Selective Effects of Light Exposure on Distribution of Motility in the Chick Embryo at E18

Nina S. Bradley and Dongwon Y. Jahng
Department of Biokinesiology and Physical Therapy, University of Southern California, Los Angeles, California 90033

Submitted 21 April 2003; accepted in final form 20 May 2003

Bradley, Nina S. and Dongwon Y. Jahng. Selective effects of light exposure on distribution of motility in the chick embryo at E18. J Neurophysiol 90: 1408–1417, 2003. First published May 21, 2003; 10.1152/jn.00393.2003. It is well established that orderly patterns of motor neuron activity, muscle recruitment, and limb movement are generated in chicks during motility by embryonic day (E)9, the midpoint in embryonic development. However, our recent work suggests that some attributes of motility, such as the rhythm of repetitive limb movements and distribution of activity, become less orderly after E9. In this study, we extend these observations by performing continuous force recordings over a 24-h period in ovo at E18 with augmented sampling of synchronized video and electromyogram (EMG) recordings. We report the distribution of three repetitive behaviors, rapid limb movement, respiratory-like movement, and beak clapping, identified in force recordings, and the general distribution of motility. We also test a model recently proposed to account for age-related changes in motility parameters. In the model, we proposed that circadian networks contribute to the age-related changes in distribution of motility. As a first test of this hypothesis, we examine whether light exposure contributes to the variable distribution of motility by comparing motility parameters at E18 for embryos incubated and tested under either a 12-h light/dark cycle or continuous light. Results suggest that exposure to light increases the total amount of activity and hastens the onset of extended respiratory-like movement sequences but does not impact expression of repetitive limb movement or beak clapping at E18. The possible influence of circadian mechanisms on embryonic behavior and insensitivity of repetitive limb movements to light exposure are discussed.

INTRODUCTION

Because of its potential diagnostic value, fetal movements are observed by ultrasound in routine prenatal care. Owing to its ready access during experimentation and extensive use in developmental studies, the chick embryo is a valuable model for advancing our understanding of embryonic behavior and its relationship to clinical progress of the human fetus. Hamburger and colleagues provided the first extensive description of motility as embryos progress toward hatching at E21 (Hamburger 1963; Hamburger and Balaban 1963; Hamburger and Oppenheim 1967; Hamburger et al. 1965). Limb movements, which accompany motility beginning E3.5, exhibit coordinated patterns of muscle activity (Bekoff 1976; Bradley and Bekoff 1990; Landmesser and O’Donovan 1984) that produce several reliable kinematic features by E9 (Chambers et al. 1995). However, EMG activity and kinematic attributes for repetitive joint excursions and inter-joint coordination appear to gradually degrade beyond E9 (Bekoff 1976; Bradley 1999; Rose et al. 1998; Sharp et al. 1999), and the distribution of motility becomes increasingly variable (Bradley 2001b).

This study focuses on the variability in motility observed at E18 because embryos begin to exhibit prehatching motility in preparation for escape from the egg. Prehatching motility includes an array of behaviors, such as rotary postural thrusts to tuck the head under the right wing (type III motility), beak clapping (repetitive opening/closing of the mandibles), and respiration (see Oppenheim 1973). The variability is of particular interest to us because it has been proposed that variable behavior has functional value in the immature nervous system, shaping existing movement options and refining movement selection (Hadders-Algra et al. 1996; Sporns and Edelman 1993). Movement options and ability to select among them appear to be established by the time of hatching, when condition-dependent sensory input from the legs can constrain interlimb coordination (Bekoff et al. 1987) and neck afferents can activate hatching (Bekoff and Kauer 1984; Bekoff and Sabichi 1987). Normal descending neural input also constrains motor-pattern variability in hatchlings, for hatching and post-hatching behaviors are more variable if this input is removed by cervical spinal transection (Bekoff et al. 1989). Refinement of movement options continues beyond hatching as more efficient kinetics during walking are acquired (Muir et al. 1996). How movement options and selection processes are established and refined in preparation for hatching remains to be determined.

