Ankle Restraint Modifies Motility at E12 in Chick Embryos

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The chick’s relationship to its environment changes dramatically over 21 days of embryonic development. At early ages embryos are buoyant; their posture and movements are relatively unconstrained. As embryos grow and fluid level in ovo decreases, movements are increasingly constrained by gravitational forces and reactive forces due to body contact with the shell wall. The issue of how age-related changes in the constraints on movement in ovo may affect embryonic motility is addressed in this paper. Our long-term goal is to determine whether experience imposed by these conditions contributes to development of posthatching motor behaviors. Because previous work indicated that parameters of motility can be modified by a reduction in buoyancy at embryonic day (E) 9, we sought to determine whether a restraint localized to a single joint could also alter either the episodic distribution of activity or the spatiotemporal patterns of limb movement at either E9 or E12. Thus a restraint was applied to the right ankle of embryos prepared for kinematic recordings. Video and kinematic analyses indicated that the restraint had minimal effect at E9, but significantly modified several motility parameters in both the wing and leg at E12. Ankle restraint decreased episode duration. Restraint also decreased most joint excursion parameters, including excursion range, cycles per sequence, and excursion velocity. Restraint increased cycle period duration and signal frequency content under 1.0 Hz. Parameters of intralimb and interlimb coordination exhibited small mixed effects. Results provide support for the hypothesis that environmental constraints contribute to features of embryonic motility. Further, significant modifications of wing excursions in ankle restrained embryos suggest that sensory feedback arising from mechanical perturbations of leg movements may entrain rostral spinal circuits for preservation of interlimb coordination at E12. Potential mechanisms and implications are discussed.

INTRODUCTION

Early studies testing the effects of somatosensory stimuli provided important evidence that motility is a centrally generated behavior in chick embryos. In these studies, body movements were counted before and after a brief (Hamburger 1963) or intermittent tactile stimulus (Oppenheim 1972), a tap or flip of a limb (Oppenheim 1972), excision of amniotic membranes (Oppenheim 1966, 1972), or surgical deafferentation (Hamburger et al. 1966; Narayanan and Malloy 1974). In intact embryos, the first application of a stimulus produced a startle or withdrawal, but embryos quickly habituated to repeated stimulus applications (Hamburger 1963; Oppenheim 1972). These findings appeared to suggest that sensory stimuli are not critical events in the development of embryonic motility (Hamburger 1963; Hamburger et al. 1966; Narayanan and Malloy 1974; Oppenheim 1966). However, initial responsiveness to a phasic stimulus followed by resumption of normal movement may be evidence that embryonic networks producing motility are intrinsically stable, as observed in pattern generators that are naturally mutable to feedback (Schöner et al. 1992). Habituation and nonresponsiveness to stimuli may indicate that sensory inputs are gated during motility in chicks as during swimming in frog tadpoles (Sillar and Roberts 1992) and locomotion in mature organisms (Pearson 1993).

There is substantial argument that the interactive dynamics of neural control, body biomechanics, and environment shape experience and behavior during development (Chiel and Beer 1997; Sporns and Edelman 1993; Thelen and Smith 1994). In the chick, movement conditions in ovo vary dramatically over the embryonic period. At early stages, the embryo is fully buoyant, and posture and movement are minimally constrained. The biomechanics of movement are progressively modified during development as body size increases, buoyancy diminishes, and posture and movement are increasingly constrained by egg volume. Cutaneous receptor coding of movement experience begins by embryonic days 6 through 7 (E6–E7) (Scott 1982), evoking movement by E7 (Hamburger and Balaban 1963; Oppenheim 1972). Cutaneous mechanoreceptors exhibit phasic, graded, rapid adapting, or slow adapting properties by E17, and because cutaneous nociceptors have low thresholds at early embryonic ages, they may also respond to limb movements (Koltzenburg and Lewin 1997). It is not known at what age muscle spindle afferents begin coding mechanical stimuli, but central synapses for primary afferent pathways are functional at E7.5 (Davis et al. 1989; Lee et al. 1988), and ankle muscle afferents innervate intrafusal fibers by E11–E13 (Maier 1992, 1993). There is no information available on the development of joint afferents or tendon organs in the chick embryo.

