

## RESEARCH ARTICLE

# Entraining to the polar day: circadian rhythms in arctic ground squirrels

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## ABSTRACT

Circadian systems are principally entrained to 24 h light–dark cycles, but this cue is seasonally absent in polar environments. Although some resident polar vertebrates have weak circadian clocks and are seasonally arrhythmic, the arctic ground squirrel (AGS) maintains daily rhythms of physiology and behavior throughout the summer, which includes 6 weeks of constant daylight. Here, we show that persistent daily rhythms in AGS are maintained through a circadian system that readily entrains to the polar day yet remains insensitive to entrainment by rapid light–dark transitions, which AGS generate naturally as a consequence of their semi-fossorial behavior. Additionally, AGS do not show ‘jet lag’, the slow realignment of circadian rhythms induced by the inertia of an intrinsically stable master circadian clock in the suprachiasmatic nucleus (SCN). We suggest this is due to the low expression of arginine vasopressin in the SCN of AGS, as vasopressin is associated with inter-neuronal coupling and robust rhythmicity.

**KEY WORDS:** Biologging, Body temperature, Chronobiology, Jet lag, Polar vertebrates, *Urocitellus parryii*, Zeitgeber

## INTRODUCTION

The Earth’s rotation about its axis drives a 24 h rhythm in the environment that influences almost every biome on the planet. Circadian clocks, endogenous molecular feedback loops with a period close to 24 h, allow organisms to adjust their physiology and behavior in a rhythmic fashion in anticipation of these daily changes, rather than simply responding to changes as they occur (Sharma, 2013; Yerushalmi and Green, 2009). The importance of circadian clocks is evidenced by the expansive range of organisms in which they occur, from prokaryotic, unicellular cyanobacteria to higher vertebrates, including mammals (Kondo et al., 1993; Buhr and Takahashi, 2013). These internal circadian clocks, however, must be entrained to an external cue, known as zeitgeber, for rhythms to remain synchronized with the environment.

In mammals, the master circadian clock, located in the suprachiasmatic nucleus (SCN) within the hypothalamus, is entrained by signals transduced from retinal photoreceptors (Reppert and Weaver, 2002; Lucas et al., 2014). This master

clock maintains synchrony among peripheral circadian clocks found throughout the body through a variety of signals including body temperature ( $T_b$ ), humoral messengers and metabolic factors (Buhr et al., 2010; Dibner et al., 2010). Although energy metabolism and scheduled feedings can influence clock mechanisms of the SCN (Castillo et al., 2004; Mendoza, 2007), the master clock is thought to be principally entrained by the transitions between light and dark that occur during dusk and dawn (Lowrey and Takahashi, 2000).

Almost since the discovery of circadian rhythms, scientists have been intrigued by the importance and function of endogenous circadian clocks in polar environments where strong transitions between light and darkness are seasonally absent (Karplus, 1952; Cullen, 1954; Swade and Pittendrigh, 1967). Does daily rhythmicity persist in environments where 24 h environmental rhythms are absent or severely dampened? And if circadian rhythms persist, what function do they serve and how do they remain synchronized with the environment? Rock ptarmigan and reindeer in the high Arctic on Svalbard (78°N) lose circadian organization of physiology and behavior in mid-summer and mid-winter (Reierth and Stokkan, 1998; van Oort et al., 2005), and it has been argued that weak circadian clocks may be a general feature of resident polar vertebrates (van Oort et al., 2005). However, arctic ground squirrels (hereafter: AGS; *Urocitellus parryii*) in northern Alaska (68.5°N) exhibit strong, daily (24 h) rhythms of above-ground activity and  $T_b$  throughout the summer months, which includes 6 weeks of continuous daylight (Williams et al., 2012, 2014). The persistence of daily activity rhythms may function to minimize thermoregulatory costs, as summertime standard operative temperature at these high latitudes is only within the thermoneutral zone of AGS during the midday interval (Long et al., 2005). The persistence of entrained rhythms in this species under conditions of continuous light is remarkable given that their semi-fossorial habits expose them to rapid transitions between light and dark as they exit and re-enter their burrows multiple times each day. AGS are therefore exposed to square-wave light–dark (LD) cycles as a consequence of their own behavior and are not exposed to dusk or dawn, or even to ambient lighting conditions when the sun is at its lowest point in the sky during the polar day.

In the present study, we examined how rhythms of  $T_b$  and activity are affected by temporary removal of AGS from the wild and exposure to square-wave LD cycles that are either in phase with their natural self-imposed exposure to light (generated by their semi-fossorial behavior) or 6 h and then 12 h phase delayed. We anticipated AGS exposed to a phase-delayed photoperiod would entrain their rhythms to the new LD cycle, but that this entrainment process would take close to a week owing to inertia of the molecular clock within the SCN (i.e. ‘jet lag’; Yamaguchi et al., 2013). However, we found that their circadian rhythms did not phase delay and instead free ran with a period <24 h and consequently AGS phase advanced their rhythms of  $T_b$  and activity. We then examined how AGS whose free-running rhythms had become phase advanced

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**List of abbreviations**

AGS	arctic ground squirrel
AVP	arginine vasopressin
LD	light–dark
ODBA	overall dynamic body acceleration
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
$T_b$	body temperature

by an average of 10 h responded to release back into their natural environment under conditions of continuous daylight. We monitored core  $T_b$  using implanted temperature loggers and measured time spent above ground and activity levels using collar-mounted light and acceleration loggers. Although the spectral composition and intensity of light exhibit 24 h rhythmic fluctuations at this latitude (Ashley et al., 2013), we predicted animals would re-entrain slowly without access to a robust LD cycle. Surprisingly, we found that rhythms of physiology and behavior rapidly (1–2 days) re-entrained to the polar day with little evidence of jet lag, the symptoms that typically arise when the internal circadian clock is temporally misaligned with solar time. Given previous findings that disruption of arginine vasopressin signaling in the circadian master clock leads to accelerated recovery from jet lag (Yamaguchi et al., 2013), we subsequently examined patterns of expression of vasopressin in AGS and found that vasopressin mRNA (*Avp*) and peptide (AVP) expression in the SCN is very low throughout the day/night cycle in this species, perhaps contributing to its ability to rapidly re-entrain to subtle environmental cues.