Increasing variability with embryonic age is likely attributable to several factors. In vivo and in vitro studies have established that spinal networks produce the limb movements in chick embryos (Hamburger et al. 1965; O’Donovan and Landmesser 1987). Through at least the first half of embryonic development when motility parameters are most consistent, in vitro studies suggest that two spinal network properties account for the initiation and duration of motility sequences: intrinsic excitability produced by recurrent excitation and synaptic depression (O’Donovan 1999; O’Donovan and Chub 1997; Tabak et al. 2000). In vivo evidence suggests that synaptic depression contributes little to variability beyond E9, for pause durations between bouts of activity drop to lowest values by E9 and remain unchanged through E18 (Bradley 2001b). Between E9 and hatching, variable motility appears to be attributable to...
modulation of spinal motility circuits by distant sources. Shorter activity bouts in spinal embryos compared with controls suggest that descending pathways provide some of the excitatory drive for motility (Bradley 2001b; Hamburger et al. 1965). Lengthening of activity sequences with age in chronic spinal embryos and embryos experiencing greater mechanical loads suggest proprioceptive afferents also provide excitatory drive (Bradley 2001b; Hamburger et al. 1965). Developing circuits for respiratory control (Akiyama et al. 1999) and circadian regulation of activity (Akasaka et al. 1995) may also modulate activity.

Thus the purpose of this study was twofold. One, we sought to determine if there are patterns in the distribution of embryonic motility that might identify the mechanisms responsible for normal variability during motor development. Two, we sought to determine whether light exposure impacts the distribution of motility. To this end, we describe a method suitable for extended recordings that captures three distinct repetitive behaviors as well as the extent of motility. We also report findings suggesting that light exposure may selectively impact the age-related onset of sustained respiratory-like movement and the total amount of activity at E18. Preliminary findings were published in abstract form (Bradley 2001a).

METHODS

Fertile chicken eggs (Gallus domesticas) were maintained in a standard incubator modified for either 12-h light/12-h dark exposure (12L) or 24-h light exposure (24L) throughout the incubation period preceding experiments at E18. Incubating eggs were illuminated (20–130 lx) by room light (clear Plexiglass door) plus two 25-W bulbs at intensities typically present in our laboratory. We selected normal light levels to determine whether our lab conditions impact the variability we observe during experimentation. We selected 24L exposure, rather than constant dark because although both produce free-running circadian rhythms (Yamada et al. 1988), 24L conditions afford greater ease in preparing and monitoring experiments, and there was evidence to suggest increased light exposure might increase activity levels (Wu et al. 2001). During experiments, embryos were maintained in a humid, temperature-controlled recording chamber (39°C) with the same light exposure schedule (12L or 24L) experienced during incubation. Recording was continuous and total recording time was parcelled into three intervals: interval 1 (1:00–8:00 PM), interval 2 (8:00 PM to 8:00 AM), and interval 3 (8:00–11:00 AM). All recording intervals were conducted under normal room light plus a 60-W bulb at 2 ft (800 lx), except interval 2 during 12L experiments when all lab lights were turned off. Age was verified at the end of experiments using established staging criteria (Hamburger and Hamilton 1951; reprinted 1992). All procedures were approved by the University Institutional Animal Care and Use Committee.

Synchronized force, video, and EMG recordings

A small window was placed in the shell to view the lateral aspect of the embryo, and membranes were deflected to expose the surface of the thigh for in ovo recording. A probe attached to a force transducer (Grass Instruments, FT03C) was lowered through an opening in the Plexiglas lid of the chamber until it made firm contact with the ventrolateral surface of the proximal thigh. Placement was adjusted to detect all visible movement. AC force output was balanced, amplified (×4,000), and filtered (30 or 100 Hz, Grass Instruments, P122), then computer sampled (500 Hz) concurrent with an event pulse (Datapac 2000; RUN Technologies). Force recording was continuous for 24 h. Video samples were also obtained for each of the three recording intervals or just prior to Interval 2 (lights out) in 12L experiments. Video samples were synchronized with the force recordings using the event pulse and a SMPTE time code that ran continuously over the 24 h.

Additional embryos were prepared for synchronized EMG, video, and force recording. In these embryos, four muscles were implanted with fine wire silver electrodes (50 µm OD, California Wire). Muscles selected included the femorotibialis (FT), tibialis anterior (TA), and lateral gastrocnemius (LG) of the leg and the trapezius (TZ) or latissimus dorsi (LD) of the wing. EMG signals were amplified (×1,000), high-pass filtered (100 Hz; Grass Amplifier P511K), and computer sampled (3 kHz) concurrent with force data and the event pulse.

