Exposing avian embryos to light affects post-hatch anti-predator fear responses
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ABSTRACT

Environmental stimuli present during incubation can impact the behavior of birds post-hatch. To determine the effect of exposing broiler chicken embryos to light on fear-related behavior post-hatch, we conducted two experiments in which we incubated eggs under various light schedules, and then measured fear responses when the chickens (N = 720) were 3-6 wk of age. In Expt. 1, the incubation photoperiods were 0L:24D, 12L:12D, and 24L:0D, and tonic immobility (TI) and inversion (INV) tests were administered. In Expt. 2, the incubation photoperiods were 0L:24D, 1L:23D, 6L:18D, and 12L:12D; and an approach test (APPR) and an emergence (EMRG) test were administered in addition to TI and INV. In Expt. 1, both 12L:12D and 24L:0D had shorter latencies to right during TI (213.5 ± 23.7 and 231.8 ± 24.2 sec, respectively) than 0L:24D (305.5 ± 26.1 sec) and also wing flapped less intensely during INV (12L:12D 5.0 ± 0.1 wing flaps; 24L:0D 5.4 ± 0.2) than 0L:24D (5.7 ± 0.1). In Expt. 2, the 12L:12D birds once again had shorter latencies to right during TI (120.0 ± 16.5 sec) and wing flapped less intensely during INV (4.7 ± 0.1 wing flaps) than 0L:24D (201.2 ± 24.9 and 5.5 ± 0.1 , respectively). They also had shorter latencies to exit the dark box in EMRG (28.9 ± 3.3 seconds), and were less active ($28 \pm 2\%$), vocalized less (178.8 ± 9.3 times/3min) and spent more time closer to the observer during APPR ($63 \pm 3\%$) than 0L:12D (42.9 ± 5.0 seconds, $35 \pm 3\%$, 211.2 ± 10.4 times/3 min, 51 ± 3). The 1L:23D and 6L:18D showed some reductions in fearfulness compared to 0L:24D, but these were not consistent across tests. The 6L:18D and 12L:12D birds demonstrated lateralization in the direction to leave the box in EMRG, whereas 1L:23D and 0L:12D exited left or right at chance levels. The results of these experiments indicate that providing at 12 hours of light stimulation daily g

embryogenesis results in long-term reductions in fearfulness as measured by multiple tests, and that this may be related to cerebral lateralization. In conjunction with other research, these findings show that light exposure during embryogenesis has important implications for behavioral phenotypes and welfare in chickens.

Keywords: light, avian, embryo, fear, incubation, lateralization

1. Introduction

Avian behavioral phenotypes are influenced by both the internal and external environment during embryogenesis. Maternal deposition of androgens into the eggs can alter offspring phenotypes (e.g. related to boldness or alertness) such that the offspring are better suited for current environmental conditions (Groothuis et al., 2005). Avian embryos also respond to external stimuli such as olfactory, auditory and photoperiodic cues (Reed and Clark, 2011). For example, exposure to species specific call during embryonic development is important for post-hatch species recognition in some avian species (Gottlieb, 1976, 1985). Olfactory stimulation can also shape later behavior. Bertin et al. (2010) observed that chickens exposed to odors during incubation preferred food bearing those odors post-hatch.

Photoperiodic cues are particularly important factors influencing post-hatch phenotypes. Embryos receive regular brief light exposures when their parent(s) leave the nest to feed (Buschmann et al., 2006; Mrosovsky and Sherry, 1980) or respond to distress calls from the embryos by rising to turn the eggs (Rogers, 1996). This light can penetrate the eggshell and reach the embryo where it is sensed either by developing retinal cells or the pineal gland (Cooper et al., 2011), resulting in melatonin synthesis. Melatonin affects embryonic growth rates as well as the development of the visual, skeletal and immune systems (see Reed and Clark, 2011).

Prenatal light exposure has also been shown to affect post-hatch behavior. Domestic fowl chicks exposed to light during incubation have a different diurnal rhythm of feeding activity than dark-incubated chicks (Archer et al., 2009), and also differ from dark-incubated chicks in terms of discrimination learning ability (Rogers, 1990), memory

retention for a passive avoidance task (Sui and Rose, 1997), and aspects of social behavior including social exploration, social recognition, and competitive success (Riedstra and Groothuis, 2004; Rogers and Workman, 1989). Chicks exposed to light during incubation also show more avoidance of an unfamiliar imprinting object than dark-incubated chicks during the first 12 hours post-hatch (Dimond, 1968).

