PHYSIOLOGICAL INTEGRATION OF SEASONAL AND DAILY CUES IN THE NORTH AMERICAN BROWN BEAR

By

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Timekeeping enables organisms to accurately and reliably perform functions integral to survival, such as reproduction, migration, and hibernation. The grizzly bear (*Ursos arctos horribilis*) is a highly adaptable species that must cope with changing seasonal conditions, food resources, and human disturbance. Despite these pressures, the North American population of grizzly bears is currently expanding into human inhabited landscapes and humans are encroaching on grizzly habitat. Together, these events require a better understanding of the proximal and ultimate factors shaping the grizzly’s activity and physiology. In the current series of studies, I evaluated the role of light, food, and hormonal signals on modulating daily and seasonal activity in a captive grizzly bear population at the Washington State University Bear Research, Education and Conservation Center. The results of these studies reveal that the grizzly bears respond to photic and non-photic cues in a seasonally dependent manner. Specifically, light serves as a strong entraining agent when food is absent (during hibernation).
but when food was present, its role usurped that of the light:dark cycle. Furthermore, circulating hormone concentrations were observed to fluctuate with seasons, but their manipulation did not dramatically affect activity patterns, suggesting a less prominent role in modulating behavior patterns. Taken together, the results of these experiments indicate that the brown bear is a behaviorally flexible species that has likely evolved specific adaptations to cope with annual changes in food availability and, more recently, human pressure.
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CHAPTER 1

INTRODUCTION

Overview

The phenomenon of biological timekeeping is important for ensuring the fitness and longevity of individuals (Moore-Ede 1986; Sharma 2003). Timekeeping has resulted in the expression of biological rhythms that enable animals to accurately and reliably perform functions integral to survival, such as migration, reproduction, and hibernation because of their predictive value (Sharma 2003). Much research has described the existence of circadian (circa-dian - about a day) and circannual (about a year) rhythms (Zucker 2001; Gwinner 2003; Refinetti 2006).

However, much less is known about these rhythms in animals whose behavior removes them from the ambient environment and thereby from human observation, such as is found in hibernating species. Indeed, what data are available remain highly controversial (French 1977; Vanecek, Jansky et al. 1985; Grahn, Miller et al. 1994; Hut, Barnes et al. 2002; Ruby 2003; Revel, Herwig et al. 2007). Furthermore, research has focused on species that exhibit the classic features of hibernation, such as reduced metabolic rate, deep torpor with periodic arousal, and severely decreased body temperature. Yet, for the grizzly bear, with the exception of metabolic rate, these adaptations are not as extreme (Nelson, Folk et al. 1983; Hissa, Siekkinen et al. 1994). Additionally, grizzly bears are currently facing challenges from human encroachment and changing climate conditions. Together, this highlights the need for additional knowledge of bear physiology, especially that related to the generation of annual rhythms.
Circadian Biology

Overview

Circadian biology is the study of biological processes that occur repeatedly on a roughly (circa) 24 h basis in concert with the rotation of the earth about its axis (Refinetti 2006). In this way, biological processes such as daily sleep-wake cycles, food anticipation, and time-compensated navigation (migration), just to name a few, can be organized in a way most beneficial to the organism (DeCoursey 2004). In fact, circadian rhythms are so well integrated into normal physiology that they can be observed to operate at the level of gene expression, behavior, and higher cognitive functions (Van Someren and Riemersma-Van Der Lek 2007). Circadian processes have been identified in species in all kingdoms of organisms, including plants, bacteria, and animals (DeCoursey 2004).

Although rhythmic events have been observed by humans for thousands of years, it is only within the last century that the topic became a bona fide field of scientific study (DeCoursey 2004; Refinetti 2006). The following discussion provides a general overview of the principles and definitions applied to the study of circadian rhythms and circannual rhythms.

