acetabulum (Figure 2B) compared to controls, replicating previous work.[9] Earlier onset of paralysis led to more severe joint deformities in both the femur (Figure 2A) and the acetabulum (Figure 2B) compared to paralysis initiated later. Qualitatively, the femoral heads, fossa depths and greater trochanter features of the I4–8 and I5–8 groups appeared similar to those of the I3–8 group (Figure 2A). From the shape profiles, a trend toward an increasingly normal femoral head and greater trochanter was apparent as the duration of immobility decreased (Figure 2A).

In terms of femur development, paralysis initiated from day 6 onward (I6–8, I7–8, and I8) produced only small effect sizes on femoral geometry (Mann-Whitney U tests detected no significant differences from the control group [$P > .05$] for femoral head to piriformis, femoral head to greater trochanter and epiphyseal width), indicating that femoral development is somewhat robust to abnormal movements from day 6 (Figure 2). For context, day 6 is when the shapes of the hip joint begin to become recognisable,[8] while limb movements commence at e6.5.[21] In contrast, paralysis initiated prior to day 6 had a substantial effect on all femoral measurements, with effects sizes between control and sustained paralysis (I3–8) of −270 μm [95%CI −318, −223] for the femoral head to piriformis, −194 μm [95%CI −223, −156] for the femoral head to greater trochanter and −137 μm [95%CI −171, −106] for the epiphyseal width (Figure 2A; Table 1).

In contrast to the femur, the impact of paralysis on acetabulum geometry gradually increased with earlier onset (Figure 2B). Any onset time before day 8 produced statistically significant differences compared to control groups ($P < .05$ in a Mann-Whitney U test), and the effects sizes for each of the height, width, and depth increased as paralysis onset was initiated earlier, leading to maximum effects sizes of −345 μm [95%CI −418, −244], −178 μm [95%CI −215, −138], and −169 μm [95%CI −198, −140] for acetabular height, width, and depth, respectively, between control and sustained paralysis (I3–8) groups (Table 1). We theorize that the finer, more delicate structure of the acetabulum leaves it more vulnerable to deformation in the presence of abnormal fetal movements, in a similar way to the pronounced susceptibility to short term paralysis of the small joints of the spine.[22,23]

Taken together, these results show that chick hip joint development is affected by the timing of sustained paralysis initiation, with the shape of the acetabulum being more temporally dependent than that of the femur. We theorize that acetabular development may be particularly sensitive to loading, or lack thereof, because of its delicate structure.

The effect of sustained paralysis on joint cavitation depended on the timing of paralysis onset (Figure 4A). Cavitation (which typically occurs around day 8.5) was normal in all samples in which paralysis was initiated after day 7 (I8, N = 3). Cavitation was disrupted in all samples in which paralysis was initiated prior to day 6 (I3–8, I4–8, and I5–8, N = 3 for each group). Between these extremes, cavitation was present in 2 out of three samples for the I6–8 and I7–8 groups (Figure 4A). These results suggest that the mechanical "ground work" for cavitation occurs prior to day 8.

2.2 | Both the timing and duration of temporary paralysis affect joint development

It is not possible to independently vary the timing and duration of sustained paralysis experiments, assuming a fixed end-point at day 9. To separate the effects of paralysis timing and duration on early hip joint development, we systematically varied duration (1, 2, and 3 days) and start-time (days 3–8), as illustrated in Figure 1, while

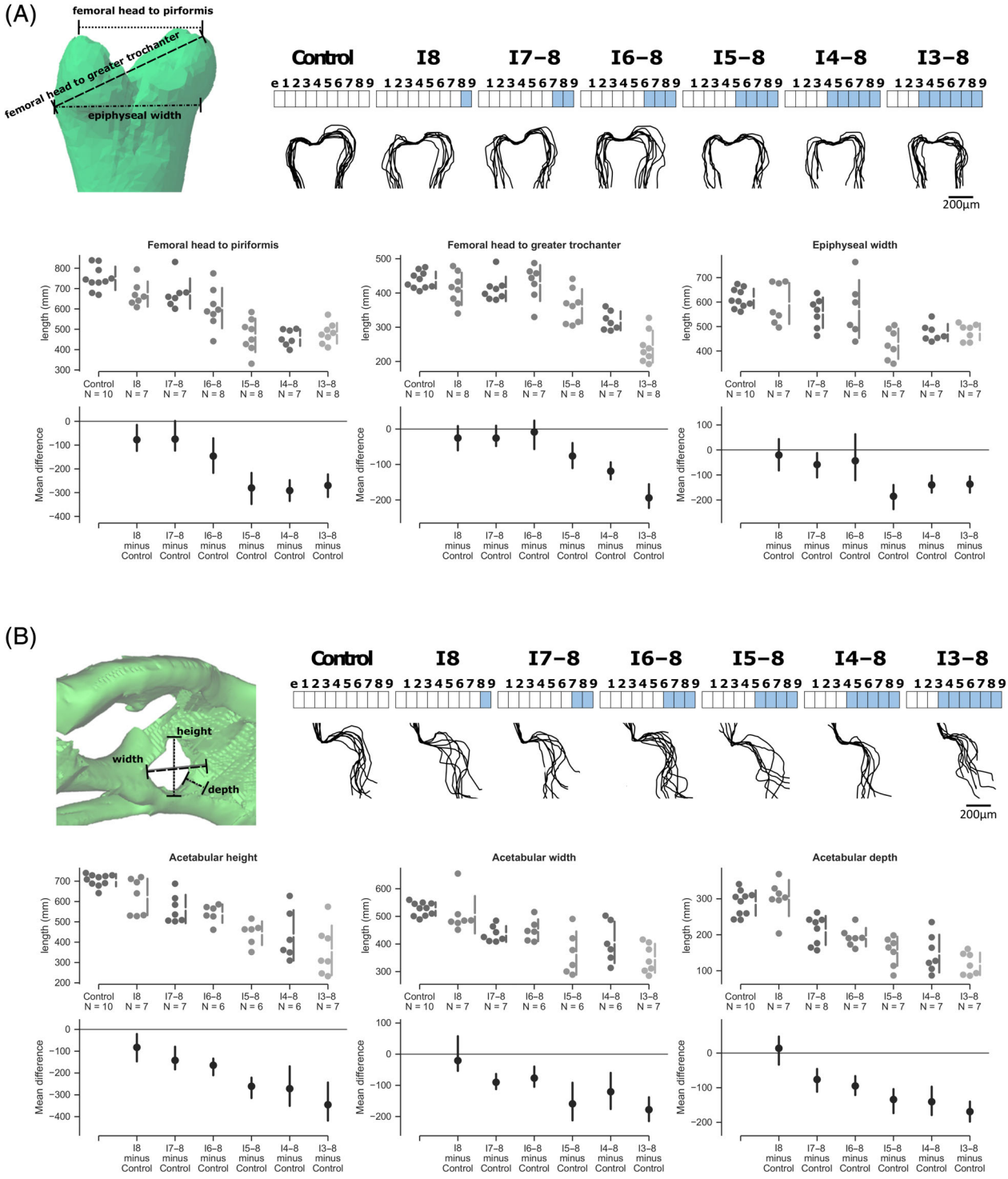


FIGURE 2 Earlier sustained paralysis is more detrimental to joint development. Sustained paralysis was induced at varying start-points from embryonic day 3 (I3–8) to day 8 (I8). Femoral (A) and acetabular (B) anatomical measurements were measured at day 9 (dot plots). Mean differences between each experimental group and the control group are represented beneath each dot plot. The error bar represents the 95% bootstrapped confidence interval for the mean differences relative to control. Scale bars 200 μm

performing the same morphological and cavitation analyzes as before. Paralysis duration was controlled by administering decamethonium bromide daily over a specified duration. For example, experimental group "I5–7" received decamethonium bromide on days 5, 6, and 7.

