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

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ABSTRACT

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 hr period in ovo at E18, with augmented sampling of synchronized video and electromyogram (EMG) recordings. We report the distribution of 3 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 hr 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 et al. 1965; Hamburger and Oppenheim 1967). Limb movements, which accompany motility beginning E3.5, exhibit coordinated patterns of muscle activity (Bekoff 1976; Landmesser and O’Donovan 1984; Bradley and Bekoff 1990) that produce several reliable kinematic features by E9 (Chambers et al. 1995; Bradley 1997). However, EMG activity and kinematic attributes for repetitive joint excursions and inter-joint coordination appear to gradually degrade beyond E9 (Bekoff 1976; Rose et al. 1998; Bradley 1999; Sharp et al. 1999), and the distribution of motility becomes increasingly variable (Bradley 2001).

This study focuses on the variability in motility observed at E18 because embryos begin to exhibit pre-hatching motility in preparation for escape from the egg. Pre-hatching motility includes an array of behaviors, such as rotatory 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 (Sporns and Edelman 1993; Hadders-Algra et al. 1996). 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 posthatching 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 2 spinal network properties account for the initiation and duration of motility sequences: intrinsic excitability produced by recurrent excitation and synaptic depression (O’Donovan and Chub 1997; O’Donovan 1999; 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 2001). 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 to controls suggest that descending pathways provide some of the excitatory drive for motility (Hamburger et al 1965; Bradley 2001). Lengthening of activity sequences with age in chronic spinal embryos and embryos experiencing greater mechanical loads suggest proprioceptive afferents also provide excitatory drive (Hamburger et al. 1965; Bradley 2001). 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 two-fold. 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 3 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 2001).

METHODS

Fertile chicken eggs (*gallus domesticas*) were maintained in a standard incubator modified for either 12 hr light/12 hr dark exposure (12L) or 24 hr light exposure (24L) throughout the incubation period preceding experiments at E18. Incubating eggs were illuminated (20 to 130 lux) by room light (clear plexiglass door) plus 2, 25 watt 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, as both produce free-running circadian rhythms (Yamada et al. 1988), but 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 parceled into 3 intervals: Interval I (1 PM-8 PM), Interval II (8 PM-8 AM), and Interval III (8 AM-11 AM). All recording intervals were conducted under normal room light plus a 60 watt bulb at 2 ft (800 lux), except Interval II
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 place 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 (x 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 hrs. Video samples were also obtained for each of the 3 recording intervals, or just prior to Interval II (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 24hrs.

Additional embryos were prepared for synchronized EMG, video, and force recording. In these embryos, 4 muscles were implanted with fine wire silver electrodes (o.d. 50 µm, 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 (x 1000), 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 3 distinct repetitive behaviors in the non-rectified 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 less than 10 sec, 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 (Oppenheim 1973; Chiba et al. 2002). 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 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 3 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 10x baseline. Upon 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 hrs (Fig. 1D). Modifying parameters adapted from previous studies (Hamburger et al. 1965; Bradley 1999), a force threshold twice baseline amplitude was set to detect the onset and offset of activity. Force exceeding threshold for at least 0.1 sec was treated as an activity sequence, and force remaining below threshold for at least 10.1 sec 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/hr was also calculated, but because activity sequences could span 2 or more hours at E18, total activity/hr was calculated by subtracting the sum of all pause durations/hr from 3600 sec.

We restricted analyses to data collected between 1 pm-11 am to control for behavioral effects that might be associated with time of day and to perform comparisons across an equal number of hourly samples. Linear regression statistics were performed to examine the relationships between episode parameters within subject across the 24 hrs and Pearson correlation coefficients (R²) are reported. Hourly totals for each parameter were collapsed into interval averages (I, II, III) for each embryo to perform within and between group comparisons. The ANOVA parametric statistics and Friedman two-way ANOVA by ranks (Siegel and Castellan, 1988) were used to test for differences between groups (p<0.05), and t-tests were used for post hoc comparisons using a Bonferroni correction (p<0.05/number of comparisons). Group averages and standard deviations (±) are reported.
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 to 25 hrs at E18, yielding recordings sufficient for analyses. The sum total activity analyzed over 22 hrs averaged 947 ±108 min for 12L embryos (N = 10) and 939 ±149 min for 24L embryos (N = 10). Synchronized EMG and force recordings for 3, E18 embryos are also presented to extend E18 findings. We first consider findings for the 3 repetitive behaviors under 12L conditions, and then examine the overall distribution of activity under 12L and 24L conditions.

Distribution of 3 repetitive behaviors over 22 hrs

From review of synchronized video records, 3 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 3, E18 recordings of force synchronized with EMG and video (Fig. 2). Repetitive, large amplitude force excursions between 0.5-10 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 3 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 3 intervals or between light conditions (Fig. 3A). Repetitive limb movements were observed an average of 39 ±6 (12L) and 35 ±6 events/hr (24L). Two-way ANOVA statistics also indicated that the duration of repetitive limb movements did not vary across the 3 intervals. However, a small (200 ms) difference in event duration between groups was significant (12L: 1.5 ±0.4 sec; 24L: 1.3 ±0.3 sec).