**MATERIALS AND METHODS****Study site and phase-shift experiment**

We studied free-living AGS (*Urocitellus parryi* Richardson 1825) near the Atigun River (68°27'N, 149°21'W; elevation 812 m), 20 km south of Toolik Field Station in Northern Alaska. Protocols involving animals were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC no. 340270-41). Between 13 and 16 May 2015, we live-trapped 14 adult ( $\geq 1$  year old) male AGS using Tomahawk live-traps baited with carrot. Squirrels were subsequently transported to Toolik Field Station, where they were implanted with  $T_b$  loggers/transmitters and subjected to LD cycles that were either in phase or phase delayed relative to self-imposed light exposure of free-living ground squirrels (Williams et al., 2014). Within 12 h of capture, we surgically implanted loggers (~5 g; iButton model DS1922L, Maxim Integrated Products, Sunnydale, CA, USA), programmed to record temperature every 10 min, into the peritoneal cavity. Additionally, eight animals (four phase-shift treatment and four controls) were implanted with temperature-sensitive radio transmitters (~7 g; model TA10TA-F40-LF, Data Sciences International, St Paul, MN, USA), which allowed real-time monitoring of  $T_b$  and activity to determine whether and by how much the  $T_b$  and activity rhythms had shifted prior to release back into the field.

Animals were housed in 43 cm×27 cm×19 cm plastic tubs (Nalgene, Rochester, NY, USA) lined with pine shavings on the floor and cotton batting (Perfect Fit, McDonald, Tukwila, WA, USA) provided as nesting material. Wet shavings were removed and replaced with fresh dry shavings daily; every 4 days, animals were transferred into a clean tub. Each day, we provided animals with 8–10 pellets of Mazuri® rodent chow (PMI Nutrition International,

St Louis, MO, USA), along with slices of carrot and apple, and Napa Nectar™ gel packs (Systems Engineering Lab Group Inc., Napa, CA, USA) for hydration. Animal care was performed and food was provided within the first 2 h of lights on.

The morning after surgeries, animals were transferred into environmental chambers (rodent incubation chambers, Powers Scientific, Pipersville, PA, USA) and held under a 13 h:11 h LD cycle at constant temperature (15°C) for 28–31 days. Each chamber was equipped with two F32T8 bulbs (3500K, 2800 lumens, Phillips Lighting, Amsterdam, The Netherlands). Lights were on from 08:00 h to 21:00 h for the control animals ( $n=7$ ), whereas lights-on for the experimental animals ( $n=7$ ) was first delayed by 6 h (14:00 h to 03:00 h) from 14 to 26 May, and then by 12 h (20:00 h to 09:00 h) until the release date (13 or 14 June). The timing and duration of the lights-on period for control animals was designed to mimic the daily above-ground interval when these animals expose themselves to sunlight in the wild (see Williams et al., 2014). We continually monitored the  $T_b$  rhythms of the subset of control and experimental squirrels implanted with transmitters and released animals after 28–31 days, when the acrophase (peak in the cycle) of the circadian  $T_b$  rhythm for experimental squirrels was, on average, 9.8 h earlier than that of control squirrels (see Results).

**Animal release and recapture**

On the day of their release, squirrels were removed from their chambers immediately after lights on, briefly anesthetized (~3 min) using isoflurane, and outfitted with a tri-axial accelerometer (Axy-3, TechnoSmart Europe SRL, Rome, Italy), light logger (Intigeo-C56, Migrate Technology Ltd, Cambridge, UK) and radio transmitter (Advanced Telemetry Systems, Isanti, MN, USA) affixed to a collar (total package mass <9 g). Accelerometers measured acceleration (g) once per second along three orthogonal axes ( $x$ ,  $y$  and  $z$ ). Light loggers sampled light (lux) once every minute and then saved the highest reading for each 5 min interval. Animals were then released at the site where they were originally captured. Control animals were released in the morning, between 09:15 h and 09:40 h, while experimental animals were released in the evening, between 21:10 h and 21:25 h. The timing of release was chosen such that animals were first exposed to natural light at the start of their subjective day, based on their chamber lighting regime. In addition to control animals that were subjected to the same captive environment and artificial lighting as the treatment group, we also used light loggers to measure patterns of above- and below-ground activity in four AGS that were never held in captivity; hereafter, we refer to this as the 'free-living' group.

We began recapturing animals 19 days after they were released. Animals were tracked to their burrows using hand-held radio-transmitter receivers and recaptured using carrot-baited Tomahawk traps. We subsequently transported animals to Toolik Field Station, anesthetized them using isoflurane, explanted the devices using the same surgical procedures, and removed the collar and downloaded the loggers. Animals were returned to their capture sites for release the following day. We successfully recaptured and recovered all loggers from all seven animals in the phase-shift group and from five of the seven animals in the control group. One of the control squirrels was predated by a raptor 20 days following release; we tracked the radio-transmitter to a mountain perch several kilometers away where we found the remains of several ground squirrels and ptarmigan, as well as a broken collar with a light logger and radio transmitter still attached; the accelerometer and  $T_b$  logger were not recovered. We did not detect a radio-signal from the final control animal and it was never recaptured.