Qualitatively, there were no obvious effects on femoral or acetabular shape for any of the one-day paralysis periods (Figure 5A,B). With 2 days of paralysis, some flattening of the proximal femur (reduction in fossa depth), reduction in femoral head outgrowth (Figure 5A) and

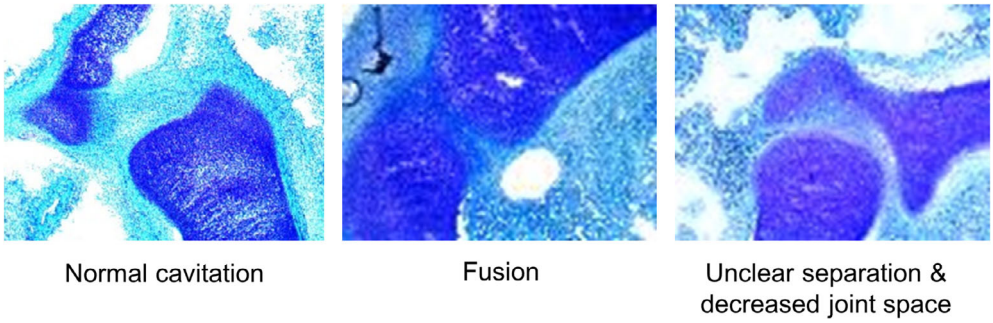


FIGURE 3 Cavitation was deemed to be "failed" if there was obvious fusion (middle) or both decreased joint space and a lack of clear separation (right)

TABLE 1 Mean effects sizes of paralysis groups compared to control group

	Paralysis group			
Measurement	**Single-day**	**Two-day**	**Three-day**	**Sustained**
Acetabular depth	13.6 (−9.4, 38.8)	−121.7 (−145.5, −97.6)	−128.4 (−154.5, −101.3)	−169.0 (−197.9, −139.9)
Acetabular height	−125.4 (−150.4, −93.5)	−178.5 (−211.5, −144.1)	−270.5 (−315.3, −230.1)	−345.4 (−418.2, −243.5)
Acetabular width	−39.3 (−62.2, −15.2)	−140.4 (−170.6, −112.7)	−103.5 (−133.6, −77.4)	−178.0 (−214.8, −138.2)
Epiphyseal width	−61.6 (−93.8, −30.6)	−82.3 (−110.3, −53.1)	−98.7 (−136.5, −56.9)	−136.7 (−170.6, −105.8)
Femoral head to greater trochanter	−13.1 (−33.6, 4.5)	−45.7 (−69.8, −24.0)	−70.1 (−102.3, −39.8)	−194.2 (−222.6, −155.6)
Femoral head to piriformis	−98.9 (−139.6, −63.5)	−165.0 (−208.5, −123.0)	−172.7 (−222.8, −125.5)	−269.9 (−318.4, −223.1)

Note: Values presented are the bootstrapped mean effect size (in μm) relative to control with 95% confidence intervals.

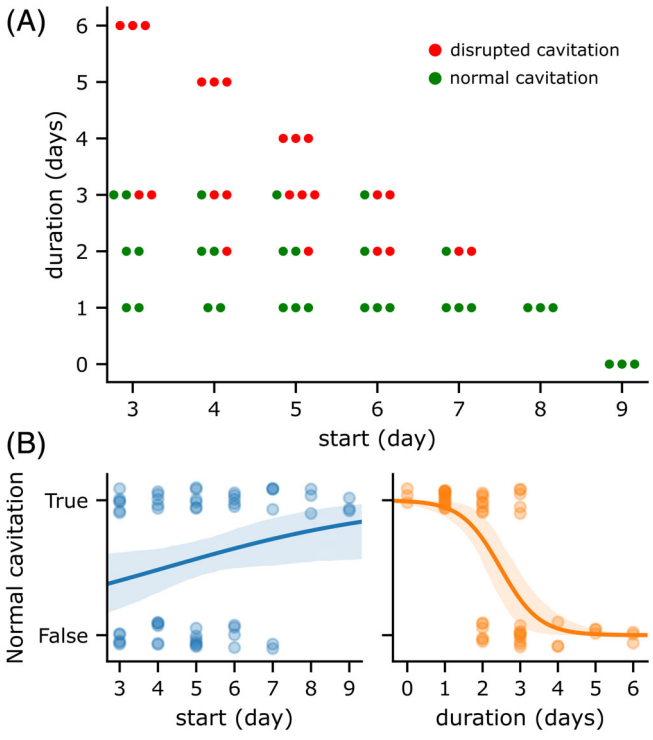


FIGURE 4 Effect of timing and duration of paralysis on cavitation. Presence or absence of cavitation as a function of start day and duration for all analyzed samples, A. Logistic regression fits for the probability of cavitation vs start day and duration, B. Raw data represented by points. Logistic regression best-fit (solid line) with error (filled area)

decreasing depth of the acetabulum (Figure 5B) became apparent in all groups apart from the I7–8 and I3–4 groups. With 3 days of paralysis, the shape effects were even more pronounced, with the I3–5, I4–6, and I5–7 groups exhibiting shapes approaching those of complete paralysis (Figure 5A,B).

Quantitatively, the duration of paralysis had a statistically significant effect on all joint measurements ($P < .001$) except epiphyseal width ($P = .058$) when tested using multivariate analysis of variance (Supporting Information S1). Tukey HSD posthoc analysis revealed statistically significant differences between the control group and two-day and three-day paralysis ($P < .001$ for all measurements), but not single-day paralysis (except for acetabular height and femoral head to piriformis, $P < .05$ for both). Effects sizes relative to the control group were substantially smaller in the single-day group compared to both the two-day and three-day groups (Table 1), and this was consistent across all measurements.

The timing of paralysis onset had a statistically significant ($P < .001$) effect on all measurements independent of paralysis duration, based on multivariate analysis of variance (Supporting Information S1). Tukey HSD posthoc analysis revealed that paralysis started prior to day 6 induced statistically significant differences from the control group ($P < .01$ for all measurements). Paralysis between days 4 and 7 produced the most severe effects on the femur