Investigations in our laboratory are exploring the contributions of environmental conditions during embryonic movement and whether the environment-related experiences exert an instructive role in the development of motor control. One study, extracting amniotic fluid to reduce the extent of buoyancy in E9 embryos, indicated that some parameters of motility are modified by environmental manipulations (Bradley 1997). However, because reducing buoyancy increased gravitational loading at all joints, it was not possible to determine whether effects were due to generalized mechanical damping or also altered motion-dependent feedback. In adult cats and several invertebrates, mechanical restraint of a single joint during locomotion alters the timing of flexor and extensor muscle activity (Andersson and Grillner 1983; Pearson 1993) and reduces the frequency of repetitive movements (Prochazka 1989). Limb loading increases extensor burst duration (Duysens and Pearson 1980; Hiebert and Pearson 1999), and
unloading decreases burst duration (Giuliani and Smith 1985). Similar trends are observed in neonatal kittens before maturation of sensory pathways and muscle properties (Bradley and Smith 1988a,b). It is not known whether loading modifies muscle recruitment in chick embryos; however, close co-variation of extensor burst duration and cycle duration in older embryos (Bekoff 1976) may be due to limb loading as embryos make more extensive contact with the shell wall in ovo.

In this study, to further explore whether environment-dependent experiences exert an instructive role during embryonic development, we perturbed movements by mechanically restraining the ankle in E9 and E12 chick embryos. Significant modifications in motility parameters were obtained at E12 and support previous work suggesting that environmental variables shape attributes of motility. Further, significant trends in wing parameters provide tentative evidence that by E12, condition-dependent feedback may modify the neural control of motility. Mechanisms that may account for these findings and implications of the findings are discussed. An abstract of preliminary findings was recently published (Bradley and Sebelski 1999).

METHODS

Leghorn chicken embryos were incubated for experiments at either E9 or E12, and age was verified using staging criteria established by Hamburger and Hamilton (1951, reprinted 1992). Approximately 24 h before experiments, embryos were transferred from rotating shelves to a stationary shelf in the incubator so that they would present a right sagittal view when the egg was opened at the time of the experiment. Pulse rate and rhythm were monitored throughout the experiment, preparation time was limited to a maximum of 30 min, and total exposure time was limited to 90 min to optimize behavioral recordings (Chambers et al. 1995). All procedures were approved by the University Institutional Animal Care and Use Committee.

Preparation for kinematic recording

Embryos were placed in a temperature-controlled chamber (38°C) and exposed for preparation by making a window in the shell and deflecting the membranes. An ankle restraint was first applied to the right lower leg of experimental embryos at E9 or E12 and worn for the duration of the experiment. The ankle restraint was fabricated from a nylon cable tie, bent 90°, and secured to the right lower leg with Superglue (Fig. 1, B and D). Experimental embryos were then prepared for kinematic recording following procedures previously established (Chambers et al. 1995; Orosz et al. 1994). In brief, the sagittal aspect of the shoulder, elbow, wrist, hip, knee, ankle, and tarsometatarsal joints of the right leg were estimated and marked with a dot of nail enamel. Additional marks were placed on the thoracolumbar spine and on the shell dorsal to the thoracolumbar marker, and a reference stick (2–6 mm) was floated on the amniotic fluid adjacent to the embryo (Fig. 1).

Video recordings and kinematic analyses

Video recordings were continuous to capture all movement and pauses in movement over the entire experiment for each embryo. Video was collected at 30 frames/s using a camera shutter speed of 1/2,000 s and stored on VHS tape. VHS recordings were first reviewed frame-by-frame to determine the onset and offset of activity (Fig. 2). With the use of methods established by Hamburger et al. (1965), bouts of continuous movement in the right wing and/or leg lasting $\geq 10$ s were operationally defined as motility sequences; pauses in motility lasting $>10$ s were defined as motility pauses (Bradley 1999; Chambers et al. 1995). Combined, a motility sequence and the subsequent motility pause formed one motility episode. Pauses $\leq 10$ s where treated as part of the concurrent motility sequence. Movements $<10$ s were treated as isolated movements and included in the concurrent motility pause.

