5-2014

Effects of light treatment on fecal corticosterone levels in captive European Starlings (Sturnus vulgaris)

Thomas A.J. Ryan

Brandi Van Roo

Effects of light treatment on fecal corticosterone levels in captive European Starlings (*Sturnus vulgaris*)

Thomas A.J. Ryan[1] Brandi Van Roo[2]

1SUNY College of Environmental Science and Forestry

Department of Environmental Forest Biology

Candidate for Bachelor of Science with Honors

May 2014

2Framingham State University – Department of Biology

Abstract

Incandescent light sources are increasing replaced by other forms of light, such as fluorescent and light-emitting diode (LED) lights, which have greater longevity and offer greater energy efficient. These sources of light differ in more than their cost and efficiency, and have many different physical properties, for instance, their degree of flickering. Low-frequency fluorescent lighting flickers below the critical flickering fusion frequency (CFF) of some birds and may be a source of stress, unlike a non-flickering light source, such as LED. Our study measured levels of glucocorticoid metabolites (GCM's) in fecal samples of captive European starlings (*Sturnus vulgaris*) both across treatments and over a period of days to demonstrate the effects of different forms of light on the stress levels of birds in a laboratory setting. Concentration of GCM's were not significantly different either across light treatments (P =0.441) or over time (P= 0.209), suggesting flickering properties of low-frequency fluorescent light is not a major source of stress for captive birds over an alternative light source.

List of Figures

List of Tables

Acknowledgements

First I would like to thank Dr. Brandi Van Roo of Framingham State University. She was willing to allow me to tag along with her for a summer and truly learn what it means to be both a field ornithologist and a behavioral endocrinologist. I'd like to thank my sister, Madeline Ryan, and Dr. Alex Weir. Without them, I would have never attended SUNY-ESF and discovered my passion for birds was more than just a hobby. I thank my academic advisor Dr. Scott Turner, a man with whom I shared few, but always professionally and academically important conversations. I thank my mother, Susan Gallagher, who has always pushed her son to achieve to his potential while allowing him to keep his head lost in the clouds.

Finally, I'd like to thank three men who have collectively served as both mentors and role models.

Bill Shields took me under his wing from my first day in college, and has displayed a gratuitous degree of patience with me. We share an addiction to behavior and learning, and for that, he will always be, primarily, a friend.

I thank my father, John Ryan. His attention to detail and infectious compassion, as a physician and as a parent, is the only thing I've found worthy of imitation.

Lastly, I thank Guy Baldassarre for the best advice I've ever received. You continue on as a reminder to never let my education get in the way of me learning something.

Introduction

Stress response in birds is primarily measured through levels of corticosterone, a glucocorticoid mediated through the HPA axis (Axelrod and Reisine, 1984; Wingfield 1994). In an acute sense, corticosterone aids avian metabolism and behavior in the presence of a stressor. Corticosterone, however, can have adverse impacts on avian health if the stressor persists chronically (Wingfield et al., 1992; Sapolsky, 1992). To date, studies have demonstrated the impact upon avian stress and corticosterone expression due to logging, tourism, and climate change, to name a few (Wasser et al., 1997; Mullner et al., 2004; Cockrem, 2013).

Another well-studied potential stressor to birds is light, in particular, fluorescent light. Fluorescent light offers advantages in efficiency and duration over the more traditional incandescent light. Fluorescent light also exhibits a flicker effect, not perceived by humans but visible to certain species of birds. At a species-specific threshold, this flickering appears continuous, known as the critical flicker fusion frequency (CFF) (D'Eath, 1998). The standard frequency for US fluorescent lights is typically 120Hz, well above human CFF, but not always above the CFF of wild or domestic animals (Jarvis et al., 2002). In captive birds, European starlings (*Sturnus vulgaris*) have been used to demonstrate the impacts of light treatment on stress. Maddocks et al. (2001), found no discernable differences in plasma corticosterone after 1 or 24 hours. Greenwood et al. (2004), similarly found no difference in plasma corticosterone levels over 6- or 14-day trials, but, did find that, given a choice, starlings demonstrated a preference for higher frequency fluorescent light over a lower frequency below the CFF. Smith et al. (2005a, b), however, found muscle spasms, malaise, and elevated corticosterone as a result of fluorescent flicker. Beyond stress, fluorescence seems to have other potential adverse effects on starlings, including mate choice (Evans et al. 2006), and is of particular interest for fast,

gregarious species, which need to make split-second directional changes on the wing (Greenwood et al. 2004).

