Effect of testosterone on the synchronization of activity rhythms to low amplitude *zeitgebers* in an arctic breeding songbird

Abstract
A key feature of polar environments is the continual presence of the sun above the horizon; at Barrow, AK this equates to ca. 12 weeks of polar day conditions lasting from the middle of May to early August. During this time, many arctic animals, such as caribou (*Rangifer tarandus tarandus*), abandon diel rhythms of activity. However, arctic migrant birds continue to display a distinct circadian rhythm despite continuous daylight experienced on breeding grounds. It is hypothesized that the persistence of diel rhythms involves entrainment to low amplitude *zeitgebers* that include small changes in light intensity or spectral quality of sunlight that occur over the polar day. We determined previously that captive Lapland longspurs (*Calcarius lapponicus*), an arctic migrant songbird, failed to entrain to light regimes that mimicked daily changes in light intensity or color temperature of polar-day conditions in Barrow, Alaska, (71° N), where birds were captured. Because birds were in non-breeding condition, it is possible that sensitivity to polar-day *zeitgebers* is increased during the breeding season and is proximately regulated by sex steroids. My current experiment tests this hypothesis through administration of either exogenous testosterone (experimental group) or empty implants (control group) to non-breeding male Lapland longspurs and exposing them to the light regimes described above. Results of this study will elucidate a potential hormonal role in the regulation of entrainment within the biological clock of a high-latitude breeding species.

Specific Aims: *The specific aims of this proposed investigation will test the **hypothesis** that sensitivity to low amplitude zeitgebers is under activational control of steroid hormones.*

Specific Aim 1: Evaluate the effect of chronic administration of exogenous testosterone on photosensitivity and entrainment of captive Lapland longspurs.

Specific Aim 2: Uncover potential endocrine and molecular correlates of entrainment in captive Lapland longspurs under various simulated photo-regimes.

Introduction
Many of the behavioral and physiological patterns that animals display oscillate with a 24-hour period. Known as circadian rhythms, these oscillations allow organisms to prepare for predictable changes in the environment, such as seasonal and diel changes in photoperiod. The degree to which circadian rhythms are synchronized is largely dependent upon the availability and exposure of the individual to external cues, or *zeitgebers*. In temperate latitudes, the light-dark (LD) cycle within a 24-hour period synchronizes diurnal rythmicity in many species (Aschoff, 1989).The observed spectral composition of light, dependent upon the rotation of the earth and photo-filtration of differing wavelengths of light, is thought to be an effective astronomical *zeitgeber* that entrains endogenous daily rhythms (Krüll, Demmelmeyer & Remmert, 1985). In mammals, molecular clocks within cells of the hypothalamic suprachiasmatic nucleus (SCN) act as timekeepers, taking in photic input through the retina of the eyes (Menaker, Moreira et al., 1997). Avian molecular clock systems are more involved, though some components are homologous to the mammalian SCN (Takahashi & Menaker, 1982). In both systems, light-cues are transduced in the brain to elicit systemic changes in physiology and behavior.

Polar environments are characterized by periods of continuous light or dark during the summer and winter, respectively. This extreme variation in annual photoperiod has led many arctic residents to abandon diel rhythms. For example, animals such as caribou (*Rangifer tarandus*) and ptarmigan (*Lagopus mutus)* have been shown to become arrhythmic during conditions of constant light (Eloranta, Timisjaervi et al., 1995). Arctic ground squirrels (*Urocitellus parryii*) and the arctic migrant bird, Lapland Longspur (*Calcarius lapponicus)*, are exceptions to the arrhythmic behavior seen in many arctic animals; both maintain rythmicity despite the constant light of the polar summer. After arriving from their wintering grounds in southern Canada and other parts of the contiguous United States, Lapland Longspurs (LALO) maintain diel rhythms in singing and feeding, indicating they are entrained to a *zeitgeber* (Karplus, 1952). Because their breeding grounds reach as far north as Barrow, Alaska (71° N), where the polar day (constant light) lasts for 83 days--from the middle of May to early August--it has been hypothesized that the LALOs' ability to remain rhythmic involves entrainment to subtle deviations in light intensity or color temperature that occur within a 24-hour polar day.