Video and force analyses

In preparation for force analyses, each video recording was reviewed to determine the SMPTE codes for onset and offset of all visible movement (SMPTE events) and to describe the movement observed. Guided by the SMPTE events and synchronizing pulses, we identified three distinct repetitive behaviors in the nonrectified force trace: limb movements (Fig. 1A), respiratory-like movements (Fig. 1B), and beak clapping (Fig. 1C). Repetitive limb movement was operationally defined as a series of rapid wing and/or leg excursions exhibiting an abrupt onset and termination that typically included startle and myoclonic features of Type II motility (Oppenheim 1973). A series of limb movements typically lasted <10 s and could occur in isolation or in clusters over several minutes. Respiratory-like movement was characterized by slow, small excursions of the wing and chest that increased in amplitude and frequency over the recording period (Oppenheim 1972, 1973). We here use the term respiratory-like because these movements were present though membranes were intact and the beak was submerged in amniotic fluid, suggesting respiration had not yet begun (Chiba et al. 2002; Oppenheim 1973). Nonetheless, respiratory-like movements were also associated with beak clapping and vocalization near the end of some experiments, and they typically occurred in sequences lasting minutes to hours. Beak clapping was characterized by a series of rapid openings/closings of the mandibles and usually occurred in clusters every few seconds for many minutes (Oppenheim 1972, 1973).

From the force recordings synchronized with video, we established selection criteria for reliable detection of the events over the remainder of the force recording. Analysis criteria included the following: a minimum of three repetitions of the behavior per event, an approximate frequency range (i.e., limb movements: 0.5–10 Hz; respiratory-like: 0.1–2 Hz; beak-clapping: 3–4 Hz), and in the case of repetitive limb movements, a second threshold: 10 times baseline. On completing manual analyses of repetitive limb movements in 12L embryos, detection was automated and 12L data re-analyzed for comparisons between 12L and 24L embryos.

Force recordings were also computer-rectified and analyzed automatically (DATAPAC 2000, RunTechnologies) to detect onset, offset, and duration of all activity over 24 h (Fig. 1D). Modifying parameters adapted from previous studies (Bradley 1999; Hamburger et al. 1965), a force threshold twice baseline amplitude was set to detect the onset and offset of activity. Force exceeding threshold for ≥0.1 s was treated as an activity sequence, and force remaining below threshold for ≥10.1 s was treated as a pause in activity (Fig. 1D). Combined, an activity sequence and the subsequent pause formed an episode. Activity duration, pause duration, episode duration, and percent activity were calculated for each episode, averaged for each hour of recording, and referenced to time of day. Total activity/h was also calculated, but because activity sequences could span two or more hours at E18, total activity/h was calculated by subtracting the sum of all pause durations/h from 3,600 s.

We restricted analyses to data collected between 1:00 PM and 11:00 AM to control for behavioral effects that might be associated with time
totals for each parameter were collapsed into interval averages (1–3 between groups (by ranks (Siegel and Castellan 1988) were used to test for differences. The ANOVA parametric statistics and Friedman two-way ANOVA for each embryo to perform within and between-group comparisons.

R$^{2}4$ h and Pearson correlation coefficients were performed to examine the relationships between episode parameters within subject across the samples. Linear regression statistics were performed to determine both the incidence and average duration of movement events per hour for each behavior over the entire recording under 12L conditions. Two-way ANOVA statistics indicated that the incidence of repetitive limb movements per hour did not vary either across the three intervals or between light conditions. Further, the duration of beak clapping events did not vary across the three intervals. However, a small (200 ms) difference in event duration between groups was significant (12L: 1.5 ± 0.4 s; 24L: 1.3 ± 0.3 s).

There was also minimal difference in comparisons for beak clapping (Fig. 3B). Two-way ANOVA and post hoc comparisons (P < 0.05/3) indicated that incidence of beak clapping varied significantly across intervals in (Fig. 3B), increasing from interval 2 (30 ± 21 events/h) to interval 3 (65 ± 61). However, the incidence did not vary between 12L and 24L conditions. Further, the duration of beak clapping events did not vary either across recording intervals or between light conditions.