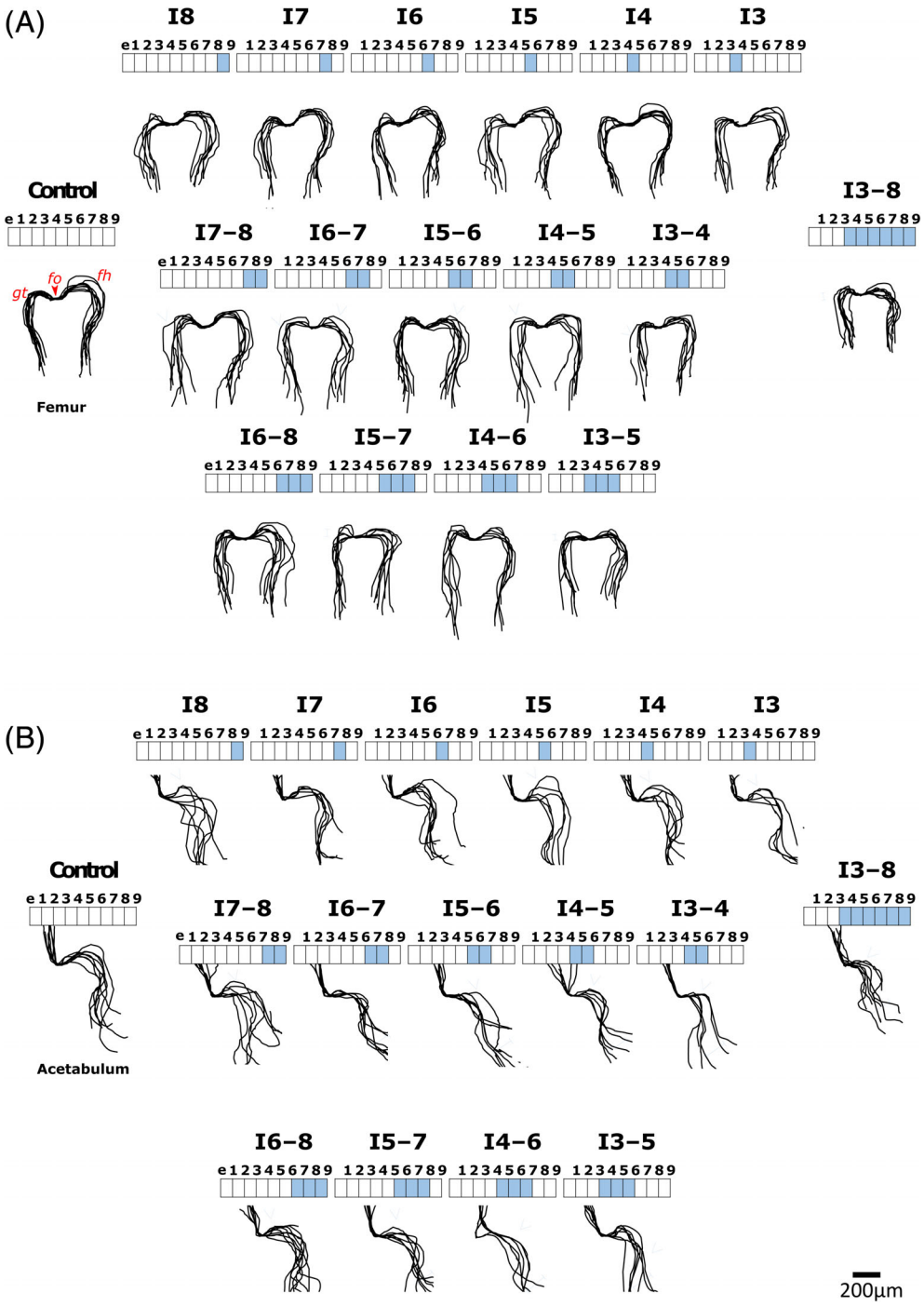


FIGURE 5 Traces of chick hip femurs, A and acetabula, B after various periods of paralysis. Replicate traces were superimposed and aligned. Shaded bars indicate the timing and duration of induced paralysis. fh, femoral head; fo, fossa; gt, greater trochanter. Scale bar 200 μm

(Figure 6) and the acetabulum (Figure 7) in both the two-day (I6–7, I5–6, and I4–5) and three-day groups (I5–7 and I4–6). This effect was particularly pronounced in measurements of femoral head to greater trochanter and epiphyseal width (Figure 6) and of acetabular width and depth (Figure 7). Meanwhile, temporary paralysis initiated either side of days 4, 5, and 6 produced relatively lesser effects, indicating that restricted movement during embryonic days 4 to 7 may be particularly harmful to joint development. Counter-intuitively, acetabular width was more affected in the two-day group than in the three-day group (Figure 7). We suspect this may be due to the delicate structure of the acetabulum being deformed by the opposing (more rigid) proximal femur after the resumption of movements.

The incidence of failed cavitation was greater with longer duration of paralysis, as illustrated in Figure 4A. We analyzed the effect of timing and duration of paralysis on cavitation using logistic regression analysis (Figure 4B). Duration of paralysis had a statistically significant effect on the probability of cavitation ($P < .001$), while timing did

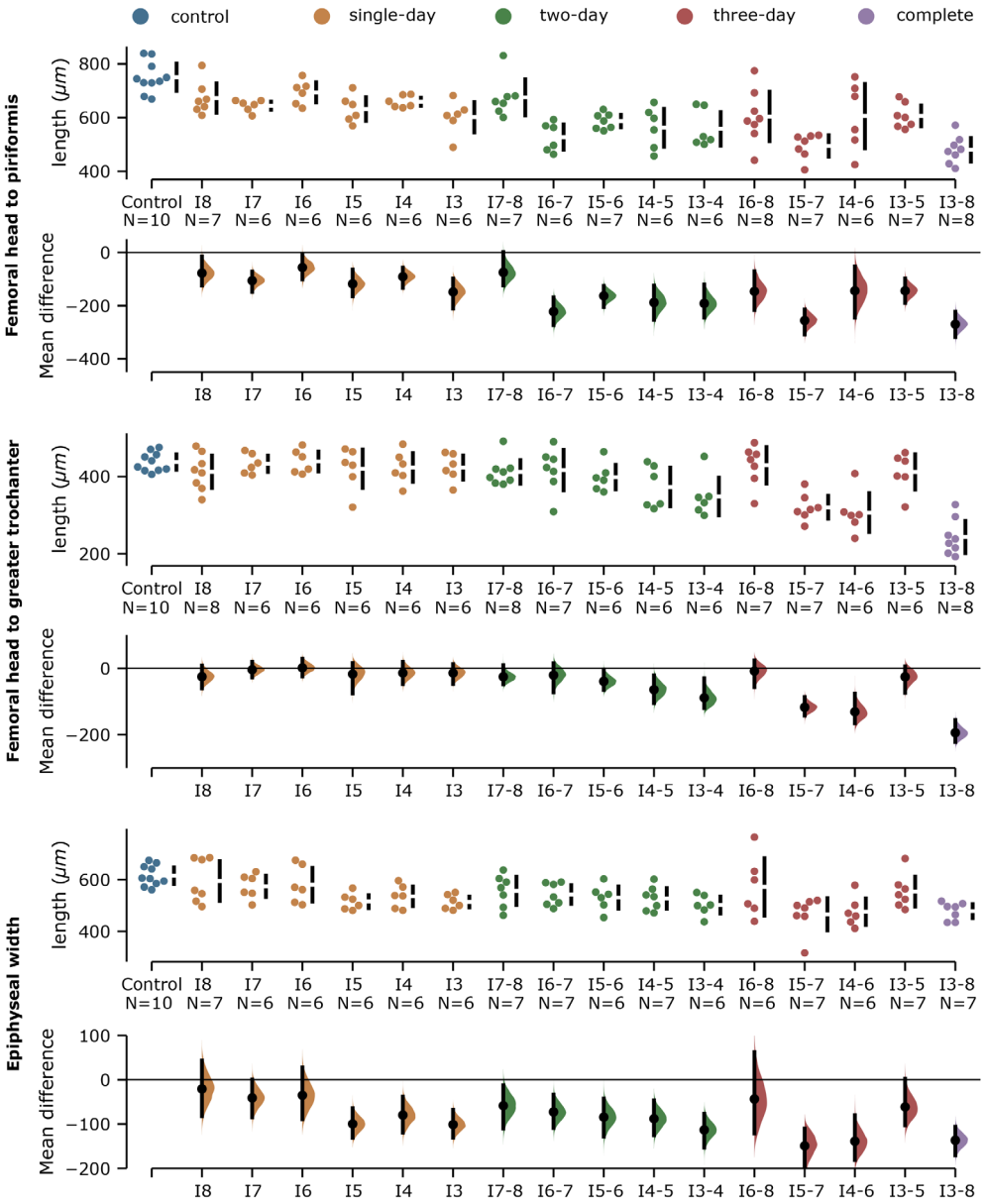


FIGURE 6 Timing and duration of paralysis influence femur development. Dot plots showing the measurements of femoral head to piriformis (top), femoral head to greater trochanter (middle) and epiphyseal width (bottom). Color represents the experimental group single-day, two-day, three-day, and complete paralysis). Effects size plots indicate the mean difference between each group and the control group (black dots). Black bars indicate the 95% confidence interval for the mean difference, while the shaded region represents a kernel density estimate for the mean difference. All effects sizes calculated using the dabest python package

not ($P = .11$). The logistic model predicted an odds ratio of .08 for paralysis duration, representing a 92% decrease in the odds of cavitation for every 1-day increase in paralysis duration. Since cavitation was sensitive to duration of limb paralysis but not timing, it is tempting to suggest that fetal movements act cumulatively to promote cavitation, rather than triggering specific events.

