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TECHNICAL COMMENT

Validating a Noninvasive Technique for Monitoring Embryo Movement In Ovo

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ABSTRACT

Avian embryos are a commonly used model system for developmental studies, but monitoring of physiological parameters such as heart rate (HR) and movement in ovo poses a challenge to researchers. These are also increasingly common research objectives for ecological and embryo behavior studies in oviparous species. We therefore explored the validity of a new digital egg-monitoring system for the noninvasive monitoring of these parameters. We tested the relationship between frequency-of-movement values gathered by digital monitoring and those gathered by the current standard method, which is comparatively invasive and requires egg windowing, and demonstrated that the digital monitoring method effectively distinguishes individual movements but cannot reliably monitor HR in actively motile embryos. We therefore provide recommendations for the appropriate use of this technique for avian physiologists. We also applied the digital monitoring method to reveal how frequency of movement varies throughout prenatal ontogeny in the chicken and showed that commonly used protocols in developmental studies can themselves alter motility; egg windowing and application of light modulate frequency of movement. Recent work has revealed the importance of embryo motility in regulating gene expression and cellular activity during developmental processes. Together with our data, this highlights the value of noninvasive monitoring methods and the importance of controlling for altered embryo motility/behavior in developmental studies.

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Introduction

Birds are a commonly used model system for studying embryonic development and, increasingly, embryo behavior. Chicken embryos in particular have been used to elucidate the molecular mechanisms underpinning the morphogenesis of many systems (Guillot and Lecuit 2013; Le Douarin and Dieterlen-Lievre 2013; Hirst and Marcelle 2015; Hu et al. 2015). The popularity of chicken egg–based models is due to their availability, short 21-d incubation period, and relative ease of manipulation compared to mammalian embryos. Monitoring of physiological parameters such as heart rate (HR) and embryonic movement in ovo, however, still poses a challenge to researchers.

Recently, a digital egg-monitoring system has been developed to allow breeders of oviparous species to noninvasively monitor embryo development and to screen for healthy hatchlings. The digital egg monitor detects disturbances in infrared light transmission through the eggshell caused either by pulsing of blood vessels or by embryo movement. Although not developed for research purposes, it has many potential applications in applied scientific research; the device has already been used to monitor the health of embryos to inform conservation work (Lemus et al. 2009; Braune et al. 2012; Angilletta et al. 2013) and may facilitate research in egg physiology and embryo development. Recently, the digital monitor has been used in behavioral studies, for example, to record the response in HR of embryonic passerines to conspecific and heterospecific adult calls (Colombelli-Négrel et al. 2014). Previously, measurement of embryonic HR in oviparous species has been achieved by methods including acoustocardiogram, electrocardiogram, and viewing blood vessels through an egg window (Haque et al. 1994). These techniques, however, are invasive or require relatively sophisticated imaging setups that cannot easily be used in the field. Despite its growing use in research, the digital egg monitor method for monitoring HR has thus far not been fully validated by comparison with current standard methods, and its measurement of embryonic movement has not been validated or studied at all.

The importance of embryo movement in typical development is becoming increasingly apparent (Pitsillides 2006; Nowlan et al. 2014; Pollard et al. 2014). There is abundant evidence that musculoskeletal development is impaired, blood flow altered,

and gene expression modified in the absence of normal movement (Hosseini and Hogg 1991; Pitsillides 2006; Nowlan et al. 2010; Rolfe et al. 2014). Moreover, embryo movement can be influenced by many factors, including pharmacological agents (Fanconi et al. 1995; Heywood et al. 2005; Jensen et al. 2014) and external cues such as temperature and circadian rhythms (Hammond et al. 2007; Ozkaya et al. 2012). This growing understanding of the extent to which altered embryo motility can influence development means that robust methods for monitoring movement in ovo are increasingly valuable. The standard method for monitoring embryo movement involves removal of a section of shell, or windowing, and illumination of the embryo so that movements can be observed and counted. This introduces an infection risk and is not suitable for most field-based studies or for projects involving threatened species. Noninvasive methods for monitoring motility are therefore an incredibly valuable tool traversing multiple interdisciplinary fields, and the measurements of embryo movement and HR are routine research objectives for which a noninvasive technique would be ideal.

We aimed to validate the digital egg-monitoring system, which is entirely noninvasive, as a method for monitoring embryo movement and HR. Herein, we compare embryo movement values obtained by the digital monitor to those gathered using traditional invasive windowing and compare measurements of HR to previously measured values from the literature. A number of studies have already used the digital monitor system to monitor embryo HR. A secondary aim was therefore to determine whether periods of embryo movement interfere with HR recording when using the digital monitor system to measure both HR and embryo movement and whether this is likely to pose a problem to researchers using the device to monitor only HR. Finally, we applied the digital monitor to answer two questions that could not readily be addressed using visual monitoring: first, how does embryo motility vary throughout development when it is monitored noninvasively, and, second, do the windowing of eggs and application of an external light themselves alter embryo motility?