Respiratory-like movement was the only repetitive behavior that differed both between 12L and 24L conditions and across recording intervals (Fig. 4). Significant main effects in two-way ANOVA comparisons indicated that respiratory events

**RESULTS**

Based on the state of an embryo at the end of the experiment, quality of force recording, and review of video samples across the experiment, a total of 20 embryos remained viable 22–25 h at E18, yielding recordings sufficient for analyses. The sum total activity analyzed over 22 h averaged 947 ± 108 min for 12L embryos (n = 10) and 939 ± 149 min for 24L embryos (n = 10). Synchronized EMG and force recordings for three E18 embryos are also presented to extend E18 findings. We first consider findings for the three repetitive behaviors under 12L conditions and then examine the overall distribution of activity under 12L and 24L conditions.

**Distribution of three repetitive behaviors over 22 h**

From review of synchronized video records, three repetitive behaviors were identified in force records: rapid limb movements (Fig. 1A), respiratory-like movements (Fig. 1B), and beak clapping (Fig. 1C). The behavioral groupings were confirmed in three E18 recordings of force synchronized with EMG and video (Fig. 2). Repetitive, large-amplitude force excursions between 0.5 and 10 Hz were accompanied by repetitive EMG bursts at similar frequencies in leg muscles (Fig. 2A) and/or wing muscles (Fig. 2B). Force traces associated with respiratory-like movements were occasionally accompanied by co-incident bursts in wing musculature (Fig. 2C) and associated with wing displacements on video. No wing or leg EMG activity appeared to be associated with beak clapping.

Because we were interested in whether expression of these three repetitive behaviors varies with the duration of experiment exposure, time of day, or lighting conditions, we determined both the incidence and average duration of movement events per hour for each behavior over the entire recording under 12L conditions. Two-way ANOVA statistics indicated that the incidence of repetitive limb movements per hour did not vary either across the three intervals or between light conditions (Fig. 3A). Repetitive limb movements were observed an average of 39 ± 6 (12L) and 35 ± 6 events/h (24L). Two-way ANOVA statistics also indicated that the duration of repetitive limb movements did not vary across the three intervals. However, a small (200 ms) difference in event duration between groups was significant (12L: 1.5 ± 0.4 s; 24L: 1.3 ± 0.3 s).

Because we were interested in whether expression of these three repetitive behaviors varies with the duration of experiment exposure, time of day, or lighting conditions, we determined both the incidence and average duration of movement events per hour for each behavior over the entire recording under 12L conditions. Two-way ANOVA statistics indicated that the incidence of repetitive limb movements per hour did not vary either across the three intervals or between light conditions (Fig. 3A). Repetitive limb movements were observed an average of 39 ± 6 (12L) and 35 ± 6 events/h (24L). Two-way ANOVA statistics also indicated that the duration of repetitive limb movements did not vary across the three intervals. However, a small (200 ms) difference in event duration between groups was significant (12L: 1.5 ± 0.4 s; 24L: 1.3 ± 0.3 s).

There was also minimal difference in comparisons for beak clapping (Fig. 3B). Two-way ANOVA and post hoc comparisons (P < 0.05/3) indicated that incidence of beak clapping varied significantly across intervals in (Fig. 3B), increasing from interval 2 (30 ± 21 events/h) to interval 3 (65 ± 61). However, the incidence did not vary between 12L and 24L conditions. Further, the duration of beak clapping events did not vary either across recording intervals or between light conditions.

Respiratory-like movement was the only repetitive behavior that differed both between 12L and 24L conditions and across recording intervals (Fig. 4). Significant main effects in two-way ANOVA comparisons indicated that respiratory events

![Fig. 1. Examples of force recordings obtained E18. Nonrectified force tracings were used to identify 3 distinct repetitive behaviors.](image-url)
The average duration of repetitive respiratory-like events was greater under 24L (911 ± 1,723 s) compared with 12L conditions (143 ± 283 s). Average duration of respiratory-like events also appeared to vary over recording intervals, peaking during interval 2 under 24L conditions and during interval 3 under 12L conditions (Fig. 4B). However, one-way ANOVAs did not achieve significance for either 24L or 12L conditions. Given the magnitude of variability in event duration, we also used the Friedman two-way ANOVA by ranks to test for trends within groups. Friedman ANOVAs were significant for both groups. Post hoc comparisons ($P < 0.05/3$) indicated that the increase in event duration from interval 2 to 3 under 12L conditions, and the from interval 1 to 3 under 24L conditions were significant.