The five longest motility sequences meeting our digitizing criteria (Orosz et al. 1994) were selected from each E9 and E12 ankle-restrained embryo for kinematic analyses. This sampling strategy was
To test the effects of ankle restraint at E9 and E12, data for each experimental group were compared with age-matched controls. Group means and standard deviation of the mean (means ± SD) are specified in RESULTS for each experimental and control age group. To determine whether ankle restraint altered motility, the two-way ANOVA for repeated measures was used for age-matched comparisons of subject means. The Student’s t-test assuming unequal variances was used for single comparisons and as a post hoc with a Bonferroni correction (P < 0.05/number of comparisons) for testing ANOVA main effects. It should be noted that the results for 16 of 18 control embryos used in this study to test the effects of ankle restraint were the focus of a previous study examining age-related transformations in motility (Bradley 1999). Thus control data will not be separately considered.

RESULTS

Approximately 6 h of recordings were analyzed for each of the two experimental groups wearing the ankle restraint (9 E9 embryos and 9 E12 embryos), and findings were compared with similar samples from 18 age-matched controls. On average, 34–39 min of video recording was collected and analyzed per embryo across experimental and control groups. In general, it appeared that episode duration for experimental embryos tended to be shorter than for age-matched controls (Table 1). Average episode duration at E12 varied from 61 to 117 s and was significantly shorter for E12 experimental embryos than for E12 control embryos. Average episode duration at E9 varied from 110 to 259 s and was not significantly different between groups. The reduction in episode duration for E12 experimental embryos could not be attributed to selective modulation of either motility sequence duration or pause duration. Motility sequence duration and pause duration were also similar between experimental and age-matched controls.

General features of motility sequences digitized

In all, 90 motility sequences (5 samples/embryo) from ankle-restrained embryos were digitized, totaling 36.9 min (E12) and 34.4 min (E9) of continuous activity. Similar samples (n = 88) were drawn from E12 (44.6 min) and E9 (32.6 min) control embryos for comparisons. Individual sequences for ankle-restrained embryos ranged from 31 to 162 s, samples averaging 49 ± 10 s (mean ± SD, E12) and 46 ± 10 s (E9).

Plots of motility sequences for ankle-restrained embryos were readily distinguished from plots for control embryos at both E9 and E12 (Fig. 3). In general, plots for ankle-restrained embryos (Fig. 3, B and D) appeared smoother, and less complex or varied than for controls (Fig. 3, A and C). At E12, time-position series for each joint in experimental embryos differed substantially in appearance from those for control embryos; ankle restraint nearly eliminated ankle motion in most plots, but excursions exceeding 10° were occasionally

TABLE 1. Distribution of continuous movement

<table>
<thead>
<tr>
<th></th>
<th>E9C</th>
<th>E9AR</th>
<th>E12C</th>
<th>E12AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Episode duration, s</td>
<td>185 ± 47</td>
<td>150 ± 22</td>
<td>94 ± 16</td>
<td>78 ± 10*</td>
</tr>
<tr>
<td>Motility sequence, s</td>
<td>35 ± 4</td>
<td>35 ± 4</td>
<td>42 ± 15</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>Motility pause, s</td>
<td>150 ± 48</td>
<td>116 ± 25</td>
<td>51 ± 21</td>
<td>46 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SD for E9 and E12 controls (E9C and E12C) and ankle-restrained embryos (E9AR and E12AR). * Value is significantly different from that for the age-matched control group.

FIG. 2. Parceling of continuous video recordings for analysis of motility parameters at E9–E12. Video recording was continuous during experiments to capture all instances of activity (upward deflections) and inactivity (downward deflections). A motility sequence (a) plus the subsequent motility pause (b) formed an episode. Pauses lasting ≥10 s (c) were included within the concurrent motility sequence (a). Motility sequences were selected for digitizing to generate time-position plots to complete kinematic analyses (shoulder and elbow plots are from Fig. 3A).
observed. At the other four joints, excursion amplitude, number of cycles, duration of cycles, and frequency of abrupt movements appeared substantially reduced across most sequences. At E9, ankle excursions for experimental embryos were very small for a substantial portion of all sequences (Figs. 3B and 4A) and minimal throughout a few sequences (Fig. 4B). However, application of the ankle restraint did not completely eliminate ankle motion in most motility sequences digitized, and a few excursions exceeded 10° midsequence in the majority of samples. Abrupt movements, occasionally present in sequences for E9 controls, were rarely detected in samples for E9 experimental embryos. Times series for other joints did not appear to differ from those for E9 controls.

