Research Note: Retinal cryptochrome gene expression is not altered by presence of light in incubators

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ABSTRACT Cryptochromes are blue-, or ultraviolet-, light-absorbing proteins involved in the circadian clock, blue/ultraviolet light perception and potentially magnetoreception. At least 4 separate cryptochrome genes have been identified in avian species. The purpose of this study was to first determine if cryptochrome genes are expressed in the developing duck retina, and second to determine if the presence of lights in incubators affects the expression of cryptochrome genes. To accomplish these goals, duck eggs were placed in one of 2 commercial incubators (Buckeye, Single Stage Incubator, Model, SS-112) at Maple Leaf Farms, Inc., one with "poultry" LEDs obtained from a commercial source (Once Innovation, Agrishift) and the other in the absence of light (dark). Eggs in the incubators were placed on a reciprocating tray, tilting to 45° to simulate the rotation of eggs; thus all eggs spent 50% facing the light source and the other 50% of time facing 45° away from light source. Temperature gradients and humidity were maintained at industry standards. Retinal tissue samples from light and dark incubators were collected on days 3, 7, 11, 16, and 21 of incubation (extraction day, **ED**) known to be anatomical hallmarks of visual system development (n = 9−18 treatment group/ED timepoint). Samples were prepped and assayed for Cry2 and GAPDH gene transcription using qRT-PCR. Data were analyzed by using 2^{-ddCt} method and a 2-way ANOVA was performed. No significant differences in Cry2 gene expression were observed between the lighted or dark incubator ($P > 0.10$). When combining light and dark treatment groups there is a significant 9 $P < 0.05$) increase in retinal Cry2 at ED 21, compared to ED 3 and 7. The presence of cryptochrome does not necessitate a migratory drive as evidenced by the fact that the Cry2 expression has been shown in non-migratory birds. However, since blue/ultraviolet wavelengths also activate the Cry2 photoreceptor, its presence could explain reports that suggest duck welfare can be improved if housed under lights that include ultraviolet wavelengths.

Key words: photoreception, magnetoreception, embryonic development

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INTRODUCTION

Research has focused on the use of lights in incubators to improve post-hatch performance (Rozenboim et al., 2004). Unfortunately, there are as many corroborative as conflicting findings. As yet, no consensus exists as to the value of lighting in an incubator, nor what wavelengths are necessary to optimize post-hatch performance. In order to better understand how light may affect an embryo, it is critical to understand what photoreceptors are expressed during development and at what timepoints each is expressed. A recent study from our labs showed that the presence of lights had no effect on the ontogeny of retinal photoreceptors that respond to red, blue, or green wavelengths or to gray scale. However, in that study we were not able to evaluate a novel (to poultry science) photoreceptor class known as cryptochrome photoreceptors. Cryptochrome photoreceptors have been found in numerous bird species, including chickens (Wiltschko et al., 2007), and may be critical for migratory birds.

The Pekin duck was domesticated between 4,000 and 10,000 years ago from Mallard ducks and it appears that Pekin ducks have maintained much of their wild-type physiological properties (Cherry and Morris, 2005, 2008). Mallards are migratory birds and during migration birds need to navigate great distances. One theory of how they navigate is by the use of cryptochrome receptors in the retina. Cryptochromes are blue-, or ultraviolet-, light-absorbing proteins involved in the circadian clock (Vigh et al., 2002). They have

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also been proposed to be the receptor molecules of the avian magnetic compass (Fu et al., 2002). There is aevidence in the literature that cryptopchrome receptors could be involved in physiological processes that control migratory restlessness and enable birds to perform their nocturnal flights. Clearly Pekin ducks do not migrate, but that does not infer that the migratory drive has not been maintained despite domestication. The purpose of this study was to first determine if the cryptochrome mRNA could be identified in the developing duck retina. And second, we set out to determine if lights in the incubators have an effect on cryptochrome gene expression in the developing embryo.

MATERIALS AND METHODS

Animals and Housing

Duck eggs were obtained from Maple Leaf Farms, Inc. (Leesburg, IN) from standard commercial breeder strains of Pekin duck. Duck eggs were placed in one of two commercial incubators (Buckeye, Single Stage Incubator, Model, SS-112) at Maple Leaf Farms, Inc., one with "poultry" LEDs obtained from a commercial source (Once Innovation, Agrishift) and the other in the absence of light (dark). All lights in both the incubator and hatcher were from LED bulbs and the energy of all lights was normalized at the level of the eggs, as determined by spectrophotometry (~4.3×10^{13} uM/cm^2/s, USB5000UV, Ocean Optics, Inc., Raleigh, NC). Eggs in the incubators were placed on a reciprocating tray, tilting to 45° to simulate rotation of eggs, thus all eggs spent 50% facing the light source and the other 50% of time facing 45° away from light source. Temperature gradients and humidity were maintained at industry standards. All study procedures were approved by the Hope College Animal Care and Use Committee following the Institutional Animal Care and Use Committee guidelines.