Material and Methods

Animals

All procedures complied with the Animals (Scientific Procedures) Act 1986 and local ethics committee. Fertilized chicken eggs were commercially sourced (Henry Stewart, Norfolk, UK) and incubated on their side at 37°C with 45% relative humidity. At embryonic day 4 (E4), a group of eggs were windowed as in Fisher and Schoenwolf (1983). Briefly, a section of shell and shell membrane was removed from the uppermost surface and the egg resealed with clear adhesive tape to allow for embryo observation and drug injection onto the chorioallantoic membrane, taking care not to damage any embryonic structures. This results in survival rates comparable to those of nonwindowed eggs (Fisher and Schoenwolf 1983). On each subsequent day of incubation, each egg was placed into the Avitronics Buddy digital egg monitor and, following the manufacturer's instructions, observed for any measurable HR or embryo movement. A 3-min period was used for each episode of monitoring in each egg throughout this study, as we observed no obvious decrease in frequency of movement or HR due to possible cooling during this time frame (Lierz et al. 2006). At the end of the experiments, the embryos were euthanized by schedule I decapitation in accordance with the Animals (Scientific Procedures) Act 1986.

Validation of Digital Monitor Embryo Movement Measurements

Accuracy of digital monitor measurements was validated by comparison with the current standard, visual-based window methods, for quantifying movement. At E11, the earliest stage at which movement was consistently detected by the digital monitor, movement was first quantified in windowed but otherwise untreated eggs ($n = 12$) by direct visualization of embryos through the window. The egg/embryo was illuminated using an external light and the number of visible movements during a 3-min period counted. Detection of embryo movement by the digital monitor was quantified immediately thereafter by placing the egg inside the device and recording the monitor display, which visually displays HR values and embryo movements with a video camera, before counting recorded movements from this video footage using a tally counter.

To test whether digital monitoring is capable of detecting changes in embryo motility, we altered movement with pharmacological intervention. Embryos were treated with decamethonium bromide (DMB), which induces rigid skeletal muscle paralysis, or 4-aminopyridine (4-AP; $n = 12$ in each group), which stimulates skeletal muscle contraction and increases the frequency of movement; 0.5 mg/mL DMB or 0.2 mg/mL 4-AP in Tyrode's solution was administered by injection through the window onto the chorioallantoic membrane and the window resealed with adhesive tape. This was performed at E15, as previous studies indicate that treatment with DMB between E11 and E13 significantly decreases embryo survival rate but is relatively well tolerated before E11 and from E15 onward (Osborne et al. 2002; Lamb et al. 2003; Pitsillides 2006), an observation that has yet to be explained.

Movement was first recorded, both visually through the egg window and using the digital monitor, in windowed but otherwise untreated E15 embryos ($n = 24$). Eggs were then returned to the incubator for 30 min to avoid excessive cooling and then randomly assigned into groups of 12, and either DMB or 4-AP was administered. Eggs were returned to the incubator for 5 min to allow the drugs to take effect and embryo movement and HR measurements repeated thereafter.

Quantifying HR with the Digital Monitor in Moving and Nonmoving Embryos

Quantification of HR in windowed, otherwise untreated E15 embryos ($n = 24$) showed that HR values varied considerably during each 3-min monitoring period, and this appeared to be related to periods of embryo movement. We therefore recorded

the HR from the video footage at 10-s intervals throughout the entire 3-min monitoring period (18 values per egg) and categorized the HR values into measurements taken while the embryo was moving or nonmoving.

Digital Monitoring to Evaluate Influence of Windowing and Light on Motility

To determine how frequency of embryonic movement varies throughout typical development and whether the window method of monitoring motility itself elicits modified embryo activity, the digital monitoring system was used to quantify frequency of movement at daily intervals between E11 and E18 in the following experimental groups: no window, window, and window plus external light ($n = 12$ for each group). Frequency of movement was monitored in the no window and window groups with the digital monitor as previously described and repeated in the window plus external light group, with the addition of an LED producing white light (but no measurable heat) positioned within the monitor chamber.

Statistics

The Shapiro-Wilk normality test was used to confirm that all frequency-of-movement data were normally distributed. Unpaired *t*-tests were initially used to compare mean frequency-of-movement values gathered using the digital monitor and window methods. Pearson's correlation coefficient was selected as the most appropriate test to investigate the relationship between values obtained by the two methods. This was used instead of comparing mean absolute values measured by each method, as these clearly differed, with values recorded using digital monitoring consistently approximately double those attained by visual scoring (see "Results"). Pearson's correlation coefficients for measurements from E11 and E15 embryos both before and after 4-AP treatment were therefore calculated, where +1, 0, and −1 indicate the strongest possible positive correlation, no correlation, and the strongest possible negative correlation, respectively. Linear regression analysis was performed to describe the relationship between the two sets of measurements. To address our final aim, mean frequency of embryonic movement values for each group of eggs (no window, window, and window plus external light) were compared. The repeated daily measurements from the three groups were compared using a repeated-measure two-way ANOVA with correction for multiple comparisons ($\alpha = 0.05$). Average HR values from untreated E15 embryos categorized as moving or nonmoving were compared by Wilcoxon signed-rank test ($\alpha = 0.05$). All statistical analyses were performed using Graph Pad Prism.

Results

Chicken embryos exhibit movement from E4 onward, and by E7 their muscle activation can be considered coordinated (Hamburger and Balaban 1963). However, we found that the digital monitor did not consistently register embryo movement (defined as detection of multiple movements during the monitoring period in all embryos where movements have been observed visually at this stage) until E11, although movement could be seen visually through the egg window before this stage. HR was consistently detectable using the digital monitor (defined as successful detection of HR in all viable embryos at this stage) in all embryos from E8 onward, slightly later than the earliest-detectible HR reported in previous studies (Lierz et al. 2006). We observed an 80% survival rate in nonwindowed eggs and a 75% survival rate in windowed eggs. In total, 24 windowed eggs were incubated up to E15, and 12 nonwindowed and 24 windowed eggs were incubated up to E18.