The mechanisms underlying the behavioral effects of light stimulation during incubation in birds probably involve both melatonin effects on behavioral rhythms and the direct effect of embryonic light stimulation on brain development via lateralization of the thalamofugal and tectofugal visual pathways (Rogers 1982). The latter results in hemispheric specialization, with the left hemisphere specialized for visual discrimination tasks, food-searching and vocal production and recognition, while the right is specialized for situations that involve a strong negative affective component, such as fear or aggression (Phillips and Youngren, 1986; Rogers, 2008). It has been hypothesized that strong lateralization is adaptive in that it facilitates dual processing of sensory information, allowing individuals to efficiently perform two tasks simultaneously. This hypothesis has been tested in domestic fowl (Dharmaretnam and Rogers, 2005) using simultaneous presentations of food acquisition tasks and predator stimuli. These studies showed that lateralized light-incubated chicks were able to more efficiently obtain food while continuing to maintain vigilance than less lateralized individuals.

Despite the presumed adaptive value of the patterns of lateralization found in light-incubated chicks, there is actually limited evidence that the behaviors observed at young ages persist throughout development. Shifts in lateralization for fear behavior in domestic chicks have been reported to occur between 5 and 15 days of age post-hatch

(Andrew and Brennan, 1983), but the effects of providing light during incubation on lateralization and fear responses in older birds have not been evaluated.

Our previous studies suggest that providing light during incubation can have beneficial effects on the welfare of broiler chickens 3-6 weeks post-hatch (Archer et al., 2009; Archer and Mench, 2009), decreasing composite asymmetry, reducing the corticosterone response to crating stress, and enhancing antibody response to challenge. We conducted two experiments to evaluate whether providing light during incubation also had developmentally persistent effects on fearfulness. Since anti-predator responses have been shown to be the most reliable measures for assessing fear (Miller et al., 2005, 2006), we utilized four different fear tests involving anti-predator responses (inversion, tonic immobility, emergence, and approach). We also assessed the degree of laterality in light and dark-incubated chicks by determining the direction of movement in the emergence test.

2. Materials and methods

2.1. Animals and husbandry

We obtained fertilized chicken eggs (Cobb 500 strain) from a commercial hatchery, and randomly assigned them to be incubated under different lighting schedules. In Experiment 1 (N = 664 eggs) we used three lighting schedules: 0 hours of light and 24 hours of darkness (0L:24D), 24 hours of light and 0 hours of darkness (24L:0D), or 12 hours of light and 12 hours of darkness (12L:12D). In Experiment 2 (N = 1512 eggs) we used four lighting schedules: 0 hours of light and 24 hours of darkness (0L:24D), 1 hour of light and 23 hours of darkness (1L:23D), 6 hours of light and 18 hours of darkness

(6L:18D), or 12 hours of light and 12 hours of darkness (12L:12D). The lighting durations in Experiment 1 were used to determine if there was an effect of light at the extremes (constant or no light) and/or the mid-point. Experiment 2 used shorter lighting durations to determine if there was a minimal amount of light needed during incubation to obtain the results observed in Experiment 1. The intensity of light at the egg level within the incubators was 550 lux as measured with a photoreceptor sensor of a light meter (LT Lutron, model LX-100™; Das Distribution Inc., East Granby, CT). In Experiment 1 lighting was controlled manually by covering or uncovering the clear incubator top with cardboard to block any light entering, while in Experiment 2 each treatment was contained within a ventilated environmentally controlled light-tight box that allowed the photoperiod to be automatically controlled by timers. Temperature and humidity inside the incubators (Hovabator; G.Q.F. Manufacturing Co., Savannah, GA) were monitored to insure that the conditions in the different incubators were similar. We candled eggs (Cool-Lite tester, GQF, Savannah, GA) once a week and removed non-viable eggs. Each experiment was conducted in three trials, with three incubators per treatment per trial.