Study Design and Sample Collection

Retinal tissue samples from light and dark incubators were collected on d 3, 7, 11, 16, and 21 of incubation (extraction day, **ED**); days that are known to be anatomical hallmarks of visual system development (n = 9−18 treatment group/ED timepoint). Embryos from the dark incubator were removed from the egg and dissected under dark conditions. Samples were placed into TRIzol at time of dissection and were immediately frozen on dry ice.

RNA and DNA Extraction, Quantification and Purity Determination

Frozen samples were thawed and incubated at room temperature (**RT**) for 5 min then homogenized in TRIzol. Samples were centrifuged at 11, 8,000 rpm for 15 min a 4°C. Samples were spun and the supernatant was removed and placed inside the new tube. Depending on the initial volume of TRIzol used (200 μL, 400 μL, 500 μL, and 800 μL) either 80 μL, 100 μL, 160 μL, or 200 μL of chloroform, respectively (4:1), was added to the supernatant and vortexed for 2 min at 2,000 rpm on MixMate. Samples were then incubated at RT for 3 min and then centrifuged at 11,800 rpm for 15 min at 4°C. A density gradient was created with which the top clear aqueous phase of RNA was separated into a fresh tube. Following this, 1 μL of Glycogen was added to the aqueous phase followed by an aqueous layer to isopropanol (1:1, isopropanol:TRIzol). Samples were vortexed for 1 min, incubated for 10 min at RT, and centrifuged at 11,800 rpm for 10 min at 4°C. The supernatant was discarded carefully without disturbing RNA pellet.

Pellets were washed 2 times with 200 μL, 400 μL, 500 μL, or 800 μL of 75% ethanol (1:1, ethanol:TRIzol, ThermoFisher Scientific, Waltham, MA). Tubes were inverted on a clean Kimwipe and pellets were allowed to air dry until translucent/clear. Pellets were then resuspended in RNase-free water, based on the pellet size and mixed carefully by flicking. Samples were placed on ice for a minimum of 10 min to mix and stored in −20°C freezer for short term (e.g., going to cDNA synthesis the same day) or −80°C freezer for long term. Preliminary RNA quantification and purity determination was done using a NanoDrop 2,000 Spectrophotometer (Thermo Scientific, Wilmington DE). Afterward, 1 uL of RNA sample was loaded on the spectrophotometer for measurement. RNA concentration was recorded at 260 nm wavelengths and RNA purity was determined by the 260/230 and 260/280 ratios. RNA was considered pure if 260/280 (RNA:protein contamination) ratio was in the range of 1.7 to 2.0, and 260/230 (RNA:Ethanol Contamination) was between 2.0 and 2.2. For all samples RNA was normalized to 100 ng/μL and stored in a −80 freezer for no more than 2 wk.

qPCR

Cry2 (Table 1) was cross-referenced with NCBI BLAST using the ref seq genome of *Anas platyrhynchos* (XM_027458086.1) and analyzed with OligoAnalyzer to determine optimal free energies. Quantitative

Table 1. Primer sequences.

Tissue	Gene	Forward	Reverse
Retina	Cry2	CTGTAGTGCTGTTGCCTCCA	AACATCCACACAGGACGGAC
Housekeeping	*GAPDH*	GGTTGTCTCCTGCGACTTCA	TCCTTGGATGCCATGTGGAC

real-time PCR (**qPCR**) was carried out using Luna Universal One-Step RT-qPCR Kit (New England BioLabs Inc., Ipswich, MA) and qPCR primers were designed in-house and purchased from Integrated DNA Technologies (Coralville, IA). GAPDH was averaged and used at every time point for all gene expression analyses. GAPDH was chosen as it does not differ statistically across developmental time points (data not shown).

Plate Design and Analysis

Each gene was assessed in duplicate using a MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 mL (Applied Biosystems, Carlsbad, CA). Mastermix was composed of 10μL Luna blue reaction dye, 1μL Luna WarmStart, 1.6 μL of primer, 5.4 μL of nuclease-free water, and 2 μL of template RNA. For gene expression analysis, a single sample was assayed in duplicate. Plates were sealed, and qPCR was run for 40 cycles with a cover temperature of 105.0°C. A 2^{-ddCt} method, including a normalization to GAPDH expression levels, was used to quantify gene expression levels of Cry2. Outliers were removed from the data set following ROUT parameters (Q = 1%).