Pharmacological Manipulation of Embryo Movement

To address whether the digital monitor is capable of detecting alterations in motility, we induced immobilization with DMB or hypermotility with 4-AP. As expected, no evidence of movement was detected either by digital monitoring or by the traditional window method in embryos injected with DMB (fig. 1*A*). These data confirm that the digital monitor does not falsely detect movement in immobile embryos. An increase in frequency of movement was detected by both methods after 4-AP treatment (fig. 1*B*): a 175% increase observed by the visual method ($t = 9.027$, df $= 11$, $P < 0.0001$) compared to only 72% using the digital monitor ($t = 9.55$, df $= 11$, $P < 0.0001$).

Validation of the Digital Monitor Method for Measuring Frequency of Embryo Movement

Both visual and digital monitor methods identified a significant increase in frequency of movement following 4-AP administration, but these values did not directly correspond. No significant difference was found between absolute frequency-of-movement values obtained by both methods at E11 ($t = 0.77$, df $= 22$, $P = 0.45$), but these did differ at E15 ($t = 9.315$, df $= 46$, $P < 0.001$). However, we observed at E15 that digital monitor values were consistently approximately double those values obtained by the traditional window method. We therefore sought to investigate the relationship between values from both methods, which revealed a positive correlation between visual and digital monitor measurements at E11 (fig. 2*A*; correlation coefficient $= 0.64$, $P < 0.05$, 95% confidence interval [CI] $= 0.10$–0.89). Digital monitor measurements were more variable at E11, the first day when embryo movement could be detected. Linear regression analysis revealed that the number of discrete movements detected using the digital monitor was generally double that detected by direct visualization, but relatively high variability was present (fig. 2*A*; regression coefficient $= 0.447 \pm 0.17$, $r^2 = 0.41$, $P < 0.05$). This variation may indicate that the digital monitor does not reliably measure movement at relatively early time points.

We observed stronger correlation between visual and digital monitor measurements at E15 (fig. 2*B*; correlation coefficient $= 0.75$, $P < 0.0001$, 95% CI $= 0.49$–0.88). Linear regression analysis revealed, again, that digital monitor values were approximately double those measured visually (fig. 2*B*; regression coefficient =