A common denominator in each of these previous starling stress studies is the use of corticosterone sampling via plasma. Venipuncture and handling, two necessary steps in the acquisition of blood samples, both may impart a large degree of stress onto the subject (Miller et al. 1991). To the contrary, our work involved the extraction of hormones from fecal samples, which serve as a valuable means to collect hormones in a non-invasive manner. While it is noted these previous studies standardized their venipuncture and handling across treatments, a less invasive means of hormone sampling may elucidate small differences in corticosterone across light treatment, given the already high baseline for stress in a captive setting (Romero and Wingfield, 1999). Fecal samples typically contain very little intact corticosterone, as it is metabolized in the liver, but modern ELIZA kits are capable of detecting extracted glucocorticoid metabolites (GCMs).

Our main aim of the study was to extract and analyze glucocorticoid metabolites from a collection of fecal samples of European starlings subjected to differential light treatments and how these metabolites changed over the course of 9 days. The fecal samples were to serve as supplementary data to a previous work in the Van Roo lab, examining behavioral cues of stress in captive starlings across these treatments and over time (Flood and Van Roo, unpublished). Both works follow a previous study as a model, Greenwood et al. (2004), with minor differences in sampling, analysis, and light treatment. Rather than high-frequency fluorescent light, LED light was compared against low-frequency fluorescent light. LED light does not exhibit a flicker effect and shares fluorescent light's economic advantages over incandescent light.

It was hypothesized that fecal samples of birds directly exposed to LED light would exhibit lower concentrations of GCMs than fecal samples of birds exposed to low-frequency fluorescent light. Habituation to the light treatment, as reflected in a significant relative decline in GCMs over time, was hypothesized to be more pronounced in low-frequency fluorescent light than LED.

Methods

Outline of direct exposure to light treatments

The following is adapted from Kalina and Van Roo, 2013 (unpublished):

Ten (n=10) wild-caught European starlings from a variety of sites in the Framingham, Massachusetts area will be exposed to the following lighting treatments: sun light, low frequency fluorescent light (100 Hz), and LED light. Under chronic exposure to each treatment, fecal and plasma corticosterone samples, changes in body weight, behavioral reactions and corticosterone assays will determine the level of stress to the light treatments. Once captured, two weeks of acclimation will allow birds to adjust to captivity (Rich & Romero 2005; Millspaugh & Washburn 2004). One cage positioned in front of the window will receive sunlight for the duration of the experiment, serving as the negative control. A curtain of black cloth placed in between the holding cages and the control will prevent the control bird from visually seeing the other birds, yet allow it to receive the natural light for the entirety of the experiment.

Two separate rooms had one of two light treatments: low-frequency fluorescent light (20 watts, 0.60 meter, Phillips F20T12 ran with GE LFL Magnetic Rapid Start Ballast) or LED light (12.5 watts, Phillips EnduraLED 800 series A19). A single bird will be transferred to an experimental room. Each bird will spend 14 days under the designated light treatment. Light

treatments will rotate between the two rooms after every experimental treatment, rather than each light treatment remaining fixed to one room location. Fecal samples will be collected on day 1, day 5, and day 9. The samples were frozen in plastic tubes upon collection for later analysis. The bottom of the cages were lined with cling wrap to ensure full fecal collection and cleaned daily to prevent cross-contamination.

Hormone extraction and analysis

Methods for extraction of GCM's from fecal samples adapted from combination of protocols from Wasser et al. (2010) and Dantzer et al. (2010). Fecal samples were thawed at room temperature for 3 hours. Samples were stirred with wooden spatulas to promote homogeneity before portions of each were used for analysis. ~0.5g of each sample was removed from each test tube and placed in separate petri dishes. The samples were smeared to allow for greater drying surface area and the mass of the smeared samples was recorded. 24 hours was afforded to allow for the drying of each sample and a fan was blown over the samples to prevent overheating which may decompose the sterols. After 24 hours, the samples were re-weighed to obtain a dry mass. 0.1g of dry fecal sample was isolated and placed in separate 15mL Eppendorf tubes along with 2mL of 70% methanol. The tubes were vortexed for 60s and rotated at 135rpm for 10 minutes on a plate shaker. After 10 minutes, the samples were centrifuged on an IEC Clinical Centrifuge (setting 3) for 15 minutes. The resulting supernatant was drained into a clean Eppendorf tube and frozen for later analysis.