### Light and acceleration data

Light logger data were used to assess whether animals were above ground (exposed to light >5 lx) or below ground (<5 lx; a base of zero is not used because of slight drift in the baseline) according to the methods described in Williams et al. (2014). Acceleration data were used to calculate overall dynamic body acceleration (ODBA), a measure of movement that correlates with metabolic rate and is therefore considered a useful index of activity-based energy expenditure (Wilson et al., 2006; Halsey et al., 2009a). For each axis, the static effect of gravity on acceleration was removed from the acceleration data by subtracting the 11 s running mean. We then calculated ODBA using the method of Wilson et al. (2006), which sums the absolute values of the calculated dynamic acceleration for each axis. Although ODBA is typically calculated using measurements of acceleration at a frequency of 10 Hz or higher, sampling frequencies as low as 1 Hz, as in our study, provide reasonable estimates, even in small animals (Halsey et al., 2009b).

### Immunohistochemistry and *in situ* hybridization

The expression of arginine vasopressin (AVP) mRNA and peptide was examined in captive AGS, and patterns of staining were compared with those of another diurnal rodent species, the Nile grass rat (*Arvicanthis niloticus*). For both species, animals ( $n=4-5$  per species) were housed under 12 h:12 h LD cycles and killed with sodium pentobarbital (200 mg kg<sup>-1</sup>) at midday and midnight, when the expression of *Avp* mRNA is expected at peak and trough, respectively, in the SCN (Jin et al., 1999). The animals were then perfused intracardially using 100 ml saline followed by 200 ml 4% paraformaldehyde in 0.1 mol l<sup>-1</sup> phosphate buffer. Brains were post-fixed for 12–18 h and cryoprotected in a 20% sucrose solution. We then collected 40 µm coronal sections through the entire SCN and paraventricular nucleus (PVN) from each brain using a cryostat. Two separate sets were collected from each animal that were processed for detecting the expression of mRNA or peptide.

### Immunohistochemistry

Free-floating sections were incubated in the primary antibody raised in guinea pig against AVP (1:10,000, Peninsula Laboratories, San Carlos, CA, USA) and processed with avidinbiotin-peroxidase technique using 3,3-diaminobenzidine (DAB) as the chromogen, as in Yan (2011). Following the reaction, the sections were mounted on slides, rinsed with ethanol and xylene, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

### *In situ* hybridization

*In situ* hybridization of *Avp* mRNA was performed as described previously (Yan et al., 1999; Ramanathan et al., 2009). Briefly, sections were processed with proteinase K at 37°C followed by 0.25% acetic anhydride at room temperature for 10 min. The sections were then incubated in hybridization buffer containing digoxigenin-labeled *Avp* cRNA probes (0.1 µg l ml<sup>-1</sup>) overnight at 60°C. After a high-stringency post-hybridization wash, sections were treated with RNase A, and then were further processed for immunodetection with a nucleic acid detection kit following the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

Images of sections containing mid-SCN and PVN from the same animal were captured (10×) using a light microscope (Nikon) and a CCD video camera (CX9000, MBF Bioscience, Williston, VT, USA). The rich expression of both AVP mRNA and peptide in the PVN serves as an internal positive control for the SCN.

### Statistical analysis

Actograms were constructed and Lomb–Scargle periodogram analysis was performed using Clocklab software (Actimetrics, Evanston, IL, USA). We display normalized actograms, in which each line of the actogram is scaled individually between the minimum and maximum value. For each animal, the daily acrophase for  $T_b$  rhythms (i.e. the time period in a cycle during which the cycle peaks) was calculated using Clocklab software. A *t*-test for unequal variances was used to compare  $T_b$  acrophase of treatment and control groups prior to release. We compared  $T_b$  acrophase as well as the daily onset and termination of above-ground activity in animals in the control versus treatment groups during the first 7 days following release using repeated measures mixed models in SAS (Proc Mixed; v. 9.4, SAS Institute, Cary, NC, USA). The covariance structure for mixed models (unstructured for  $T_b$  acrophase and activity onset; simple for activity offset) was selected using Akaike's information criterion for small sample sizes (AICc; Littell et al., 2006). Simple effects tests (LSMEANS/SLICE) were used to examine significant two-way interactions (i.e. treatment×day).