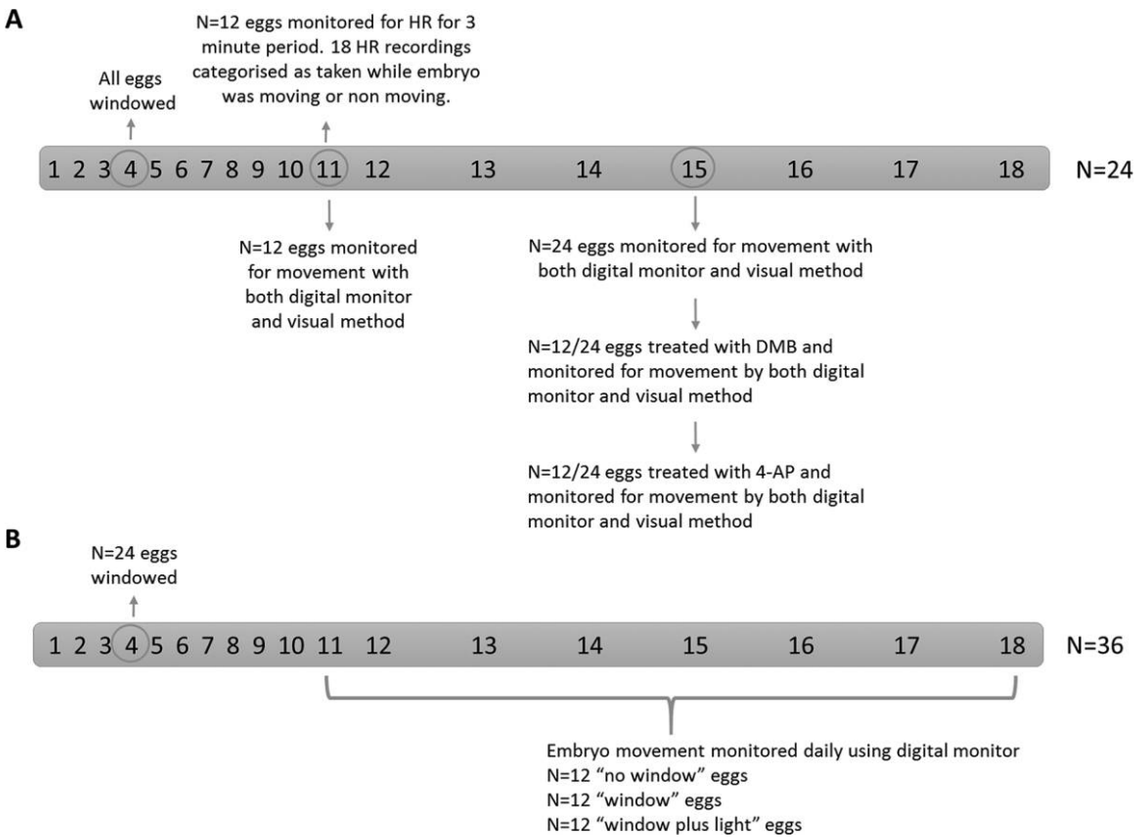

Figure 1. Experimental design. *A*, Twenty-four chicken eggs were windowed at E4. At E11, embryo movement was monitored using both visual and digital monitoring methods in 12 eggs. At E11, heart rate (HR) was also monitored in 12 eggs. Eighteen HR measurements were made from each egg during a 3-min period and categorized as measurements taken during periods of movement and nonmovement. *B*, The digital monitoring method was applied to monitor embryo movement daily between E11 and E18 in three groups of eggs: no window, window, and window plus light ($n = 12$ per group). Window and window plus light eggs were windowed on E4. Window plus light eggs had an LED placed inside the digital monitor chamber during monitoring between E11 and E18. A color version of this figure is available online.

0.421 ± 0.08, $r^2 = 0.56$, $P < 0.0001$). The digital monitor registers approximately two movements for every one recorded by the visual method. This is likely explained by the digital monitor counting limb flexion and extension, for example, a kicking motion, as two separate movements, whereas the observer in the visual method likely records this only once. Correlation coefficient was not calculated for DMB-treated embryos, as no movement was observed by either method.

While movement was observed to increase by both digital monitor and visual techniques following 4-AP administration (fig. 1*B*), there was no correlation between these values (fig. 2*C*; correlation coefficient $= -0.25$, $P > 0.05$, 95% CI $= -0.72$ to 0.38). This reflects an increased variability of visual measurements after 4-AP treatment, which we suggest is due to observer error in distinguishing individual movements when embryos are moving multiple body parts or moving very frequently.

Quantification of HR with the Digital Monitor

HR was monitored in windowed but otherwise untreated actively moving or nonmoving E15 embryos. HR values recorded from nonmoving embryos were significantly faster than in moving embryos ($P < 0.0001$, lower than expected). HR values recorded from moving embryos were also considerably more variable, with a range of 196 compared to 74 beats per minute (bmp) recorded for moving embryos (see fig. 3).

Mean HR predicted by the literature for a normal E15 embryo is 240 bmp (Cain et al. 1967), which closely matches the values obtained from the digital monitor in nonmoving embryos at this stage. We would not expect HR to decrease in more active embryos; in fact, we would expect the opposite, as HR has been shown to accelerate with fetal movement in healthy human embryos (Rochard et al. 1976). This indicates that the lower HR recorded by the digital monitor in moving embryos is likely due to interaction between measurement of embryonic movement and HR and does not reflect a real decrease in HR.

Embryo Movement Is Altered by External Cues

Having established that the digital egg-monitoring system reliably monitors embryo movement, we applied the device to address how frequency of movement changes during typical

