Limb Movements During Embryonic Development in the Chick: Evidence for a Continuum in Limb Motor Control Antecedent to Locomotion

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Bradley, Nina S., Dhara Solanki, and Dawn Zhao. Limb movements during embryonic development in the chick: evidence for a continuum in limb motor control antecedent to locomotion. J Neurophysiol 94: 4401–4411, 2005. First published September 14, 2005; doi:10.1152/jn.00804.2005. New imaging technologies are revealing ever-greater details of motor behavior in fetuses for clinical diagnosis and treatment. Understanding the form, mechanisms, and significance of fetal behavior will maximize imaging applications. The chick is readily available for experimentation throughout embryogenesis, making it an excellent model for this purpose. Yet in 40 yr since Hamburger and colleagues described chick embryonic behavior, we have not determined if motility belongs to a developmental continuum fundamental to posthatching behavior. This study examined kinematics and synchronized electromyography (EMG) during spontaneous limb movements in chicks at four time points between embryonic days (E) 9–18. We report that coordinated kinematic and/or EMG patterns were expressed at each time point. Variability observed in knee and ankle excursions at E15–E18 sorted into distinct in-phase and out-of-phase patterns. EMG patterns did not directly account for out-of-phase patterns, indicating study of movement biomechanics will be critical to fully understand motor control in the embryo. We also provide the first descriptions of 2- to 10-Hz limb movements emerging E15–E18 and a shift from in-phase to out-of-phase interlimb coordination E9–E18. Our findings revealed that coordinated limb movements persist across development and suggest they belong to a developmental continuum for locomotion. Limb patterns were consistent with the half center model for a locomotor pattern generator. Achievement of these patterns by E9 may thus indicate the embryo has completed a critical phase beyond which developmental progression may be less vulnerable to experimental perturbations or prenatal events.

INTRODUCTION

Ultrasound recordings indicate human fetuses begin stepping or kicking at 13-14th gestational week (de Vries et al. 1982; Ianniruberto and Tajani 1981), and neonatal infants take alternating steps when supported upright on a treadmill (Thelen et al. 1987; Yang et al. 1998). Leg kinematic patterns for infant stepping are similar to patterns in cats during treadmill locomotion; this inspired the view that neonatal stepping may be produced by a central pattern generator (CPG) (Forssberg 1985; Forssberg and Dietz 1997). Such findings also suggest a developmental continuum from fetal kicking to locomotion in humans (Bradley 2003; Forssberg and Dietz 1997; Thelen 1985). Thus extending our understanding of the relationship between fetal movements and development of locomotion can serve to improve clinical examinations of fetal behavior. The chick, also a biped, expresses a continuum of spontaneous limb movements extending from embryonic day (E) 3.5 to hatching and walking (Hamburger 1963). Because the chick embryo is readily accessible, study of its movements can assist in understanding the continuum of motor control development in infants.

We know spinal circuits produce the earliest leg movements in chick embryos (Hamburger et al. 1965, 1966; O’Donovan and Landmesser 1987). We also know spinal circuits produce leg movements having electromyographic (EMG) and kinematic features by E9 that could be produced by a CPG (Bekoff 1976; Bradley and Bekoff 1990; Landmesser and O’Donovan 1984; O’Donovan and Landmesser 1987). However, studies in isolated spinal cord have required us to rethink whether motility is produced by a CPG (Bradley 1999, 2001; O’Donovan and Chub 1997). In chick spinal cord, the excitatory drive for spontaneous activity changes from cholinergic at E4 to glutamatergic at E10–E12 (Hanson and Landmesser 2003; Milner and Landmesser 1999). If these excitatory systems are pharmacologically blocked, GABAergic pathways provide compensatory drive and restore spontaneous activity (Chub and O’Donovan 1998; Hanson and Landmesser 2003; Milner and Landmesser 1999). The spontaneous activity is associated with waves of excitation that broadly spread from ventral to dorsal horn (O’Donovan et al. 1994). Such findings lead to the proposal that embryonic motility is produced by population dynamics arising from recurrent excitation and synaptic depression within an immature network rather than a CPG (O’Donovan and Chub 1997; Tabak et al. 2000). Nonetheless recent findings in mutant fetal mice lacking choline acetyltransferase for acetylcholine synthesis indicate that flexor/extensor and left/right motor output patterns are altered, suggesting that acetylcholine both drives motility and configures the locomotor CPG (Myers et al. 2005).