Terms and principles of circadian biology

In circadian biology, the term rhythm refers to a measureable variable, while the seemingly similar term, oscillation, is usually reserved for more mechanistic studies (Refinetti 2006). Moreover, while an oscillator always produces a rhythm, a rhythm (or what appears to be a rhythm) is not necessarily produced by an oscillator. Thus, caution is required in interpreting the results of experimental manipulations designed to address one aspect versus the other. Once a rhythm is determined to be present, it can be described by its period (tau),
amplitude, and phase. Period refers to the duration of the rhythm or distance between peaks in the rhythm (Refinetti 2006). Amplitude is defined as the “extent of an oscillatory movement, measured from mean to extreme value” (DeCoursey 2004). Finally, the phase, or a point on the rhythm can also be envisioned as reflecting the relationship between a periodic quantity, such as locomotor activity, and a reference rhythmic event or entrainment signal, such as dawn (Daan 2001; DeCoursey 2004; Refinetti 2006).

Rhythms must meet several criteria to be considered circadian in nature. Rhythms must be 1) generated endogenously (within the organism), 2) have a free-running period close to, but not exactly, 24 hours, and 3) can be synchronized to 24 h environmental signal (Aschoff 1981). In general, rhythms with a period between 19 and 28 hours are considered circadian (Refinetti 2006). The endogenous nature of circadian rhythms is confirmed when they persist in the absence of external entrainment cues (Van Someren and Riemersma-Van Der Lek 2007). Typically, the daily light:dark cycle is the predominant external cue that entrains the clock to exactly 24 hrs. In the absence of external cues, the endogenous clock will no longer be entrained to a precise 24 h solar rhythm; rather, it will free-run with a period that is either slightly longer or shorter than 24 h (DeCoursey 2004; Refinetti 2006). Intriguingly, these constant conditions are experienced normally by some species living at extreme latitudes and by those hibernating in burrows. Yet, the role of the circadian clock under these conditions is controversial. For example, arctic dwellers, including reindeer (Rangifer sp.) and Svalbard ptarmigans (Lagopus mutus hyperboreus) exhibit a complete absence of circadian rhythmicity during polar summers and winters (Reierth and Stokkan 1998; van Oort, Tyler et al. 2005). The final criterion for a rhythm to be circadian is that the rhythm must be able to be entrained to a
24 h rhythmic external cue. A rhythmic external cue is said to “give the time” to the internal (endogenous) clock and thus is referred to as a Zeitgeber, a German word for time-giver (Refinetti 2006). The endogenous clock is synchronized to the external cycle, and is thereby reset (or phase-shifted) to match the 24 h environmental rhythm. The close proximity of the endogenous clock period to 24 h facilitates more robust entrainment to the 24 h day because entrainment is optimal when the two oscillators have similar frequencies (Johnson, Elliott et al. 2004). In fact, there are limits to entrainment. For example, if a Zeitgeber’s period is much different (i.e., longer or shorter in duration) than the internal clock’s period, the Zeitgeber will fail to successfully entrain the internal rhythm (Johnson, Elliott et al. 2003). The ability of an organism to entrain to the external 24 h rhythm is vital to ensure the predictive validity for behaviors such as feeding, migrating, and sleeping-waking.

**Organization of the circadian system**

The circadian system is composed of a complex set of substrates and pathways that is not completely understood. Nevertheless, circadian systems have been observed in unicellular organisms, invertebrates, nonmammalian vertebrates, and mammalian vertebrates. This review will focus on the mammalian vertebrate organization of the circadian system.

On its most basic level, the circadian system can be visualized as a flow of information beginning with an input, then integrated by a processor, and finally relayed to targets via a series of outputs (Figure 1.1) (Moore and Leak 2001). In the biological realm, input is from external cues in the form of photic cues (light) and nonphotic cues (food, temperature, etc.) (Refinetti 2006). The external cue is carried to the pacemaker, or clock, of the system (the
processor). Finally, after the pacemaker processes the information, it is relayed to effectors (organs and cells in the organism) (Moore and Leak 2001). This conceptual organization can be applied to both central oscillators (central nervous system) and peripheral oscillators, i.e., those found in other organs.

