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Ontogeny of Behavioral Responsiveness to Sound in the Chick Embryo as Indicated by Electrical Recordings of Motility

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The primary purpose of these experiments was to gather normative behavioral data regarding the ontogeny of responsiveness to sound in the chicken embryo. As a prerequisite, a sensitive and accurate method for recording embryonic motility was developed (Experiment 1). By means of platinum electrodes inserted just beneath the shell membrane, potentials resulting from heartbeat and movement were recorded on a polygraph. The technique was found to be effective when applied to chick embryos 6 days and older. Correlations between visual observations of activity and the records produced by the electronic technique substantiated its accuracy. Behavioral responses of chick embryos (Stages 39–43) to acoustic stimulation (Experiment 2) were then recorded. High-intensity (115-dB SPL) tones of 400, 700, and 1400 Hz were used as stimuli. The earliest consistent responses were recorded from Stage 40 (ca. Days 14–15) subjects; the 700 and 1400 Hz tones produced statistically reliable inhibition of movement during the stimulus period compared with the post-stimulus period. Reliable increases in movement during the stimulus period were first recorded at Stage 42 (ca. Days 16–17) in response to 700 and 1400 Hz and at Stage 43 (ca. Days 17–18) in response to 400 Hz.

We have previously reported the results of several studies describing characteristics of the avian auditory system and its development (Parks & Rubel, 1975; Rubel & Parks, 1975; Rubel & Rosenthal, 1975; Rubel, Smith, & Miller, 1976); this article, describing the behavioral responsiveness of chick embryos to acoustic stimulation, represents another in that series. The purpose of this investigation is to establish, for the normal communally incubated embryo, the relation between developmental stage and behavioral responsiveness to tones of various frequencies.

Before behavioral testing could commence, it was necessary to have an adequate method for measuring behavioral responses. For 40 yr the most widely used method has been to observe the embryo visually through a hole in the shell. This method has several inherent drawbacks, however. First, it involves a major modification of the embryo's environment; recent evidence (Oppenheim, Levin, & Harth, 1973) indicates that this modification may affect the behavior of the embryo, and it almost certainly has acoustic consequences. A second weakness is that it is impossible for the human observer to mark each individual movement or even see each movement. Only periods of movement can be reliably marked by this technique. Finally, it is difficult with this method to determine an objective standard for what is to be called one movement and to maintain that standard throughout an observation session or between observers.

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Experiment 1

A potentially useful method for recording motility of avian embryos was reported by McCafferty, Pressman, and Knisely (1965; also see Bautzmann, Dunker, & Schröder, 1954/55). Using implanted silver electrodes to record contractions of the amnionic membrane, they also noted that embryonic motility could be recorded by this means. The following is a description of our refinement of that method and the results of tests undertaken to validate its effectiveness.

Method

Subjects. Over 300 White Leghorn chicken embryos (*Gallus domesticus*) were recorded from in the course of developing and testing this technique. Of that number, approximately 50 were visually observed at the time of recording. A final series of eight embryos, ranging in age from 7 to 18 days of incubation (the first 24 hr of incubation being Day 0), provided the quantitative data presented below.

Unincubated eggs were obtained from Spafas, Inc., Norwich, Connecticut, and incubated in a forced-air incubator maintained at 37.5 °C and 50%–60% humidity. Eggs were automatically turned four times daily.

Apparatus. Electrodes consisted of a 4-mm length of uninsulated platinum wire, .12 mm in diameter, held in a 2-cm-long section of 22-ga. hypodermic tubing.

Recordings were made with a Grass (Model 7) polygraph equipped with Grass (Model 7P1A) dc preamplifiers and Grass (Model 7DAB) driver amplifiers.

The majority of the visual observations of embryos were made with the aid of a Zeiss operating microscope.

Procedure. Prior to implantation of the electrodes, eggs were candled to determine the position of large blood vessels close to the surface of the egg. Avoiding such vessels, we made two small holes in the shell with a 26-ga. hypodermic needle. These holes were positioned approximately 180° apart and halfway along the longitudinal axis of the egg. The 4-mm length of platinum wire was bent at right angles to the electrode shaft and inserted just under the shell surface. The shaft was secured to the shell surface with a mixture of paraffin and stearic acid, the latter being added to raise the melting point of the paraffin.

During the recording sessions eggs were kept in a 14 × 10 × 7 cm Plexiglas chamber maintained at 37–38 °C by a forced-air heating system. This chamber was located in an IAC electrically shielded, sound-attenuating room. Eggs were supported on two parallel rubberized bars to minimize vibration. Wires were attached to the electrode shafts with miniature alligator clips and led out of the soundproof room to the differential inputs of the polygraph preamplifier.

For experiments involving visual monitoring of behavior, lateral observation windows were made in the eggs according to the technique described by Oppenheim et al. (1973). This resulted in a hole in the shell of approximately 3 cm^2 through which the embryo was observed. An event-marker switch, held by the experimenter observing the embryo, was linked to the timing module of the polygraph. Using this system, the observer marked periods of *inactivity* directly on the polygraph record. In one case, that of the 7-day-old embryo, activity rather than inactivity was marked.

A microphone system provided a means of communication between the experimenter observing the embryo and the experimenter monitoring the polygraph record. This arrangement allowed qualitative observations regarding the activity of the embryo to be noted on the polygraph record as it was being produced. At no time was the observer given information about the polygraph record being generated by the embryo.

Observation periods lasted 5–10 min, the length of each being predetermined and arbitrary. Windows in the eggs were covered with Parafilm between observation periods.

Results and Discussion

Qualitative findings. Our experience with this technique indicates that it is an accurate and sensitive method for recording heartbeat, amnionic contractions, and body movements from chick embryos 6 days and older. Each of those three types of activity produces a characteristic pattern of deflection which is readily distinguishable: heartbeat—high frequency, low amplitude (~150 μV); amnionic contractions—low frequency and rhythmic; body movement—fast and arrhythmic. Although our experience with subjects younger than 6 days is limited, it appears that a progressive decrease in signal amplitude makes recording increasingly difficult.