**Kinematic features of motility sequences digitized**

The extent of restraint at the ankle and potential effects at other joints were first estimated by determining the maximum extension and maximum flexion position achieved at each joint for each motility sequence and taking the difference. Calculations suggested ankle restraint reduced both wing and leg joint excursion ranges at E12, but only ankle excursions at E9. A two-way ANOVA comparison indicated that the reduction in total joint excursion range at E12 was significant across all five joints, and post hoc t-tests, using a Bonferroni correction for five comparisons, confirmed that the effect was significant at each joint except the elbow (Table 2). Although a Student’s t-test confirmed that the restraint significantly reduced ankle excursion range at E9, no differences in range were observed at the other joints (Table 2).

Times series for the ankle, knee, and elbow were separately parceled into excursion cycles (i.e., peak-to-peak extension) for kinematic analyses to test the effects of the ankle restraint on spatiotemporal features of motility. Because the restraint did not totally eliminate ankle motion, ankle excursions intermittently exceeded the 5° threshold for detecting joint excursion cycles (Fig. 5). Nonetheless, two-way ANOVA comparisons indicated that application of the restraint significantly reduced the number of cycles per sequence at both E12 and E9. Post hoc comparisons using a Bonferroni correction for three comparisons indicated that the effect was significant for all three joints at E12, but only for the ankle at E9.

Reductions in ankle excursion amplitude and frequency were accompanied by a lengthening of cycle time at both ages. As illustrated in Fig. 6 and confirmed in post hoc comparisons (Bonferroni correction, 3 comparisons), restraint of ankle excursions more than doubled ankle cycle period duration from that of controls at both ages. More importantly, in E12 experimental embryos, knee cycle periods were ~2 s longer and elbow cycle periods were 1.3 s longer than in E12 controls. Knee and elbow cycle periods were similar between groups at E9.

Time series for the wing were parceled by elbow cycles, and time series for the leg were parceled by ankle cycles to calculate maximum velocity/cycle, maximum velocity/sequence, and average velocity/sequence. Because there were significantly fewer ankle cycles for experimental embryos, and therefore fewer samples per calculation, time series for the leg were also parceled by knee cycles; however, the two sampling
methods did not yield differences $>7^\circ$/s in group calculations. Thus velocities reported in Table 3 are based on ankle cycles for consistency in comparison to previously published data (Bradley 1997, 1999). At E12, two-way ANOVA comparisons including all five joints were significantly different for both extension and flexion. Post hoc tests, using a Bonferroni correction for 10 comparisons (5 joints; extension, flexion), indicated that average maximum velocities at all joints were significantly less for E12 experimental embryos compared with E12 controls. At E9, two-way ANOVA comparisons including all five joints indicated that only average maximum extension velocities were significantly different between groups. However, two-way ANOVA comparisons including only leg (3) joints indicated that average maximum velocities were significantly less for E9 experimental embryos compared with controls in both directions. Post hoc tests for six comparisons (3 leg joints; extension, flexion) indicated that only ankle velocities (flexion and extension) were significantly less from those

**TABLE 2. Average maximum joint excursion range**

<table>
<thead>
<tr>
<th>Joint</th>
<th>E9C ± SD</th>
<th>E9AR ± SD</th>
<th>E12C ± SD</th>
<th>E12AR ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoulder</td>
<td>43 ± 10</td>
<td>44 ± 9</td>
<td>60 ± 8</td>
<td>48 ± 11*</td>
</tr>
<tr>
<td>Elbow</td>
<td>50 ± 16</td>
<td>46 ± 8</td>
<td>66 ± 13</td>
<td>55 ± 11</td>
</tr>
<tr>
<td>Hip</td>
<td>20 ± 10</td>
<td>20 ± 7</td>
<td>35 ± 10</td>
<td>14 ± 4*</td>
</tr>
<tr>
<td>Knee</td>
<td>31 ± 8</td>
<td>26 ± 5</td>
<td>40 ± 10</td>
<td>29 ± 9*</td>
</tr>
<tr>
<td>Ankle</td>
<td>38 ± 7</td>
<td>14 ± 4*</td>
<td>47 ± 14</td>
<td>11 ± 4*</td>
</tr>
</tbody>
</table>

Values are means ± SD for E9, E12 controls (E9C and E12C) and ankle-restrained embryos (E9AR and E12AR) and are expressed in degrees. *Value is significantly different from that for the age-matched control group.