![Figure 1.1. Circadian system organization with input from a variety of environmental signals relayed to a central pacemaker where it is processed and then output to various effector sites to modulate behavior and physiology.](image)

**Input**

Input into the circadian system can be from a variety of sources and can act as Zeitgebers. These include photoperiod, temperature, and the presence of food. This information is carried to the central pacemaker in the suprachiasmatic nucleus (SCN) of the
hypothesis via several afferent neural projections including the retinohypothalamic tract (RHT), geniculohypothalamic tract (GHT), pretectohypothalamic tract (PHT), and nonvisual afferents. These afferents serve to set the precise timing of the central pacemaker in an organism (Moore and Leak 2001). The most potent form of external input into the circadian system is believed to be light (Moore and Leak 2001; DeCoursey 2004; Refinetti 2006). Both the RHT and GHT are composed of afferents originating in the retina (Pickard 1985). Retinal ganglion cells are stimulated by light, and their axons project from the eye bilaterally, terminating in the central SCN of the hypothalamus (Moore, Speh et al. 1995; Berson, Dunn et al. 2002). The same retinal ganglion cells whose axons create the RHT comprise the afferents that form the GHT (Pickard 1985). The afferents of the GHT project to the intergeniculate leaflet (Hickey and Spear 1976). The intergeniculate leaflet then projects to the SCN creating a pathway of secondary visual input to the SCN (Hickey and Spear 1976; Card and Moore 1989; Moore and Leak 2001). The third visual afferent pathway to the SCN, the PHT, has been less extensively studied, but it is thought to provide additional modulatory visual information to the SCN (Mikkelsen and Vrang 1994). Finally, there are numerous projections to the SCN that are collectively grouped into the “nonvisual afferent” designation. These afferents come from such areas as the cerebral cortex, thalamus, hypothalamus, and brainstem (Moore, Speh et al. 2002).

Pacemaker

The SCN serves as the central pacemaker, or master clock, in virtually all mammals. It lies in the anterior hypothalamus dorsal to the optic chiasm and ventral to the paraventricular nucleus and third ventricle (Gurdjian 1927). Ablation of this area produces arrhythmicity in many circadian behaviors (Moore and Eichler 1972; Stephan and Zucker 1972; Moore and Leak...
2001). Further studies demonstrate that SCN neurons continue to express rhythmicity in vitro and that SCN transplants restore rhythms to previously arrhythmic animals (Inouye and Kawamura 1979; Sawaki, Nihonmatsu et al. 1984; Ralph, Foster et al. 1990). Anatomically and cellularly, the SCN is divided into two general divisions: the core which is comprised primarily of vasoactive intestinal peptide (VIP) expressing neurons and the shell containing arginine vasopressin (AVP) expressing neurons (Figure 1.2) (Card, Brecha et al. 1981; Card and Moore 1984; Watts, Swanson et al. 1987). Early electrophysiology work identified a circadian rhythm of firing rate for neurons within the SCN (Inouye and Kawamura 1979), and later studies expanded those findings with the discovery that core VIP neurons become arrhythmic in constant conditions (Shinohara, Tominaga et al. 1993) while shell AVP neurons display an endogenous rhythm in constant conditions (Tominaga, Shinohara et al. 1992). Because the VIP neurons receive the majority of their input from retinal pathways, it is believed that these neurons are responsible for the phase-shifting and entrainment of the SCN (Watanabe, Vanecék et al. 2000; Reed, Meyer-Spasche et al. 2001). Shell AVP neurons receive input from, and are thought to be entrained by, VIP neuronal activity (Moore, Speh et al. 2002). Output from the shell AVP neurons is considered to be an entrainer for the rest of the brain and body. Within the SCN neurons is a molecular network of so-called “clock genes” (Dunlap 1999). Two of these genes, circadian locomotor output cycles kaput (clock) and brain and muscle arnt-like (bmal1), are important for mammalian circadian clock function (Dunlap 1999). Clock and bmal1 are transcribed in the nucleus and then translated into the proteins CLK and BMAL1, respectively. Forming a dimer, CLK:BMAL1 then activate the transcription of the circadian genes, period (per) and cryptochrome (cry) in a positive feedback loop by binding to E-box
binding sites within the promoter regions of these genes (Refinetti 2006). The accumulation of the gene products of per and cry, PER and CRY, serve as a negative feedback signal to the CLK:BMAL1 complex creating the full regulatory loop. The duration of this transcription/translation feedback loop is the determining factor for the endogenous period of an organism (Dunlap 1999).