The verbal characterizations of activity corresponded quite well to the polygraph record. Reports of large movements were concomitant with large deflections and reports of smaller movements corresponded to smaller deflections (see Figure 1). This relation between the magnitude of movement and the size of deflections produced is not surprising given the principle on which the technique is based. As in the case of the electrocardiograph and electromyograph, muscle activity results in an ionic concentration gradient which is recorded as a potential difference between the two electrodes (Berne & Levy, 1972). Contraction of a greater number of muscle fibers, then, would result in greater ionic flux and a larger potential being recorded.

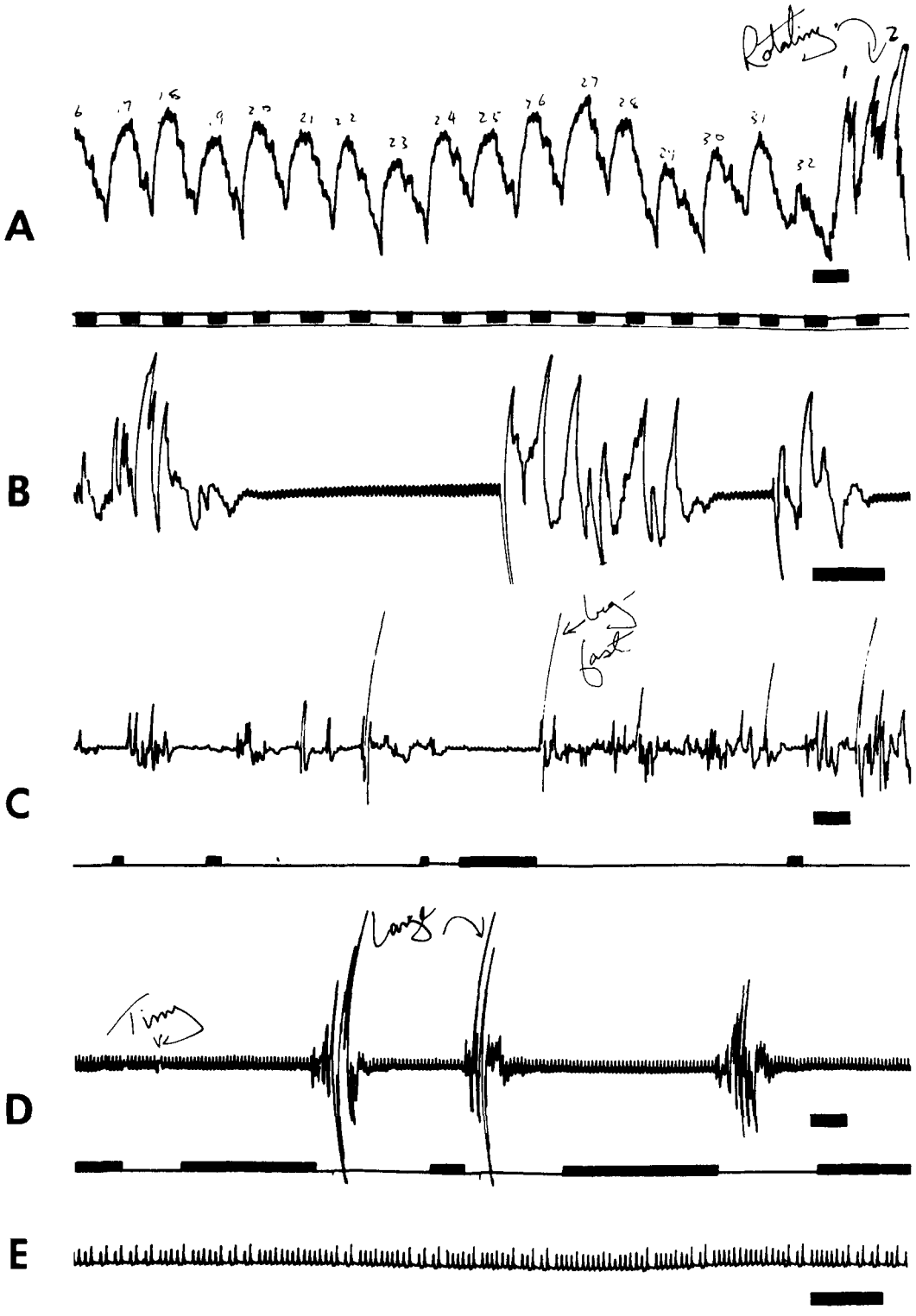
A
B
C
D
E

Table 1
Percentage of Inactivity Observations During Which Deflections of Given Magnitudes Were Recorded

Magnitude of deflection	Age (in days)/Observation time (in min) 12/12	14/12	15/31	17/8	18/12	Deflections recorded/total observations	Cumulative %
≥6× heartbeat	0	5	0	0	0	1/215 total obs.	.5
≥3× heartbeat	0	23	7	0	0	9/215 total obs.	4
≥2× heartbeat	5	32	17	2	7	21/215 total obs.	10
≥1.5× heartbeat[a]	10	—	—	4	13	10/141 total obs.	7

[a] When reliably measured.

Very few preparations yielded unusable recordings, i.e., recordings that did not show heartbeat or any but the largest body movements. Such recordings also had a noise level two to four times greater than normal. One consistent feature of these preparations was a high resistance, greater than 50 kΩ, across the electrodes. Normal resistance was 20–30 kΩ. We suspect that the high resistance resulted from one or both of the electrodes' being between the shell and inner shell membrane rather than in the albumin of the egg. Approximately 10% of the preparations were unusable in terms of the above criteria.

Quantitative findings. Quantitative data were gathered in an attempt to answer three questions: (a) What is the survival rate of embryos implanted with electrodes? (b) Does this technique indicate movement when none is perceived visually? (c) Does this technique manifest all visually observed movements?

The hatch rate of embryos implanted early in development not only is important for chronic application of the technique but also provides some measure of implantation's injurious effects. For the purpose of estimating survival rate, 20 embryos were implanted with electrodes on Days 7–8 and allowed to mature to hatching (Day 21). Twenty fertile control eggs from the same batch were incubated under identical conditions. Of the 20 controls 14 hatched, and 13 implanted subjects did so. The hatch rate of implanted embryos, then, was 92% of the control hatch rate. As our experience indicates that embryos older than 11 days virtually never die subsequent to implantation, the data presented estimate the minimum survival rate to be expected.