**Distribution of activity**

Force recordings indicated that the duration of activity sequences varied greatly within an experiment, and because repetitive movements collectively represented only a subset of total activity at E18, we examined the distribution of activity to determine if the extended duration of an experiment or light exposure conditions contributed to the variability in activity. Descriptive statistics were similar between 12L and 24L conditions and are here combined. Based on analyses for 697–1,617 episodes per embryo, an episode consisted largely of an activity sequence (57 ± 17 s), followed by a brief pause (21 ± 5 s). Activity duration fluctuated markedly across consecutive episodes, whereas pause duration exhibited minimal variability (Fig. 5A). Thus in all embryos, activity duration co-varied closely with episode duration, whereas pause duration did not (Fig. 5B). Pearson correlation coefficients ($R^2$) for activity and episode duration exceeded 0.94 in all experiments, whereas coefficients for pause and episode duration fell <0.03. Averages for the sum of all movement per hour yielded relative distributions similar to those for activity sequences, and indicated that embryos were active ~72% of the time (2,597 ± 323 s/h), individual totals ranging 53–80%.

To determine whether the distribution of activity varied with time of day or light exposure, activity sequences (Fig. 6A) and total activity (Fig. 7A) were averaged for each hour of recording, then collapsed into interval 1–3 averages (Figs. 6B and 7B) for comparisons. The two-way ANOVA comparison indicated activity sequence duration progressively increased across the three intervals but did not differ between 12L and 24L conditions. Combining groups, post hoc comparisons indicated that both the increase from interval 1 (72 ± 86 s) to interval 2 (841 ± 1726 s) and interval 2 to interval 3 (2,061 ± 2,838 s) were significant ($P < 0.05/2$). As indicated in Fig. 6A, activity sequence duration varied substantially during interval 2 under 24L conditions. Analyses suggested that the extreme variability was primarily attributable to an earlier onset of extended respiratory-like sequences (Fig. 4B). During interval 2, eight embryos under 24L conditions (lights on) initiated respiratory-like sequences lasting 10 min or more compared with only three embryos under 12L conditions (lights out).

Given activity duration could vary dramatically across consecutive sequences (Fig. 5A), we also examined total activity per hour. Trends for hourly averages appeared to differ between groups, dipping slightly under 12L conditions during interval 2 (dark), but progressively increasing across intervals under 24L conditions (Fig. 7A). The two-way ANOVA was

---

**FIG. 2.** EMG for limb muscles synchronized with force recordings of repetitive movement at E18. A: force recordings for repetitive limb movements were accompanied by EMG bursts of similar frequency in leg muscles, most notably the tibialis anterior (TA) and femorotibialis (FT) and occasionally the lateral gastrocnemius (LG). B: force recordings for repetitive limb movement were also accompanied by EMG bursts of similar frequency bursts in wing muscles, such as the latissimus dorsi (LD). C: occasionally force recordings for repetitive respiratory-like movements were accompanied by bursts of similar frequency in wing muscles, such as the trapezius (TR).
significant for recording intervals, but not light condition, and there was a significant interaction. Post hoc one-way ANOVAs ($P < 0.05/2$) indicated that the progressive increase for 24L data were significant (Fig. 7B), and the incremental increases from interval 1 to 2 and interval 2 to 3 were also significant ($P > 0.05/3$). The post hoc ANOVA for 12L conditions fell just short of significant, and because there appeared to be a small drop in total activity during interval 2 (dark), we reviewed each recording. In a subset of six experiments, total activity/hr appeared to vary with light exposure (Fig. 7C). A one-way ANOVA for total activity in this subset ($P < 0.05/3$) varied significantly across intervals, averaging 2,807 ± 255 s (interval 1), dropping to 2,289 ± 291 s (interval 2, lights out), then increasing to 3,220 ± 249 s (interval 3). Correcting for all 12L post hoc comparisons ($P < 0.05/5$), the decrease from interval 1 to 2 fell just short of significance. Finally, a $t$-test correcting for all post hoc tests ($P < 0.05/9$) also indicated that total activity during interval 1 was greater under 12L versus 24L conditions (Fig. 7B).