Figure 1.2. Cellular organization of the SCN with A. lighter (green) stained neurons expressing VIP peptides residing primarily the core and darker (red) stained neurons expressing AVP located in the shell. B. Cartoon representation of unilateral SCN. Part A courtesy of (Welsh, Takahashi et al. 2010).

Output

Once processed by the SCN, signals are conveyed to various effector sites and organs via both neural and humoral outputs (Stephan, Berkley et al. 1981; Watts and Swanson 1987; Watts, Swanson et al. 1987). GABAergic projections from the SCN terminate in three major areas: the hypothalamus, thalamus, and septal area (Buijs, Hou et al. 2004; Refinetti 2006). Specifically,
hypothalamic projections mainly terminate in the paraventricular nucleus (PVN), preoptic area (POA), and dorsomedial hypothalamic nucleus (DMH) (Watts, Swanson et al. 1987).

Functionally, the PVN is considered the predominant pathway by which photic information reaches the pineal gland to govern melatonin secretion (Klein, Smoot et al. 1983; Perreau-Lenz, Kalsbeek et al. 2003). In contrast, the POA is an important site for thermoregulation in the mammalian brain while the SCN is the site of circadian control of temperature via neural connections between brain regions (Briese 1989; Refinetti 2006). Ablation of the POA results in loss of thermoregulatory function, but not rhythmicity, and ablation of the SCN results in loss of temperature rhythmicity but not thermoregulation, suggesting some independent function of these two nuclei (Carlisle 1969; Toth 1973; Refinetti, Kaufman et al. 1994; Refinetti 1995).

Lastly, the DMH belongs to a circuit that carries information from the SCN to the locus coeruleus and ventrolateral preoptic area, both of which are involved in the control of sleep/wakefulness (Chou, Scammell et al. 2003; Mistlberger 2005; Refinetti 2006). It is important to note, however, that time-of-day information can also be transmitted via humoral signals (Aronson, Bell-Pedersen et al. 1993; Silver, LeSauter et al. 1996; Sollars and Pickard 1998).

**Entrainment**

Entrainment involves the adjustment of the frequency and phase of an endogenous oscillator to the earth’s 24 h cycle of rotation (Daan 2001; Johnson, Elliott et al. 2003).

Entraining agents, or Zeitgebers, are not only photic, but can include temperature, humidity, feeding, air pressure, species-specific song, and even nitrate concentrations (Menaker and Eskin
1966; Zimmerman, Pittendrigh et al. 1968; Hayden and Lindberg 1969; Roenneberg and Rehman 1996). In order to establish whether a Zeitgeber has entrained an endogenous rhythm, three criteria must be met (Johnson, Elliott et al. 2004). First, the free-running period of the internal rhythm must be close to that of the entraining cycle. Second, the internal rhythm must maintain a stable temporal relationship, or “phase-angle,” with the Zeitgeber’s rhythm. Finally, once the influence of the Zeitgeber is removed, the free-running rhythm of the organism must persist precisely in-phase with the previously applied Zeitgeber. That is, the free-running activity must begin very close to the time of the last signal from the Zeitgeber. If this third criterion is not met, then the rhythm is said to have been “masked” rather than truly entrained. Entrainment of the circadian clock combined with the direct masking effects of light and dark are thought to represent the major determinants of activity patterns in animals (Daan 2000; Roenneberg, Daan et al. 2003; Redlin, Hattar et al. 2005; Refinetti 2008; Erkert, Fernandez-Duque et al. 2012). Masking occurs when photic or non-photic cues directly affect the output of the clock without changing the period or phase of the circadian rhythm (Aschoff 1960). Masking also allows an animal to respond acutely to a proximal change in the environment (Aschoff 1999). Positive masking and negative masking serve to enhance or suppress the output, respectively, depending on the temporal niche or chronotype of the animal (Aschoff 1999; Shuboni, Cramm et al. 2012). For example, in diurnal animals, direct effects of light serve to increase activity while in nocturnal animals, light serves to decrease activity (Cohen, Smale et al. 2010; Shuboni, Cramm et al. 2012). Food is another strong masking agent and can alter the temporal niches of animals when restricted to a particular
phase of the day (Stephan 2002) although debate remains as to whether the phase of the clock is altered as well (Saper, Lu et al. 2005).