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 II (30 ±21 events/hr) to Interval III (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 occurred more frequently under 12L (23 ±9 events/hr) compared to 24L conditions (11 ±8). The incidence tended to peak during Interval II (8 pm to 8 am) under both conditions, but post hoc comparisons between intervals (12L and 24L data combined, p<0.05/3) did not achieve significance. ANOVA comparisons for event duration also indicated that the average duration of repetitive respiratory-like events was greater under 24L (911 ±1723 sec) compared to 12L conditions (143 ±283 sec). Average duration of respiratory-like events also appeared to vary over recording intervals, peaking during Interval II under 24L conditions and during Interval III 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 II to III under 12L conditions, and the from Interval I to III 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 to 1617 episodes per embryo, an episode consisted largely of an activity sequence (57 ±17 sec), followed by a brief pause (21 ±5 sec). 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, while pause duration did not (Fig. 5B). Pearson correlation coefficients (R²) for activity and episode duration exceeded 0.94 in all experiments, while coefficients for pause and episode duration fell below 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 approximately 72% of the time (2597 ±323 sec/hr), individual totals ranging 53% to 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 I, II, and III averages (Fig. 6B, 7B) for comparisons. The two-way ANOVA comparison indicated activity sequence duration progressively increased
across the 3 intervals, but did not differ between 12L and 24L conditions. Combining groups, post hoc comparisons indicated that both the increase from Interval I (72 ±86 sec) to Interval II (841 ±1726 sec) and Interval II to Interval III (2061 ±2838 sec) were significant (p<0.05/2). As indicated in Fig. 6A, activity sequence duration varied substantially during Interval II 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 II, 8 embryos under 24L conditions (lights on) initiated respiratory-like sequences lasting 10 min or more, compared to only 3 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 II (dark), but progressively increasing across intervals under 24L conditions (Fig. 7A). The two-way ANOVA was 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 was significant (Fig. 7B) and the incremental increases from Interval I to II and Interval II to III 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 II (dark), we reviewed each recording. In a subset of 6 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 2807 ±255 sec (Interval I), dropping to 2289 ±291 sec (Interval II, lights out), then increasing to 3220 ±249 sec (Interval III). Correcting for all 12L post hoc comparisons (p<0.05/5), the decrease from Interval I to II 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 I 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 3 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 to 12L conditions. Under both conditions, the amount of residual activity progressively decreased across intervals and post hoc comparisons indicated the decreases from Interval I to II and III were significant (p<0.05/3).

DISCUSSION

In this study we provide the first descriptions of motility drawn from continuous 24 hr force recordings of individual embryos under relatively unconstrained conditions during a period when behavior is transitioning in preparation for hatching. Though 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 2D 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 3 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 2-fold increase in sequence duration (57 sec) and relative activity (72%) compared to findings in previous studies (e.g., Hamburger and Balaban 1963; Hamburger and Oppenheim 1967; Oppenheim 1973; Bradley
2001). In the earlier studies motility measures were based on either direct vision of ongoing behavior over 5-15 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 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 hrs 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, while the variability in pause duration decreases as the range collapses toward its lower limit (Bradley 2001). By tracking the incidence and duration of the 3 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 3 repetitive behaviors) decreased over 24 hrs. 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 I), 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 8-10 sec 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 II and III 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-E19 (e.g., Kuo and Shen 1937; Corner and Bott 1967, Oppenheim 1972; Akiyama et al. 1999; Chiba et al. 2002). 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 et al. 1937; Oppenheim 1972). During recording Intervals II and III, 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 upon 3 lines of research. 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 BD 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 arylalkylamine-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 (Hericová 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, 3 measures suggested that motility varied modestly with light exposure. One, during 12L conditions total activity tended to decrease during Interval II (lights out) in 6 experiments. Two, respiratory-like events were exponentially longer under 24L compared to 12L conditions. Three, fusing of respiratory-like events into longer continuous sequences began several hours earlier under 24L conditions. During Interval II, respiratory-like events exceeding 10 min were observed in 6 experiments under 24L conditions (lights on), compared to only 3 experiments under 12L conditions (lights off). It was not until Interval III 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 BD and Christensen 2000).

Our findings also indicated that of the 3 behaviors studied, only respiratory-like events varied between light conditions. In nearly all experiments, respiratory events fused and increased in duration over 24 hrs, however onset of sustained respiratory-like events was hastened under continuous 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 (Ockleford 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 Oppenheim 1973; Bartness et al. 2001).