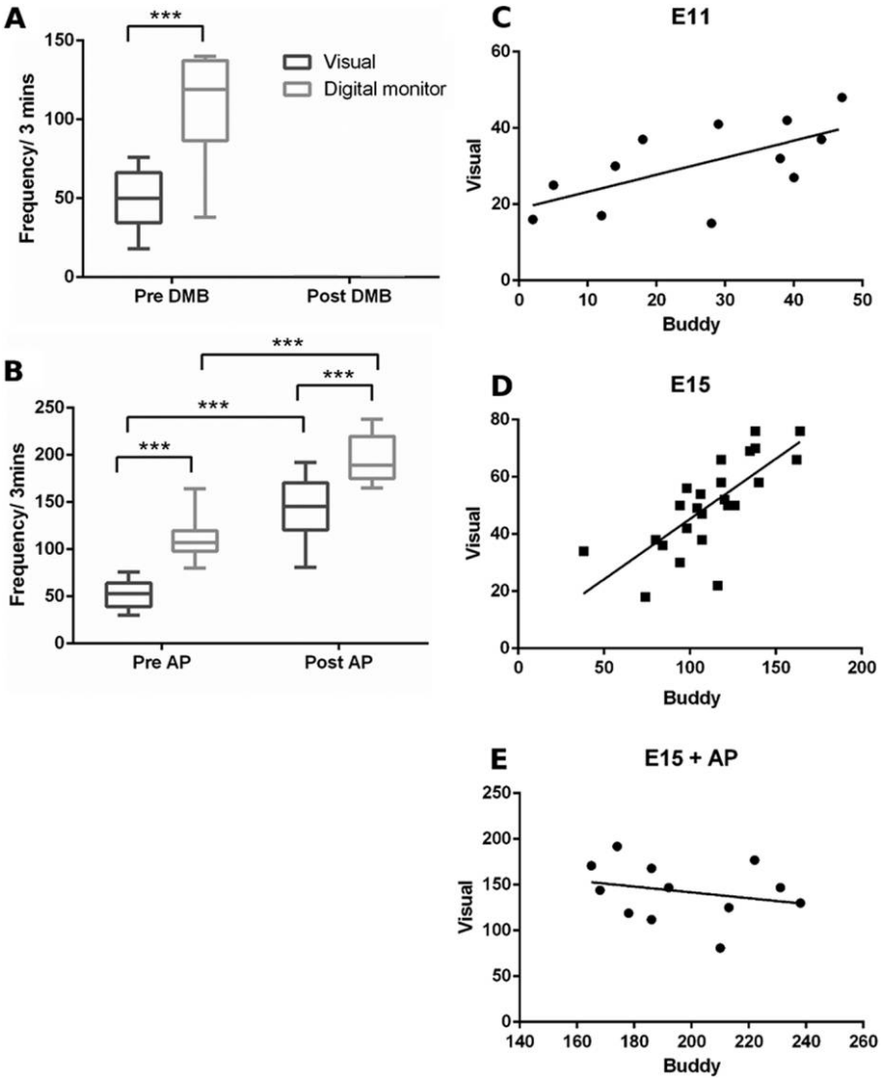

Figure 2. Frequency of movement in chicken embryos before and after treatment with drugs that modify muscle contraction (*A*, *B*) and correlation between frequency of movement measured by either visual or digital monitor–based methods (*C*–*E*). Effect of DMB (*A*), a depolarizing neuromuscular blocking agent, and 4-AP (*B*), a skeletal muscle stimulant ($n = 12$ for each group), on the frequency of movement (no./3 min) measured by both visual (dark gray) and digital (light gray) monitor–based methods. The relationship between frequency of movement measured by either visual or digital monitor–based methods at E11 (*C*; Pearson's correlation coefficient = 0.64, indicating a moderately strong positive correlation between the two methods), E15 in windowed but otherwise untreated embryos (*D*; Pearson's correlation coefficient = 0.75, indicating a strong positive correlation), and E15 after treatment with 4-AP (*D*; Pearson's correlation coefficient = 0.25, indicating no correlation between the two methods).

development and whether commonly used windowing protocols themselves alter chicken embryo development and behavior. Daily monitoring revealed a trend for increasing frequency of movement between E12 and E15. By E16, the mean number of movements was 128 ± 21.9 discrete movements per 3-min period. The frequency of embryonic movement did not increase significantly at any later time point, suggesting that movement levels plateau or become restricted after this developmental stage due to the size of the embryo and the egg (fig. 4).

Our data confirm that embryo motility is influenced by external cues that are often not controlled for in developmental studies. We observed increased motility in the window compared to the no window group, which was statistically significant at E12 and E14 (45% increase, $P < 0.01$ at E12; 25% increase, $P < 0.05$ at E14; fig. 4). In the window plus external light group, movements were significantly more frequent than in nonwindowed eggs at E12–E15 (fig. 4) and tended also to be increased relative to window eggs, although the latter was not statistically significant. No difference in frequency was observed between any of the different experimental groups after E15. We suspect that this is again due to restricted embryo movement as a result of embryo/egg size. These data demonstrate that embryo

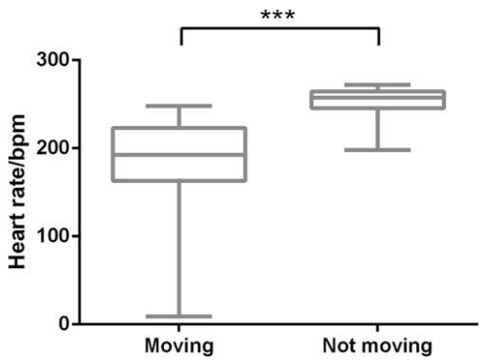

Figure 3. Heart rates in 24 windowed but otherwise untreated chicken embryos measured via the digital monitor at incubation day 15. Measurements were made by the digital monitor at 10-s intervals from 3-min recordings and the embryos categorized as moving or not moving during this interval. Values from nonmoving embryos were less variable and significantly larger than those from moving embryos ($P < 0.0001$).

motility is influenced by the traditional windowing and light technique.

Discussion

This study validates the digital monitor system as a suitable method for measuring the frequency of embryo movement in ovo. It has several advantages over the current standard method, which involves visual detection of movement through an egg window, for many, but not all, types of developmental, behavioral, and ecological study. Our data demonstrate that the digital monitor is capable of distinguishing between individual embryonic movements. We found a strong correlation between frequency-of-movement measurements made by the existing window-based and digital monitor methods at E15, although the digital monitor detects approximately two movements for every one movement detected visually. This is likely due to the categorization of flexion and extension of a limb (i.e., a kicking movement) as one movement visually but two by the digital monitor. It is not possible, however, to distinguish different types of movements using the digital egg-monitoring system alone. For example, flexion and extension or limb and jaw movements cannot be distinguished without windowing. Studies wishing to monitor just one type of movement or use interventions such as local immobilization of a body region perhaps by ablation of specific muscles would still require the more invasive window method. However, normal embryo motility involves repetitive limb, jaw, and whole-body movements (Hamburger and Balaban 1963), and so general increases in embryo motility measured by the digital egg monitor could reasonably be assumed to involve an increased frequency of each of these movements.

This difference in the definition of a discrete movement between methods is a potentially confounding factor, which means that frequency-of-movement values obtained by the digital monitor method should not be directly compared to values obtained by other methods that may be available in the literature. However, most studies that have monitored embryo movement thus far have required only relative measurements, for example, between control and treated groups of individuals in a study. Observation of embryo movement via windowing is a subjective method and is likely considerably more prone to human measurement error and interobserver variation, due to the difficulty of visually distinguishing between individual embryonic movements. We suspect that the lack of correlation we observed between the digital method and the visual method after application of a muscle stimulant results from an increase in human measurement error when monitoring very motile embryos by the window-based method. For this reason, we would recommend that the digital monitoring system be used to monitor embryo movement whenever possible.