Continuous (parametric) entrainment

The continuous model of entrainment states that “the rate of motion of the clock mechanism changes proportionally to the intensity of light present” (Pittendrigh and Daan 1976; Aschoff 1979). The presence of light continuously throughout the daylight hours is thought to modulate the endogenous free-running period of the organism, allowing it to better match and stably entrain to the periodicity of the Zeitgeber (Daan 2001; Johnson, Elliott et al. 2004). Thus, the continuous model is based on the assumption that it is the magnitude of the Zeitgeber (i.e. light intensity) which affects the speed of the central pacemaker. The free-running period of the circadian pacemaker accelerates or decelerates in response to daily changes in light intensity in order to mimic the environmental cycle (Aschoff 1979; Johnson, Elliott et al. 2003). For example, in humans, the free-running period is significantly greater than 24 h (25-26 h). However, examination of the experimental methods revealed that participants had been allowed to turn on a light during the subjective daytime, thereby increasing light intensity (magnitude of the Zeitgeber). This extra daytime illumination altered the free-run period and after correction, the endogenous period was shown to be much shorter than previously measured (24.18 h) (Czeisler, Duffy et al. 1999). Changes in light intensity affect both tau and alpha predictably in nocturnal animals (Meijer 2001) and in diurnal mammals as well, provided the light intensity is great enough (>1000 lux) (Lee and Labyak 1997). Moreover, the diurnal European ground squirrel (Spermophilus citellus) remains stably entrained to the ambient photocycle despite the fact that they emerge from their burrows after dawn and
return before dusk and are never exposed to discrete dawn and dusk cues, indicating that only the subtle changes in light intensity experienced when the animal is out of the burrow are sufficient to entrain the circadian clock (Hut, van Oort et al. 1999; Daan 2000).

Discrete (nonparametric) entrainment

Originally proposed by Pittendrigh, the discrete model of entrainment occurs when light results in a phase-shift of the oscillator causing it to advance or delay and thereby match the period of the entraining stimulus (Pittendrigh 1981; Daan 2001; Johnson, Elliott et al. 2004; Refinetti 2006). Therefore, entrainment results from daily resetting of the clock rather than continuous modulation of the clock’s speed as the continuous model predicts (Pittendrigh and Minis 1964). This daily resetting of the endogenous clock prevents the natural drift of the free-running period leaving period of the expressed rhythm to be matched precisely to 24 h light:dark cycle (Refinetti 2006). It must be noted that in order for the discrete theory of entrainment to be upheld, the circadian oscillator must respond uniquely to pulses during different times of its respective cycle (Johnson, Elliott et al. 2003). In other words, light must exert differential effects on the endogenous clock at different times during the subjective day so that the appropriate phase shifting can occur to match the exogenous periodicity of the Zeitgeber. In nature, these discrete light pulses are in the form of dawn and dusk transitions (Johnson, Elliott et al. 2003; Johnson, Elliott et al. 2004). Numerous studies substantiate the entraining ability of brief light pulses to the light-dark cycle (Pohl 1983; Czeisler, Allan et al. 1986; Glass, Tardif et al. 2001). Effects of dark pulses are more variable (Redlin and Mrosovsky 2004; Shuboni, Cramm et al. 2012), but in general, cause effects that are mirror images to light pulses (Boulos and Rusak 1982). Light or dark pulses are an effective way to evaluate
entrainment without the confounding effects of potential masking (Roenneberg, Daan et al. 2003). For example, animals exposed to ‘skeleton photoperiods’ in which two light or dark pulses are given during a 24 hr period, entrain as if they were under complete photoperiods (Pittendrigh and Daan 1976). In modeling experiments, entrainment was most accurate when both phase shifts (discrete entrainment) and tau changes (continuous entrainment) were incorporated (Beersma, Daan et al. 1999). With animals and humans exposed to both discrete and continuous effects of light, it is reasonable to assume that both methods of entrainment are used.