2.3 | The effects of paralysis can be reduced by applying external mechanical stimulation

Having quantified the effects of reduced movement (through paralysis) on joint development, we next sought to offset or ameliorate those effects by applying external manipulation of the paralyzed limb. The developing embryo was accessed *in ovo*, and one limb was manipulated on day 8 while the contralateral limb remained immobile. We chose to apply external manipulation at day 8, prior to the physiological timing of hip joint cavitation at e8.5.[8] We aimed to test if "artificial cavitation" (if it were to occur) would enable more normal shape development even in the continued absence of spontaneous movements. This set-up enabled paired comparisons of joint shape and cavitation between un-manipulated and manipulated limbs (Figure 8-10).

We found that the manipulated limbs of immobilized chick fetuses had more normal joint shapes compared to the contralateral un-manipulated limbs. All measurements of femoral and acetabular geometry were significantly greater in the manipulated limb relative to the contralateral

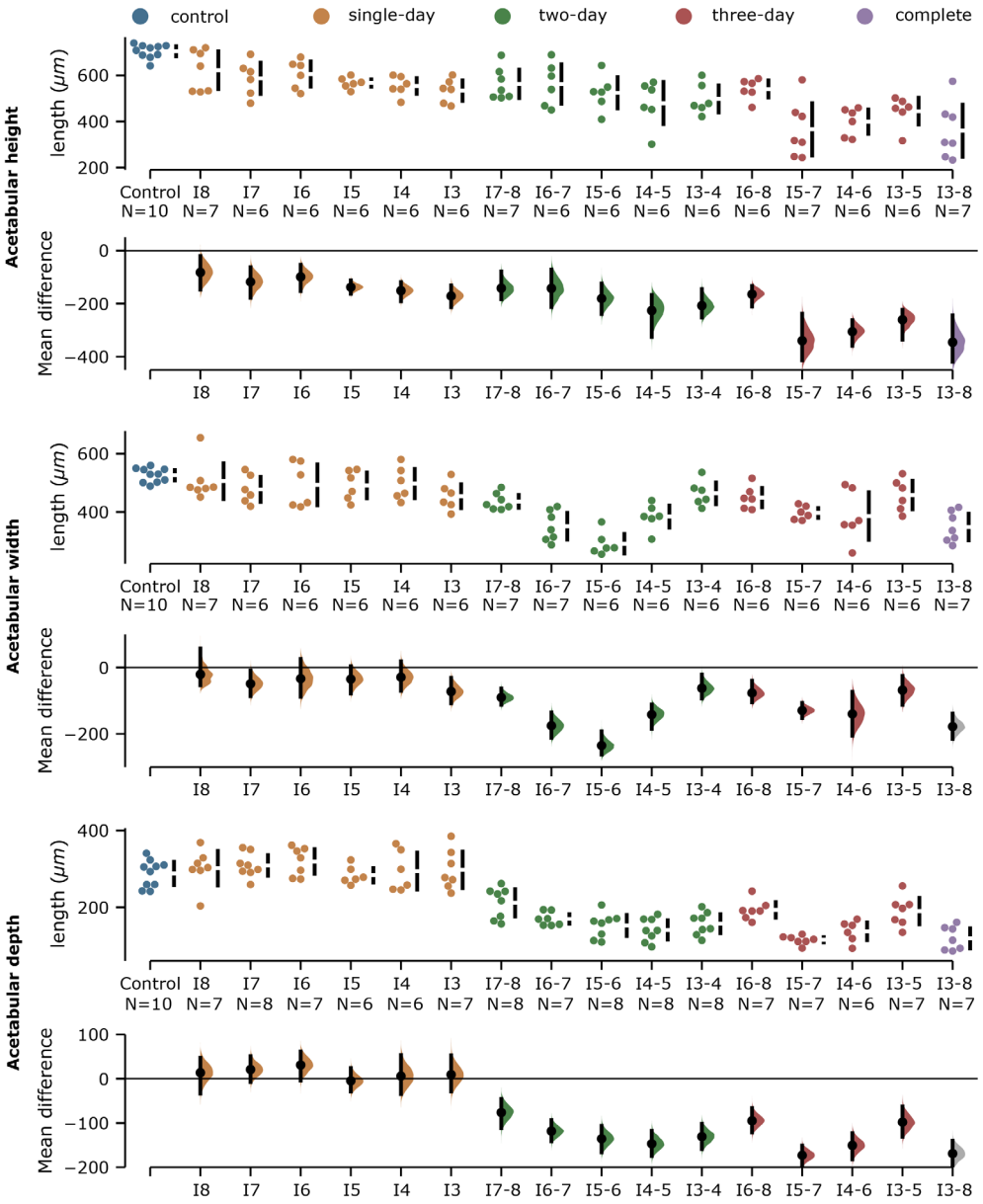


FIGURE 7 Timing and duration of paralysis influence acetabulum development. Dot plots showing the measurements of femoral head to piriformis (top), femoral head to greater trochanter (middle), and epiphyseal width (bottom). Color represents the experimental group (single-day, two-day, three-day, and complete paralysis). Effects size plots indicate the mean difference between each group and the control group (black dots). Black bars indicate the 95% confidence interval for the mean difference, while the shaded region represents a kernel density estimate for the mean difference. All effects sizes calculated using the dabest python package

limb ($P < .01$ for all measurements; Figure 8). There were noticeable differences qualitatively between the manipulated and un-manipulated sides, with apparent increases in acetabular depth and femoral head size in the manipulated side (Figure 9). To quantify the ability of manipulation to "rescue" joint shape, we compared the positive effects of external manipulation to the negative effects of immobilization when compared to normal controls. The negative effects of immobilization were defined as the mean differences between the I5–8 group (sustained paralysis from day 5, as described in the first section of Section 2) and the control (mobile) group, while the positive effects of manipulation were defined as the mean differences between manipulated and un-manipulated paralyzed limbs. We found that external manipulation rescued 56% of the immobilization effects for the femur on average (33%, 85%, and 51% for the femoral head to piriformis, femoral head to greater trochanter and epiphyseal width, respectively), and 37% of the immobilization effects on the acetabulum on average (23%, 46%, and 40% for the acetabular height, width, and depth, respectively).

Cavitation was assessed by sectioning through the hip joint of six specimens (Figure 10). Cavitation occurred in four of the six manipulated limbs, compared to two of the six limbs on the un-manipulated side. However, even the two cavitated limbs on the un-manipulated limbs exhibiting some signs of hindered cavitation (decreased joint spacing or lack of clear boundaries between opposing rudiments) (Figure 10).

These results indicate that external manipulation promoted joint shape morphogenesis and cavitation more in line with control groups.