To determine whether the electronic method indicated movement when none was observed visually, we analyzed the records as follows: First, only observer marks (signifying inactivity) 2 sec or longer were scored; shorter marks typically resulted from the observer's beginning to mark an inactivity phase just as the embryo moved, and the correspondence of these short marks to polygraph deflections or their absence was difficult to determine objectively. Each of the marks that were 2 sec in length or longer was checked to determine whether at any time during the corresponding period there was a pen deflection equal to or greater than five times (5×) the deflection caused by the heartbeat of the embryo. The number of such incidents was totaled, and the same procedure was carried out with 3× and 2× heartbeat criteria. If the amplitude of heartbeat was large enough that 1.5×

Figure 1. Examples of polygraph recordings. (Subjective observer comments [A, C, D] were noted on the traces at the time of recording. Solid bar on the right of each trace represents 3 sec. A: 7-day embryo. Observer marks below polygraph trace indicate amnionic contractions. Note that embryonic motility, as observed visually, produced irregular, high-frequency deflections which can be discriminated from the rhythmic low-frequency pattern of amnionic contractions. B: 10-day embryo. Heartbeat is clearly recorded as regular, low-amplitude oscillations. C: 15-day embryo. Observer marks below trace indicate inactivity. D: 18-day embryo. Observer marks indicate inactivity. Heartbeat is clearly recorded. E: Heartbeat of 18-day embryo approximately 10 min after Nembutal injection. Body movement has ceased and heartbeat is noticeably irregular.)

Table 2
Percentage of Inactivity Periods Scored from Polygraph and Not Marked by Observer

Length of inactivity period	Age (in days)					Periods not marked/ total periods	Cumulative %
	12	14	15	17	18		
≥3 sec	12	6	14	0	36	43/244	18
≥5 sec	0	0	3	0	12	7/121	6

heartbeat could be reliably measured, that criterion was also applied. The results of this analysis are summarized in Table 1.

The data presented in Table 1 show that the number of discrepancies is extremely small when only large deflections are considered and that it increases as smaller deflections are scored.[1] It is important to note that the observer was never able to see the entire embryo and that at times only a very small portion was clearly visible. This fact, together with the noted increase in errors as deflection size decreased, strongly suggests that errors resulted from the inability of the observer to see many of the small movements.

An alternate explanation, that some of the smaller deflections were not caused by movement of the embryo but resulted from noise in the recording system or some other cause, is refuted by results obtained from two types of controls. First, embryos injected with an overdose of Nembutal during the recording session stopped moving within 1 min of the injection, and at the same time, pen deflections other than heartbeat ceased. Heartbeat was recorded for up to 45 min after the injection, gradually becoming less frequent (less than 1 beat/sec) and more irregular (see Figure 1E). The amplitude of the heartbeat record diminished during that time, finally decreasing to background noise levels. The second type of control, infertile eggs, produced no deflection.

In an attempt to resolve the third question, i.e., whether this technique manifests all movements, we first classified continuous segments of the polygraph record as either activity or inactivity periods, depending on whether they contained one or more deflections equal to or greater than the movement criterion. That criterion was 1.5× heartbeat amplitude or, if that level was not reliably measurable, 2× heartbeat. We then calculated for each observational session the percentage of inactivity periods lasting 3 sec or longer that were not marked as such by the observer. The same procedure was carried out on inactivity periods lasting 5 sec or longer. The results of this analysis are summarized in Table 2.

The data presented in Table 2 show that an absence of deflection other than heartbeat is highly correlated with the visual observation of inactivity. The fact that the correlation increases with the length of the quiescent period being scored suggests that errors are largely the result of the time lag between the cessation of movement and the beginning of inactivity observations. This lag was sometimes 5 sec or longer (see Figure 1) and resulted from the observer's waiting to see whether the subject was entering a prolonged inactivity phase or was simply momentarily inactive between movements.

Since the movement criterion used in this analysis was a deflection 1.5× or 2× heartbeat, periods during which small movements resulted in a break in the heartbeat record were scored as inactivity phases. If the observer did not mark these as such (see Figure 1D), an error was scored. As this occurred several times in the case of one of the 18-day subjects, the number of errors for that group

[1]This increase in errors does not parallel the increase in the frequency of smaller movements. One-minute polygraph record samples from a 15-day and an 18-day embryo were analyzed to determine the frequency of movements of various magnitudes. Results from the 15-day embryo were as follows: 5× heartbeat—25%; 3×—34%; 2×—40% (total number of movements counted = 114). Results from the 18-day embryo were as follows: 5×—15%; 3×—24%; 2×—30%; 1.5×—31% (total number of movements = 103). These differences in frequency are several times smaller than the differences in error rate reported in Table 1.

is more than twice what it would otherwise have been.

Although these data support the contention that this technique manifests all movement, an absolute statement to that effect is impossible as there is no control for observer fallibility and variability. Certainly, however, the sensitivity, speed, and attentiveness of the electronic technique are superior to those of the human observer.

The one case in which activity rather than inactivity was marked, that of a 7-day subject, provides further evidence of the technique's sensitivity and accuracy. During most of the observation period the amnion was moving in a series of rhythmic contractions. The period of these contractions (approximately 1 cycle/3 sec) was long enough that each could be marked by the observer. A section of the resulting polygraph record is presented in Figure 1A. With the observer marks covered from view, 191 oscillations were counted during a 10-min period. During the same period there were 193 observer marks.

There are four apparent advantages of this technique over visual observation as a method of recording motility of avian embryos. First, this technique involves a minimal manipulation of the embryo's environment. Whereas we found the hatch rate of implanted subjects to be more than 90% of the control hatch rate, Oppenheim et al. (1973) found only a 50% hatch rate, relative to controls, with windowed eggs. This advantage is particularly important for long-term studies and those that involve testing of hatchlings that were also tested as embryos. The second advantage of this technique is its accuracy. Fewer movements are missed with this method, and also, the reaction time of the observer is eliminated as a factor. Third, this technique provides a standardized method for defining what will be considered movement and for quantifying movements in a particular time period. Finally, the described technique allows heartbeat and movement to be continuously recorded for days at a time, whereas accurate visual observation can be maintained for only a period of minutes (cf. Vince, Reader, & Tolhurst, 1976). The most serious shortcoming of this technique is that specific behaviors, such as movement of a particular body part, cannot typically be differentiated.