**Food entrainment**

While light is a reliable entraining agent for circadian and seasonal rhythms, other non-photic cues, such as food, exert powerful effects. The ability to anticipate feeding times or food availability makes ecological sense as food resources are usually available at certain times of the day or year (Mistlberger 1994). The first evidence of entrainment to feeding occurred when rats were observed to increase activity several hours prior to a once-a-day feeding (Richter 1922). Suprachiasmatic nucleus output pathways synapse within multiple hypothalamic structures that regulate feeding behavior and metabolism, including the periventricular, paraventricular, and dorsomedial nuclei (Watts 1991; Canteras, Ribeiro-Barbosa et al. 2011). Additionally, receptors for food regulation and metabolic hormones, including leptin, cholecystokinin, and ghrelin, are found in these same hypothalamic nuclei potentially allowing for direct modulation by feeding on the circadian system (Beinfeld and Palkovits 1981; Elmquist, Ahima et al. 1998; Horvath, Diano et al. 2001; Saper, Lu et al. 2005). Based on the presence of large projections into these regions, the hypothalamus has been proposed as an
integrator of multiple inputs such as circadian signals, food availability, temperature, and social interactions (Saper, Lu et al. 2005). Integration from multiple environmental signals allows an adaptive phenotype, which would be especially prudent for seasonal animals (Saper, Lu et al. 2005). Intriguingly, overwhelming evidence suggests that food entrainment is not mediated by the SCN. When a restricted feeding regimen is instituted, clock gene rhythms in tissues outside the SCN, including the stomach, liver, intestines, pancreas, heart, lungs, and muscle, shift to align with the feeding paradigm, while the SCN remains locked onto the light:dark cycle (Damiola, Le Minh et al. 2000; Stokkan, Yamazaki et al. 2001; Schibler, Ripperger et al. 2003). Attempts to localize the food-entrainable oscillator, or FEO, via lesion studies have been unsuccessful, in part because many of the hypothalamic regions associated with food intake are also heavily involved in metabolism, leading to heavily altered physiological animal models (Mistlberger 2011). Additionally, food anticipation remains, albeit sometimes dampened, following ablation of nuclei or mutation of various clock genes (Mistlberger 2011). Taken together, these diverse results suggest a redundant, compensatory, and potentially diffuse FEO (Mistlberger 2011).

**Photoperiodic influence on circadian and seasonal rhythms**

Modulation of physiological processes by changes in duration or amount of light is known as photoperiodism (Elliott 1976; Goldman 2001). Photoperiodism allows organisms to use the daylength as a predictive cue for timing seasonal events and initiating physiological and developmental processes necessary for fitness and survival (Bradshaw 2007). Because of these features, photoperiod is often a very strong Zeitgeber for many animals and plants (Bunning
However, the effects on physiology attributed to photoperiodism are likely a result of multiple Zeitgebers exerting their influence in the natural environment, such as changing temperatures and metabolic state (Roenneberg and Merrow 2007).