Assuming motility is dependent on population dynamics does not necessarily exclude the possibility that it is part of a behavioral continuum for locomotion. Walking ability of hatchlings hours after cervical transection or deafferentation is evidence that locomotion in the chick is controlled by a CPG (Bekoff et al. 1987, 1989; Jacobson and Hollyday 1982b), so presumably the CPG is assembled during embryogenesis. Embracing these assumptions, we hypothesized that there is a behavioral continuum in control of limb movements between early and late embryogenesis and that discontinuities might reveal the emergence of the locomotor CPG in the chick embryo. To that end, we recorded motility at four time points,

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provide the first detailed study of kinematics and EMG at E15 and E18, and report age-related continuities and discontinuities between E9 and E18. Findings lead us to propose that fundamental attributes of motility extend across embryonic development and may be driven by population dynamics enlisting an immature locomotor CPG. Raw kinematic data for a portion of E9 and E12 embryos included in this study were drawn from an earlier study (Bradley 1999) and are here analyzed using methods that could be applied uniformly across all ages. These analyses have not previously appeared elsewhere.

**METHODS**

Fertile Leghorn chicken eggs were incubated in a force draft, humidified incubator under standard conditions until experimentation. E9, E12, E15, or E18. Eggs were maintained in thermostat-regulated baths (40°C) during preparation and recording. Pulse rate and intervals between sequences of motility were monitored to verify optimal behavioral status. Staging criteria (Hamburger and Hamilton 1951; reprinted 1992) were used to verify embryonic age. All procedures were approved by the University Institutional Animal Care and Use Committee.

**Kinematic and EMG recordings**

Embryos were prepared for behavioral recordings in ovo by placing a window in the shell and reflecting egg membranes to obtain a sagittal view of the entire body. The estimated surface locations for eight joints (shoulder, elbow, wrist, lower thoracic spine, hip, knee, ankle, foot) in the ipsilateral wing and leg were marked for digitizing by either applying spots of white nail enamel or inserting modified 5 mm long pins. A point was placed on the outer shell for X-Y registration of digitized points across consecutive pictures, and a 5- to 6-mm stick for distance calibration was placed adjacent to the embryo. Muscles of the ipsilateral leg and wing were implanted with bipolar fine wire electrodes (25 μm platinum or 50 μm silver) for electromyographic recording (EMG). Four of the following muscles were implanted: sartorius (SA), hip flexor; femorotibialis (FT), knee extensor; tibialis anterior (TA), ankle dorsiflexor; lateral gastrocnemius (LG), ankle extensor; latissimus dorsi (LD), shoulder retractor; triceps brachii (TR), elbow extensor.

Video acquisition at 30 fps was continuous along with output from a SMPTE time code generator (SR50, Horita) that uniquely identified each video picture. A synchronizing pulse was manually triggered at 5- to 6-mm stick for distance calibration was placed adjacent to the embryo. Muscles of the ipsilateral leg and wing were implanted with bipolar fine wire electrodes (25 μm platinum or 50 μm silver) for electromyographic recording (EMG). Four of the following muscles were implanted: sartorius (SA), hip flexor; femorotibialis (FT), knee extensor; tibialis anterior (TA), ankle dorsiflexor; lateral gastrocnemius (LG), ankle extensor; latissimus dorsi (LD), shoulder retractor; triceps brachii (TR), elbow extensor.

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**Kinematic and EMG analyses**

Motility sequences typically increase with age, so we selected three to five video sequences of continuous activity amenable to computer digitizing to sample 4–5 min of movement per embryo. Each joint marker and reference was automatically digitized at 60 Hz to generate x and y (2D) coordinates (Motus, Peak Performance Technologies). Coordinates were low-pass filtered using a Butterworth (double pass, 6 Hz at E9–E15, 12 Hz at E18 based on best fit to raw data) for linear trend analyses, or fast Fourier transform (FFT, smoothing factor of 4) for peak frequency analyses. Filtered coordinates were entered into an algorithm that first calculated changes in distance between adjacent joint markers to estimate a z coordinate for movement out of plane. The three coordinates were used to calculate joint angles (Oroz et al. 1994). Joint angles were calculated for the ipsilateral wing (shoulder, elbow) and leg (hip, knee, ankle) employing methods previously described (Bradley 1999; Bradley and Sebelski 2000; Chambers et al. 1995).