Because of the advantages of an electronic method of recording behavior of avian embryos, there have been in the last several years three published reports of attempts to develop such a technique (Kovach, Callies, & Hartzell, 1970; Salter, 1966; Tremor & Rogallo, 1970). Although Sedlacek (1976) used the method developed by Kovach et al. with embryos 10–12 days old, the sensitivity of those previously reported techniques is such that they are not generally applicable to embryos younger than 17 days (Oppenheim et al., 1973). It is surprising that the technique reported by McCafferty et al. (1965) has not previously been adapted to this purpose. The data presented here show that our modification of that method provides an effective means of recording embryonic motility over at least the last two thirds of incubation.

Experiment 2

We investigated the behavioral responses of chick embryos to acoustic stimulation, employing the recording technique described in Experiment 1. This study was designed to provide normative behavioral data regarding (a) the developmental stages at which embryos will respond to an acoustic stimulus, (b) the basic characteristics of the responses, and (c) the manner in which the time of onset of responsiveness and/or the type of response elicited is related to the frequency of the tone stimulus. Embryos, Stages 39–43, were presented with a pure-tone stimulus of 400, 700, or 1400 Hz, and movement was recorded during and after stimulation.

Method

Subjects. Two hundred six White Leghorn (*Gallus domesticus*) chicken embryos were used in this study. Eggs were obtained from a commercial breeder and incubated as described in Experiment 1. Subjects were tested between 13 and 18 days of incubation (the first 24 hr of incubation was considered Day 0).

Apparatus and procedure. Recording procedure. Movement of the embryos was recorded by the electronic method described in Experiment 1. Response histograms were compiled by leading the output of the

polygraph to an Ortec (Model 4620/4621) time histogram analyzer. The voltage discriminator of the histogram analyzer was set to a level approximately twice that generated by the subject's heartbeat. Thus, a single count was recorded each time a pen deflection exceeded that movement criterion. Bin width was .5 sec and each histogram consisted of 127 bins (63.5 sec total).

Stimulus presentation. Each stimulus consisted of a series of three pure-tone bursts of 800-msec duration, with rise and fall times of 10 msec. As the interburst interval was 200 msec, the total duration of the stimulus was 2.8 sec. Tones of 400, 700, and 1400 Hz were used.

Tones were generated by a Wavetek (Model 134) function generator and presented by means of a Wisconsin-type electronic switch, a Hewlett-Packard (Model 350D) attenuator, a Southwest Technical Products (Model .01) 60-W amplifier, and a Realistic (Model 40-1238) loudspeaker. The frequency and intensity of the sound at the egg were measured with a General Radio (Model 1962-9601) electret condenser microphone, a GR (Model 1560-P42) preamplifier, and a GR (Model 1900-A) wave analyzer. In all cases the intensity of the stimulus at the egg was 115 dB (SPL) and that of harmonics was less than 80 dB. Background noise level in the chamber was 60 dB.

Testing procedure. Subjects were allowed at least 15-min adaptation time after being implanted with electrodes and placed in the recording chamber. Five test trials were given to each subject, a new trial being initiated every 6 min independent of the subject's behavioral state. Stimulus presentation was concomitant with triggering of the histogram analyzer, and within subjects, the histogram resulting from each trial was automatically summed with those from preceding trials.

As the stimulus lasted a total of 2.8 sec and the histogram bin width was .5 sec, the number of counts recorded in the first six bins of the histogram represented the amount of movement during the stimulus.

In addition to the five test trials, five mock trials were presented, two before testing and three after. Mock trials were identical to test trials with the exception that there was no sound stimulus.

Each subject was tested only once and with only one of the three stimulus frequencies. Subjects were killed for staging immediately after testing. Staging was performed according to the procedure of Hamburger and Hamilton (1951). Stages corresponded to days of incubation in the following manner: State 39—ca. 13–14 days; Stage 40—ca. 14–15 days; Stage 41—ca. 15–16 days; Stage 42—ca. 16–17 days; and Stage 43—ca. 17–18 days.

Results

The first level of analysis involved gaining a qualitative impression of whether, within a given age group and stimulus condition, the tone stimulus resulted in an increase or decrease in movement during its presentation.

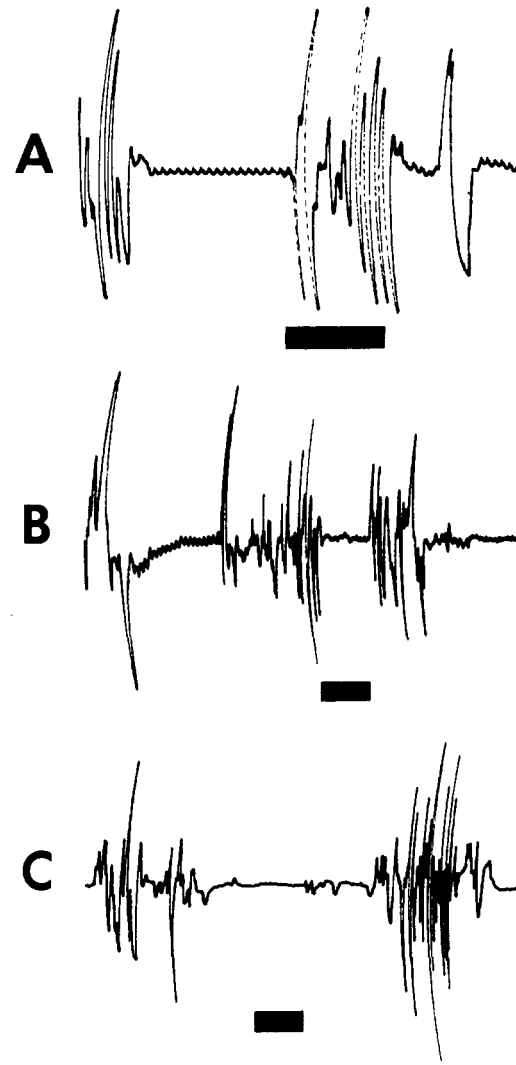

Figure 2. Examples of response types. (Solid bar indicates stimulus period. A: Excitation. Stage 43 [400 Hz]. B: Inhibition. Stage 40 [1400 Hz]. C: No response. Stage 39 [1400 Hz].)