## RESULTS

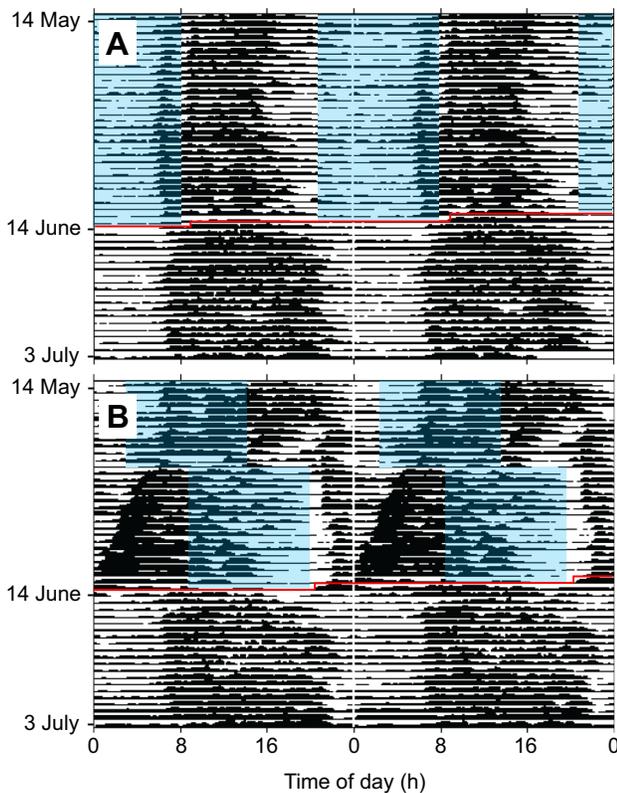
### $T_b$ rhythms

Of the five control animals for which we obtained  $T_b$  data, four maintained entrained daily rhythms of body temperature during their 28–31 days in the environmental chamber (Fig. 1A; Fig. S1a–c). The fifth control animal (Fig. S1d) exhibited a free-running  $T_b$  rhythm with a period of 23.8 h; we do not include this squirrel in the comparisons between control and treatment group animals reported below. All seven treatment group animals failed to shift their  $T_b$  rhythms to match the phase-delayed LD cycle provided in the chambers. Instead,  $T_b$  rhythms free ran for six of the seven animals, with a period <24 h (range: 23.6–23.8 h; Fig. 1B; Fig. S1e–i), similar to what was observed in the free-running control. The seventh experimental animal had disrupted  $T_b$  rhythms without a clear phase shift (period: 23.9 h); this animal was not included in the comparisons between groups reported below. Examination of the actograms revealed splitting of the  $T_b$  components for phase-shifted squirrels; a second daily interval of  $T_b$  warming occurred shortly after lights on following feeding and cage cleaning each day (Fig. S1).

On the day prior to release, the acrophase of  $T_b$  rhythms for phase-shifted squirrels was  $9.8\pm 1.4$  h (mean±s.d.) earlier than that of control squirrels ( $T_{3,6}=8.9$ ,  $P=0.001$ ). Following their release back into the wild, the acrophase for  $T_b$  rhythms of control squirrels did not change whereas the  $T_b$  rhythms of experimental squirrels rapidly (1–2 days) became re-entrained with geophysical time to match those of control squirrels (Fig. 1; Fig. S1). The acrophase of  $T_b$  rhythms during the 7 days following release was significantly affected by treatment ( $F_{1,8}=22.0$ ,  $P=0.001$ ), day ( $F_{7,8}=1057.0$ ,  $P<0.0001$ ), and the interaction between treatment and day ( $F_{7,8}=1128.0$ ,  $P<0.0001$ ). Simple effects tests indicated  $T_b$  acrophase differed between groups on the day animals were released ( $F_{1,8}=279.2$ ,  $P<0.0001$ ); this difference was no longer significant on the day following their release ( $F_{1,8}=4.4$ ,  $P=0.07$ ) and there was no significant difference on the second day following release into the field ( $F_{1,8}=0.4$ ,  $P=0.55$ ; Fig. 2).

### Activity rhythms

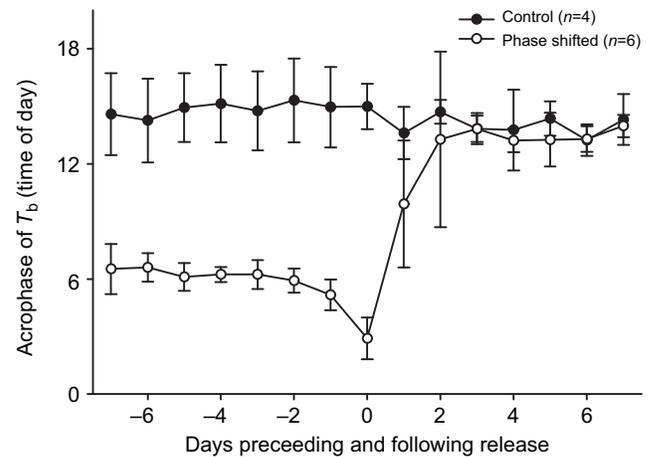
The implanted radio transmitters indicated daily activity rhythms during captivity aligned closely with  $T_b$  rhythms (Fig. S2). The mean period of the activity rhythms for phase-shifted AGS was 23.7 h (range: 23.6–23.7 h) compared with 24.0 h for control AGS (range: 23.9–24.1 h; excluding the one control squirrel that free ran



**Fig. 1. Body temperature ( $T_b$ ) rhythms of arctic ground squirrels (AGS).** Double-plotted actograms of  $T_b$  from (A) a representative control animal and (B) a representative experimental (phase-shifted) animal. Each line represents 2 consecutive days of data. Black bars indicate intervals when  $T_b$  was above the 24 h mean. The control animal was maintained in captivity on a 13 h:11 h light–dark (LD) cycle with lights off (blue shading) between 21:00 h and 08:00 h from 14 May to 14 June. The experimental animal was maintained on a 13 h:11 h LD cycle with lights off (blue shading) between 03:00 h and 14:00 h from 14 to 26 May (a 6 h delay) and between 09:00 h and 20:00 h from 27 May to 14 June (a 12 h delay). Data below the red line depict patterns following release on 14 June until 3 July, when recaptures began. Following their release, all animals rapidly re-entrained with the natural day despite continuous daylight. Data for additional animals are shown in Fig. S1.