Joint angles were imported into analyses software to construct time-displacement plots, merge EMG data, and complete analyses (Datapac R2K, Run Technologies). Wing and leg joints displayed similar spatiotemporal changes within a motility sequence, so linear trend analyses were performed to obtain measures of intralimb (shoulder/elbow; hip/knee; ankle) and interlimb coordination (shoulder/hip). ANOVA and post hoc Student’s t-test were applied to Pearson correlation coefficients (R) produced by trend analyses to test for age-related changes in coordination patterns. Significance testing for the ANOVA was set at P < 0.05, and post hoc Student’s t-test employed the Bonferroni correction of P < 0.05/3 comparisons (E9 vs. E12, E12 vs. E15, and E15 vs. E18). We examined temporal attributes of repetitive joint motion by applying FFT analyses to identify peak frequencies. The time series for each joint was parceled into sequential frames of 4.25 s, and 256 points were sampled per frame (zoom ratio 1:1) to identify the amplitudes and power for frequencies between 0.2 and 30 Hz. The three greatest peak frequencies within a frame were pooled across frames by joint per embryo to complete power trend analyses. ANOVA and post hoc Student’s t-test were applied to power coefficients (R²) produced by the trend analyses to test for age-related changes in the spectral content of the time series. Numerical summaries of group data report means ± SD.

**RESULTS**

Results summarize findings for 12 embryos per age and digitized samples totaled 46 min (E9), 69 min (E12), 57 min (E15), and 52 min (E18). Representative samples are shown in Fig. 1. At all ages, motility sequences typically began with near-synchronous onset of joint excursions in the ipsilateral wing and leg. Also, rotations of joints within a limb tended to share spatiotemporal characteristics. For example, the time series for shoulder and elbow angles at E9 (Fig. 1A) were similar to one another, and this trend was also seen in plots at E12–E18 (Fig. 1, B–D). Sequences frequently included a few more or less rhythmic excursions (horizontal bar, Fig. 1C). Most sequences also included abrupt excursions in one direction followed by gradual return to a resting position (horizontal arrows, Fig. 1, B and D).

The more obvious age-related modifications in motility kinematics were the changes in joint excursion amplitude and the extent to which excursions at two joints shared spatiotemporal features. The amplitude of excursions for all wing and leg joints significantly varied with age (Fig. 2, *). Significant post hoc comparisons indicated that a single pattern of change was characteristic of all five joints: excursion amplitudes first increased from E9 to E12, then progressively decreased from E12 to E15 and from E15 to E18. Only the post hoc comparison for ankle excursions at E9 and E12 fell short of significant (P < 0.03). The increasing joint range E9 to E12 coincided with substantial musculoskeletal refinement of the limb segments and joints (Hamburger and Hamilton 1992) during an interval when the embryo remained fully buoyant in the center of the egg and movements appeared unconstrained by the environment. The decreasing joint ranges E12 to E18 coincided with substantial body growth and increasing space limitations, such that any portion of the body might intermittently contact the shell wall. The foot frequently contacted the shell, but not all limb extensions resulted in contact or appeared mechanically unconstrained. However the embryo was also
less buoyant beyond E12 and contacts or constraints acting on the contralateral half of the body could not be reliably tracked.

Age-related modifications for intralimb coordination

Joints within a limb tended to move together and the extent of intralimb coordination was reliable as indicated by significant linear co-variations between joints within embryo at all ages. However, the linear trends exhibited complex age-related changes between E9 and E18. Age-related changes in wing coordination are characteristic of the complexity. Trend analyses for the wing time series in Fig. 1, A and D, are shown in Fig. 3 to serve this point. In these two sequences, wing linear trends at E9 in A1 and E18 in B1 appear similar with Pearson coefficients exceeding 0.7. ANOVA and post hoc comparisons for Pearson coefficients confirmed that shoulder and elbow excursions co-varied to a similar extent from E9 to E15 but decreased from E15 to E18 (Fig. 4A). The down turn at E18
FIG. 3. Plots for linear regressions illustrate the close association between joints within a limb during a sequence of spontaneous motility at E9 (A) and E18 (B). The close associations between shoulder and elbow traces for time-displacement kinematics in Fig. 1, A and D, are here illustrated in A1 and B1, respectively. The linear trend line and parameters, including Pearson correlation coefficient \( R \) for each plot are also indicated. All linear trends were significant. A1–A4 were constructed from 3,180 paired data points (e.g., shoulder and elbow angle), representing the entire motility sequence (53 s). B1–B4 were constructed from 4,740 paired data points (79 s). Close associations for concurrent excursions of the hip and knee (A2, B2) as well as knee and ankle (A3, B3) were also common. As seen here, linear correlation parameters for shoulder and elbow appeared similar at E9 and E18 despite changes in joint excursion range. In contrast, similar strength associations for knee and ankle excursions were observed at E9 and E18, but the direction of the linear trend shifted from positive to negative, indicating the knee and ankle rotated in opposite directions of flexion vs. extension at most time points in the sequence. Linear associations between shoulder and hip were weak (A4, B4).
was attributable to eight sequences yielding negative coefficients, indicating that the shoulder and elbow tended to rotate in opposite directions. These sequences were distributed among four embryos (Fig. 4B), and in all four cases, elbow excursion range rarely exceeded 10°. Video clips for two of these embryos also revealed that the head was tucked under the wing, abducting the shoulder and elevating the elbow. These findings suggested to us that the negative co-variations may have arisen from postural configurations and/or constraint of the wing but that overall intralimb coordination of the wing was similar between E9 and E18.