Three examples of recordings obtained during stimulus trials are presented in Figure 2: Panel A shows an excitatory response, B an inhibition of movement, and C no response. Although we saw many clear robust responses, more often the high level of ongoing embryonic activity and its variability over time made it difficult to distinguish true responses from nonevoked activity or quiescence. To improve the response-to-background ratio, we averaged histograms from subjects within the same

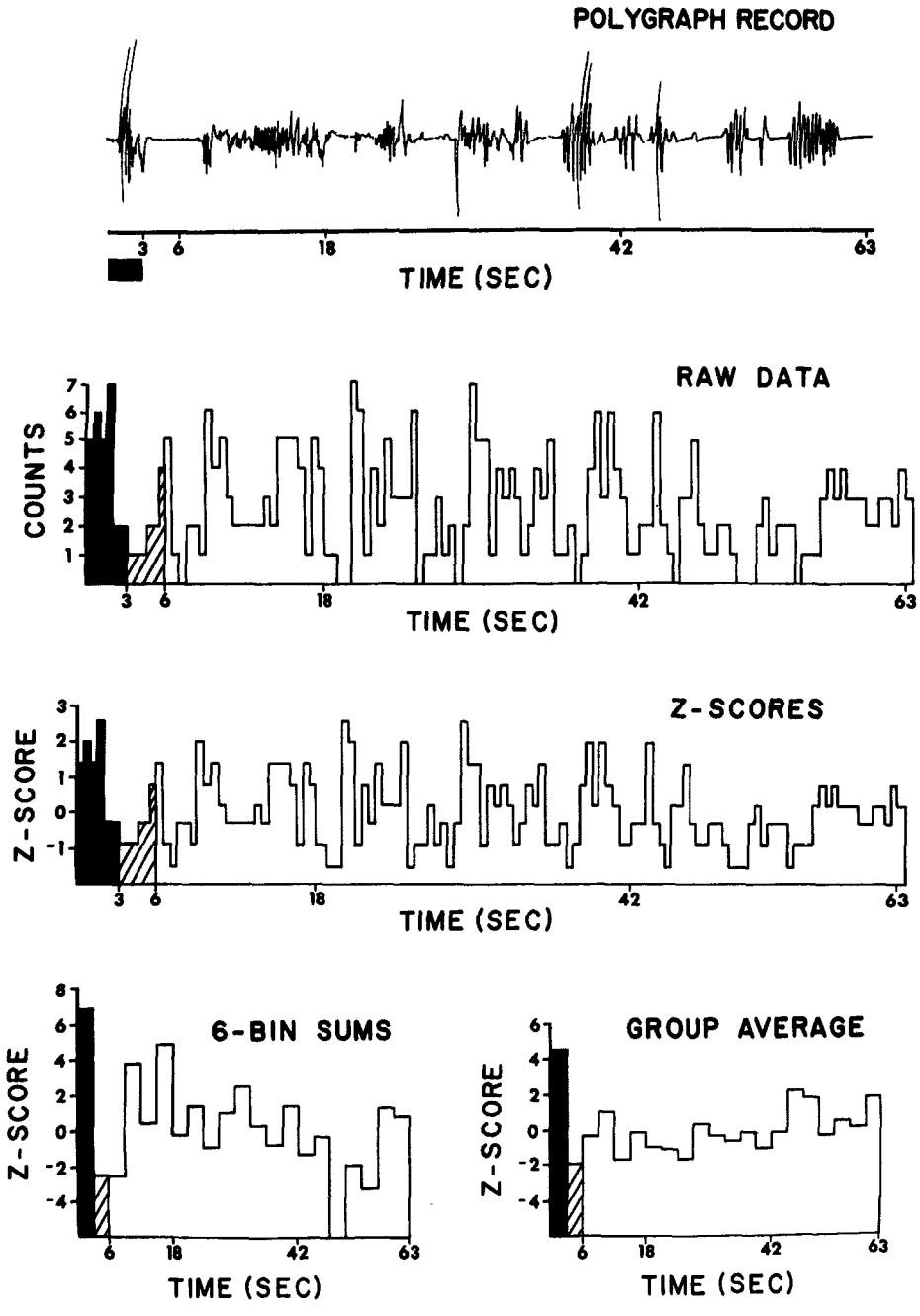

Figure 3. Data reduction procedure. (Data were taken from Embryo H74E113, a member of the Stage 43 1400 Hz group. The polygraph record shows the subject's output during one of the five test trials [solid bar on left indicates stimulus period]. The time histogram analyzer summed over trials the counts generated in each .5-sec time bin, producing the raw data histogram [solid bins indicate the 3-sec stimulus period; striped bins indicate the 3-sec poststimulus period]. The count total in each bin was then converted to a *z* score on the basis of the mean and standard deviation of the last 107 bins. A histogram of 6-bin sums was then compiled by adding the *z* scores within successive groups of six bins, the 127th bin being dropped. That histogram was averaged with those of all Stage 42 [1400 Hz] subjects to produce the group average.)

age and stimulus group. The manner in which data were prepared prior to averaging is described below and illustrated in Figure 3.