and had a period of 23.8 h). The phase-shifted squirrels (treatment group) rapidly realigned the timing of their above-ground activities after release, as indicated by light loggers, to match that of control squirrels and free-living squirrels that were never brought into captivity (Fig. 3; Fig. S3). The onset of above-ground activity during the 7 days immediately following release was significantly affected by treatment ( $F_{1,9}=26.2$ ,  $P=0.0006$ ), day ( $F_{6,9}=21.3$ ,  $P<0.0001$ ), and the interaction between treatment and day ( $F_{6,9}=25.24$ ,  $P<0.0001$ ). Simple effects tests indicated the onset of above-ground activity differed between control and treatment groups on the first ( $F_{1,9}=78.0$ ,  $P<0.0001$ ) and second day ( $F_{1,9}=11.0$ ,  $P=0.009$ ) following their release; the difference in activity onset was no longer significant on the third day ( $F_{1,9}=4.6$ ,  $P=0.06$ ) and was clearly not different by the fourth day following release ( $F_{1,9}=1.3$ ,  $P=0.29$ ; Fig. 4A). The one control animal that phase shifted despite exposure to a LD cycle synchronized to its initial  $T_b$  rhythm (Fig. S1d) exhibited a similar pattern to phase-shifted animals except that this individual had a later onset of above-ground activity on the day following its release from captivity.

In contrast to activity onset, the offset of above-ground activity changed over time to approach what was observed in free-living



**Fig. 2. Mean ( $\pm$ s.d.) acrophase of  $T_b$  rhythms during the 7 days preceding (negative numbers) and following (positive numbers) release from captivity.** Control animals were maintained on a LD cycle with light occurring coincident with when they are typically above ground. Phase-shifted animals were subjected to two successively delayed LD cycles such that the LD cycle was 12 h out of phase for 17 days prior to release. Following release, phase-shifted squirrels quickly realigned their  $T_b$  rhythms with geophysical time and control animals. The day following release (day 1), the difference in  $T_b$  acrophase approached significance ( $P=0.07$ ) but by day 2, no difference was detected ( $P=0.55$ ).

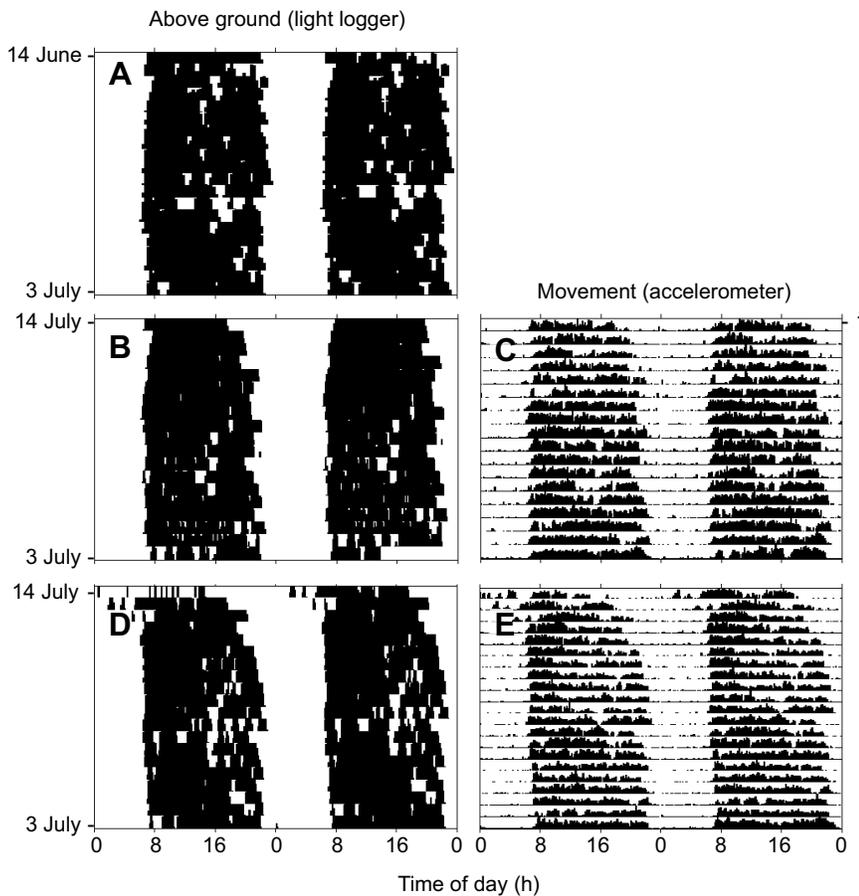
animals but did not differ between control and phase-shifted animals (day:  $F_{6,54}=7.1$ ,  $P<0.0001$ ; group:  $F_{1,9}=1.1$ ,  $P=0.31$ ; day $\times$ group:  $F_{6,54}=0.13$ ,  $P=0.99$ ; Fig. 4B). Similar to what was observed for time spent above ground based on light logger data, accelerometers revealed that squirrels in the treatment group were active throughout much of their first night but, by day 2, had shifted over to a 24 h ODBA rhythm that was synchronized with control squirrels (Fig. 3; Fig. S3).

#### **In situ hybridization and immunohistochemistry**

The expression of AVP peptide was extremely low in the SCN, with only lightly labeled fibers in the dorsal region of the nucleus, while the expression of *Avp* mRNA was barely detectable within the SCN collected during either the day or night (Fig. 5, left panel). In contrast, strong expression of AVP peptide and *Avp* mRNA was detected in the PVN (Fig. 5, middle panel) as well as supraoptic nucleus (data not shown). The strong signals of both peptide and mRNA in the PVN from the same animals or brain sections, as a positive control for the methods of immunohistochemistry or *in situ* hybridization, indicate the lack of AVP expression in the SCN was not due to any potential technical issue, but rather reflected diminished levels of endogenous expression within the SCN in AGS brains. In diurnal grass rats, strong *Avp* and AVP expression was detected in the SCN (Fig. 5, right panel).