Statistical Analyses

qPCR data was analyzed with GraphPad PRISM version 8.1.2 (GraphPad Software, San Diego, CA) using a Brown-Forsythe and Welch ANOVA analysis to correct for unequal standard deviations. Post-hoc analyses were performed using the Dunnett T3 multiple comparisons test.

RESULTS AND DISCUSSION

Cry2 gene expression was observed in retinas at all ages assessed. No differences ($P > 0.1$) were observed in Cry2 gene expression in embryos incubated in the light compared to dark incubators (data not shown). However, when light and dark groups are combined, the overall Cry2 gene expression level across development increases (Brown-Forsythe ANOVA, $F_{(4.0, 33.24)} = 5.636$, $P = 0.0014$) at the oldest time point investigated (ED 21), compared to ED 3 (t = 3.261, df = 15.11, $P = 0.047$) and 7 (t = 3.389, df = 14.16, $P = 0.039$). These results are illustrated in Figure 1.

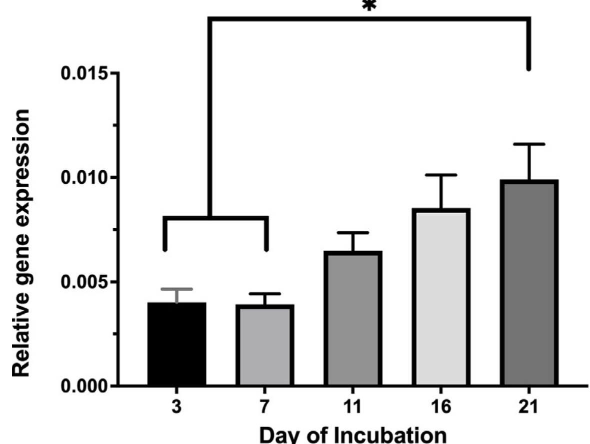

Figure 1. Cryptochrome (Cry2) gene expression in the retina during embryonic development. An increase in Cry2 gene expression is observed at 21 days of incubation, compared to earlier 3 and 7 days of incubation. No significant differences were observed in Cry2 gene expression in the retina between embryos incubated in dark or lighted incubators (data not shown). Statistical analysis performed using a Brown-Forsythe and Welch ANOVA. *$P < 0.05$ by a Post-hoc Dunnett T3 multiple comparisons test. Mean ± SEM.

Cryptochromes (blue-light photoreceptor proteins) have been suggested as the primary magnetoreceptors in the avian compass. Several different cryptochromes have been found in the eyes of migratory birds: Cry1a, Cry1b, Cry2, and Cry4 (Wiltschko et al., 2007). Unfortunately, it does not appear that members of anseriformes have as yet been investigated for cryptochrome expression until our current study. In our embryonic tissues, we were only able to detect Cry 2 expression. The light source in our study did not contain UV, however, it did include blue wavelengths. It is possible, however, that the addition of UV may have resulted in differential expression of Cry2 during development. A much more extensive study that includes many post-hatch and adult ages of ducks must be undertaken to fully understand cryptochrome expression in the Pekin duck.

The ability of birds to obtain directional information from the earth's magnetic field was first described in the 1960s in migratory birds. Migratory birds show a strong directional preference during the spring and autumn in the wild and also in suitable test cages. When the North direction of the magnetic field in a test cage is shifted, the bird changes its heading accordingly. Although first demonstrated in a small songbird (Wiltschko et al., 2007), it has since been shown in scores of other bird species from various avian lineages that are not closely related, and including non-migratory species such as the domestic chicken (for a list, see (Wiltschko et al., 2007)). Using the geomagnetic field as a compass thus seems to be a general feature of birds (for review, see (Wiltschko et al., 2015)). Although Pekin ducks are considered domesticated Mallards, there is no evidence that they have retained any migratory drive. The presence of cryptochrome does not necessitate a migratory drive as an evidenced by the fact the Cry2 expression has been shown in non-migratory birds. However, since the Cry2 photoreceptor is also activated by blue/ultraviolet wavelengths, it could begin to explain recent reports that suggest that duck welfare can be improved if housed under a lighting system that includes UV (House et al., 2021; Huth and Archer, 2015). Further investigations into the physiological role of Cry2 expression in the duck eye and how it relates to ducks' ability to perceive their environment will be critical for the future housing designs to maximize duck welfare

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DISCLOSURES

The authors declare no conflicts of interest.

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