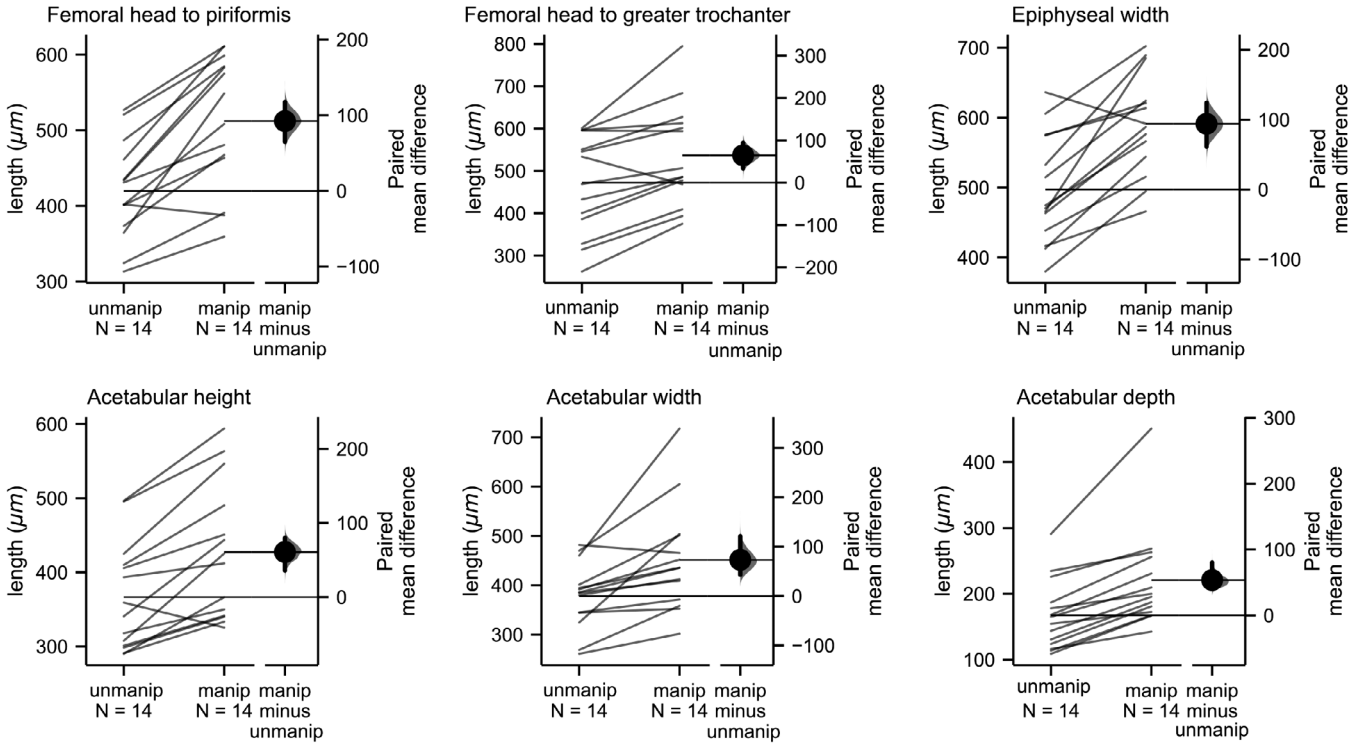
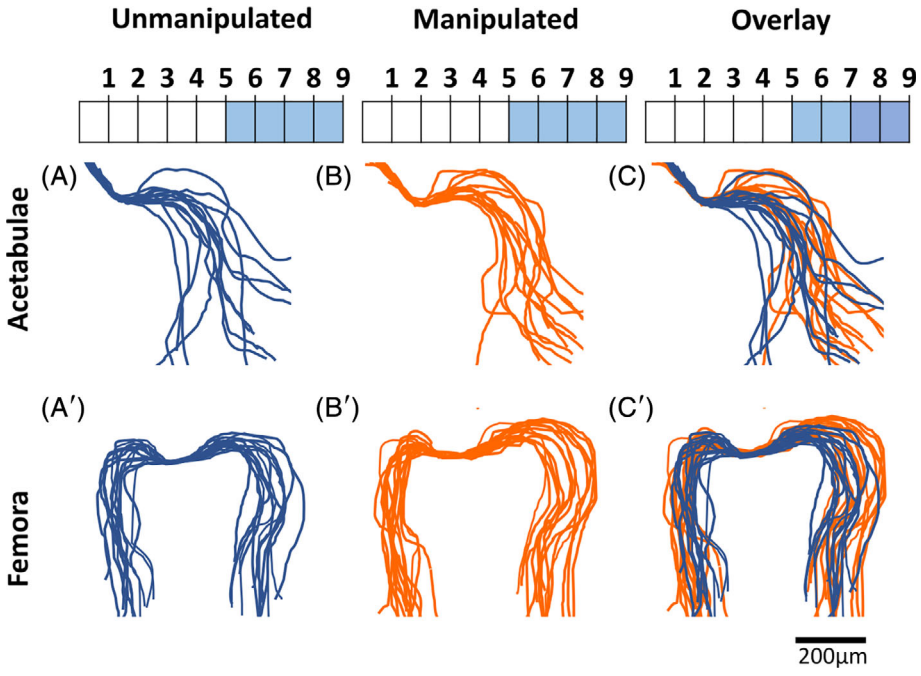


FIGURE 8 External manipulation improves the morphogenesis of immobilized limbs. Paired plots of femoral and acetabular measurements in un-manipulated and manipulated limbs. Effects size plots indicate the mean difference between immobile un-manipulated and manipulated limbs (black dots). Black bars indicate the 95% confidence interval for the mean difference. All effects sizes calculated using the dabest python package

FIGURE 9 External manipulation rescues joint development. Shape profiles of the femoral head and acetabulum comparing un-manipulated (left side, blue) and externally manipulated (right side, orange) contralateral hip joints of 14 immobilized chicks. Right (manipulated) side mirrored for visual comparison



3 | DISCUSSION

Abnormal early fetal movements are known to adversely affect the development of synovial joints, yet the developmental windows most sensitive to fetal movements have never been identified. In this paper, we systematically varied the duration and timing of imposed immobility while quantifying the effects on joint cavitation and shape.

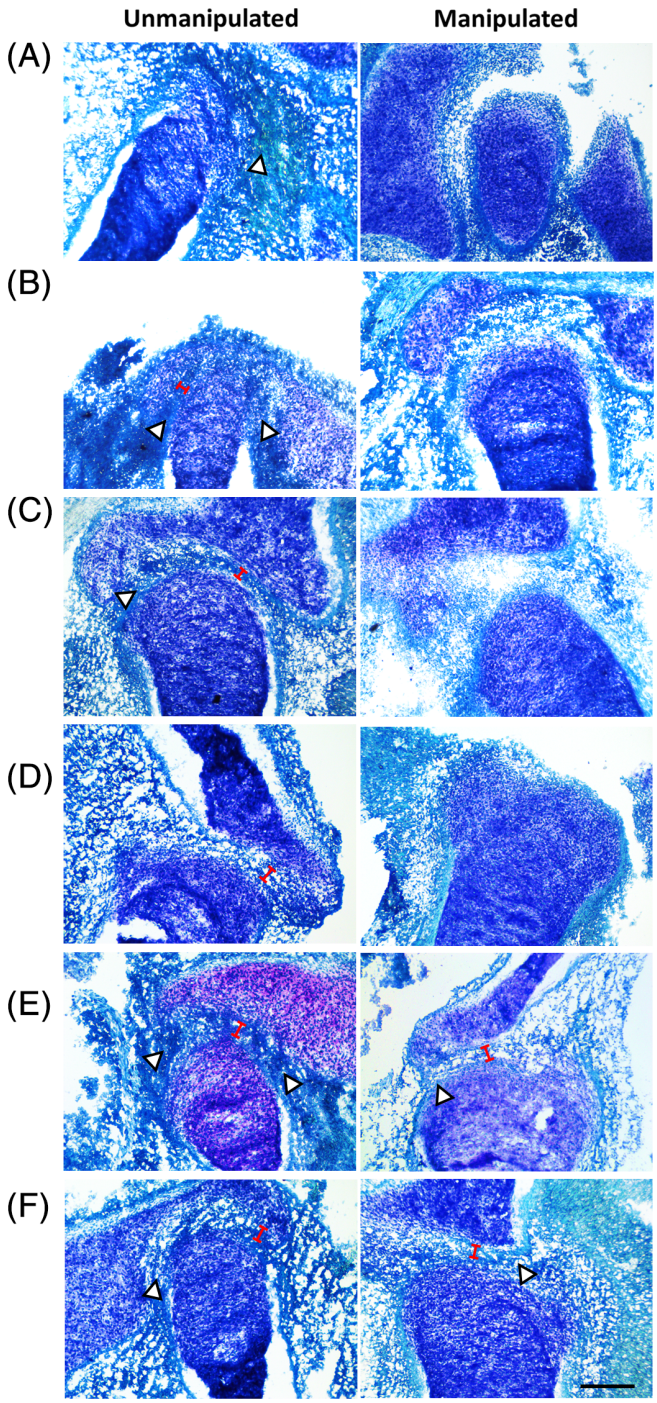


FIGURE 10 Histological sections of externally manipulated (right limb) and un-manipulated immobile (left limb) hip joints. Un-manipulated hip joints had more signs of cavitational failure (triangles, I-bars) than manipulated hip joint. Closed triangle: no clear separation between joint rudiments; red I-bars: decreased joint space between joint rudiments. Scale bar = 200 μm