Leg excursions exhibited two age-related trends. Hip and knee excursions moved in phase with one another producing modest positive coefficients at all ages, where as knee and ankle excursions shifted from an in-phase to out-of-phase pattern between E9 and E18 (Fig. 4A). The range and distribution of correlation coefficients for hip and knee excursions were similar at all ages with subject averages ranging from weakly to strongly positive in nearly all embryos. The general trend suggested that hip and knee motions are moderately coupled at all four time points. Hip and knee coefficients tended to be strongest for E18 embryos (e.g., Fig. 3, A2 and B2) and may indicate coupling of hip and knee motions is further strengthened during prehatching.

At E9–E12, knee and ankle traces exhibited similar spatiotemporal patterns (Fig. 1, A and B) that frequently yielded moderate to strong positive coefficients (Fig. 3A3). The knee and ankle rarely rotated in opposite directions at E9 or E12 (Fig. 4C). At E15, there were many instances when the ankle rotated out of phase with the knee (vertical arrow, Fig. 1C), but subject averages for 50% of E15 embryos fell within the E9–E12 range (Fig. 4C). By E18, the out-of-phase knee/ankle pattern was characteristic in 8 of 12 embryos, many sequences yielding moderately or strongly negative linear parameters (Fig. 3B3). Post hoc comparisons for ankle and knee excursions indicated that the increase in negative co-variations between E12 and E15 was significant (⁎, Fig. 4C). Here too, video for some motility sequences appeared to indicate mechanical constraint might account for out-of-phase limb kinematics. In some sequences, plantar flexion (ankle extension) during foot contact with the shell appeared to generate reactive forces that displaced the knee in a rostral direction, synchronously flexing the knee and hip. However, shell contact was not always apparent and in many cases the foot clearly did not contact the shell wall.

We asked whether age-related changes in leg EMG patterns might account for the kinematic transformation from an in-phase to out-of-phase knee/ankle pattern at E15–E18. Over numerous attempts to obtain synchronized EMG at E9–E12, we found that fine wire electrodes only occasionally captured the distinct repetitive muscle bursting seen in suction electrode recordings at E9–E10 (Bekoff 1976; Bradley and Bekoff 1990). TA frequently exhibited tonic low-amplitude activity, whereas extensor muscles appeared to be inactive except during abrupt extensions. When repetitive TA bursting was apparent, the activity coincided with ankle flexion (→, Fig. 5A); and if LG or other extensors were active, bursting coincided with joint extension (⁎, Fig. 5A). At E15–E18, repetitive muscle activity was readily detected, and during repetitive limb motions, alternating ankle flexor (TA) and extensor (LG) activity was common (Fig. 5, B and D–F). If multiple muscles
were active, hip flexor (SA) bursting was coincident with TA bursts, forming a flexor synergy (\( \downarrow \), Fig. 5B), and FT bursts were typically paired with LG, forming an extensor synergy (\( \ast \), Fig. 5B). FT activity was notably the most variable, and there were many instances when FT onset shifted, increasing the extent of coactivity with TA, forming a “mixed synergy” (Fig. 5, E and F). We at first speculated that the mixed FT+TA synergy might account for the out-of-phase knee/ankle kinematics but observed that the out-of-phase kinematic pattern also occurred when FT activity was synchronous with LG (\( \ast \), Fig. 5B). More extensive study of these EMG and movement patterns will be required to determine if small latency shifts in FT can induce inertial lags between leg and foot segments.