Like other entrainers, photoperiod serves to “reset” the endogenous clock to the 24 h light-dark environmental cycle, both through continuous and discrete modulation (Daan 2001; Gunawan and Doyle 2007). The most obvious effects of photoperiod are the expansion or compression of expressed circadian rhythms (Refinetti 2006). In electrophysiological and gene expression studies, internal pacemaker changes occurred in accordance with changing photoperiod (Messager, Ross et al. 1999; Mrugala, Zlomanczuk et al. 2000; Sumova, Jac et al. 2003). For example, in Drosophila, the circadian expression of per mRNA remained stably entrained to a range of photoperiods using light-dark ratios of 4:20, 8:16, 12:12, 16:8, and 20:4. Evidence suggests that while circadian rhythms may be entrained by photoperiod, they themselves may be the basis for photoperiodism (Bünning 1936; Pittendrigh and Minis 1964; Oster, Maronde et al. 2002; Hazlerigg and Wagner 2006; Paul, Zucker et al. 2008), but may have evolved independently of photoperiodism (Bradshaw 2007). As the potential basis of photoperiodism, circadian rhythms provide daily coding of the prevailing photoperiod via the dark rise of melatonin (Goldman 2001; Hazlerigg and Wagner 2006; Lincoln 2006; Paul, Zucker et al. 2008; Zawilska, Skene et al. 2009). This seasonal entrainment by photoperiod is believed to act upon an endogenous circannual clock. Animals such as sheep, ground squirrels, and stonechats, exhibit recurring seasonal cycles for several years even in the absence of photoperiodic cues (Gwinner 2003; Kondo, Sekijima et al. 2006; Lincoln, Clarke et al. 2006).
However, exactly how organisms internalize photoperiod to entrain various physiological changes such as reproductive status, molting, and migration, remain unclear. Two opposing hypotheses seek to explain this process; both propose that the timing of the light exposure rather than absolute duration is essential for encoding the appropriate daylength. Büning proposed the *external coincidence* theory which states that day length is measured by the “coincidence of external photic stimulation and internal sensitivity to light” (Bunning 1960). This model revolves around the premise that the circadian period is made of two half-cycles which differ in their sensitivity to light; one half being light-sensitive and the other half dark-sensitive (Bunning 1960). Therefore, the half-cycle upon which a light exposure falls determines whether the expressed rhythm exhibits characteristics of either short photoperiods or long photoperiods. Evidence suggests this phase-dependent entrainment may also occur for circannual clocks in which animals experience sensitive and non-sensitive phases across the year (Monecke, Saboureau et al. 2009). A second model for photoperiodic time measurement, the *internal coincidence* theory, states that there are two separate circadian oscillators, a morning (M) and evening (E) oscillator coupled to dawn and dusk, respectively (Pittendrigh and Minis 1964). The two-oscillator theory was proposed after observations of locomotor activity rhythm ‘splitting’ following an abrupt change in light intensity during constant conditions (Pittendrigh 1960; Pittendrigh and Daan 1976). The ‘split’ rhythm occurs when a single daily period of locomotor activity separates into evening and morning bouts which oscillate independently until they merge once again into a stably coupled single bout. As daylength changes, shorter or longer, the phase relationship of the two oscillators varies to determine the behavior and physiology of the animal (Pittendrigh and Daan 1976). The M oscillator is
entrained to dawn and controls the timing of night offset while the E oscillator is entrained to dusk and controls the timing of night onset. Subsequent reports have attempted to isolate and test the M and E oscillators and while evidence indicates independent morning and evening responses of Aa-NAT activity, melatonin production, and clock gene responsiveness to light manipulations, none to date have conclusively identified the two oscillators (Illnerová and Vaněček 1982; Elliott and Tamarkin 1994; Daan, Albrecht et al. 2001).