We discovered that proximal femur shape is relatively robust to sustained paralysis after day six, while the severity of effects in the acetabulum were relative to the duration of sustained paralysis. We found that chick hip development up to day 9 is relatively robust to a single day of paralysis, while longer spells of paralysis are most detrimental between days 4 and 7. We reveal that successful or failed cavitation is more dependent on the duration- rather than the timing- of paralysis. Having identified the times most sensitive to abnormal movements, we attempted to mitigate the effects of immobility by applying an external mechanical stimulus. We found that even a short period of external manipulation was sufficient to rescue some of the effects of immobility on joint morphogenesis and cavitation.

The period most sensitive to immobility corresponds to the timing of several important developmental events. By day five, the cartilaginous femur, ischium, and ilium are present, but the boundaries between them are not easily identified.[8] By day 6, the pubis is also present and the future acetabular space is already identifiable (ie, interzone is present). At this point, the elements of the hip joint begin to develop more refined shapes. Soon after this (e6.5[21]), independent limb movements begin. Refinement of shape continues until, and beyond, cavitation at HH34 (around day 8/8.5).[8] The interzone forms regardless of immobilization,[16] which suggests that immobilization may affect the biophysical characteristics of this zone, rather than arresting interzone formation.

Our results indicate that the most detrimental periods of immobilization mostly preceded the time at which limb movements have been reported to commence (e6.5[21]). These findings suggest that early, whole body movements play a critical role in the early mechanically-mediated aspects of hip joint cavitation and morphogenesis. Given that a single day of paralysis from e8 (just prior to cavitation at e8.5) does not hinder successful cavitation, this would indicate that the interzone region has been sufficiently weakened by movements over the preceding days allowing cavitation to progress even in the absence of movements over that period. The theory that several days of early movements are needed to weaken the interzone region in preparation for cavitation is strengthened by our findings that even when movement is allowed to resume after one of the more "critical" periods of paralysis (eg, I4–6, in which movement would likely have occurred from day seven onward), cavitation does not normally occur. Meanwhile, an externally applied movement is likely forceful enough to rupture an unweakened interzone region. We believe that our data- particularly the external manipulation study- corroborates the hypothesis that cavitation is a "rupture" type event as proposed by Drachman and Sokoloff,[16] with weakening of the interzone region occurring independently of fetal movements, and movements providing the impetus for physical tearing, forming the cavity.

This study provides insight into the potential for the developing joint to recover after a period of immobility. Most previous studies of chick immobilization have

not allowed movement to resume after a period of immobilization,[9,15,16] and therefore such models represent the more severe forms of conditions in which fetal movements are abnormal for sustained periods of time (such as fetal akinesia deformation sequence[1]). It is believed that conditions such as arthrogryposis are caused by transient periods of reduced or absent movement during development, rather than a complete absence of movements. For example, amyoplasia, the most common form of arthrogryposis, is not associated with genetic mutations or pathology of the muscular or neurological systems, and therefore there is the physiological capacity for fetal movements.[1] The only previous chick immobilization study to enable recovery of movement after immobilization that we are aware of is that of Drachman and Coulombre in 1962.[20] Their study applied either 1 or 2 days of paralysis at varying timepoints, and analyzed joint development at hatching. In contrast to the results of this study, they found significant effects, at hatching, of one day of paralysis during the same time frame as our study (e7–9). While the 1962 study analyzed only joint range of motion and ankylosis, and not specific shape effects or cavitation, it is entirely possible that the nonsignificant shape effects we see after one day of paralysis could have long-lasting effects leading to joint angulation later in development.

In this study, two different forms of movement "recovery" after paralysis were assessed: temporary immobilization and externally-applied manipulation. In all of the timed immobilization groups in which immobilization was not applied up until the last day of treatment (e8), at least one day of movement would have occurred prior to harvest at e9, as the decamethonium bromide drug is effective for roughly a day following administration (spontaneous movement was not assessed after immobilization treatment had ended in order to maximize survival, but movement was consistently observed at harvest at e9 in all groups which were not treated at e8). However, in the most severely affected three-day immobilization groups (e4-6 and e5-7), the observed effects on shape and cavitation persisted after two or three days of movement recovery prior to harvest, indicating that resumption of natural movements after a critical period of fetal immobility is insufficient to rescue joint development. In contrast, limb manipulation on e8, during maintained immobilization started at e5, was able to recover some aspects of shape and cavitation at e9. The experiment shows that a very short period of external manipulation can increase the frequency of cavitation compared to the un-manipulated side, and lead to noticeable and statistical effects on joint shape. Therefore, a severe reduction in early whole-body movements can be compensated for to some degree by applied, localized movements. This *in vivo* recovery supports the conclusions of *in vitro* tissue explant models, in which externally applied movements affect the shapes of developing limb rudiments.[18,19,24]

Our results demonstrate that shape at e9 is tightly linked with successful cavitation, as previously proposed.[9] When cavitation was consistently classified as normal (most notably in the single-day paralysis group), there were limited effects on joint shape compared to controls, whilst when cavitation was consistently absent in the I3–8, I4–8, and I5–8 sustained paralysis groups, all aspects of joint shape measured were substantially decreased relative to controls (Figures 2 and 4). There were no groups in which abnormal cavitation was associated with completely normal morphogenesis, or vice-versa.

Our findings have the potential to enhance prenatal screening for musculoskeletal conditions, and may open avenues to *in utero* preventative, or ameliorative, treatments. Arthrogryposis, also known as multiple congenital contractures, is a syndrome which occurs in 1 in 3000 to 5000 births,[5] and is associated with reduced fetal movements. It is very poorly diagnosed prenatally, with an estimated 22% of cases being diagnosed prenatally.[25] This research indicates that the time prior to joint cavitation may be a period during which fetal movements are particularly important for joint development. Therefore, between 10 and 12 gestational weeks[26-28] could be a useful time window for targeted screening for arthrogryposis, and this window would also be an opportunity to potentially induce or increase movements if there was a safe way to do so. *In utero* surgical interventions are currently only performed for very severe congenital defects, for example the heart or pulmonary system. However, in the future it may be feasible to develop a noninvasive procedure to safely promote limb movement in the fetus, for example using pulsed ultrasound.[29] Considering that arthrogryposis can be life-threatening, and even when it is not, it is associated with many complications and life-long consequences,[30] a treatment to reduce its severity would make a significant difference to affected patients' lives.

This study has some limitations. Numbers of samples are low for some groups, particularly for the assessment of cavitation in some of the timed-immobilization studies due to high mortality rates common in membrane dissection.[31] A further limitation of our methodology is that we could not characterize cavitation and morphogenesis in the same limb, as the cavity is not evident in the 3D scans. The immobilized chick limb samples were challenging to section, due to the low muscle tone and due to (we believe) altered mechanical properties of the tissues. The sections shown are the best ones obtained throughout each limb, where "best" was defined as both rudiments of the hip joint being visible, and as comparable as possible a view as the other sections (bearing in mind the immobilized limbs often had abnormally angled joints or

contractures). The designation of cavitation or absent cavitation was consistent between multiple sections—that is, all sections throughout each limb either showed normal cavitation or lack thereof. Another limitation of our approach is that we did not systematically monitor resumption of movements, or lack thereof, on a daily basis, as to do so would have reduced viability further. However, we know that each administration of decamethonium bromide leads to paralysis for at least 24 hours,[32] and we saw movements at harvest for specimens immobilized 48 hours previously (I7; I6–7; I5–7 when harvested at e9). Therefore, in these experiments the drug was active for between 24 and 48 hours. Finally, we used a single endpoint for all experiments in this study, e9, or just after cavitation, as we wanted to assess the effects of varying periods of immobilization, and the effects of external manipulation, on cavitation without having to consider the confounding effects of resumption of movement after the (potentially failed) cavitation event. Having identified a potential intervention to rescue some of the adverse effects, it would certainly be interesting to enable movements to resume after the intervention, to assess the long-term effects of artificially enhanced cavitation via external manipulation on the joint.