EMG patterns and physical constraints did not appear to fully account for the shift from an in-phase to out-of-phase knee/ankle kinematic pattern. Owing to small excursion amplitudes at E15–E18, it was not feasible to segment data into cycles for analyses as in our earlier studies. As an alternative

FIG. 5. Examples of kinematics and EMG for repetitive leg movements during spontaneous motility. A: in the mid region of this E12 plot, repetitive rotations of leg joints occur at intervals of \( \approx 6 \) s. Repetitive bursts of the ankle flexor (TA) occur during ankle flexion (\( \uparrow \), A). TA bursts are preceded by brief synchronous bursts of the knee (FT), ankle (LG), and elbow extensor (TR) during extension of leg joints (\( \ast \), A). B: in this E18 plot, 10 cycles of flexion and extension are accompanied by synchronous bursts in TA and SA (flexor synergy, \( \downarrow \), B), alternating with LG. FT is intermittently active, at times coactive with LG (\( \ast \), B). However, knee rotations are nonetheless out of phase with ankle excursions (\( \ast \), B). B is an expansion of a segment in C where it is identified by the 1st horizontal bar. C: many E18 motility sequences exhibited both slow and fast oscillatory limb movements. The slower oscillating movements of 2–3 Hz (1st horizontal bar), resembled tremor, and occurred in a series that was separated either by pauses or faster, repetitive ballistic limb movements (RBLM, 2nd horizontal bar). Faster movements (3–10 Hz) were more abrupt and brief, and often larger in amplitude, resembling a sudden shiver. D: onset of consecutive TA bursts was selected as reference for averaging SA, FT, and LG activity during repetitive leg movements. In this example, EMG traces are the average for 35 TA bursts during the slower tremor-like segments in C. Signal averaging indicated the segments of tremor were characterized by the alternating activity of flexors (TA+SA) and extensors (LG+FT). E: during RBLM, TA and LG activity was primarily alternating, but FT activity often shifted and was co-active with TA. F: activity in E is averaged relative to onset of the 3 TA bursts and illustrates the phase shift in FT activity. TR, triceps brachii; FT, femorotibialis; LG, lateral gastrocnemius; TA, tibialis anterior; SA, sartorius.
strategy we sampled segments of the time series where the two joints closely co-varied, either positively or negatively, using angle-angle plots as snapshots of the limb segments rotating about one another (Enoka 2002). We constructed 713 plots (15 ± 2 plots/embryo) from which we identified 4 patterns: elliptical (Fig. 6, A–C), diagonal (D and E), polygonal, and complex (not shown). In most cases the angle-angle pattern varied substantially across consecutive excursions, generating a combination of patterns such as one elliptical and two diagonal plots (Fig. 6F). The number of elliptical and diagonal plots increased from E9 to E12 and most were rightward leaning (ER, DR, Table 1), conveying the close in-phase scaling of amplitude and time between knee and ankle motions.

The total number of elliptical and diagonal plots did not vary E12–E18, but the number of leftward leaning plots (EL, DL) progressive increased, conveying a close out-of-phase scaling of amplitude and time for knee and ankle motions. Disregarding lean direction, plots within a category appeared similar in pattern across age even as duration of elliptical and diagonal plots decreased from ~1.7 s (E9) to 0.8 s (E18).

**Emergence of rapid limb oscillations**

Motility sequences on E18 were distinguished by extended bouts of limb oscillations that resembled tremor (Fig. 5, B and C). Oscillations of the leg were generally 2–3 Hz and only a few degrees in amplitude. The amplitude and timing attributes of kinematics were particularly stable over multiple tremor-like excursions and were the only samples of motility generating several overlapping elliptical plots (Fig. 6G). Note that knee and ankle excursions were out of phase with one another, generating leftward leaning plots. During these sequences FT activity was paired with LG and/or slightly phase advanced (Fig. 5, B and D).

The 2- to 3-Hz oscillations were at times abruptly interrupted by explosive 3- to 10-Hz movements resembling shivering (right horizontal bar, Fig. 5C). We refer to the latter events as repetitive ballistic limb movements (RBLM). RBLM also frequently erupted during otherwise inactive stretches of recording, often several times in sequence with only a few seconds of pause in between. It was during RBLM we most frequently noted a distinct shift in timing of FT bursts to form the mixed FT+TA synergy, alternating with LG (Fig. 5, E and F). All E18 embryos exhibited tremor-like and/or RBLM movements accompanied by one or more of the EMG features we describe. These kinematic and EMG features were not readily apparent at E15. However, less-organized oscillations in kinematic traces were found in eight embryos, and in four of these embryos, oscillations were occasionally accompanied by irregular bursts in the TA, LG, FT, or IF EMG.