A possible factor that allows for photoentrainment in LALOs is elevated concentration of sex steroids. Like many other animals, the upregulated production of testosterone (T) facilitates male LALO reproductive physiology and behaviors needed to compete for mates and defend territories during the breeding season. Moreover, T has been shown to modulate photosensitivity and locomotor activity in birds (Turek & Gwinner, 1982). Another key hormone linked to circadian rhythms is melatonin. Secreted by the pineal gland with the onset of low ambient light (dusk), melatonin is thought to be an internal time indicator by acting as a cue for sleep in diurnal animals, including humans (Cajochen, Krauchi, & Wirz-Justice, 2003). With the return of higher levels of photointensity (dawn), transcription factors for melatonin are degraded resulting in oscillations of plasma melatonin with a 24-hour period. If diurnal rhymicity in LALOs involves entrainment to subtle deviations in light intensity that occurs within a 24-hour polar day, it is likely that the synthesis and degradation of melatonin corresponds to these perceived astronomical *zeitgebers*.

The physiological mechanisms that underlie the persistent circadian rhythms observed in LALOs at high polar latitudes have yet to be identified. An understanding of mechanisms used for entrainment to low amplitude *zeitgebers* in an arctic migrant bird may allow researchers to anticipate consequences of environmental changes in LALO breeding grounds. For example, if the extent to which endogenous mechanisms increase photosensitivity were known, these data could be used to predict the magnitude of environmental changes that LALOs can tolerate and persist. A known potent driver and one of the predicted consequences of climate change is the increased accumulation of carbon dioxide in the atmosphere. This increase in carbon dioxide may have an impact on the observed composition and quality of sunlight by altering cloud cover and the overall makeup of gases in the atmosphere that sunlight must penetrate. If changes in sunlight composition and quality are shown to act as *zeitgebers* allowing LALOs to entrain, then it is probable that global changes in behavior and relative abundance of birds will be observed. With support from the Center for Global Change and National Science Foundation, experiments investigating the ability of LALOs to exhibit a circadian rhythm under varying light conditions are being conducted in the lab of Dr. C. Loren Buck (Department of Biology). Briefly, LALOs were transported from Barrow, Alaska, to the vivarium at the Conoco Phillips Integrated Science Building where they were implanted with crystalline testosterone or shams (controls). Currently, these birds are being exposed to various light regimes that mimic the variation in photo-intensity or color temperature experienced at their breeding grounds. A more detailed account of this procedure and preliminary results are discussed below.

This proposed research builds upon the past 1.5 years of investigations I have been conducting with captive LALOs to investigate mechanisms of entrainment to low amplitude *zeitgebers*. Results from these experiments indicate that captive LALOs photo-entrain to a LD cycle. However, captive LALOs failed to entrain to low amplitude changes in photo-stimulation (e.g., photo-intensity and color temperature). Therefore, I have set out to test the hypothesis that steroid hormones are necessary for photo-entrainment to low amplitude photic cues. Moreover, I will determine if plasma melatonin levels correspond to a perceived *zeitgeber.* To date, I have completed two rounds of experiments in which LALOs administered with exogenous T where exposed to 12-hours of high photo-intensity and 12 hours of low photo-intensity as well as 12-hours of white light and 12 hours of red light. Birds failed to entrain. Since photo-intensity can be modified by common atmospheric perturbations such as clouds and fog, variations may not be a reliable cue for time of day. Subsequently, I am testing the hypothesis that testosterone is necessary for activation of photo-entrainment of LALOs to changes in color temperature *and* photo intensity.

Experimental Design

*The **hypothesis** of this study is that sex steroids have activational effects on an arctic migrant bird's sensitivity to light, allowing entrainment to low amplitude zeitgebers.* I will test this overarching hypothesis by way of the two specific aims listed above.

Experimental Objectives

- Design and replicate the varying light intensities and color temperatures present at the breeding grounds of Lapland Longspurs.
- Evaluate locomotor activity in male Lapland Longspurs.
- Provide data and tissue samples for subsequent analyses.

Objective 1. Design and replicate the varying light intensities and color temperatures present at the breeding grounds of Lapland Longspurs.

Data acquired from the Barrow Environmental Observatory have been used to establish daily summer minimum and maximum light intensities and color temperatures. These parameters are being used as boundaries during light and color temperature manipulations.