Following completion of hatch all chicks were moved to a housing room at the Hopkins Avian Research Facility at the University of California, Davis. They were managed according to the guidelines set forth in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 2010). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

In the housing room we placed chicks (N = 506 in Experiment 1, N = 1006 in Experiment 2) from each trial in one of nine pens (Experiment 1) or one of twelve pens (Experiment 2) by incubator, with treatments randomized by block (incubator) throughout the room. Each pen was 6.0 m² and was bedded with wood shavings (approximately 10 cm deep). Chicks were given access to feed and water *ad libitum* throughout the study. They were fed a pre-starter mash (23.21% crude protein, 3.10kcal/g metabolizable energy) for the first 3 weeks, and then Purina Mills Flock Raiser Sunfresh Crumble™ (St. Louis, MI; 20% protein, 3.00kcal/g metabolizable energy) for the remainder of the 6 week experiment.

The birds were raised under a 12L:12D photoperiod with a photophase light intensity of 250 lux. Light intensity was measured along a horizontal plane at 25cm above the floor with the photoreceptor sensor of the light pointed toward the light sources. There were no dawn-dusk transitions between the light and the dark phase of the photoperiod. Light intensity during the dark phase was 0 lux (complete darkness).

2.2. Fear measurements

We used tonic immobility (TI) and inversion (INV) tests to measure anti-predator responses in both experiments. Tonic immobility testing was carried out as described by Archer and Mench (2014) on 10 randomly selected birds (5 males and 5 females; determined by secondary sex characteristics which were confirmed at 5 weeks of age.) per pen when the birds were five weeks old. In brief, we placed birds on their backs in a wooden cradle and held them there for 15 seconds. If the bird righted before 10 seconds TI was re-induced up to three times. If the bird could not be induced in three attempts it

was scored as 0. We recorded latency to first head movement, latency to right, and number of induction attempts. The test was terminated in 600 seconds if a bird failed to right, and that bird was scored as 600.

When the birds were 42 days old we subjected the same ten birds that had been used for TI testing to an INV test, as described by Newberry and Blair (1993) and Archer and Mench (2014). We caught each bird and then inverted it by holding it by its legs with one hand until the bird ceased to wing flap, or for 30 seconds. We determined the duration of and number of wing flaps, the number of vocalizations, and the number of body curls from video recordings (Cannon, ZR900, Melville, NY, USA; 24 frames per second).

In Experiment 2 we administered two additional fear tests, the approach test (APPR) and the emergence test (EMRG), when the birds were three weeks of age. Ten randomly selected birds (5 males and 5 females) were selected per pen to be used in both of these tests and in the TI and INV tests that followed. Both tests were videotaped (Cannon, ZR900, Melville, NY, USA; 24 frames per second). For APPR we placed the bird in a 1m x 0.5m x 0.6m enclosure that had three solid sides made of wood and a front made of chicken wire. The observer (GSA) then immediately sat approximately 0.5m from the front of this enclosure, visible to the bird. The enclosure was painted black and marked with white paint into three equal areas and the bird's proximity to the observer, its activity (% of time moving), and the number of vocalizations given (distress calls) were recorded via video for three minutes after the bird was placed in the enclosure. The enclosure was cleaned between birds.

For EMRG we placed a bird in a wooden dark box (0.3 x 0.3m), via a hatch in the top of the box, for thirty seconds. We then removed the front partition of the box so that the bird could exit either left or right down one of two perpendicularly attached wooden alleyways (0.6m in length by 0.3m height, covered with chicken wire). Latency to exit the box was determined from video, with the camera placed out of the sight of the bird to limit potential disturbances that would affect emergence. Directionality (Left = -1, Right = 1) was recorded as a measure of laterality. Each bird was tested twice with a two day period between testing. An average directionality score significantly different from zero ($P < 0.05$) indicated that the directionality of exit was not at chance levels. The test apparatus was cleaned between birds.

2.3. Statistical analysis

We used a one-way ANOVA to analyze treatment effects for the TI, INV, and APPR tests, and a GLM with treatment, testing day, and their interaction to analyze the EMRG test. We performed LSD *post hoc* tests comparing each light treatment group to the controls (0L:24D) when we found significant differences. All data were tested to ensure that they met the assumptions of ANOVA (Shapiro-Wilk test for normality, Levene's test for homogeneity of variance); no transformations were required to meet these assumptions. All analyses were performed using SAS 9.2 for Windows (SAS Institute Inc.). We also used a T-test to determine whether directionality when exiting the dark box in the EMRG test differed from chance (mean = 0, $P < 0.05$).