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 (Cassone 1990; Chabot and Meneker 1992; Bartness et al. 2001; 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-E11, and subsequent decrease between E12-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 12L 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 pre-hatching 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 (Personius and Balice-Gordon 2000; Kiehn and Tresch 2002). 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 hr 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 post-hatching behaviors is an elusive problem remains true today.
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FIGURE LEGENDS

Figure 1. Examples of force recordings obtained E18. Non-rectified force tracings were used to identify 3 distinct repetitive behaviors. A. Repetitive limb movements were characterized by abrupt force displacements at a frequency of 0.5-10 Hz. A threshold 10x baseline noise and exceeding peak voltage for respiratory-like movements was used to discriminate these events. B. Force traces for repetitive respiratory-like movements had a characteristic waveform readily distinct from repetitive limb movements, including a slower rise and fall, smaller amplitude, regularly spaced excursions, and a frequency of 0.1-2 Hz. C. Force recordings for repetitive beak clapping consisted of 3 or more very small amplitude, stereotypic oscillations at 3-4 Hz. Repetitive beak-clapping usually occurred in clusters spaced a few seconds apart, as illustrated here by 2 beak-clapping events. D. The rectified force trace was used to detect activity sequences (a) and intervening pauses (p). Activity was operationally defined as force exceeding a threshold 2x baseline noise. Pauses marked the end of activity sequences and were operationally defined as drops in force below threshold for 10.1 sec or more.

Figure 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).
Figure 3. Incidence and duration of repetitive limb and beak-clapping movements. Plots of between-subject averages (± s.d.) summarize trends across hours and between light exposure conditions (12L, closed diamonds; 24L, open squares). Lights were out between 8 pm and 8 am under 12L conditions only. A. The occurrence and duration of repetitive limb events did not vary over 22 hrs of recording or between light exposure conditions. B. The occurrence of beak clapping events significantly increased from Interval II to III, but did not differ between light conditions. The duration of beak-clapping events did not vary over the recording intervals or between light conditions.

Figure 4. Incidence and duration of repetitive respiratory-like movements. Logarithmic plots of between-subject averages (± s.d.) summarize trends across hours and between light exposure conditions (12L, closed diamonds; 24L, open squares). Lights were out between 8 pm and 8 am (Interval II) 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 II under 24L conditions and Interval III under 12L conditions.

Figure 5. Durations of activity sequences and pauses forming consecutive episodes for one E18 experiment, and their relationships to episode duration. A. Activity sequences (light vertical bars) and pauses (dark vertical bars) are serially ordered by episode number and summarize 24 hrs of recording for a 12L experiment. Interval I extended from episode 1 through 474. Interval II (lights out), delineated by heavy horizontal bar below the X-axis, extended from episode 475
to 1280. Interval III extended from episode 1281 to 1400. B. The durations for all activity sequences (light filled diamonds) and pauses (dark filled diamonds) in A are plotted against their respective episode duration. The Pearson correlation coefficient indicated activity duration strongly co-varied with episode duration ($R^2 = 0.98$), while pause duration did not ($R^2 = 0.00$).

Figure 6. Variations in activity sequence duration over 22 hrs 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 ($\pm$ s.d.) for sequence duration are plotted following conventions in earlier figures. B. Hourly averages were collapsed into 3 intervals to perform two-way ANOVA comparisons. A significant main effect and post-hoc comparisons indicated that sequence duration significant increased across consecutive recording intervals (*). Activity sequence duration did not differ between 12L and 24L conditions.

Figure 7. Variations in total activity over 22 hrs of recording under 12L and 24L conditions. A. Based on hourly averages, trends for total activity tended to differ between light conditions, producing a significant interaction in two-way ANOVA comparisons. Between-subject averages ($\pm$ s.d.) for sequence duration are plotted following conventions in earlier figures. B. Post hoc comparisons indicated that total activity progressively increased across recording intervals under 24L conditions, and during Interval I, total activity was less under 24L than 12L conditions (*). C. Under 12L conditions, total activity tended to decrease during Interval II (lights out) and was most apparent in the data plotted for 6 experiments. The post hoc ANOVA for this data subset was significant (*). Dotted vertical lines mark the onset and offset of Interval II (lights out).
Figure 2
A  Repetitive Limb Movements

**EVENT OCCURRENCE**

**EVENT DURATION**

B  Repetitive Beak-Clapping Movements

**EVENT OCCURRENCE**

**EVENT DURATION**
Repetitive Respiratory-like Movements

A  EVENT OCCURRENCE

B  EVENT DURATION

Figure 4
24 hr Recording Under 12L Conditions

A  DURATIONS FOR CONSECUTIVE ACTIVITY SEQUENCES AND PAUSES

B  CO-VARIATION OF ACTIVITY DURATION WITH EPISODE DURATION
Figure 6
Figure 7

A  TOTAL ACTIVITY

B  TOTAL ACTIVITY

C  TOTAL ACTIVITY*

Figure 7