Thawed samples were analyzed via the instructions of Enzo Life Sciences Corticosterone Kit. The instructions are briefly outlined below:

A serial dilution of corticosterone standard was prepared in Assay Buffer (diluted 1:10), yielding standards of 200,000, 4,000, 800, 160, and 32ug/mL. The supernatants (samples) were

mixed with steroid displacement reagent (40:1). Wells were filled in duplicate, according to protocol. Assay buffer was added to NSB and Bo wells (0pg/mL). Three corticosterone standards of 66.6uL were added to wells in duplicate. These sample duplicates were dispersed at random locations throughout the well, to determine uniform plate reading. Standards and samples were pipetted into appropriate wells. Starling plasma samples were combined and a serial dilution was prepared and administered directly to wells (to later demonstrate parallelism, see below). Blue conjugate was added to all except TA & Blank wells. Yellow antibody was added to all except Blank, TA, and NSB wells. Wells were incubated for 2 hours at room temperature and 200rpm. Wells were then washed with 400uL of wash buffer in triplicate and padded dry, vigorously, while inverted, with paper towels. Conjugate was added to TA wells and pNpp substrate was added to all wells and incubated for 1 hour at room temperature. After the hour, 50uL of Stop Solution was added to each well and the plate was immediately read with BIO-RAD Benchmark Plus microplate spectrophotometer.

Calculations

The spectrophotometer gave values in terms of optical density (OD). Optical density values were averages among duplicates and NSB OD values were subtracted from standards and samples. Percent Bound was calculated according to formula:

%Bound = Net OD/Net Bo OD *100

The standard curve of %Bound of standards vs. their concentrations was used to arrive at a line of best fit (R2 = 0.989), from which the concentrations of the samples could be determined from their OD. Listed below are the reported precision, accuracy, cross-reactants with percentages, and sensitivity of the kit (Table 3).

Results

Average concentrations of corticosterone and their respective standard deviations can be found in Table 1 and Figure 1. These were analyzed with a two-way ANOVA, across treatments and over time (Table 2). There was no significant differences across light treatment (P= 0.441, df=2), over time (P=0.209, df=2) or considering both simultaneously (P=0.839, df=4).

Table 1. Average Concentrations and Standard Deviations

1	33.03 +/- 10.28	25.86 +/- 8.46	27.11 +/- 12.49
5	41.6 +/- 13.43	38.7 +/- 18.05	42.65 +/- 30.74
9	54.16 +/- 12.49	36.24 +/- 6.71	34.1 +/- 18.97

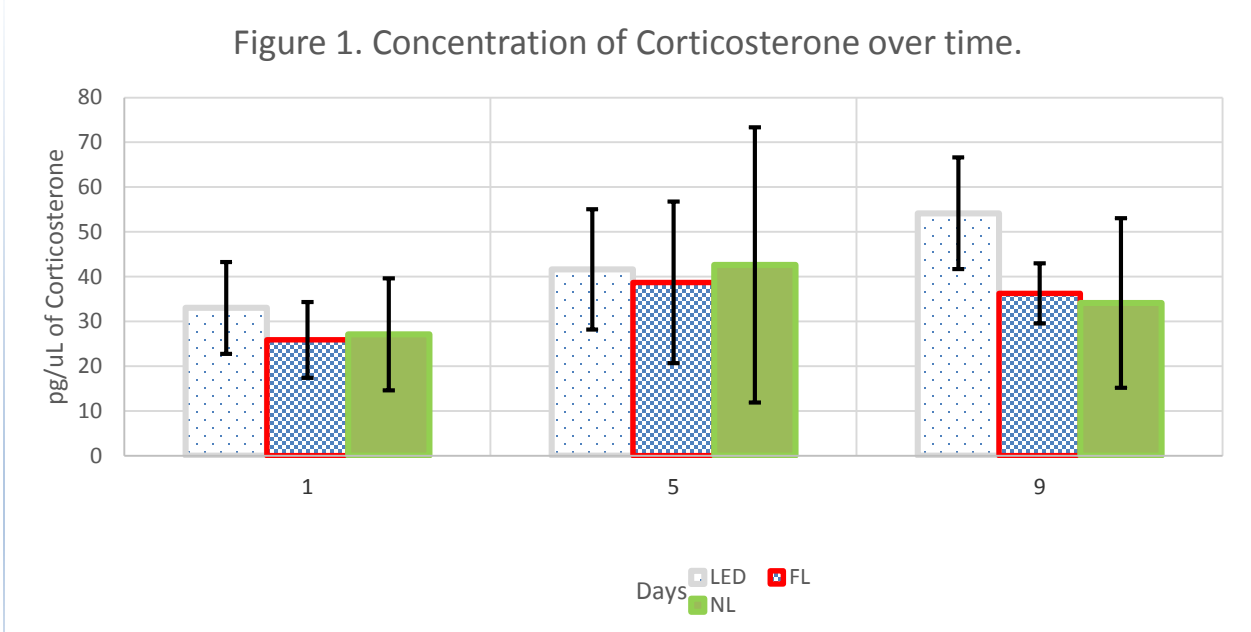

Table 2. ANOVA Summary

Source	Degrees of Freedom	P-value
Light Treatment (rows)	2	0.441
Overt time (columns)	2	0.209
Rows x Columns	4	0.839