We have shown that a period of immobilization between embryonic days 4 and 7 led to abnormalities of hip joint cavitation and shape which were almost as severe as those caused by a complete lack of fetal movements. We have also shown that external manipulation of one hindlimb at e8 in otherwise immobilized chicks led to pronounced improvements in joint shape and cavitation compared to the contralateral, un-manipulated limb. These results indicate that the most critical time window for the influence of fetal movements on early joint development is likely to be prior to cavitation, which occurs at around 12 gestational weeks in humans,[28] opening avenues for targeted screening for musculoskeletal conditions linked with abnormal fetal movements. Furthermore, this study indicates that gentle manipulation of fetal limbs following a period of immobility could reduce the severity of the effects on joint shape and angulation later in development.

3.1 | Experimental procedures

All chick immobilization experiments were performed in accordance with European Legislation (Directive 2010/63/EU).

3.2 | Timed immobilization study

Fertilized eggs were incubated, removed of albumen, and windowed on day 3, as performed previously.[9] Paralysis was induced by administering 100 μL of 0.5% decamethonium bromide (DMB) *in ovo*. In order to elucidate a critical time period for morphogenesis and/or cavitation, we analyzed four experimental groups: "sustained" paralysis (varying day of initiation of immobilization and continuing until harvest), "single-day" paralysis (varying day of initiation of immobilization for a single 24-hour period), "2-day" paralysis (varying day of initiation of immobilization for a single 48-hour period), and "3-day" paralysis (varying day of initiation of immobilization for a single 72-hour period), as illustrated in Figure 1. We use the notation "I3–4" to represent immobilization on day 3 and 4 (2-day paralysis), immobilization on day 3 only is denoted group "I3," and immobilization starting on day 5 and continued until harvest at day 9 is denoted group "I5–8" (where the last day of treatment is day 8, lasting until day 9). A sham-immobilization group was used as a control for all timed immobilization groups, where embryos underwent identical experimental steps as the sustained' immobilized regime, but with 100 μL of sterilized PBS dropped onto the embryonic membranes instead of 100 μL of 0.5% DMB. Three distinct experiments were performed to obtain adequate replicates. Hindlimbs only were assessed. Limbs that were damaged at any point during processing were not included in the final analysis. Left limbs were processed for 3D shape analysis, and right limbs processed histologically to detect cavitation (see below). A minimum of six limbs per group were analyzed for morphology, and between two and four limbs analyzed histologically, as detailed in Table 2.

3.3 | External manipulation study

As above, fertilized eggs were incubated, removed of albumen, and windowed on day 3. Chicks were immobilized starting on e5 and continued up to an including day 8 (equivalent to the I5 to 8 regime shown in Figure 1). To enable access to the hind limb, membrane dissection was performed at e7, following previously described methods.[31] At e7, sterilized curved and pointed forceps were used to carefully perforate a hole in the chorion above the forelimb. Below this, a hole was then made in the amnion above the spine of the chick embryo. The membranes were pulled over the head of the embryo exposing the torso and hind limb. On e8, immobilization was applied as normal. Next, sterilized curved forceps were inserted between the body cavity and uppermost hindlimb. The forceps were opened, abducting the limb away from the body between a 40° and 45° angle (measured using a goniometer). The limb was then returned to the rest position, and was adducted a total of five times following,[24] over the course of approximately 10 seconds. Eggs were then sealed, returned to the

TABLE 2 Number of chick hind limbs analyzed for shape changes (using optical projection tomography, OPT) and histologically (for assessment of cavitation)

	Sustained			Single-day			Two-day			Three-day			Manipulation	
	OPT	Hist		OPT	Hist		OPT	Hist		OPT	Hist		OPT	Hist
I3–8	8	3	I3	6	2	I3–4	6	2	I3–5	7	4	Un-manip	14	6
I4–8	7	3	I4	6	2	I4–5	6	3	I4–6	6	3	Manip	14	6
I5–8	8	3	I5	6	3	I5–6	7	3	I5–7	7	4			
I6–8	8	3	I6	6	3	I6–7	6	3	I6–8	8	3			
I7–8	7	3	I7	6	3	I7–8	7	3						
I8	7	3	I8	7	3									

Note: Only limbs from the manipulation study were paired, where the manipulated right side was compared with the un-manipulated left side.

incubator, and harvested at e9. Embryos were harvested from two manipulation experiments, and pooled. Again, only hind limbs were assessed. Limbs damaged due to processing were not included in the final analysis. The right limb of each embryo was manipulated, while the left limb acted as a paired control throughout this experiment. A total of 14 pairs of hind limbs were analyzed for morphology, and six pairs of hind limbs were analyzed for cavitation.

3.4 | Joint shape analysis

Limbs for 3D shape analysis were stained with 0.055% Alcian blue and imaged with OPT, as described previously.[18] 3D reconstructions of hip joints made using Mimics (version 17.0) were virtually aligned into the frontal plane for the femoral head, and virtual sections through the lateral plane of the acetabulum, from which their shape profile outlines were drawn and overlain. The chick acetabulum at e9 is perforated at the articulation with the femoral head, that is, is not closed. As the precise location of the perforation was not always easy to detect in virtual sections of the 3D scans, the external boundaries of the acetabulum were traced when obtaining shape profiles of the region. Femoral profiles were aligned with the lowest point of the femoral neck, and acetabular profiles with the labrum. Outlines from virtual sections were compared according to timed immobilization groups, and between manipulated and contralateral control groups. Outlines of the manipulated (right) hind limbs were mirrored to enable visual comparison with outlines of the unmanipulated (left) hind limbs. Six measurements, as illustrated in Figure 2A,B, were taken using Paraview (v 4.4.0, Sandia National Laboratories, Kitware Inc, Los Alamos National Laboratory, USA) based on easily identifiable anatomical landmarks.

3.5 | Cavitational analysis

Limbs for cavitational analysis were prepared for cryosectioning by submerging in 15% sucrose in PBS for 2 hours, followed by infiltration with optimum cutting temperature (OCT) compound in 30% sucrose, and embedded in fresh OCT-30% sucrose. Limbs were flash-frozen and lateral 8 or 12 μm sections were cut using a cryostat (NX70 Cryostat, Cryostar, Thermo Fisher Scientific, UK) and mounted onto Superfrost Plus slides (Thermo Fisher Scientific, UK). Sections were rehydrated for five minutes in 1X PBS at room temperature, and then immersed in 1% Toluidine blue for one minute. Slides were then rinsed off, and imaged at ×4 and ×10 magnifications. A joint was classified as failing to cavitate when there was obvious fusion between cartilaginous regions, or when there was both no clear separation between rudiments, and decreased joint space between the rudiments. Images illustrating examples of each of these categories are shown in Figure 3.

3.6 | Statistical analysis

We used one-way multivariate analysis of variance (MANOVA) tests followed by Tukey's Honest Significant Difference post hoc test using R (code provided in Supporting Information S1) to test for significant differences ($P < .05$) between each group for the six geometric measurements. The results of the MANOVA are reported in Supporting Information S1.