**FIG. 4.** Examples of near-maximal and maximal ankle restraint at E9. Despite near-maximal restraint of the ankle (A), hip and knee cycles typically exhibited spatiotemporal patterns that closely co-varied with concurrent shoulder and elbow excursions. Even when ankle restraint was maximal over the entire motility sequence (B), hip and knee excursions co-varied with shoulder and elbow excursions for some portion of the sequence.

**FIG. 5.** Average number of cycles per motility sequence for ankle-restrained and age-matched control embryos. Stars note significant differences between ankle-restrained embryos and age-matched controls. Vertical bars represent ± SD from the group mean.

**FIG. 6.** Average cycle periods for ankle-restrained and age-matched control embryos. Stars note significant differences between ankle-restrained embryos and age-matched controls. Vertical bars represent ± SD from the group mean.
for E9 controls. Average joint velocities were also compared, but no differences were found for either age group.

Given the apparent effects of ankle restraint and earlier work indicating that generalized gravitational loading dampens joint excursions, we were also interested in examining the frequency content of joint excursions during ankle restraint. FFT analyses were used to calculate the percent of total signal under 0.5 and 1.0 Hz for each time series. Analyses for E12 embryos revealed consistent differences between ankle-restrained and control groups in two-way ANOVA and post hoc t-tests (5 comparisons). On average, 85% of the signal fell below 0.5 Hz, and 90% below 1.0 Hz in E12 experimental embryos, compared with 74% (0.5 Hz) and 82% (1.0 Hz) for E12 controls. FFT analyses for E9 data indicated that 48–54% of the total signal fell below 0.5 and 66–73% fell below 1.0 Hz with no differences between experimental and control embryos.

Parametric estimates of intralimb and interlimb coordination were based on the percent of all cycles per pair of time series yielding a Pearson correlation coefficient of $r \geq 0.7$. For example, estimates of intralimb coordination for the wing were based on regression of the shoulder time series against the elbow time series, parceling by elbow cycles. Because there were far fewer ankle cycles per sequence for experimental embryos, intralimb estimates for the leg and interlimb (wing/leg) estimates were parcelled by knee cycles. Although this method has been a useful tool in earlier studies, the results of comparisons between control and ankle-restrained groups proved difficult to interpret. As summarized in Fig. 7, restraint of the ankle did not alter the incidence of close co-variation in concurrent shoulder and elbow excursions (S/E) at either age. As expected, ankle restraint significantly reduced the incidence of close co-variations in ankle and knee excursions (A/K) at E12, but not at E9. However, ankle restraint increased the incidence of close co-variations in hip and knee excursions (H/K) at E9, but not at E12. We also asked whether ankle restraint increased the incidence of negative co-variations ($r \leq -0.7$) and found no effect. Few cycles yielded strong negative co-variations (E9: 2–4%; E12: 2–7% of cycles). The effects of ankle restraint on interlimb coordination were also mixed. The small increased incidence of close co-variations in concurrent elbow and knee excursions (E/K) was significant at E9, but not at E12 (Fig. 7). There was no effect on incidence of negative co-variations in elbow and knee excursions (E9: 3%; E12: 9% of cycles).

Finally, given the significant effects observed in wing excursions of E12 ankle-restrained embryos, we sought to determine whether the wing was indirectly constrained by a loss of forces normally emergent with unfettered intersegmental dynamics during rapid, large amplitude movements. Reexamining all E12 control time series, we identified the sequence (across joints) of abrupt movement onsets to determine whether leg excursions typically began before wing excursions (Fig. 8). Results indicated that onset of hip, knee, and/or ankle excursions preceded shoulder and/or elbow excursions in only 27 ± 16% of abrupt movements. In 68 ± 24% of abrupt movements, onset of wing excursions preceded leg excursions. In the remainder of cases, excursions of one or more joints in the wing and leg began synchronously.