Methodology

After completion of quarantine and surgery, birds were divided into 2 groups of 12 (6 treated, 6 controls per group) and placed in environmentally controlled chambers. The chambers are equipped with fluorescent lights and individual bird cages with mounted infrared sensors to monitor locomotor activity (perch hopping). Birds have been provided with food (PetCo. wild bird seed) and water *ad libitum* throughout the experiment. Light intensity and color temperature are controlled by an externally mounted control panel interfaced to a computer. Initially, both groups were exposed to 12 hours of light (1300 lux) and 12 hours dark, referred to as the LD treatment, until the birds entrained (approximately 4 days). Once entrainment was demonstrated, the following protocols were used for each group during the experiment:

The first group was subjected to a daily change in light intensity for 10 days, while the second group experienced a daily shift in color temperature (light quality) for 10 days (see below). Groups were then returned to the original LD treatment for 10 days to ensure entrainment and then the light treatments were switched between groups (for 10 days) such that all birds received both experimental light cycles.

Light Intensity group: 12 hours of bright light (1300 lux): 12 hours of low light (300 lux) with a constant color temperature (6500 K).

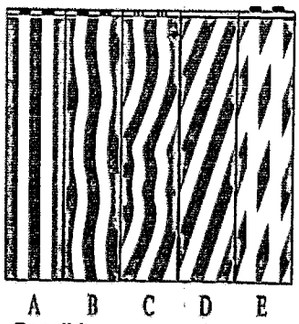
Color temperature group: 12 hours of white light (6500 K): 12 hours of red light (3500 K) with a constant light intensity (1300 lux).

As mentioned above, birds failed to entrain to manipulations in either photo-intenstiy or color temperature. Now, I will modify the aforementioned protocol by incorporating changes in photo-intensity *and* color temperature.

High-intensity white light / Low- intensity red light group: 12 hours of white light (1300 lux) at 6500k: 12 hours of red light (300 lux) at 3500k.

High-intensity red light / Low-intensity white light group: 12 hours of red light (1300 lux) at 3500k: 12 hours of white light (300 lux) at 6500k.

Figure 1



Possible correspondence between a behavior and a zeitgeber. Top=zeitgeber. adopted from (Krüll, 1985)

Objective 2. Evaluate locomotor activity in treated male Lapland Longspurs.

Sex steroids have been shown to modulate circadian rhythms in birds (Subbaraj, & Gwinner, 1985). For example, European starlings (*Sturnus vulgaris)* with exogenous testosterone showed a lengthening in circadian activity time such that perch hopping behavior shifted into later portions of the night (Gwinner, 1975). A shift in circadian activity will indicate a behavioral change that corresponds to synchronization to a weak *zeitgeber*. The actogram software will generate a history of the behavior throughout the experiment.

Figure 1 depicts a relationship between a zeitgeber and the entrained behavior. Activity patterns in both A and B indicate synchronization of activity with B having less precision with respect to timing of activity than A. C represents activity pattern of an animal exposed to an even weaker *zeitgeber* with some of the behavior "free running." D has no *zeitgeber*; behavior is free running without any known external stimuli and in E, an external cue deteriorates free-running. (Krüll,Demmelmeyer, & Remmert, 1985).

Methodology

Birds are individually housed and each cage is equipped with two perches and an infrared sensor to detect perch hopping activity. These data are computer recorded and converted to actograms for each bird (activity over time). If animals entrain to the subtle changes in the photic environment, patterns of the behavior and *zeitgeber* will correspond; actograms will be similar to that of **Figure 1**, panel A. Though behavioral profiles will be evaluated after the experiment, the software allows for real-time analysis of locomotor activity.

Objective 3.Evaluate data for patterns of entrainment of activity and tissue samples for circulating levels of T and melatonin.

Blood samples collected (200-250µl) from a wing vein before and after the implants were inserted will enable me to ascertain that the T implants succeeded in raising plasma testosterone levels. In addition, at the end of the photo-entrainment experiments, birds will be sampled at 4 time points across the circadian day for analysis of plasma concentration of melatonin. Diethyl Ether will be used to extract hormones from the plasma samples and will later be analyzed for T and melatonin concentrations using commercially competitive binding radioimmunoassay (RIA).

Future grant awards will allow me to use the currently held LALOs to investigate other possible *zeitgebers* such as temperature and food availability. Furthermore, additional funding is actively being pursued (e.g., Angus Gavin Memorial Bird Research Grant) which will allow me to spend a summer at the lab of a collaborator, G. Bentley, at University of California, Berkley, developing the techniques required to conduct tissue analysis. Specifically, I am proposing to sequence the *Per 1* gene of the avian clock to enable *in situ* hybridization experiments such that I will be able to develop a mechanistic understanding of clock gene function on circadian rhythms in LALOs.