The emergence of tremor-like oscillations and RBLM at E18 raised the possibility of age-specific changes in the frequency content of motor output commands and potential cues to changes in motility circuitry. Therefore we performed FFTs on each time series (joint). The three frequencies having largest amplitude and power peaks per frame of the FFT were compiled across all sequences per embryo; examples of two knee data sets are plotted in Fig. 7. After determining there were no differences in mean peak frequency between joints, we selected knee peak frequencies to test for age-related variations in light of its stable co-variation with hip across age but

![Fig. 6. Angle-angle plots for concurrent knee and ankle excursions occasionally formed kinematic loops. Angle-angle plots were generated from segments of kinematic data containing 1 instance of flexion and extension, in either order, where knee and ankle traces most closely followed one another. Representative plots were drawn from embryos at E9 (A and B), E12 (C and D), E15 (E and F), and E18 (G). Two patterns were readily identified at all ages, closed elliptical loops (A–C), and diagonal lines (D and E). Sequential flexion and extensions of knee and ankle were also occasionally examined and were characterized by both loops and lines (F). Sequences of tremor-like oscillations occasionally generated overlapping elliptical trajectories; 5 completely closed elliptical loops are shown (G). At E9–E12, nearly all plots were right leaning (A, B, and D). At E15–E18, plots were more commonly left leaning (C, E, and G). The beginning of each plot is identified by "s" (start) and the direction of motion is identified by an arrow.](http://jn.physiology.org/)

<table>
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Values indicate percent distribution of angle-angle plots classified at each age as elliptical leaning right (ER) or left (EL), diagonal to right (DR) or left (DL); polygon (PG); complex (CX). Examples in Fig. 6 are indicated in parentheses; ns, not shown.

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variable relationship with ankle (Fig. 4A). Contrary to our predictions, FFTs revealed that the mean peak frequency and range of 0–10 Hz were similar across ages. The only distinct trends were a decline in power at lower frequencies and power coefficients for trend analyses of peak frequencies with increasing age. Exemplary power plots for knee excursions in an E9 (Fig. 7A) and E18 embryo (Fig. 7B) are shown. To test for age-related differences, we parceled the peaks into four bandwidths: frequencies <1, 1–1.99, 2–2.99, and 3–10 Hz. Age-related declines in power between E9 and E18 were significant for frequencies <2 Hz. Post hoc tests were mostly nonsignificant, indicating the trends were progressive rather than stepwise. Absolute power for frequencies 2–10 Hz did not vary with age, but they represented a significantly greater percent of total power with increasing age.

**Age-related changes in interlimb coordination**

Time series for shoulder and hip excursions also shared spatiotemporal features in many samples at all ages (Figs. 1 and 3, A4 and B4). Again, the relationship between shoulder and hip varied significantly with age (Fig. 4A). At E9, embryos exhibited a moderately strong pattern of in-phase shoulder and hip excursions; but at E12 and E15, only three to four embryos exhibited an in-phase pattern. At E18, a moderately strong out-of-phase pattern predominated. The out-of-phase shoulder and hip excursions at E18 were common during tremor-like movements (Fig. 8), and intermittent during less regular excursions (*, Fig. 1D). EMG activity for wing muscles at E12 and E18 appeared consistent with kinematic traces, i.e., latissimus dorsi (LD) was active during shoulder retraction, and the triceps brachii (TR) was active during elbow extension. During repetitive bursts in two experiments at E12, TR bursts were coincident with FT and LG bursts during leg extensions (*, Fig. 5A). In three experiments at E18, LD bursts were observed during RBLM (Fig. 5E). Distinct LD bursting accompanied tremor of the wing that could last for many seconds (Fig. 8). No one pattern of LD and leg muscle activity was apparent during faster movements and leg EMG tended to decay or cease over the course of wing excursions.

**DISCUSSION**

Chick embryos are spontaneously active beginning E3.5, and by the end of embryonic development at E20–E22, they escape the egg with precocious walking skill (Jacobson and Hollyday 1982a; Muir et al. 1996). Spinal transection and deafferentation in hatchlings indicates walking is controlled by a CPG (Bekoff et al. 1987, 1989; Jacobson and Hollyday 1982b). Whether motility is also controlled by a CPG is not yet clear. By E9, chick embryos generate motility sequences characterized by coordinated flexion and extension of leg joints and alternating flexor and extensor muscle synergies (Bradley and Bekoff 1990; Chambers et al. 1995). Between E9 and E12, both the wing and limb exhibit an increase in intralimb coor-
dination (Bradley 1999). However, leg movements exhibiting a
dissociation of knee and ankle excursions at E13 raise the
possibility that intralimb coordination breaks down during
subsequent development (Sharp et al. 1999). Our findings
extend these observations to E18 and reveal five critical points
for advancing our understanding of embryonic leg movements.
One, coordinated kinematic and/or EMG patterns observed at
E9 were expressed at all four time points E9–E18. Two, variable
knee and ankle excursions at E15–E18 sorted into
in-phase and out-of-phase patterns. Third, leg EMG patterns at
E18 did not directly account for out-of-phase coupling between
the knee and ankle. Fourth, 2- to 10-Hz limb excursions
emerged at E15–E18, and during the faster movements, mixed
muscle synergies were produced resembling EMG patterns
during the paw shake in cat. Fifth, wing and leg movements
were intermittently coordinated E9–E18, but an in-phase pat-
tern at E9 and out-of-phase pattern at E18 were the most
distinct, confirming and extending an earlier observational
study of motility in ovo (Provine 1980).