**DISCUSSION**

Our results indicate that sustained restraint of ankle motion significantly modifies parameters of motility at E12. Episode duration was reduced and excursion parameters for both the

**TABLE 3. Average maximum joint velocity**

<table>
<thead>
<tr>
<th></th>
<th>E9C</th>
<th>E9AR</th>
<th>E12C</th>
<th>E12AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoulder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>133 ± 42</td>
<td>164 ± 75</td>
<td>228 ± 42</td>
<td>112 ± 47*</td>
</tr>
<tr>
<td>Flexion</td>
<td>147 ± 44</td>
<td>174 ± 95</td>
<td>286 ± 63</td>
<td>158 ± 53*</td>
</tr>
<tr>
<td>Elbow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>191 ± 59</td>
<td>178 ± 38</td>
<td>271 ± 66</td>
<td>126 ± 31*</td>
</tr>
<tr>
<td>Flexion</td>
<td>135 ± 32</td>
<td>130 ± 34</td>
<td>263 ± 79</td>
<td>115 ± 40*</td>
</tr>
<tr>
<td>Hip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>85 ± 45</td>
<td>77 ± 32</td>
<td>181 ± 57</td>
<td>41 ± 13*</td>
</tr>
<tr>
<td>Flexion</td>
<td>81 ± 49</td>
<td>76 ± 22</td>
<td>119 ± 36</td>
<td>37 ± 17*</td>
</tr>
<tr>
<td>Knee</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>145 ± 25</td>
<td>114 ± 40</td>
<td>213 ± 55</td>
<td>58 ± 20*</td>
</tr>
<tr>
<td>Flexion</td>
<td>102 ± 16</td>
<td>83 ± 23</td>
<td>177 ± 52</td>
<td>62 ± 17*</td>
</tr>
<tr>
<td>Ankle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>181 ± 37</td>
<td>64 ± 32*</td>
<td>267 ± 41</td>
<td>50 ± 21*</td>
</tr>
<tr>
<td>Flexion</td>
<td>139 ± 33</td>
<td>62 ± 23*</td>
<td>188 ± 54</td>
<td>35 ± 13*</td>
</tr>
</tbody>
</table>

Values are means ± SD for E9 and E12 controls (E9C and E12C and ankle-restricted embryos E9AR and E12AR) and are expressed in deg/s. * Value is significantly different from that for the age-matched control group.
wing and leg were altered. Joint excursion ranges, number of excursion cycles per sequence, average maximum excursion velocities, and incidence of close co-variation in concurrent ankle and knee excursions were less in E12 ankle-restrained embryos than in age-matched controls. Additionally, cycle period duration and the portion of frequency content falling below both 0.5 and 1.0 Hz in wing and leg excursions were greater than in E12 controls. At E9, the only modifications in motility parameters beyond those at the ankle were increased incidences of close co-variation for hip/knee and elbow/knee comparisons.

Because we did not eliminate ankle rotations, we cannot rule out the possibility that modifications observed at E12 can also be obtained at E9. Failure to fully restrain the ankle was likely due to attributes of the joint, e.g., ankle resting posture was substantially >90° (Fig. 1A), and the joint was less compliant than at E12. Nonetheless, in some portion of most records, the ankle exhibited little or no rotation, and during these cycles, no effect was observed at other joints. For example, in the first portions of Figs. 3B and 4A, ankle motion was substantially dampened, yet excursions at all other joints were similar in amplitude and cycle period duration to values for E9 controls (Fig. 3A). Even when ankle motion was minimal, wing excursions appeared undisturbed, and both hip and knee excursions co-varied with wing excursions (Fig. 4B). However, given modifications in motility at E9 following more general restraint (Bradley 1997; Sharp and Bekoff 1996) and application of pyridoxine (Sharp et al. 1998), the minimal effects obtained during ankle restraint at E9 must be viewed as preliminary.

Failure to obtain more extensive modifications at E9 could also be a consequence of differences in conditions between E9 and E12. The restraint, fabricated from a plastic cable tie, may have made the leg more buoyant at E9 due to less limb mass, because the foot appeared to float at the fluid line. Increased buoyancy could account for normal hip and knee excursion ranges, as well as increased hip/knee and elbow/knee co-variations, because the reverse effects are observed after reducing buoyancy at E9 (Bradley 1997). Indeed, an observation drawn from this study is that application of an experimental manipulation across ages does not guarantee that the same mechanisms are being tested. In chick, the relationship between embryo and environment is constantly changing as total body mass and its relative distribution, extent of buoyancy, and posture change within a fixed volume. Thus it is likely that motion-dependent dynamics induced by ankle restraint also vary even with small incremental changes in age.