3.0 Results

3.1. Tonic immobility

There was a significant effect of lighting duration during incubation on latency to right (Experiment 1: $F_{2, 177} = 4.2$, $P = 0.02$; Experiment 2: $F_{3, 336} = 4.37$, $P = 0.005$, Table 1). In Experiment 1, both the 12L:12D and 24L:0D birds had shorter latencies to right than the 0L:24D birds ($P = 0.007$ and $P = 0.03$, respectively). The 0L:24D birds had the longest latency to right in both experiments. Latency to first head movement and number of induction attempts did not differ between treatments ($P > 0.05$) in either experiment (Table 2).

3.2. Inversion test

The INV response also differed between treatments in both studies (Experiment 1: $F_{2, 173} = 5.87$, $P = 0.003$; Experiment 2: $F_{3, 336} = 8.35$, $P < 0.001$, Table 1). In Experiment 1, the 0L:24D birds had the greatest intensity of wing flapping. The 24L:0D and the 12L:12D birds wing flapped less intensely than the 0L:24D birds ($P = 0.05$, $P = 0.001$, respectively). In Experiment 2, the 0L:24D birds wing flapped more intensely than all other birds ($P < 0.01$). There were no differences between treatments in number of wing flaps, duration of wing flapping, percent of birds body curling, or % of birds vocalizing in either experiment (Table 2, $P > 0.05$).

3.3. Approach and Emergence Tests

Data from APPR and EMRG are presented in Figure 1. Only distress calls were given, and 12L:12D birds vocalized less ($P = 0.02$) than 0L:24D birds. They were also less active ($P = 0.05$), and spent more time in the area closest to the observer ($P = 0.02$) than the 0L:24D birds. Treatment also affected latency to emerge from the box ($F_{3, 336} = 4.24$, $P = 0.006$, Table 1), with the 12L:12D birds having a shorter ($P < 0.03$) latency to emerge than all other treatments. The 12L:12D and 6L:18D birds turned right (0.68 and

0.52 right, respectively) when exiting the box significantly more than expected by chance (chance score = 0, $P < 0.0001$), whereas the 1L:23D and 0L:24D birds turned left or right at chance levels ($P > 0.05$, 0.11 and 0.12 right, respectively). There was no interaction between treatment and testing day ($P > 0.05$).

4. Discussion

Our experiments indicate that light stimulation during incubation is important for reducing fearfulness in 3-6 week old broiler chickens. Providing at least 12 hours of light daily throughout incubation resulted in consistent decreases in fearfulness as evidenced by a decreased latency to right in the tonic immobility test, less intense wing flapping in the inversion test, less distress calling and more proximity to the observer in the approach test, and a shorter latency to leave the box in the emergence test. Given that chicken embryos are typically incubated commercially in complete darkness, these results have important implications for welfare, since excessive or prolonged fear can result in wasted energy, injuries, behavioral inhibition, reduced ability to adapt to change, delayed maturation, decreased growth and reproduction, and death (Jones, 1986). In general, our results are consistent with those of previous studies examining the effects of light stimulation during embryogenesis on fearfulness in domestic fowl. Light incubated chicks have been reported to give fewer distress calls and interrupt feeding behavior more in response to a simulated predator than chicks incubated in darkness (Dharmaretnam and Rogers, 2005; Wichman et al., 2009). They also have shorter latencies to approach novel stimuli (Adam and Dimond, 1971; Deng and Rogers, 2002). However, these previous studies were all conducted on chicks that were 3-9 days old and so could be measuring relatively short-term effects of incubation conditions. To our knowledge, no other studies

have examined whether light stimulation during incubation results in long-lasting effects on fearfulness. The persistency of these responses, as well as their consistency across four different fear tests involving human handling or proximity, have important practical implications. They suggest that providing appropriate light stimulation during incubation could potentially reduce the fear responses of broilers to caretakers and during hand-catching prior to slaughter in the commercial setting.