#### **DISCUSSION**

We found that  $T_b$  and activity rhythms of AGS did not entrain to successive phase-delayed square-wave LD cycles with a 24 h period, yet these rhythms very rapidly re-entrained in a diurnal pattern to the dampened physical oscillations of the arctic environment under natural conditions of continuous daylight. The observed free-running  $T_b$  rhythms of captive animals were initially in phase with the timing of activity in free-living AGS, indicating that daily behavioral and physiological rhythms in AGS are controlled by an endogenous circadian clock that is synchronized



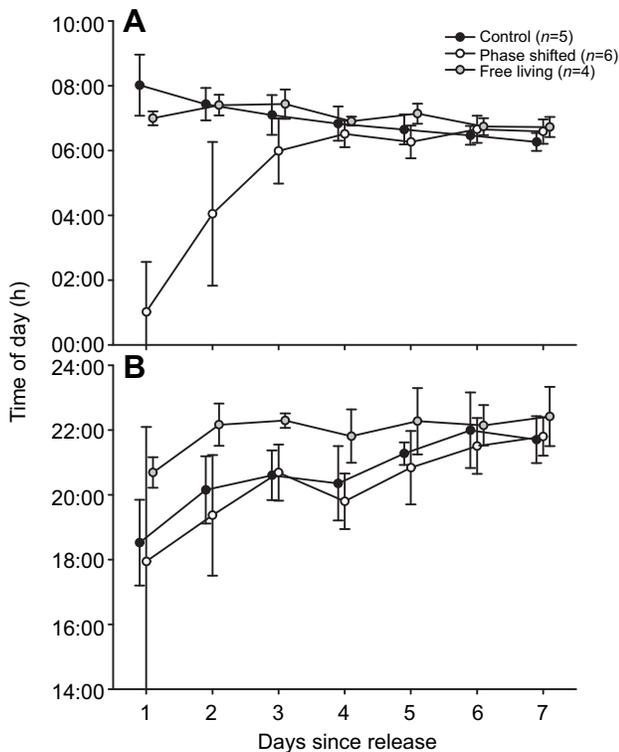
**Fig. 3. Activity rhythms of AGS.** Double-plotted actograms of time above ground (black bars) from (A) a free-living animal that was not brought into captivity, (B) a control animal that was maintained on a LD cycle where lights on matched the portion of the day when free-living animals are typically above ground, and (D) an animal that was phase advanced by 9.8 h as a result of a free-running circadian rhythm after 30 days in captivity. Double-plotted actograms of movement (overall dynamic body acceleration, ODBA) for the same (C) control animal and (E) phase-advanced animal are also shown. All data shown are following release from captivity. The y-axis shows days from release on 14 June until 3 July, when recaptures began. Phase-shifted animals rapidly developed 24 h above-ground activity rhythms that matched those of controls and free-living animals (see Figs S3 and S4 for all other animals).

with the polar day. The master clock can apparently be entrained by subtle parametric changes in the intensity or spectral composition of light across the polar day, or to other cues, yet appears to be relatively insensitive to abrupt transitions between light phases and dark phases. Despite the lack of dusk or dawn during the polar day, shifts in behavioral and physiological rhythms of AGS exhibited exceptionally little jet lag, the delay in realignment that results from inertia of the circadian master clock. We speculate that rapid re-entrainment may be a function of reduced neuronal coupling within the SCN associated with the low expression of vasopressin.

The responses of polar animals to the extended intervals where the sun remains above or below the horizon are diverse; some polar species appear to lose their circadian organization of physiology and behavior, whereas, in others, rhythms persist and are either synchronized with the environment or are free running with a period that deviates slightly from 24 h (reviewed in Williams et al., 2015). In invertebrates, it has long been postulated that persistent daily rhythms of behavior may indicate a circadian clock that is entrained to diel changes in temperature or UV light (Nordtug and Melø, 1988). In bumblebees, daily rhythms of behavior persist in the Arctic (Stelzer and Chittka, 2010), and captive experiments suggest they are capable of entraining to cycles in UV radiation (Chittka et al., 2013). However, Antarctic midges do not exhibit rhythmic oscillations of clock genes despite the persistence of daily behavioral rhythms under the midnight sun (Kobelkova et al., 2015). This highlights the difficulty in interpreting data from observational studies; the presence of daily rhythms does not necessarily indicate that circadian systems are functional and entrained to the natural environment as animals may be responding

directly to external cues, a phenomenon known as ‘masking’. For example, the masking influence of light results in daily activity cycles in Mexican blind cavefish (*Astyanax mexicanus*) maintained on a LD cycle, but such rhythmicity is not present under natural conditions of continuous darkness (Beale et al., 2013). Similarly, the absence of daily rhythms of behavior does not necessarily indicate that endogenous circadian clocks are no longer functional or entrained, as circadian clocks may simply be disconnected from output pathways. This phenomenon occurs in some arctic shorebirds, for example, which transition from arrhythmicity to ~24 h rhythms of activity coincident with the onset of incubation (Steiger et al., 2013).