We also examined whether the residual activity, total activity minus the sum of event durations for each of the three

![Repetitive Respiratory-like Movements](image)

**FIG. 4.** Incidence and duration of repetitive respiratory-like movements. Logarithmic plots of between-subject averages (±SD) summarize trends across hours and between light exposure conditions (12L, ●; 24L, □). Lights were out between 8:00 pm and 8:00 AM (interval 2) under 12L conditions only. A: repetitive respiratory-like events occurred more frequently under 12L than 24L conditions. Comparisons between recording intervals were not significant. B: event durations were longer under 24L conditions and varied over the recording, peaking during interval 2 under 24L conditions and interval 3 under 12L conditions.

![Repetitive Limb Movements](image)

**FIG. 3.** Incidence and duration of repetitive limb and beak-clapping movements. Plots of between-subject averages (±SD) summarize trends across hours and between light exposure conditions (12L, ●; 24L, □). Lights were out between 8:00 pm and 8:00 AM under 12L conditions only. A: the occurrence and duration of repetitive limb events did not vary over 22 h of recording or between light conditions. B: the occurrence of beak clapping events significantly increased from interval 2 to 3 but did not differ between light conditions. The duration of beak-clapping events did not vary over the recording intervals or between light conditions.
repetitive behaviors, accounted for variations in total activity across recording intervals. A two-way ANOVA indicated that the residual activity varied significantly both between groups and across intervals. Residual activity was reduced under 24L compared with 12L conditions. Under both conditions, the amount of residual activity progressively decreased across intervals and post hoc comparisons indicated the decreases from interval 1 to 2 and 3 were significant ($P < 0.05/3$).

**DISCUSSION**

In this study, we provide the first descriptions of motility drawn from continuous 24-h force recordings of individual embryos under relatively unconstrained conditions during a period when behavior is transitioning in preparation for hatching. Although kinematic analyses provide detailed descriptions of motility, the methods are highly labor-intensive, the data are difficult to obtain, and by necessity behavior may be constrained to meet analysis requirements such as those for two-dimensional motion analysis. Thus conventional behavioral methods typically provide valuable but limited understanding of the dynamic context from which samples of behavior are plucked for analyses. By tracking three repetitive behaviors simultaneously, the force recordings provided a dynamic contextual framework for analyses of motility over an extended period of time.

Our new methods also yield a somewhat different metric of embryonic behavior at E18 from those of earlier studies. The most notable deviation was the nearly twofold increase in sequence duration (57 s) and relative activity (72%) compared with findings in previous studies (e.g., Bradley 2001b; Hamburger and Balaban 1963; Hamburger and Oppenheim 1967; Oppenheim 1973). In the earlier studies, motility measures were based on either direct vision of ongoing behavior over 5- to 15-min intervals or repeated review of approximately hour-long video recordings. Visual and video analyses may be subject to effects of examiner fatigue, incomplete view of the

**FIG. 5.** Durations of activity sequences and pauses forming consecutive episodes for 1 E18 experiment and their relationships to episode duration. A: activity sequences (○) and pauses (□) are serially ordered by episode number and summarize 24 h of recording for a 12L experiment. Interval 1 extended from episode 1 through 474, Interval 2 (lights out), delineated by heavy horizontal bar below the x axis, extended from episode 475 to 1,280. Interval 3 extended from episode 1,281 to 1,400. B: the durations for all activity sequences (○) and pauses (●) in A are plotted against their respective episode duration. The Pearson correlation coefficient indicated activity duration strongly covaried with episode duration ($R^2 = 0.98$), while pause duration did not ($R^2 = 0.00$).

**FIG. 6.** Variations in activity sequence duration over 22 h of recording under 12L and 24L conditions. A: based on hourly averages, activity sequences tended to lengthen over the recording period, the increases first appearing under 24L conditions. Between-subject averages ($±$SD) for sequence duration are plotted following conventions in earlier figures. B: hourly averages were collapsed into 3 intervals to perform 2-way ANOVA comparisons. A significant main effect and post hoc comparisons indicated that sequence duration significantly increased across consecutive recording intervals (*). Activity sequence duration did not differ between 12L and 24L conditions.
embryo, and/or disregard of small brief movements, where as our force recordings were sufficiently sensitive and reliable to detect nearly all movement over 24 h of continuous recording. Further, the lengthy activity sequences in our study appear consistent with findings of Bollweg and Sparber 1999. In their study, electrodes inserted through holes drilled in the shell detected nearly continuous electrical activity at E18, and samples of synchronous video suggested that after controlling for heart beat, electrical events corresponded with movement. Nonetheless, we acknowledge that our methods alter the embryonic environment and may have impacted behavior (Bollweg and Sparber 1999).