The initial step in preparing the data was to normalize the bin activity scores for each subject. If this were not done, subjects with a high baseline activity level would have a

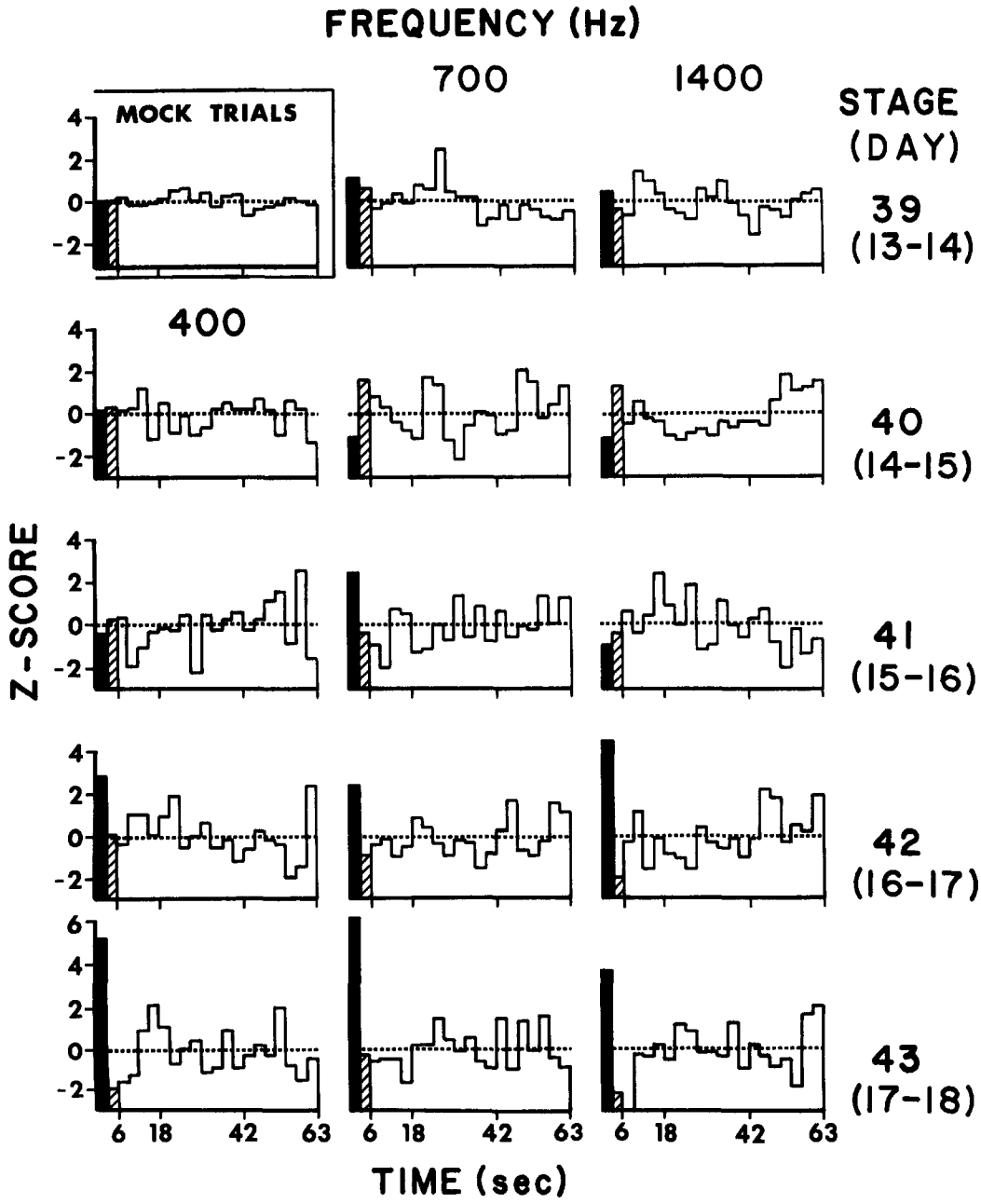

Figure 4. Group averages. (Solid bar represents the activity level [in z scores] during the 3-sec stimulus period; the diagonally striped bar represents the activity level during the 3-sec period immediately following the stimulus; the activity levels during each succeeding 3-sec period are indicated by the open portion of each histogram. Dotted line in each histogram is at mean baseline activity level [$z = 0$]. Average of all mock trials is shown in upper left.)

greater effect on the final average than would less active subjects. Therefore, we converted all 127 bin scores for each subject to z scores on the basis of the mean and standard deviation of that subject's last 107 bin scores. The first 20 bins, representing the first 10 sec after stimulus onset, were not included so as to avoid confounding the measure of baseline activity level with the immediate effects of the stimulus. Next, 21 successive groups of six z scores were summed, the first group of six .5-sec bin scores corresponding to the 3-sec stimulus period. This provided a measure of the total activity during the stimulus period and in the twenty 3-sec poststimulus periods (Bin 127 was dropped). These data were then averaged with those of all subjects of the same age and stimulus condition. It is important to note that although group data are graphically represented in their averaged form, the data from individual subjects within groups were retained for statistical analysis (see below). The final averages for each group are presented in Figure 4.

Data generated in mock trials were reduced in exactly the manner just described. However, as we were primarily interested in determining whether the triggering or recording procedure resulted in an artifactual change in baseline noise, mock-trial data were averaged over all stage-frequency groups. The resulting histogram is included in Figure 4.

Inspection of Figure 4 reveals the following: (a) Mock-trial data evidenced no triggering or recording artifact; (b) three groups, Stage 40—700 Hz, Stage 40—1400 Hz, and Stage 41—1400 Hz, show low levels of activity during the tone stimulus; (c) several groups, Stage 41—700 Hz, Stage 42—400, 700 and 1400 Hz, and Stage 43—400, 700, and 1400 Hz, show high levels of activity during the stimulus; and (d) there is a tendency for the activity level during the immediate 3-sec poststimulus period to be opposite that during the stimulus period; postinhibition excitation is seen in the Stage 40—700 Hz and Stage 40—1400 Hz groups, and postexcitation inhibition is most pronounced in the Stage 42—700 Hz, Stage 42—1400 Hz, Stage 43—400 Hz, and Stage 43—1400 Hz groups.