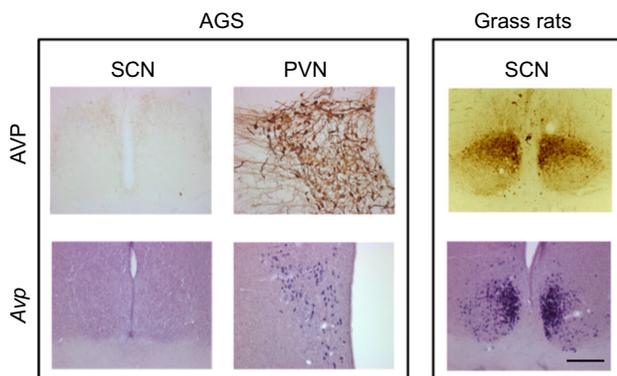
Our finding that AGS exhibit free-running circadian rhythms when removed from their natural environment and exposed to an ‘out-of-phase’ square-wave LD cycle indicates persistent daily behavior and  $T_b$  rhythms are regulated by an underlying circadian clock, yet this clock does not readily entrain to abrupt transitions between light and dark. The size of the phase shift induced by a pulse of light is dependent on the timing of the pulse relative to the phase of the clock (DeCoursey, 1960), and it is possible that the lack of responsiveness to the phase-delayed LD treatment was due to the timing of light onset. However, this seems unlikely given that one control animal exhibited a free-running circadian rhythm despite exposure to a LD cycle that closely matched its initial activity and  $T_b$  rhythms. Further, all phase-shifted squirrels readily and rapidly re-entrained once returned to the natural environment although their rhythms were advanced by 8.25–10.55 h relative to controls at the time of their release. Although the vast majority of circadian rhythms research involves captive animals held in artificial lighting,



**Fig. 4. Mean ( $\pm$ s.d.) onset and termination of daily above-ground activity in control, phase-shifted and free-living (never held in captivity) AGS.**

(A) Onset; (B) termination. The start of day 1 corresponds to 15 h after controls were released and 3 h after phase-shifted squirrels were released from captivity. Phase-shifted squirrels had an earlier onset of activity relative to controls for the first 2 days following their release ( $P < 0.05$ ). There was no difference in the termination of daily activity between phase-shifted and control squirrels, although termination was much more variable in phase-shifted squirrels on the day following their release. Groups are slightly offset from one another on each day for ease of visualization.

our study demonstrates that natural lighting can be a more potent zeitgeber for entrainment, even when the sun never drops below the horizon. This finding contrasts with the classic view that twilight periods contain the most important photic information that allows



**Fig. 5. Expression patterns for arginine vasopressin (AVP) mRNA and protein in the suprachiasmatic nucleus (SCN) of a representative AGS maintained on a 12 h:12 h LD cycle.** Scale bar, 200  $\mu$ m. Expression in the paraventricular nucleus (PVN) is shown as a positive control. Regardless of zeitgeber time (midday shown; midnight not shown), *Avp* mRNA and AVP protein expression remained low in the SCN, but not the PVN, of AGS. High expression of *Avp* and AVP in the SCN of a grass rat at midday is shown for comparison.

animals to maintain their synchrony with the external environment (Roenneberg and Foster, 1997).

The circadian system of AGS appears to be much more sensitive to the natural changes in lighting conditions that characterize the polar day than to an artificial LD cycle. The inability to mimic the intensity of natural light is one limitation of lab-based studies on circadian function. Even in *Drosophila melanogaster*, a prominent model system for studying the molecular and neurogenetic basis of circadian behavioral rhythms, many laboratory-based assumptions about circadian behavior have not been supported by observations of animals under natural conditions (Vanin et al., 2012). Differences in responsiveness between lab- and field-based studies might relate to the sensitivity of animals to gradual changes in the intensity or spectral quality of light (Vanin et al., 2012); although most circadian studies rely on square-wave LD cycles, it has long been known that captive animals are more sensitive to gradual transitions in lighting conditions (Kavanau, 1969; Boulos et al., 1996; Tang et al., 1999). Although it is possible that AGS are entraining their rhythms to changes in the intensity of light across the polar day, daily changes in the spectral composition of light are more consistent and less sensitive to cloud cover, and may therefore be a more prominent zeitgeber (Ashley et al., 2013; Krügel et al., 1985). Walmsley et al. (2015) recently showed that some clock neurons in mammals are highly sensitive to the spectral composition of light, supporting the premise that changes in the color of light can act as a signal for entrainment. The ultraviolet (UV) and near-UV spectrums exhibit particularly robust daily rhythms at polar latitudes and are likely zeitgeber for invertebrates (Nortug and Melø, 1988; Stelzer and Chittka, 2010) and birds (Ashley et al., 2013). However, as in most diurnal mammals, UV light does not penetrate the lens of the eye in ground squirrels (Hut et al., 2000) and therefore cannot act as a zeitgeber for circadian entrainment. In addition to changes in lighting conditions, there are robust daily oscillations in ambient temperature conditions at our study site in the Arctic, and we assume that persistent daily activity rhythms function to minimize daily thermoregulatory costs (Long et al., 2005; Williams et al., 2012). It is also possible, though we think unlikely, that daily rhythms in ambient temperature are used as a cue to entrain arctic ground squirrels; ectotherms can readily be entrained by temperature, but resistance to temperature entrainment is a general property of circadian clocks within the mammalian SCN (Buhr et al., 2010). Some mice, however, can be entrained by temperature cycles, although temperature is a much weaker zeitgeber than LD cycles (Refinetti, 2010). Further, we are not aware of any evidence that mammals can detect the plane of polarized light or use the position of the sun as a zeitgeber, though these could both theoretically be used as timing cues in polar environments.