**Development of respiratory-like movements contributes to variability in motility parameters**

Because methods were sufficiently sensitive to distinguish repetitive limb, beak clapping, and respiratory-like movements, we speculated that distribution differences between behaviors might account for the overall variability in motility. We knew from a previous study that between E9 and E18 activity sequence duration becomes increasingly more variable, owing to increases in its upper limit, whereas the variability in pause duration decreases as the range collapses toward its lower limit (Bradley 2001b). By tracking the incidence and duration of the three behaviors, we found that parameters for limb and beak-clapping movements exhibited relatively little variability, and residual activity (total activity duration minus duration of the three repetitive behaviors) decreased over 24 h. In contrast, parameters for respiratory-like events exhibited increasing variability over recording intervals. Oppenheim (1973) also reported a distinction between these behaviors during hatching (E20–E21) in that the frequency of beak clapping remained relatively constant while the frequency of respiration increased.

From the outset of some experiments (interval 1), we observed respiratory-like movements, e.g., small repetitive excursions of the shoulder and chest wall, while membranes were yet intact and the beak was submerged in amniotic fluid. We have also observed isolated or serial “shrugs” of the wing at 8- to 10-s intervals resembling respiratory behavior as early as E15 (unreported observations). At both E15 and E18 these shrugs were accompanied by EMG bursts in wing musculature (Fig. 2C). Between intervals 2 and 3 in several experiments, the advent of audible chirping concurrent with these movements indicated that the respiratory-like movements we tracked were also elements of functional respiration. A number of studies have documented the onset of respiration between E18 and E19 (e.g., Akiyama et al. 1999; Chiba et al. 2002; Corner and Bott 1967; Kuo and Shen 1937; Oppenheim 1972). The onset of respiration occurs just prior to or at the time the embryo penetrates the chorioallantoic membrane, the prelude to shell pipping. Onset of respiration is triggered by an increase in blood CO₂ level and can be artificially triggered as early as E15 (see Oppenheim 1973 for review). Increases in respiratory rate are associated with changes in O₂ consumption (Kuo and Shen 1937; Oppenheim 1972). During recording intervals 2 and 3, repetitive respiratory-like events began to lengthen markedly as the number of events dropped off, suggesting that the sequences were fusing into single extended events. The variability in incidence and duration of respiratory-like events were similar to the variability in activity sequence duration suggesting respiratory-like events contributed significantly to the variability in distribution of activity.

**Motility exhibited modest sensitivity to light exposure**

We hypothesized that the pattern of light exposure might impact motility parameters based on three lines of research.

![Figure 7](http://jn.physiology.org/Downloadedfrom)
One, some parameters of embryonic motility appear to increase during exposure to intense cold light illumination at E4–E14 (Wu et al. 2001). Second, light exposure during incubation has been shown to accelerate the rate of morphogenesis (Coleman and McDaniel 1976; Ghatpande et al. 1995) and time to onset of hatching (Bohren and Siegel 1975; Fairchild and Christensen 2000). Third, circadian mechanisms are established in the pineal gland prior to hatching. Under a 12L schedule, melatonin release in cultured pineal cells is phase related by E13–E14, increasing during dark and decreasing during light (Akasaka et al. 1995). Also, transcription of the gene encoding aryalkylamine-N-acetyltransferase, an enzyme that converts serotonin to N-acetylserotonin in the melatonin biosynthesis pathway, co-varies with melatonin concentrations in the pineal gland at E16 (Herichova et al. 2001). Further, embryos incubated under 12L conditions from E0 to E18 continue to exhibit a circadian-related melatonin rhythm when transferred to constant darkness E19–E20 (Zeman et al. 1999).