Continuum in motor development

Coordinated kinematic and EMG patterns observed at E9
were also expressed E12–E18, supporting the view that the leg
pattern for early motility forms the basis for mature motor
patterns such as hatchling and walking (Bekoff 1992). Sharp et
al. (1999) observed that the extent of in-phase coupling of hip
and knee did not vary E9–E13, and our results extend this
pattern to E15–E18 (Fig. 4A). In-phase hip and knee coupling
is consistent with the model of unit burst generators (half-
centers) that synchronize flexion and extension across joints
(Orlovsky et al. 1999). Consistency of hip/knee coupling during
spontaneous motility suggests hip and knee unit burst
generators are established and functionally interconnected by
E9. In addition, alternating ankle flexor and extensor EMG
activity was observed at all four time points, suggesting the
ankle burst generator is functioning E9–E18. Further, in-phase
coordination of wing excursions also suggests that limb
movements are controlled by coupled unit burst generators E9–E18.

Our kinematic findings are consistent with the variable
coupling between knee and ankle at E13 reported by Sharp et
al. (1999); and they extend the observation to E18. The
in-phase coupling of ankle and knee excursions at E9–E12 and
more variable coupling after E12 might seem to suggest knee
and ankle unit burst generators are initially synchronized but
that coupling breaks down between E12 and E18. However, a
breakdown in coordination between burst generators seems
unlikely given the continued expression of alternating muscle
synergies at E18, even as knee and ankle moved out of phase
(Fig. 5, B–D). Further, the discrepancy between EMG and
kinematic patterns at E18 may indicate that embryonic unit
burst generators are not readily decoupled by movement re-
lated feedback. Thus we propose an alternative view, one that
attributes developmental changes in limb coupling during em-
byrogenesis to biomechanical dynamics. There are seemingly
substantial changes in the musculoskeleton, force-generating
capabilities of leg muscles, and environmental constraints as
the embryo increases in size and begins to fold over itself in
tight quarters. Any of these variables alone or in combination
may exert mechanical forces sufficient to mask coordination of
knee and ankle unit burst generators. For example, the vari-
ability in knee/ankle coordination at E15–E18 was partially
attributed to a reversal from in-phase to out-of-phase coupling
rather than to a loss of coupling. The out-of-phase excursions
during 2- to 10-Hz oscillations and some startles could have
resulted from inertial interactions between limb segments such
as during paw shaking in cats (Hoy et al. 1985). Further, in the
adult cat muscle afferent input is required to reconfigure leg
muscle patterns during rapid limb movements (Koshland and
Smith 1989a,b), but muscle afferents appear to be significantly
immature over the ages of our study (Maier 1992, 1993).

Transformations in the source of drive to unit burst gener-
ators may also account for the increasing variability in limb
kinematics over age. Glycinergic and GABAergic actions
switch from excitatory to inhibitory during the later half of
embryogenesis (Chub and O’Donovan 1998). Descending
pathways (Glover 1993; Glover and Petursdottir 1991; Okado
and Oppenheim 1985) and proprioceptive afferents (Maier
1992, 1993) are undergoing refinements that may provide a
more variable source of drive to unit burst generators. Imma-
ture excitation may be intermittent and poorly modulated
resulting in the occasional rhythmic excursions that resemble
a series of startles as seen E15 (horizontal bar, Fig. 1C). Emer-
gence of 2- to 3-Hz oscillatory limb movements and 3- to
10-Hz RBLM at E15–E18 may herald the emergence of more
sustained brain stem drive of wing and leg unit burst genera-
ators.

Is motility produced by a locomotor pattern generator?