Table 3. Values from the Manufacturer

Precision	Corticosterone (pg/mL)	Intra-assay %CV	Inter-assay %CV
	Low-171	8.0	
	Medium-403	8.4	
	High-780	6.6	
	Low - 174		13.1
	Medium- 415		8.2
	High- 780		7.8
Sensitivity	26.99pg/mL		
Cross-Reactivity	Corticosterone	100%	
	Deoxycorticosterone	28.6%	
	Progesterone	1.7%	
	Testosterone	0.13%	
	Tetrahydrocorticosterone	0.28%	
	Aldosterone	0.18%	
	Cortisol	0.046%	

Discussion

Direct exposure of individual starlings to low-frequency fluorescent light, LED light, and sunlight showed no appreciable difference in stress, as measured by fecal GCMs. Further, no differences arose in how concentrations of fecal GCMs fluctuated over 9 days of exposure to these sources of light. These results match those of Greenwood et al. (2004), indicating corticosterone levels, serving as a measure of stress, do not differ significantly between light sources above and below the starling CFF.

Levels of GCMs were highly variable among each treatment. Sunlight, intended to serve as a negative control group, appears, anecdotally, to have been most variable. It is difficult to determine the source of this variability, be it the setup of the light treatments, the hormone extraction and analysis, or some combination of the two.

The light treatments and experimental setup are difficult to fully assess, given the lack of communication between lead authors, but certain aspects are worth addressing. Most notably, it is possible the stressful nature of captivity itself imparts such a large degree of stress that any signals of stress from a light treatment may simply be buried under the noise of an already stressful atmosphere. Among other possible audible stressors, construction was taking place during these experiments and may have been an irregular source of stress. Cages being cleaned out for fecal collection and movement of subjects between holding cages and experimental cages exposed the starlings to a consistent, potentially stressful set of encounters. Other studies mention other light treatment properties, including intensity, which were not measured across treatments and, therefore, cannot be ruled out as a source of variance. Information is also limited on bird age and holding cage specifics. Starlings are notably gregarious by nature, and, whereas others have paired test birds, this study did not. Isolation from other starlings for ~9 days may have imparted stress on the test birds. However, to use the non-invasive means of hormone sampling, isolation appears to be a necessary factor of the experimental design.

Various reviews have covered the necessary steps in validating hormone assay from fecal samples (Buchanan and Goldsmith 2004, Palme 2005). Validation of the Enzo Life Sciences Corticosterone ELIZA Kit is of major concern. The demonstration of parallelism was performed with serial dilutions of pooled blood plasma samples rather than pooled extracts of fecal samples. While this validation is not altogether useless, parallelism with pooled extracts of fecal samples was an important step in demonstrating the procedure as a non-major source of variance, and, regrettably, we cannot confidently report such a claim without it. Beyond parallelism, other steps in sound validation practice include biological relevance via an ACTH test and knowledge of delay times between stressor and reflection of that stressor in the fecal

samples, i.e., how long it takes for the GCM's to be excreted. Neither were feasible in our case because the starlings were no longer available for further testing once the protocol for hormone extraction and analysis was taking place. For all other potential validation concerns, the reported precision, accuracy, sensitivity, and cross-reactions are provided by the manufacturer (Table 3).

A final note on the study species is worthy of consideration. Urban birds have been shown to display a larger degree of environmental tolerance (Bonier et al. 2007), and, perhaps, this holds for tolerance toward light source. Replication of these works with a traditionally non-urban species able to tolerate a captive setting would shine light on this issue.

Conclusions

Stress, as measured by fecal glucocorticoid metabolites of captive European starlings, does not appear to be significantly different between LED light or low-frequency fluorescent light. Stress also does not seem to appreciably differ among these treatments across a moderate time interval of 9 days.

Works Cited

Axelrod, J., Reisine, T.D., 1984. Stress hormones: their interaction and regulation. *Science* 224, 452–459.

Bonier, Frances, Paul R. Martin, and John C. Wingfield. "Urban birds have broader environmental tolerance." *Biology letters* 3.6 (2007): 670-673.