To test these judgments, we performed a repeated measures within-subjects analysis of variance. The data used in this analysis were in the form of the 21 six-bin z-score sums for each subject (see Figure 3). Two statistical contrasts were implied by this design: (a) The activity level during the

Table 3
Results of Contrasts of Activity Level During the Stimulus with 3-Sec and 60-Sec Poststimulus Periods

		>Stimulus frequency											
		400 Hz				700 Hz				1400 Hz			
Stage	Contrast	*n*	*F*	*df*	*p*<	*n*	*F*	*df*	*p*<	*n*	*F*	*df*	*p*<
39	1st vs. 2nd bin[a]		—		—		.21		*ns*		.72		*ns*
		—		—		15		1, 14		14		1, 13	
	1st vs. all[b]		—		—		1.34		*ns*		.3		*ns*
40	1st vs. 2nd bin		.03		*ns*		8.0		.05		10.48		.01
		15		1, 14		15		1, 14		16		1, 15	
	1st vs. all		.02		*ns*		2.46		*ns*		2.12		*ns*
41	1st vs. 2nd bin		.37		*ns*		4.22		.06		.3		*ns*
		15		1, 14		15		1, 14		16		1, 15	
	1st vs. all		.12		*ns*		3.87		*ns*		.7		*ns*
42	1st vs. 2nd bin		3.86		*ns*		5.75		.05		8.34		.05
		15		1, 14		14		1, 13		15		1, 14	
	1st vs. all		2.84		*ns*		6.08		.05		4.65		.05
43	1st vs. 2nd bin		27.95		.01		37.0		.01		17.73		.01
		16		1, 15		11		1, 10		15		1, 14	
	1st vs. all		27.63		.01		70.3		.01		7.54		.05

[a] Contrast of activity level during 3-sec stimulus block with first 3-sec poststimulus block.
[b] Contrast of activity level during 3-sec stimulus block with following twenty 3-sec blocks.

stimulus block contrasted with the activity level during the immediate poststimulus block, and (b) the stimulus-block activity level contrasted with that during all 20 poststimulus blocks. These contrasts were performed and the results are presented in Table 3.

The first reliable responses were recorded from Stage 40 (14–15 days) subjects presented with either 700- or 1400-Hz tones. In both those groups the level of activity during the stimulus period was reliably lower ($p < .05$) than that during the first 3-sec poststimulus period. At Stage 41 statistically significant results were not achieved, although the 700-Hz tone elicited an increase in movement which fell just short of significance ($p < .06$). Stage 42 (16–17 days) subjects responded to 700 and 1400 Hz with an increase in motility (both contrasts, $p < .05$), and by Stage 43 (17–18 days) all three tones were effective in evoking movement.

General Discussion

This study yielded information about three aspects of the development of behavioral responsiveness to auditory stimuli: (a) the developmental stage at which responses are first evidenced, (b) the relation between response onset time and the frequency of the tone stimulus, and (c) the type of response elicited at different developmental times. These subjects are discussed in turn below.

Onset of Responsiveness

Although previously there has been no clear indication of the age at which behavioral responses to acoustic stimulation can first be obtained, evidence has accumulated over the last 40 yr to show that the chick auditory system becomes functional well before hatching. Gos (1935), using a bell as the conditioned stimulus, presented evidence of conditioning in 17-day-old chick embryos. Similar results were obtained by Sedlacek (1964) with 16-day embryos and a 3000-Hz tone as the CS. At 15 days, click stimuli appear to alter the pattern of embryonic motility (Vince et al., 1976), although it is uncertain whether the stimulus is acoustic or vibratory. Grier, Counter, and Shearer (1967) found that embryos exposed to a 200-Hz tone from Day 12 to Day 18 of incubation (hatching occurs on Day 21) preferentially approached that stimulus after hatching. Two investigators, Hunt (1949) and Sviderskaya (1967), reported responses in 14-day and 5-day chick embryos, respectively. The procedure used by Hunt, however, is unclear and his results are therefore difficult to interpret. Sviderskaya's findings are questionable because the brain stem auditory nuclei (n. magnocellularis and n. laminaris) are not even recognizable until Day 7 (Rubel et al., 1976) and the hair cells of the cochlea do not appear until about Day 8 (Rebollo & Casas, 1964).

We were first able to obtain reliable responses on Days 14–15 (Stage 40). It is certainly possible that with a different response measure or auditory stimulus, responses might be evoked earlier in development. However, it is important to examine these behavioral data in the context of what is known of the physiological and morphological development of the auditory system. Doing so not only provides some estimate of the reasonableness of the behavioral findings (cf. Sviderskaya, 1967) but also is essential for the attainment of some integrated notion of sensory development. In terms of receptor development, Vanzulli and Garcia-Austt (1963) recorded cochlear microphonics as early as Day 13, and histologically, the cochlea appears mature by about Day 15 (Knowlton, 1967; Rebollo & Casas, 1964). Saunders, Coles and Gates (1973) were able to record evoked potentials from the 8th nerve of embryos 12–13 days old. At that age, tones of 100–1500 Hz were effective stimuli. The brain stem auditory nuclei magnocellularis and laminaris have been shown by Rubel et al. (1976) and Rodriguez and Rebollo (1966) to be recognizable as early as Day 7. More important, Rubel et al. found that the period of morphogenetic cell death in those nuclei is over by Day 14 and that about Day 15 the nuclei begin to increase significantly in volume. Neuropil and cell body regions both increase in volume, which suggests the elaboration of terminal branches and/or receptor surfaces as well as metabolic changes within the cells.

Although further confirmation by electrophysiological or biochemical methods is lacking, the available physiological and morphological data suggest that at least the receptor and brain stem elements of the chick auditory system may become functional as early as Days 12–13 and probably not later than Day 15. Our finding that the onset of behavioral responsiveness occurs on Days 14–15 is consonant with that conclusion. Furthermore, it is interesting to note that behavioral responses can be evoked quite soon after the onset of function as defined by physiological measures. While it is known that the primary central auditory pathways are established prior to cochlear function (Ramón y Cajal, 1960; Rubel, 1978), this study indicates that the functional link between sensory and motor elements is established by the time of or very soon after the onset of sensory function.

Importance of Frequency as Stimulus Variable

In terms of the frequency range audible to hatchling chickens—about 100–4000 Hz (Rubel & Parks, 1975)—the stimuli used in this study (400, 700, and 1400 Hz) are low to middle frequencies. However, within the relatively narrow frequency range used, there were notable differences in the effectiveness of the three tones in evoking behavioral responses. Reliable responses to the 700- and 1400-Hz tones were recorded at Stage 40, but 400 Hz did not elicit such responses until Stage 43. These results indicate that the chick is first responsive to frequencies in the low to middle ranges and that responsiveness to the lowest frequencies develops later.