Chicks are precocious walkers capable of running to stay up
with a mobile brood shortly after hatching (Muir et al. 1996).
Further, chicks can walk ≥2 days earlier if time to hatching is
accelerated by rearing in continuous light (Bohren and Siegel
1975; Fairchild and Christensen 2000) or exposure to artificial
clicking (Vince et al. 1976). These findings seem to suggest the
CPG can produce adaptive locomotion as early as E19. Al-
though investigators have intermittently explored the possibil-
ity of a functional continuity between embryonic motility and
locomotion, evidence favors the view that motility does not
serve a functional role in locomotor development (Haverkamp
and Oppenheim 1986). In chicks for example, motility ap-
ppeared to fully recover after 1–2 days of neuromuscular block-
ade, and the immobilization had no impact on hatching and
posthatching development if the embryo was free of foot
deformities (Oppenheim et al. 1978). Prolonged immobiliza-
tion of Xenopus and Ambystoma embryos between the stages of
premotility and established swimming only transiently altered
swimming behavior (Haverkamp 1986; Haverkamp and Op-
penheim 1986). Results of these studies were interpreted to
indicate that the nervous system develops in forward reference
to functional activity at later stages of development indepen-
dent of experience (Haverkamp and Oppenheim 1986). How-
ever, resilience to transient paralysis may also indicate that
CPG networks are already sufficiently established to remain
robust in the face of perturbations. If this is correct, a clearer
understanding of early CPG circuitry assembly in chicks may
offer some indication as to why they are precocious walkers
and why altricial animals appear more vulnerable to transient
paralysis (Moessinger 1983).

Alternately active antagonist muscles about a joint and
alternating flexor and extensor synergists are evidence that

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half-centers or unit burst generators forming CPG for limb movements may be established by E6–E9 in chicks (Bekoff 1976; Bradley and Bekoff 1990). Spontaneous activity in isolated spinal cord may be important for establishing the CPG circuitry. The E4–E12 lumbar spinal cord produces episodic motor activity composed of cyclic bursting similar to EMG and kinematics during repetitive leg movement (Bekoff 1976; Bradley 1999; Landmesser and O’Donovan 1984). Cycle durations and alternating antagonist leg muscles also appear similar between preparations (Bradley 1999; Bradley and Bekoff 1990; O’Donovan and Landmesser 1987). However, spinal preparations produce more stereotypic activity and more consecutive cycles bracketed by longer pauses (Bradley 2001; Landmesser and O’Donovan 1984). The spinal activity is driven E4 by acetylcholine, then by glutamate and GABA between E4 and E9 (Chub and O’Donovan 1998; Milner and Landmesser 1999; Sernagor et al. 1995). In mice, the absence of acetylcholine appears to reduce activity and alter development of motor output for intralimb and interlimb coordination (Myers et al. 2005). Differences between spontaneous spinal cord activity and motility may indicate they are not one and the same, but the general repetitive structure suggests to us that the spontaneous spinal activity is important to intact motility.

The characteristics of and continuities in kinematic and EMG activity we observed at E9–E18 seem to suggest limb movements for motility are produced by coupled unit burst generators equivalent to a functional CPG for control of locomotion at hatching. Excluding negative knee/ankle correlations at E15 and E18, our regression results are consistent with the close positive co-variations between hip and knee (Bradley and Smith 1990; Buford et al. 1990). It is of note that the negative knee/ankle correlations we observed at E18 were accompanied by alternating flexor and extensor synergies. Alternating flexor and extensor synergies are also expressed during backward locomotion in cat, even as the hip moves out of phase to the knee (Buford and Smith 1990). Further, in-phase elliptical and diagonal angle-angle plots, similar to those we observed during motility E9–E18, are also observed during treadmill locomotion in cats (Buford et al. 1990) and swimming in rats (Walton et al. 2005). Finally our E18 EMG data suggest there is a physiological bridge binding E9 data with the CPG for locomotion. The frequency range for alternating flexor and extensor EMG synergies at E18 fell within ranges observed during walking, swimming and astepping 1–3 days post hatching (Johnston and Bekoff 1996).

Significance of findings

During normal embryonic development, the patterns of limb movement appear to be coordinated soon after they first emerge, suggesting that many of the key organizational elements (e.g., unit burst generators or half-centers) within the spinal cord for CPG control of locomotion are functional at the developmental onset of limb movement. Similar observations have been made regarding development of motor skills in kittens (Bradley and Smith 1988a,b). Achievement of repetitive limb movements between E4 and E9 may be emblematic of the embryo having completed a critical phase of development beyond which the developmental progression of these motor patterns may be less fragile in the face of unexpected prenatal events and therefore predictive of the capacity to attain locomotor skill. Conversely, limb movements lacking these patterns may be evidence that establishment of the requisite circuitry was altered very early in embryogenesis and likely to impact later locomotor outcome. However, our results also caution that seemingly altered motor patterns do not necessarily indicate neural control has been compromised. The embryo or fetus is just as likely as the adult to experience the biomechanical consequences of moving the limb’s segmented masses. Embryonic motor behavior may be dramatically shaped by variables such as physical constraint that change dramatically over development even as the limb and body to which it is attached are changing.

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REFERENCES