In addition to the MANOVA statistical tests, we calculated effect sizes using the dabest estimation plot package.[33] In this approach, confidence intervals for the effects sizes of treatments relative to control are computed through random sampling with replacement (bootstrapping). This analysis enabled us to visualize effects sizes relative to control, as well as quantify how effects vary between treatments.

The effect of timing and duration on the probability of normal cavitation was modeled using logistic regression (using the glm R package). We used this approach to test for statistically significant effects of timing and duration on probability of cavitation. We also used the model to calculate an odds-ratio for each predictor. The odds-ratio represents the change in the odds of cavitation with a 1-day increase in the predictor (start or duration of paralysis).

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AUTHOR CONTRIBUTIONS

Devi Bridglal: Conceptualization; formal analysis; investigation; methodology; visualization; writing-original draft; writing-review and editing. **Colin Boyle:** Formal analysis; methodology; software; visualization; writing-original draft; writing-review and editing. **Rebecca Rolfe:** Investigation; methodology; supervision; writing-review and editing. **Niamh Nowlan:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; supervision; visualization; writing-original draft; writing-review and editing.

ORCID

Colin J. Boyle https://orcid.org/0000-0002-7508-8508
Rebecca A. Rolfe https://orcid.org/0000-0003-2177-3136
Niamh C. Nowlan https://orcid.org/0000-0002-9083-6279

REFERENCES

1. Nowlan N. Biomechanics of foetal movement. *Eur Cell Mater*. 2015;29:1-21.
2. Pollet V, Percy V, Prior HJ. Relative risk and incidence for developmental dysplasia of the hip. *J Pediatr*. 2017;181:202-207.
3. Broadhurst C, Rhodes A, Harper P, Perry D, Clarke N, Aarvold A. What is the incidence of late detection of developmental dysplasia of the hip in England? A 26-year national study of children diagnosed after the age of one. *Bone & Joint J*. 2019;101(3):281-287.
4. Sandell LJ. Etiology of osteoarthritis: genetics and synovial joint development. *Nat Rev Rheumatol*. 2012;8(2):77-89.
5. Lowry RB, Sibbald B, Bedard T, Hall JG. Prevalence of multiple congenital contractures including arthrogryposis multiplex congenita in Alberta, Canada, and a strategy for classification and coding. *Birth Defects Res A Clin Mol Teratol*. 2010;88(12):1057-1061.
6. Archer CW, Dowthwaite GP, Francis-West P. Development of synovial joints. *Birth Defects Res C Embryo Today*. 2003;69(2):144-155.
7. Decker RS, Koyama E, Pacifici M. Genesis and morphogenesis of limb synovial joints and articular cartilage. *Matrix Biol*. 2014;39:5-10.
8. Nowlan NC, Sharpe J. Joint shape morphogenesis precedes cavitation of the developing hip joint. *J Anat*. 2014;224(4):482-489.
9. Nowlan NC, Chandaria V, Sharpe J. Immobilized chicks as a model system for early-onset developmental dysplasia of the hip. *J Orthop Res*. 2014;32(6):777-785.
10. Kahn J, Shwartz Y, Blitz E, et al. Muscle contraction is necessary to maintain joint progenitor cell fate. *Dev Cell*. 2009;16(5):734-743.
11. Nowlan NC, Bourdon C, Dumas G, Tajbakhsh S, Prendergast PJ, Murphy P. Developing bones are differentially affected by compromised skeletal muscle formation. *Bone*. 2010;46(5):1275-1285.
12. Nowlan NC, Dumas G, Tajbakhsh S, Prendergast PJ, Murphy P. Biophysical stimuli induced by passive movements compensate for lack of skeletal muscle during embryonic skeletogenesis. *Biomech Model Mechanobiol*. 2012;11(1–2):207-219.
13. Brunt L, Skinner R, Roddy K, Araujo N, Rayfield E, Hammond C. Differential effects of altered patterns of movement and strain on joint cell behaviour and skeletal morphogenesis. *Osteoarthr Cartil*. 2016;24:1940-1950.
14. Brunt LH, Begg K, Kague E, Cross S, Hammond CL. Wnt signalling controls the response to mechanical loading during Zebrafish joint development. *Development*. 2017;144(15):2798-2809. https://dev.biologists.org/content/144/15/2798.abstract
15. Osborne AC, Lamb KJ, Lewthwaite JC, Dowthwaite GP, Pitsillides AA. Short-term rigid and flaccid paralyses diminish growth of embryonic chick limbs and abrogate joint cavity formation but differentially preserve pre-cavitated joints. *J Musculoskelet Neuronal Interact*. 2002;2(5):448-456.
16. Drachman DB, Sokóloff L. The role of movement in embryonic joint formation. *Dev Biol*. 1966;14:401-420.
17. Roddy KA, Kelly GM, van Es MH, Murphy P, Prendergast PJ. Dynamic patterns of mechanical stimulation co-localise with growth and cell proliferation during morphogenesis in the avian embryonic knee joint. *J Biomech*. 2011;44(1):143-149.
18. Chandaria VV, McGinty J, Nowlan NC. Characterising the effects of in vitro mechanical stimulation on morphogenesis of developing limb explants. *J Biomech*. 2016;49(15):3635-3642.
19. Parisi C, Chandaria VV, Nowlan NC. Blocking mechanosensitive ion channels eliminates the effects of applied mechanical loading on chick joint morphogenesis. *Phil Trans R Soc B*. 2018;373(1759):20170317.
20. Drachman D, Coulombre A. Experimental clubfoot and arthrogryposis multiplex congenita. *The Lancet*. 1962;280(7255):523-526.
21. Hamburger V, Balaban M. Observations and experiments on spontaneous rhythmical behavior in the chick embryo. *Dev Biol*. 1963;7:533-545.
22. Rolfe RA, Bezer JH, Kim T, et al. Abnormal fetal muscle forces result in defects in spinal curvature and alterations in vertebral segmentation and shape. *J Orthop Res*. 2017;35(10):2135-2144.
23. Levillain A, Rolfe RA, Huang Y, Iatridis JC, Nowlan NC. Short-term foetal immobility temporally and progressively affects chick spinal curvature and anatomy and rib development. *Eur Cell Mater*. 2019;37:23-41.

24. Lelkes G. Experiments in vitro on the role of movement in the development of joints. *Development*. 1958;6(2):183.
25. Filges I, Hall JG. We are failing to identify disorders of fetal movement–why? *Prenat Diagn*. 2012;32(10):919-920.
26. Bardeen CR. Studies of the development of the human skeleton.(A). The development of the lumbar, sacral and coccygeal vertebrae.(B). The curves and the proportionate regional lengths of the spinal column during the first three months of embryonic development. (C). The development of the skeleton of the posterior limb. *Am J Anat*. 1905;4(3):265-302.
27. Bardeen CR, Lewis WH. Development of the limbs, body-wall and back in man. *Am J Anat*. 1901;1(1):1-35.
28. Strayer LM Jr. The embryology of the human hip joint. *Yale J Biol Med*. 1943;16(1):13-26.
29. Fatemi M, Ogburn PL Jr, Greenleaf JF. Fetal stimulation by pulsed diagnostic ultrasound. *J Ultrasound Med*. 2001;20(8):883-889.
30. Hall JG. Arthrogryposis multiplex Congenita: etiology, genetics, classification, diagnostic approach, and general aspects. *J Pediatr Orthop B*. 1997;6(3):159-166.
31. Spurlin J, Lwigale P. A technique to increase accessibility to late-stage Chick embryos for in ovo manipulations. *Dev Dyn*. 2013;242(2):148-154.
32. Germiller JA, Goldstein SA. Structure and function of embryonic growth plate in the absence of functioning skeletal muscle. *J Orthop Res*. 1997;15(3):362-370.
33. Ho J, Tumkaya T, Aryal S, Choi H, Claridge-Chang A. Moving beyond P values: data analysis with estimation graphics. *Nat Methods*. 2019;16(7):565-566.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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