Our data also suggest that there is a minimum threshold for detection of embryonic movement by the digital egg monitor. Before E11, movements that can be detected visually are not detected by the monitor. Similarly, we found that HR was also not consistently detected before E8. This limits the usefulness of the digital egg monitor for monitoring physiological parameters throughout the whole of prenatal ontogeny, and window-based methods would be required before these time points. However, thus far, studies into the role of embryo movement in development have focused on the musculoskeletal system. With this in mind, a number of studies have shown that limb-patterning events and early skeletal growth are insensitive to mechanical stimuli (Drachman and Sokoloff 1966; Hosseini and Hogg 1991; Pitsillides 2006). Although embryo movement starts as early as E4 in the chicken, joint cavitation and endochondral ossification are influenced by embryo movement only during the later phases of development, during which movement can be consistently monitored by the digital egg monitor, so it is certainly useful for the study of these phenomena. Additionally, the existing studies that have used this digital egg-monitoring system to study embryonic responses to external stimuli have also focused on relatively late developmental stages (Colombelli-Négrel et al. 2014).

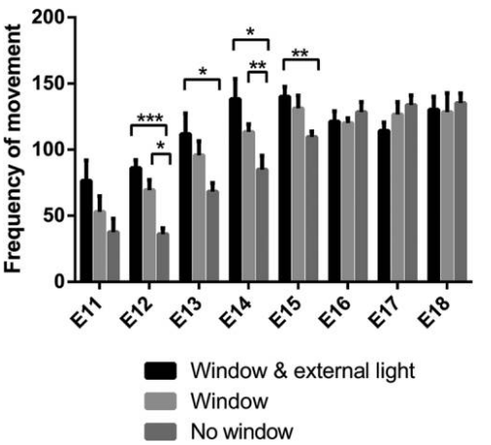

Figure 4. Frequency of movement monitored by digital monitoring system daily between incubation days 11 and 18. Data are shown as mean frequency (no./3-min recording period; mean ± SEM). One asterisk indicates $P < 0.05$; two asterisks indicate $P < 0.01$; three asterisks indicate $P < 0.001$.

The digital monitor is more suitable than existing invasive methods for monitoring particularly valuable embryos, such as threatened species. Windowing is an invasive technique, and we observed a slightly decreased survival rate of windowed eggs in this experimental study. Some windowing techniques have been shown to increase the incidence of neural tube defects and decrease hatching rate when carried out early in development, that is, E1–E4, although the use of more recently developed windowing techniques has been shown to virtually eliminate this risk (Fisher and Schoenwolf 1983). Even if the impact on survival rate is not a concern, our data indicate that windowing certainly can influence embryo behavior, which may have implications for the development of many systems and could also be a confounding factor in studies of embryo behavior. We consider that the use of less invasive methods would be advantageous in many studies that explore broader physiological questions.

Having established the suitability of the digital system for monitoring movement, we applied this method to test how embryo movement varies during prenatal ontogeny and whether factors that are often not controlled for in developmental studies can influence embryo movement. After E15, embryo movement plateaued in all experimental groups monitored. It is possible that this is a normal trend that occurs at this late stage but is perhaps more likely due to constriction of movement by the large size of the embryo relative to the egg size at late developmental stages. Indeed, late-stage embryos have been observed to extend their limbs beyond the egg and exhibit more frequent limb movements if sections of shell adjacent to the limbs are removed (Bradley et al. 2014). It is possible that this restraint may act to limit the ability of embryos to respond with an increase in motility to an external stimulus such as light. This likely explains why we observed the greatest impact of external stimuli on embryo movement between E12 and E15; after this time point, movement is restricted, and before this time point, the chick embryo sensory system may not be sufficiently developed to coordinate a response. Chicken embryos respond to proprioceptive stimuli from E7.5 onward (Oppenheim et al. 1978), and lighting regimes certainly do influence their development (Lowe and Garwood 1977), but it is not known exactly how early-embryonic responses to light occur.

Our work also reveals that the traditional windowing approach currently used in nearly all studies in embryonic chicks may itself affect embryo behavior, at least at some developmental stages. Both windowing of eggs and application of light can increase the frequency of embryo movement during a monitoring period. This impact of egg windowing on motility may account for the small amount of variation still observed between window and digital monitor measurements of embryo movement at E15 when we assume that the digital method counts two movements for every one by the visual method. It is important to consider the potential impact that windowing may have in any studies in which the research endpoints are factors that could be influenced by embryo movement. Whether this impact on embryo behavior extends to very early stages or how such alterations in behavior may influence cellular activity and gene expression patterns remains to be defined. Given the demonstrated role of movement in regulating gene expression (Dowthwaite et al. 1999; Groenendijk et al. 2005; Rolfe et al. 2014), we suggest that care should be taken when interpreting data gathered solely from windowed eggs.