Although we did not test the effectiveness of high frequencies, behavioral studies suggest that high frequencies, like very low frequencies, would become effective stimuli relatively later in development. Mice (Mikaelian & Ruben, 1965), opossums (Larsell, McCrady, & Larsell, 1944), rabbits, minks, cats, and dogs (Foss & Flottorp, 1974) all respond earliest to low to mid-range frequencies and later to progressively higher and lower frequencies. Physiological studies of the avian cochlea (Vanzulli & Garcia-Austt, 1963), 8th nerve (Saunders, Coles, & Gates, 1973), and cochlear nuclei (Konishi, 1973) also support the expectation that high frequencies would evoke responses later in development. However, those investigations found that responses were first evoked by frequencies below 500 Hz, which suggests that of our three test frequencies (400, 700, and 1400 Hz), 400 Hz would be effective earliest in development. At present we are unable to explain the discrepancy between our findings and that expectation. It is possible that the discrepancy is related to sound transmission into the egg (i.e., selective suppression of low frequencies). Whereas the preparations we used were intact, physiological investigations require removal of the shell and exposure of the ear to airborne sounds. In addition, we would point out that behavioral responses certainly involve levels of processing beyond that of the receptor and it may be naive to expect an absolute correlation between behavioral and physiological measures (cf. Raslear, 1974).

Inhibition Versus Excitation

Although we have used the terms "excitation" and "inhibition" to describe the two basic response types, characterization of response histogram data in those terms might seem presumptuous. The activity level recorded during the stimulus could represent the baseline level of activity, while the actual response of the subjects was to increase or decrease their motility during the poststimulus minute. However, a comparison of the baseline activity levels (mean of last 107 bin scores) recorded during mock and stimulus trials revealed no consistent differences within Age × Stimulus Groups, which indicates that the stimulus did not affect our measure of those levels. For that reason, in addition to the response patterns observed during the course of this study (see Figure 2), we think that the use of the terms "excitation" and "inhibition" is justified.

We have no ready explanation for the inhibitory response seen at Stage 40. However, Gottlieb (1971) reported a *decrease* in bill clapping in 22- to 23-day mallard embryos in response to the maternal call of that species. Testing prior to that time revealed

no consistent response, and on subsequent days the response was an *increase* in bill clapping. In addition, Impekoven and Gold (1973) observed a *decrease* in movement when the "kow" parental call was presented to 15-day laughing gull embryos. No response to that stimulus was recorded at later stages of development, and younger embryos were not tested. While these studies provide intriguing parallels to our results, differences in stimuli and the response measures used make any generalization difficult.

The fact that the response characteristics invert between Stage 40 and Stage 42 is probably responsible for the failure to obtain statistically significant responses at Stage 41. That stage appears to be one of transition; the developmental variation between subjects results in some showing excitation and others inhibition. It is important, however, to note the difference between the responses to 700 and 1400 Hz at Stage 41. Although, as a group, subjects still showed some inhibition of movement in response to 1400 Hz, the 700-Hz tone resulted predominantly in excitation ($p < .06$, see Table 3). This suggests that 700 Hz is capable of evoking an increase in movement slightly earlier in development than is 1400 Hz. The fact that we did not record a correspondingly earlier inhibitory response to 700 Hz may be due to the limited resolving capabilities of the experimental design. Alternatively, it may reflect actual differences in the sensory processes that lead to inhibition rather than excitation of movement; a change in the relation between frequency and developmental onset time of a given response type would suggest that the sensory mechanisms mediating the two types of responses are different.

A second possibility is that the shift from inhibition to excitation results from ontogenetic changes in neural systems mediating the motor aspects of the response. This possibility is related to a more general question regarding the developmental primacy of supraspinal restraints on embryonic activity (Oppenheim & Reitzel, 1975). In the chick embryo, Oppenheim (1975) demonstrated the existence of supraspinal influences on embryonic motility by 9–10 days of incubation. Further, spinal embryos of that age fail to show normal increases in activity following the application of strychnine, which suggests that the supraspinal input may have an inhibitory effect on somatic motility. Although supraspinal influences that inhibit movement apparently develop early (see reviews by Crain, 1974, and Oppenheim & Reitzel, 1975), it is as yet unclear whether they antedate excitatory influences. Although other interpretations are possible, the developmental sequence of response types that we reported here may provide evidence to that effect.

Importance of Prenatal Acoustic Stimulation

As discussed above, the auditory system becomes functional well before hatching in a variety of avian species (Gos, 1935; Gottlieb, 1971; Grier et al., 1967; Impekoven & Gold, 1973; Sedlacek, 1964). Several ethologically oriented investigations (Gottlieb, 1975a, 1975b, 1975c; Impekoven, 1971; Tschanz, 1968) have shown that certain prenatal acoustic stimuli play a major role in the development of adaptive auditory discrimination abilities, such as parent or species recognition. Further, Kerr, Ostapoff, and Rubel (Note 1) found that the normal developmental narrowing of frequency generalization functions in chicks (see Rubel & Rosenthal, 1975) is retarded by perinatal auditory deprivation, thus demonstrating a facilitative effect of stimulation on a primary feature of auditory perceptual development.

With regard to the present study, we have described three aspects of the development of behavioral responsiveness to simple tonal stimuli: (a) the stage at which a behavioral response is first elicited, (b) the sequence in which the three test frequencies become effective stimuli, and (c) the type of response elicited by each frequency initially and at subsequent stages of prenatal development. The first two types of information certainly reflect fundamental features of the development of audition. Regarding the type of response elicited, it is uncertain at present whether this is determined at the sensory or motor level. In any case, it is not known whether, or to what degree, any or all of these

basic developmental features can be influenced by discrete environmental stimulation. Given the descriptive foundation provided by the present study, it is hoped that experimental investigation of that question will prove productive.

Reference Note

1. Kerr, L. M., Ostapoff, M., & Rubel, E. W. *The influence of auditory experience on the ontogeny of frequency generalization in the chicken.* Manuscript in preparation, 1977.

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