Anticipated Results

If T is necessary to alter the sensitivity of birds to low-amplitude light intensity or color temperature *zeitgebers*, we would anticipate that the activity rhythms of testosterone-implanted birds would entrain to one or both of the experimental light treatments compared to subjects with empty implants (low T). I predict that captive LALOs treated with T will entrain to changes in light intensity *and* color temperature. Moreover, results obtained from the melatonin assays should corroborate behavioral (activity) findings. That is, I expect that birds entrained to low level zeitgebers will have low levels of melatonin during the active phase of the circadian day and much elevated levels during the inactive phase. A failure of birds to entrain may indicate that either **a)** specific combinations of light intensities *and* color temperatures not tested are needed to exact entrainment or **b)** upregulation of T does not correspond to an increase in sensitivity to light or **c)** LALOs do not entrain to the low amplitude *zeitgebers* tested. Whatever the outcome, this investigation will provide important information on molecular and neuroendocrine mechanisms of photoentrainment of a high latitude migrant species. This information aids in predicting organismal and populational resilience to global climate change.

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Project Schedule

The start date of the research was 1 July, 2010. Opportunities to conduct subsequent experiments with the same group of birds may be possible depending on several factors such as space availability and the acquisition of necessary permits and funding.

Task	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr
Capture free living male LALOs in Barrow, Alaska	▬										
Order assay kits/supplies					▬		▬				
Receive LALOs from Anchorage airport and transport to Vivarium at UAA		▬									
Quarantine LALOs		▬	▬								
Implant LALOs with T filled or empty capsules				▬							
Run plasma T and melatonin assay						▬	▬	▬			
Collect blood samples and expose LALOs to initial light intensity/color temperature protocols					▬	▬					
Begin light/color temperature experiment and data collection					▬	▬	▬	▬			
Euthanize LALOs and harvest tissue samples											▬
Ship tissues to Univ. of Cali, Berkley											▬
Data Analysis							▬	▬			
Write-up for publication / submit reports							▬	▬	▬		
SICB Conference									▬		
Presentation of results at UAA Undergraduate Research Forum											▬

Budget Justification and Other Sources of Project Support
Listed below is an account of the funding requested along with funding that has been used in support of this project. The requested funding will enable me to run assays for melatonin and to present results at an annual conference held by the Society for Integrative and Comparative Biology. An Itemized explanation is given at the bottom of this section.

Category	**Fran Ulmer**	**CGC**	**NSF**
Salaries and Benefits			
One month postdoctoral salary	$0	$0	$4200
One month student salary		$1500	
Total Salaries and Benefits	**$0**	$1500	$4200
Travel			
Domestic			
RT to Barrow, Alaska	$0	$800	
RT to Salt Lake (SICB Conference)	**$800**	$0	
Lodging for 3 days	**$300**	$300	
Per diem for 3 days	$0	$180	
Total Travel	**$1100**	$1280	$0
Services			
Per diem for birds in Barrow	$0	$400	
Per diem for birds at UAA	**$500**	$1440	
Shipment of birds from Barrow	$0	$200	
Shipping of tissue samples	$0	$100	
TOTAL SERVICES	**$500**	$2140	$0
Supplies			
Diethyl ether	**$ 90**	$0	
Nitrogen (for extraction)	**$150**	$0	
Testosterone, silastic tubing, silicone glue	$0	$150	
Radioimmunology assay kits (Testosterone)	$0	$800	
Radioimmunology assay kits (Melatonin)	**$1200**	$0	
Misc. lab supplies (gloves, capillary tubes)	**$100**	$200	
TOTAL SUPPLIES	**$1540**	$1150	$0
TOTAL FUNDING REQUESTED	**$3140**		
Total other funding		$6070	$4200

RT to Salt Lake/ Lodging, $1100: This money will allow me to attend a conference and present my results such that I can discuss my findings with other scientists.
Per Diem for birds, $500: This money will fund space needed for maintaining animals within the Vivarium at UAA.
Supplies, 6 RIA kits @ $200 each, $1200: This money will allow me to develop the methods needed for running melatonin assays. RIA for melatonin varies by species so enough kits need to be purchased to validate measurements in LALOs.
Supplies, $340: This money will be used to equip the lab with various items used on a daily bases (e.g. gloves, capillary tubes) and items needed to extract hormones (diethyl ether, nitrogen).