We additionally highlight a limitation of the digital monitor system. Embryonic movements occur periodically, with periods of activity alternating with periods of inactivity (although the frequency of these periods change during development; Hamburger and Oppenheim 1967). The HR measurements gathered using the digital monitor from windowed but otherwise untreated embryos during periods of no movement closely match the predicted, previously measured values for embryos of an equivalent stage from the literature (Cain et al. 1967). Our measurements made during periods of embryo movement, however, were consistently lower than predicted. Decreases in HR are not normally observed in more active embryos (Dipietro et al. 2001), suggesting that the lower HR values detected by the digital monitor do not indicate a real change in cardiac rhythm but instead reflect interference between the measurement of movement and HR. Changes in HR detected by the digital monitor may therefore reflect (1) a real change in cardiac rhythm or (2) a change in embryo movement. It is noteworthy that HR measurements using the digital monitor are increasingly used as an endpoint in defining embryo responses to external stimuli (Colombelli-Négrel et al. 2014; Noiva et al. 2014). Both possibilities that produce altered HR recordings indeed reflect embryonic responses to stimuli, and, thus, the research conclusions of these studies are upheld, but the mechanisms could be different from those assumed. We recommend that care be taken in interpreting studies using only the digital recording system to monitor HR responses as an endpoint to environmental factors or pharmacological interventions that could also involve changes in embryo movement.

We observed a reduction in embryo movement during the final stages of development. Based on our findings, we would not recommend using the digital monitoring method alone to monitor HR in embryonic chickens before E15, but the monitoring system could likely be used more reliably at late stages of development. Additionally, if the study design limits the use of other techniques (e.g., field-based studies that require a battery-powered device such as the digital egg monitor), HR measurements should at least be taken during intervals when no embryo movement is detected by the device. However, acoustocardiogram likely remains the most reliable method for monitoring embryonic HR that does not necessarily require windowing.

Our experiments tested the relationship between frequency-of-movement values gathered by digital monitoring and the current standard method, which is comparatively invasive and requires egg windowing. These demonstrated that the digital monitoring method effectively distinguishes individual movements but cannot reliably monitor HR in actively motile embryos. We would recommend that the digital monitoring system be used to monitor movement when possible, to eliminate the possibility of decreased survival rate, altered embryo behavior, and subjectivity involved in using the window method. For studies that require monitoring before E11 or for movement of indi-

vidual body parts to be distinguished, the windowing method described in Fisher and Schoenwolf (1983) should be used to minimize the impact on survival rate and results interpreted with the caveat that the impact of windowing on behavior could and likely does influence some physiological parameters. We would ideally recommend that use of the digital system to monitor HR be limited to late developmental stages, when the frequency of periods of activity relative to inactivity decreases in the days immediately before hatching.

In the circumstances we have outlined above, when the digital monitor can be used reliably, it opens up avenues of research for avian and reptilian physiologists that were not previously possible. The digital monitor system is easily transportable and can be used in the field (e.g., to monitor eggs in natural nests, when more invasive methods could introduce an infection risk or disturb the parents) to enable ecological studies, it can safely be used with the eggs of threatened species, and, unlike some invasive methods for monitoring HR, it does not require embryos to be euthanized at the end of a study. Furthermore, the digital system allows the impact of external stimuli on physiological parameters to easily be explored. In our study, we exploited this to highlight the impact of light on embryo behavior and the importance of controlling for altered embryo motility/behavior in developmental studies.

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Literature Cited

Angilletta M.J., M.H. Zelic, G.J. Adrian, A.M. Hurliman, and C.D. Smith. 2013. Heat tolerance during embryonic development has not diverged among populations of a widespread species (*Sceloporus undulatus*). Conserv Physiol 1:cot018. doi: 10.1093/conphys/cot018.

Bradley N.S., Y.U. Ryu, and M.C. Yeseta. 2014. Spontaneous locomotor activity in late-stage chicken embryos is modified by stretch of leg muscles. J Exp Biol 217:896–907.

Braune B., A. Scheuhammer, D. Crump, S. Jones, E. Porter, and D. Bond. 2012. Toxicity of methylmercury injected into eggs of thick-billed murres and arctic terns. Ecotoxicology 21: 2143–2152.

Cain J.R., U.K. Abbott, and V.L. Rogallo. 1967. Heart rate of the developing chick embryo. Exp Biol Med 126:507–510.

Colombelli-Négrel D., M.E. Hauber, and S. Kleindorfer. 2014. Prenatal learning in an Australian songbird: habituation and individual discrimination in superb fairy-wren embryos. Proc R Soc B 281:20141154.

Dipietro J.A., R.A. Irizarry, M. Hawkins, K.A. Costigan, and E.K. Pressman. 2001. Cross-correlation of fetal cardiac and somatic activity as an indicator of antenatal neural development. Am J Obstet Gynecol 185:1421–1428.

Dowthwaite G.P., A.C. Ward, J. Flannely, R.F. Suswillo, C.R. Flannery, C.W. Archer, and A.A. Pitsillides. 1999. The effect of mechanical strain on hyaluronan metabolism in embryonic fibrocartilage cells. J Int Soc Matrix Biol 18:523–532.

Drachman D.B. and L. Sokoloff. 1966. The role of movement in embryonic joint development. Dev Biol 14:401–420.

Fanconi S., S. Ensner, and B. Knecht. 1995. Effects of paralysis with pancuronium bromide on joint mobility in premature infants. J Pediatr 127:134–136.

Fisher M. and G.C. Schoenwolf. 1983. The use of early chick embryos in experimental embryology and teratology: improvements in standard procedures. Teratology 27:65–72.

Groenendijk B.C.W., B.P. Hierck, J. Vrolijk, M. Baiker, M.J.B.M. Pourquie, A.C. Gittenberger-de Groot, and R.E. Poelmann. 2005. Changes in shear stress–related gene expression after experimentally altered venous return in the chicken embryo. Circ Res 96:1291–1298.