Are neural mechanisms for motility responsive to environmental conditions?

We considered the possibility that modifications in motility observed in E12 ankle-restrained embryos could be attributable solely to mechanical dampening, because some modifications were also obtained in a study of E9 embryos following a more global constraint (Bradley 1997). In the latter study, decreases in joint excursion range, plus increases in cycle period and the proportion of frequency content <0.5 Hz were also obtained in the wing and leg following a reduction in buoyancy. Because reducing buoyancy increased gravitational loading at both the wing and leg, it was not possible to determine whether results were solely due to mechanical dampening or also feedback shaped by environmental conditions. We cannot conclusively rule out the possibility that the ankle restraint imposed mechanical dampening sufficient to account for the significant effects observed in wing parameters at E12 in this study. It is possible, for example, that a more stable posture and sustained hip flexion throughout a motility sequence in E12 ankle-restrained embryos imposed a mechanical restraint of wing motions. However, video analyses indicated that wing excursion onset typically preceded and/or began synchronous with leg excursions during abrupt movements, suggesting that wing motions were not mechanically constrained. Nor did it appear that the ankle restraint blocked excursion range at the knee and hip, because embryos occasionally exhibited knee flexion ranges similar to controls, but it is possible that ankle extensors were sufficiently stretched by ankle restraint at 90° to limit knee extension range.

The array of changes observed in wing parameters, and possibly those for the hip and knee, following ankle restraint suggest that by E12, motion-dependent feedback arising from environmental conditions is sufficient to modify attributes of motility. During locomotion in adult animals, muscle spindle and Golgi tendon afferents contribute significantly to the timing and phase transitions of repetitive stepping within (Conway et al. 1987; Hiebert et al. 1996; Whelan et al. 1995) and between limbs (Hiebert et al. 1994). Joint afferents have also been implicated in the phasing of repetitive limb movements (Andersson and Grillner 1983), and as a limb is loaded during stepping, cutaneous receptor response to contact forces appears to enhance muscle activation (Collins et al. 1999).

The mechanisms responsible for coding the effects of mechanical restraint during embryonic motility at E12 are not readily apparent. Currently available information suggests that muscle spindles are not likely candidates for inducing the effects observed. Anatomic evidence suggests that afferent endings only begin innervating intrafusal fibers in ankle muscles E11–13 (Maier 1992, 1993), and it has yet to be deter-
mined whether intrafusal fiber innervation begins earlier in wing muscles (A. Maier, personal communication). There is no information available on the development of tendon organs or joint afferents, and limited evidence in the adult chicken suggests that joint afferents may not be an important source of movement information (Gentle et al. 1995). Thus given the earlier, more rapid maturation of cutaneous mechanoreceptors (Hamburger and Balaban 1963; Koltzengen and Lewin 1997; Oppenheim 1972; Scott 1982), cutaneous mechanoreceptors appear to be the most likely candidate for coding movement at E12. Supporting this view, reexamination of videos indicated that E12 experimental embryos experienced more frequent contact between the wing and thigh than E12 controls. The increased contact appeared attributable to the thigh remaining more flexed and in closer proximity to the wing during movement. (The wing seldom contacted the thigh in either group of E9 embryos.) Although we do not know the implications of the enhanced contact, we have observed on several occasions that embryos as young as E11 initiate chewing-like movements when the foot spontaneously contacts the inner surface of the beak, suggesting that movements can be initiated or modified by body on body contact.

**Coupling of ipsilateral limbs**

It is a potential paradox that ankle restraint did not decrease the incidence of close co-variations between wing and leg movements, because interlimb coordination is diminished following a reduction in buoyancy (Bradley 1997). Descending and/or propriospinal pathways appear to be coordinating pathways, akin to commissural projections in the lamprey (Buchanan 1999; Cohen et al. 1992), because wing/leg coupling is lost after spinal transection (Hamburger and Balaban 1963; Oppenheim 1975). Reticulospinal and vestibulospinal projections are possible coordinating pathways for they reach the lumbar cord by E5 and E8, forming synaptic contacts within 1–2 days (Shiga et al. 1991), and exhibiting adultlike terminal distributions by E10 (Okado and Oppenheim 1985). Serotonin projections from raphe nuclei also reach the lumbar cord by E8 (Okado et al. 1992), and serotonin depolarizes 50% of lumbar motor neurons by E12 (Muramoto et al. 1996). Entrainment may also be induced or stabilized by primary afferents spanning up to 20 spinal segments by E10 (Eide and Glover 1995).