Buchanan, AR Goldsmith; Non-invasive endocrine data for behavioural studies: the importance of validation. *Anim. Behav*., 67 (2004), pp. 183–185.

Cockrem, John F. "Corticosterone responses and personality in birds: Individual variation and the ability to cope with environmental changes due to climate change." *General and comparative endocrinology* 190 (2013): 156-163.

Dantzer, Ben, et al. "Fecal cortisol metabolite levels in free-ranging North American red squirrels: assay validation and the effects of reproductive condition." *General and comparative endocrinology* 167.2 (2010): 279-286.

D'Eath, R.B., 1998. Can video images imitate real stimuli in animal behaviour experiments? *Biol. Rev.* 73, 267–292.

Evans, Jennifer E., Innes C. Cuthill, and Andrew TD Bennett. "The effect of flicker from fluorescent lights on mate choice in captive birds." *Animal behaviour* 72.2 (2006): 393-400.

Flood, Kalina, Van Roo, Brandi. "The effects of LED lighting versus fluorescent lighting on chronic stress in European Starlings, *Sturnus vulgaris*" unpublished.

Greenwood, V. J., Smith, E. L., Goldsmith, A. R., Cuthill, I. C., Crisp, L. H., Walter Swan, M.B. & Bennett, A. T. D. 2004. Does the flicker frequency of fluorescent lighting affect the welfare of captive European starlings? *Applied Animal Behaviour Science*, 86, 145-149.

Jarvis, J. R., Taylor, N. R., Prescott, N. B., Meeks, I. &Wathes, C. M. 2002. Measuring and modelling the photopic flicker sensitivity of the chicken (Gallus g. domesticus). *Vision Research*, 42, 99-106.

Maddocks, S. A., Goldsmith, A. R. & Cuthill, I. C. 2001b. The influence of flicker rate on plasma corticosterone levels of European starlings, *Sturnus vulgaris*. *General and Comparative Endocrinology*, 124, 315-320.

Miller, M.W., Hobbs, N.T., Sousa, M.C., 1991. Detecting stress responses in Rocky Mountain bighorn sheep (Ovis Canadensis canadensis): reliability of cortisol concentrations in urine and feces. *Canadian Journal of Zoology* 69, 15–24.

Millspaugh, Joshua J., and Brian E. Washburn. "Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation." *General and comparative endocrinology* 138.3 (2004): 189-199.

Mu¨ llner, A., K. E. Linsenmair, and M. Wikelski. 2004. Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (Opisthocomus hoazin). *Biological Conservation* 118:549–558.

Palme, Rupert. "Measuring fecal steroids: guidelines for practical application." *Annals of the New York Academy of Sciences* 1046.1 (2005): 75-80.

Rich, Erin L., and L. Michael Romero. "Exposure to chronic stress downregulates corticosterone responses to acute stressors." *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 288.6 (2005): R1628-R1636.

Romero, L. M., and J. C. Wingfield. 1999. Alternations in hypothalamic-pituitary-adrenal function associated with captivity in Gambel's White-crowned Sparrows (*Zonotrichia leucophrys gambelii*). *Comparative Biochemistry and Physiology B* 122:13–20.

Sapolsky, R. M. 1992. Neuroendocrinology of the stress response. *Behavioural endocrinology* (ed. J. B. Becker, S. M. Breedlove & D. Crews), pp. 287-324. Cambridge, MA: MIT Press.

Smith, E. L., Evans, J. E. & Pa´rraga, C. A. 2005a. Myoclonus induced by cathode ray tube screens and low frequency lighting in European starlings (*Sturnus vulgaris*). *Veterinary Record*, 157, 148-150.

Smith, E. L., Greenwood, V. J., Goldsmith, A. R. & Cuthill, I. C. 2005b. Effect of repetitive visual stimuli on behaviour and plasma corticosterone of European starlings. *Animal Biology*, 55, 245-258.

Wasser, Samuel K., et al. "Noninvasive physiological measures of disturbance in the northern spotted owl." *Conservation Biology* 11.4 (1997): 1019-1022.

Wasser, Samuel K., et al. "Non-invasive measurement of thyroid hormone in feces of a diverse array of avian and mammalian species." *General and comparative endocrinology* 168.1 (2010): 1-7.

Wingfield, J. C., C. M. Vleck, and M. C. Moore. 1992. Seasonal changes of the adrenocortical responses to stress in birds of the Sonoran Desert. *Journal of Experimental Zoology* 264: 419–428.

Wingfield, J. C., 1994. Modulation of the adrenocortical response to stress in birds. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), *Perspectives in Comparative Endocrinology*. National Research Council, Canada, pp. 520–528.