Guillot C. and T. Lecuit. 2013. Mechanics of epithelial tissue homeostasis and morphogenesis. Science 340:1185–1189.

Hamburger V. and M. Balaban. 1963. Observations and experiments on spontaneous rhythmical behavior in the chick embryo. Dev Biol 7:533–545.

Hamburger V. and R. Oppenheim. 1967. Prehatching motility and hatching behavior in the chick. J Exp Zool 166:171–203.

Hammond C.L., B.H. Simbi, and N.C. Stickland. 2007. In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (*Gallus gallus*). J Exp Biol 210:2667–2675.

Haque M.A., W. Watanabe, H. Ono, Y. Sakamoto, and H. Tazawa. 1994. Comparisons between invasive and noninvasive determinations of embryonic heart rate in chickens. Comp Biochem Physiol A 108:221–227.

Heywood J.L., G.M. McEntee, and N.C. Stickland. 2005. In ovo neuromuscular stimulation alters the skeletal muscle phenotype of the chick. J Muscle Res Cell Motil 26:49–56.

Hirst C.E. and C. Marcelle. 2015. The avian embryo as a model system for skeletal myogenesis. Results Probl Cell Differ 56: 99–122.

Hosseini A. and D. Hogg. 1991. The effects of paralysis on skeletal development in the chick embryo. I. General effects. J Anat 177:159.

Hu D., N.M. Young, X. Li, Y. Xu, B. Hallgrimsson, and R.S. Marcucio. 2015. A dynamic Shh expression pattern, regulated by SHH and BMP signaling, coordinates fusion of primordia in the amniote face. Development 142:567–574.

Jensen H.B., M. Ravnborg, U. Dalgas, and E. Stenager. 2014. 4-aminopyridine for symptomatic treatment of multiple sclerosis: a systematic review. Ther Adv Neurol Disord 7:97–113.

Lamb K.J., J.C. Lewthwaite, J.P. Lin, D. Simon, E. Kavanagh, C.P. Wheeler-Jones, and A.A. Pitsillides. 2003. Diverse range

of fixed positional deformities and bone growth restraint provoked by flaccid paralysis in embryonic chicks. Int J Exp Pathol 84:191–199.

Le Douarin N.M. and F. Dieterlen-Lievre. 2013. How studies on the avian embryo have opened new avenues in the understanding of development: a view about the neural and hematopoietic systems. Dev Growth Differ 55:1–14.

Lemus J.Á., G. Blanco, B. Arroyo, F. Martínez, and J. Grande. 2009. Fatal embryo chondral damage associated with fluoroquinolones in eggs of threatened avian scavengers. Environ Pollut 157:2421–2427.

Lierz M., O. Gooss, and H.M. Hafez. 2006. Noninvasive heart rate measurement using a digital egg monitor in chicken and turkey embryos. J Avian Med Surg 20:141–146.

Lowe P.C. and V.A. Garwood. 1977. Chick embryo development rate in response to light stimulus. Poult Sci 56:218–222.

Noiva R.M., A.C. Menezes, and M.C. Peleteiro. 2014. Influence of temperature and humidity manipulation on chicken embryonic development. BMC Vet Res 10:234.

Nowlan N.C., C. Bourdon, G. Dumas, S. Tajbakhsh, P.J. Prendergast, and P. Murphy. 2010. Developing bones are differentially affected by compromised skeletal muscle formation. Bone 46:1275–1285.

Nowlan N.C., V. Chandaria, and J. Sharpe. 2014. Immobilized chicks as a model system for early-onset developmental dysplasia of the hip. J Orthop Res 32:777–785.

Oppenheim R.W., R. Pittman, M. Gray, and J.L. Maderdrut. 1978. Embryonic behavior, hatching and neuromuscular development in the chick following a transient reduction of spontaneous motility and sensory input by neuromuscular blocking agents. J Comp Neurol 179:619–640.

Osborne A.C., K.J. Lamb, J.C. Lewthwaite, G.P. Dowthwaite, and A.A. Pitsillides. 2002. Short-term rigid and flaccid paralyses diminish growth of embryonic chick limbs and abrogate joint cavity formation but differentially preserve precavitated joints. J Musculoskelet Neuronal Interact 2:448–456.

Ozkaya E., E. Baser, M. Cinar, V. Korkmaz, and T. Kucukozkan. 2012. Does diurnal rhythm have an impact on fetal biophysical profile? J Matern Fetal Neonatal Med 25:335–338.

Pitsillides A.A. 2006. Early effects of embryonic movement: "a shot out of the dark." J Anat 208:417–431.

Pollard A.S., I.M. McGonnell, and A.A. Pitsillides. 2014. Mechanoadaptation of developing limbs: shaking a leg. J Anat 224: 615–623.

Rochard F., B.S. Schifrin, F. Goupil, H. Legrand, J. Blottiere, and C. Sureau. 1976. Nonstressed fetal heart rate monitoring in the antepartum period. Am J Obstet Gynecol 126:699–706.

Rolfe R.A., N.C. Nowlan, E.M. Kenny, P. Cormican, D.W. Morris, P.J. Prendergast, D. Kelly, and P. Murphy. 2014. Identification of mechanosensitive genes during skeletal development: alteration of genes associated with cytoskeletal rearrangement and cell signalling pathways. BMC Genom 15:48.