In our study, three measures suggested that motility varied modestly with light exposure. One, during 12L conditions total activity tended to decrease during interval 2 (lights out) in six experiments. Two, respiratory-like events were exponentially lower under 24L compared with 12L conditions. Three, fusing of respiratory-like events into longer continuous sequences began several hours earlier under 24L conditions. During interval 2, respiratory-like events exceeding 10 min were observed in six experiments under 24L conditions (lights on), compared with only three experiments under 12L conditions (lights off). It was not until interval 3 that trends began to appear more similar between 12L and 24L conditions (Fig. 4B). Collectively these findings appear to be in agreement with studies indicating that light enhances activity (Wu et al. 2001) and accelerates embryonic development in the chick (Bohren and Siegel 1975; Coleman and McDaniel 1976; Ghatpande et al. 1995; Fairchild and Christensen 2000).

Our findings also indicated that of the three behaviors studied, only respiratory-like events varied between light conditions. In nearly all experiments, respiratory events fused and increased in duration over 24 h; however, onset of sustained respiratory-like events was hastened under constant light exposure. Exposure to artificial clicking also advances the onset and rate of breathing in chick embryos and can trigger early hatching (Vince et al. 1976). Auditory-triggered responses appear to be mediated by increased triiodothyronine (T3) production (Ockelford et al. 1983) and reversed by thiourea inhibition of thyroid hormone synthesis (Wittmann et al. 1984). Thus given that light exposure accelerates development and circadian mechanisms in the pineal gland are largely established prior to hatching, accelerated respiratory-like behavior during 24L conditions may have been mediated in part by circadian regulation of thyroid hormones (Abe et al. 1979; Kalsbeek et al. 2000; also Bartness et al. 2001; Oppenheim 1973).

Repetitive limb movements did not vary between light conditions

Locomotor behavior exhibits a circadian pattern peaking with that period of day when an animal forages for food, and in most birds studied, activity levels peak during daylight, varying inversely with melatonin levels (Bartness et al. 2001; Cassone 1990; Chabot and Meneker 1992; Murakami et al. 2001). Thus we were interested in whether during embryonic development limb movements begin to exhibit a circadian pattern. Wu et al. (2001) observed an increase in number of limb and/or trunk movements in chick embryos during exposure to intense light as early as E5, followed by a peak between E9 and E11 and subsequent decrease between E12 and E14. Our findings appear to be an extension of this trend for neither the number or duration of repetitive limb movements varied between intervals under 12L conditions or between 24L and 24L conditions. It is possible that circadian influences on respiration precede those acting on limb movement and effects are not manifested until E19–E20. It is also possible that repetitive limb movements at E18 may vary with high-intensity light, though the decrement in limb movements beyond E13 observed by Wu et al. (2001) suggests this is not likely.

Alternatively, the absence of circadian effects may indicate that limb movements across the whole of embryonic development are produced by neural mechanisms other than the locomotor pattern generator. O’Donovan and Chub (1997) have argued that early embryonic limb movements are produced by neuronal population dynamics arising from immature neuron properties. By extension, repetitive limb movements at E18 may be the product of population dynamics unique to prehatching conditions. For example, age-related reductions in spontaneous limb movement may be a function of changes in expression of cation-chloride cotransporters and reduced intracellular chloride concentration (Delpire 2000), and/or gap junction coupling of motor neurons (Kiernh and Tresch 2002; Personius and Balice-Gordon 2000). In support of this view, our force and EMG recordings indicated repetitive limb movements were characterized by brief, very high-frequency excursions that more closely resembled tremor than stepping in neonatal chicks (Bekoff et al. 1987; Johnston and Bekoff 1992, 1996; Muir and Chu 2002) or step-like patterns in the isolated cord of neonatal rodents (Astuta et al. 1990; Whelen et al. 2000).

In sum, results of continuous 24-h force recordings suggest that the increasing variability in distribution of motility during embryonic development is attributable to developing respiratory-like movements. Results also indicate that repetitive respiratory-like movements, but not limb movements or beak clapping, can be influenced by exposure to light, suggesting that only the former may be governed by developing circadian mechanisms at E18. Given that chicks hatch E20–E21 and begin walking within hours thereafter, the apparent insensitivity of repetitive limb movements to light exposure may indicate that they are not an embryonic form of locomotion. Oppenheim’s (1973) observation that the relationship between embryonic motility and posthatching behaviors is an elusive problem remains true today.

We thank M. Dona and D. Wong for assistance in piloting the methods for this study, D. Zhao for assistance in data analyses, and Dr. Joe Miller for helpful discussions and suggestions regarding data presentation.

**REFERENCES**

Abe K, Kroning J, Greer MA, and Critchlow V. Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone.