We measured the direction of exit in the emergence test to determine if there was a relationship between reduced fearfulness and laterality. There is growing evidence across taxa that responses associated with fear, and particularly anti-predator responses, are lateralized (Bisazza et al., 1998), with populations or individuals showing side biases for aspects of anti-predator behavior such as vigilant gaze or escape direction (reviewed in Leliveld et al., 2013).

The visual pathways in avian embryos are known to become lateralized as a result of the positioning of the embryo's head during the last four days of incubation (Rogers, 1982). At this time, the head turns so that the right eye is facing outwards towards the shell and thus receives light stimulation when the parents leave the nest. This stimulates the development of the neural pathways associated with that eye (Rogers, 1996; Rogers and Deng, 1999; Koshiba et al., 2002) and results in lateralization of the two cerebral hemispheres. Light stimulation during early embryogenesis, before the development of a functional visual system, can also result in cerebral lateralization, possibly via changing gene expression in photosensitive regions (Chiandetti et al., 2013). Although there are differences in patterns across taxa, this lateralization results in each hemisphere becoming

dominant for certain functions, including processing of fear-inducing stimuli (Leliveld et al, 2013).

In our second experiment, the 6:L18D and 12L:12D birds both showed evidence of laterality in terms of directionality of exit from the dark box. However, fear reactions were only reduced consistently in the 12L:12D birds, suggesting that additional mechanisms could underlie the responses we observed. Other possibilities include the effects of incubation lighting on patterns of hormone secretion associated with the establishment of circadian rhythms (Zeman et al, 1992; Hill et al., 2004), or epigenetic influences (Keverne and Curley, 2008).

5. Conclusions

Our results demonstrate that light stimulation during incubation of chicken embryos has long-lasting effects on anti-predator fear responses, with at least 12 hours of light stimulation required daily. While the exact mechanism underlying these effects is unknown, this research provides evidence that providing an appropriate lighting environment during incubation could improve the welfare of broiler chickens by reducing fearfulness towards humans post-hatch. Future studies could also examine the effects of incubation lighting on laying hens, since panic due to excessive fearfulness in commercial non-cage flocks can be a significant cause of mortality

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Table 1
Measures with treatment effects for the Tonic Immobility and Inversion tests in Experiments 1 and 2 (means $\pm$ SE). Asterisks indicate significant differences between the light treatment groups (1L:23D, 6L:18D, 12L:12D, 24L:0D) and dark-incubated birds (OL:24D)

	Tonic Immobility Latency to right (sec)	Inversion Intensity (wing flaps/sec)
Experiment 1		
OL:24D	305.5 $\pm$ 26.1	5.7 $\pm$ 0.1
12L:12D	213.5 $\pm$ 23.7 **	5.0 $\pm$ 0.1***
24L:0D	231.8 $\pm$ 24.2*	5.4 $\pm$ 0.2*
Experiment 2		
OL:24D	201.4 $\pm$ 24.9	5.5 $\pm$ 0.1
1L:23D	218.2 $\pm$ 21.9	5.1 $\pm$ 0.1**
6L:18D	193.7 $\pm$ 20.8	4.9 $\pm$ 0.1***
12L:12D	120.0 $\pm$ 16.5**	4.7 $\pm$ 0.1****

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

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Table 2
Measures with no treatment effects for the Tonic Immobility and Inversion tests in
Experiments 1 and 2 (means $\pm$ SE)

		Experiment 1	Experiment 2
Tonic Immobility	Induction attempts (#)	1.2 ± 0.06	1.3 ± 0.06
	Latency to first head movement (sec)	24.1 ± 3.1	49.2 ± 10.0
Inversion	Duration of wing flapping (sec)	5.7 ± 0.3	5.9 ± 0.3
	Wing flaps (#)	30.7 ± 1.6	29.0 ± 1.2
	Vocalization (% birds)	9 ± 1	9 ± 1
	Body curls (% birds)	88 ± 2	90 ± 2

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Figure 1. Results from the approach (# Vocalizations, % Time spent active , and %S time closet to the observer) and emergence (Latency to exit box) tests. *Indicates significantly different from 0L:24D treatment (P < 0.05).