Generalized excitation is the basis of the current model for embryonic motility (O’Donovan and Chub 1997), and given inhibitory transmitters depolarize developing motor neurons (Sernagor et al. 1995), excitation likely underlies interlimb coupling in young embryos. However, intersegmental mechanisms are not well understood in the chick embryo and appear to be complex. For example, in the isolated spinal cord, when serial rostral to caudal transections are performed, caudal lumbar segments appear to lose excitatory drive producing fewer cycles of fictive motility. Whereas, when serial caudal to rostral transections are performed, rostral thoracolumbar segments appear to be released from inhibition producing more cycles (Ho and O’Donovan 1993). Thus the seeming paradox, preservation of interlimb coordination during ankle restraint versus degradation during reduced buoyancy, may be attributable to differences in the capacity of rostrocaudal and caudorostral projections to entrain distant spinal segments. Restraint appeared to be greatest at the wing during reduced buoyancy, and rostrocaudal projections were seemingly insufficient to entrain leg excursions with modified wing movements; whereas caudorostral projections may more effectively maintain interlimb coordination in response to ankle restraint.

**Mutability in parameters of periodicity and descending control of embryonic motility**

We are particularly interested in the extent of episode duration variability across studies, because it has been suggested that episodic activation of motility and cyclic activity within a motility sequence are controlled by separate mechanisms (Bradley and Bekoff 1992; O’Donovan and Chub 1997; Oppenheim 1975). Ankle restraint significantly decreased episode duration in E12 embryos, but the decrease could not be attributed selectively to change in either motility sequence duration or pause duration. Decreased episode duration, attributable to decreased selectivey to change in either motility sequence duration or pause duration, was also observed following buoyancy reduction at E9 (Bradley 1999). During normal development, episodes decrease from ~2–3 min at E9 to 1–2 min at E15, due to decreases in pause duration, for motility sequences progressively lengthen (Bradley 1999; Hamburger 1963; Rose et al. 1998). However, in the isolated spinal cord, episode durations are substantially longer due to pause durations exceeding 6 min at E6.5 (Milner and Landmesser 1999) and 12 min at E9–E12 (Fedichuk et al. 1999). It has been proposed that motility sequences are terminated by activity-dependent depression of transmitter release and reinitiated as the spinal network recovers from synaptic depression (O’Donovan and Chub 1997). The discrepancies in episode values between intact and isolated cord preparations raise the possibility that developing descending and propriospinal pathways also regulate episode duration. By 6, stimulation of reticulospinal pathways evokes rhythmic motor activity in the lumbosacral cord and application of N-methyl-d-aspartate (NMDA) to a brain stem bath reduces the pause between motility sequences from >10.6 to 2.6 min (Sholomenko and O’Donovan 1995). It has also been suggested that descending and/or propriospinal pathways stabilize episodic behavior because seizure-like activity can be observed in embryos with chronic spinal gap transections shortly after adultlike pathway distributions are normally established (Oppenheim 1975). Thus maturation of spinal pathways, as well as synaptic mechanisms, may account for age-related decreases in pause duration. Further, variability in episode parameters across conditions may be additional evidence that experience can modulate control of motility during embryonic development.

In summary, we have demonstrated that ankle restraint alters motility parameters at E12. Although some changes in leg excursions may be due to mechanical forces imposed by the restraint, significant modifications in wing excursions suggest that mechanical perturbation may induce neural commands to preserve interlimb coordination. The mechanisms supporting changes in wing motility at E12 are not currently known, but cutaneous mechanoreceptors, central primary afferent relays, and propriospinal pathways appear sufficiently mature to be possible mechanisms. Whether similar effects can be induced at earlier ages remains to be clarified. Modifications in wing excursions at E12 support our hypothesis that mechanical
attributes of the environment contribute to transformations in embryonic behavior during normal development as in ovo forces emergent with movement change. Thus it is also possible that experiences during embryonic development contribute to establishing a mature motor repertoire. Because cutaneous receptors appear to be the means for coding movement experiences at E12, tests of this hypothesis could be readily pursued by local application of anesthetics.


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