In some resident polar vertebrates, including rock ptarmigan and Svalbard reindeer, the circadian organization of behavior is lost during the polar day and polar night (Reierth and Stokkan, 1998; van Oort et al., 2005). Van Oort et al. (2005) proposed that a seasonal absence of circadian rhythms may be common to all resident polar vertebrates. Subsequent investigation revealed that melatonin secretion in arctic reindeer, which is only rhythmic during spring and autumn, is independent of endogenous circadian rhythms and is instead driven predominantly by natural changes in ambient illumination (Stokkan et al., 2007). Further, circadian rhythmicity is absent in the transduced luciferase reporters for two key clock genes within the fibroblast cells of reindeer, which led to the hypothesis that a lack of robust circadian mechanisms (e.g. weak circadian clocks) may be ‘an adaptive evolutionary consequence for life in the extreme photic environment to which these animals are

naturally exposed' (Lu et al., 2010). More recent work, however, indicates weak circadian clocks may be a general feature of ungulates, rather than necessarily representing an adaptation to the intervals of continuous lighting in polar environments (Ensing et al., 2014). Whether the capacity of AGS to entrain to the polar day reflects physiological adaptation to the Arctic is also uncertain as even the more temperate zone European ground squirrel (*Spermophilus citellus*) maintains daily rhythms without being above ground during the intervals of dusk and dawn (Hut et al., 1999). Sensitivity to parametric changes in the intensity or spectral quality of light while maintaining insensitivity to rapid transitions between light and dark, which are generated as a result of their semi-fossorial habits, may be a general characteristic of circadian function in ground squirrels, though the mechanisms that underlie this phenomenon remain unclear.

Our finding that AGS can entrain their circadian activity and  $T_b$  rhythms to the polar day was anticipated given our previous data showing these rhythms persist throughout the arctic summer (Williams et al., 2012, 2014). However the rapid shift in these rhythms under conditions of continuous daylight was unexpected and is particularly surprising given their lack of sensitivity to square-wave LD cycles. Although rapid shifts in behavior could occur independent of the phase of the clockwork in the SCN (i.e. masking), this is unlikely given surface activity of SCN-lesioned ground squirrels occurs both in the daytime and at night (DeCoursey et al., 1997). Additionally,  $T_b$  rhythms, which are generated by the SCN to entrain peripheral circadian clocks (Buhr et al., 2010), also shifted rapidly after animals were released back into their natural environment. In captive animals, abrupt shifts in the LD cycle lead to a misalignment between SCN clock signals and environmental time cues; the intrinsic stability of the master clock is what makes it a useful pacemaker, but this stability confers inertia which slows the process of re-entrainment leading to jet lag (Hastings, 2013). The stability of the SCN stems from a hierarchy of paracrine neuropeptidergic signals that act to synchronize individual SCN clock cells (Maywood et al., 2011; Brancaccio et al., 2013). Using vasopressin receptor knockout models, Yamaguchi et al. (2013) showed that vasopressin confers on the SCN an intrinsic resistance to external perturbation (i.e. jet lag). Surprisingly, we found extremely low expression of vasopressin in the SCN of captive AGS; this pattern of low expression contrasts strongly with what has previously been observed in nocturnal rodents (mouse: Jin et al., 1999; rat: Cagampang et al., 1994; hamster: Yan et al., 2005), as well as what we observed in diurnal grass rats (Ramanathan et al., 2009; present study). The captive AGS in which we measured vasopressin expression exhibited clear diurnal rhythms in their core  $T_b$ , as well as in the expression of the period circadian clock (Per) genes *PER1* and *PER2* (Ikeno et al., 2017), indicating animals had entrained circadian rhythms at the time of sampling. We speculate that the lack of vasopressin expression may facilitate the rapid shifts in  $T_b$  and activity rhythms observed in our study. However, given that rodents will re-entrain more rapidly to phase delays than to phase advances (Reddy et al., 2002), more research is needed to determine the extent to which AGS are resistant to jet lag. Further, the function and/or adaptive value of low vasopressin expression within the SCN of AGS is unclear. In vasopressin-deficient Battleboro rats, circadian rhythms are dominated by a food-entrainable oscillator (Murphy et al., 1998), but whether this is relevant for AGS, where food is available throughout the day, is unclear. Irrespective of the function of low vasopressin in AGS, this pattern does not appear to be a general feature of ground squirrels as Hut et al. (2002) report high numbers of vasopressin-expressing

neurons in the SCN of European ground squirrels once circadian  $T_b$  rhythms became re-established upon completion of hibernation.

Our study demonstrates that the persistence of daily activity patterns under the midnight sun in AGS is linked to circadian systems that are presumably sensitive to subtle parametric changes in the color or intensity of light yet resistant to entrainment by square-wave LD cycles. Sensitivity to parametric changes in light intensity and/or color temperature, combined with resistance to entrainment by square light waves, may be a universal feature of circadian clocks in ground squirrels, which must be capable of synchronizing their rhythms without exposure to dusk and dawn yet impervious to abrupt transitions between light and darkness driven by their semi-fossorial habits.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: C.T.W., B.M.B., C.L.B.; Methodology: C.T.W., L.Y.; Formal analysis: C.T.W., L.Y.; Investigation: C.T.W.; Resources: C.T.W.; Writing - original draft: C.T.W.; Writing - review & editing: B.M.B., L.Y., C.L.B.; Funding acquisition: C.T.W., B.M.B., L.Y., C.L.B.

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#### Supplementary information

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