Regardless of the coding mechanism responsible for translating photoperiodic cues, it appears that both absolute daylength and photoperiodic history affect the ability of an organism to exhibit photoperiodism. Early studies using Syrian hamster (Mesocricetus auratus) identified a critical daylength of less than 12.5 h that resulted in testicular regression while daylengths greater than 12.5 h resulted in testicular maintenance and spermatogenesis (Elliott 1976). Later studies revealed that the absolute daylength requirement differed among species and for individual traits. For example, in male Siberian hamsters, transition from 16 h of daylight to 14 h invoked testicular regression while transition to 13 h or less resulted in both testicular regression and development of their winter coat (Duncan, Goldman et al. 1985). After having experienced short daylengths for several months, animals will often become refractory to the short days and revert back to spring/summer physiology (Goldman 2001). In Siberian hamsters, when transferred from 16 to 14 h of light, gonadal regression was observed, implicating the 14 h duration of light as having inhibitory effects on reproductive status (Hoffmann, Illnerova et al. 1986). However, when the hamsters were held in 8 h of light and then transferred to 14 h, gonadal regrowth occurred (Hoffmann, Illnerova et al. 1986). Similar effects of photoperiodic history have been demonstrated for other species, including Syrian
hamsters, birds, sheep, and voles (Daan and Aschoff 1975; Robinson and Karsch 1984; Hastings, Walker et al. 1989; Goldman, Gwinner et al. 2004). Collectively, it is not only the absolute length of light that affects traits, but also the past photoperiodic history.

**Endocrine influence on circadian and seasonal rhythms**

The expression of seasonal and circadian rhythms depends heavily on the integration of environmental cues with a temporally organized neuroendocrine system (Cauter and Buxton 2001). Numerous hormone profiles follow a 24 h pattern as a result of the endogenous rhythm produced by SCN neurons (Cauter and Buxton 2001). For the sake of brevity, this discussion will only focus on the two that I looked at in my studies.

*Melatonin*

Melatonin, produced primarily by the pineal gland and secreted in a 24 h rhythm, plays an important role as a mediator of the light:dark cycle (Goldman, Gwinner et al. 2004). The rhythmic daily secretion of melatonin has been observed in many organisms including fish, reptiles, birds, rodents, and other mammals including humans (Honma, Honma et al. 1992; Thrun, Moenter et al. 1995; Tosini and Menaker 1998; Kumar, Gwinner et al. 2000; Jessop, Limpus et al. 2002; Steinlechner, Stieglitz et al. 2002; Bayarri, Munoz-Cueto et al. 2004). This daily secretion persists with a free-running period in constant environmental conditions and can be entrained by the light-dark cycle (Rosenthal 1991). This suggests that melatonin is controlled by the SCN.

Indeed, photic information primarily reaches VIP expressing neurons in the core of the SCN via the RHT pathway. Excitation via glutamate release in response to light increases VIP
neuronal firing. Interconnections between core SCN VIP neurons and shell AVP neurons activate the efferent limb of the SCN. Projections from mainly shell neurons in the SCN synapse within the paraventricular nucleus (PVT) of the hypothalamus (Klein, Smoot et al. 1983). From the PVT, the information travels via the median forebrain bundle and terminates within the intermediolateral column of the spinal cord (Klein, Smoot et al. 1983). Pre-ganglionic fibers within the spinal cord travel to the superior cervical ganglion (SCG), synapse with post-ganglionic noradrenergic fibers, which innervate the pineal gland, and stimulate the release norepinephrine (Figure 1.3) (Klein, Smoot et al. 1983; Korf, Schomerus et al. 1998). Norepinephrine binds to adrenergic receptors of the pineal gland and induces a transduction pathway that results in the activation of the enzymes arylalkylamine N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) (King and Steinlechner 1985). These two enzymes, AA-NAT and HIOMT, acetylate and methylate serotonin to create melatonin in the pineal gland (King and Steinlechner 1985; Korf, Schomerus et al. 1998). Once synthesized, melatonin is immediately released into the bloodstream.
Figure 1.3. Illustration of melatonin production pathway beginning with an environmental light cue entering the retina and proceeding via the RHT to the SCN and then on to the PVT. Activated PVT neurons travel to the spinal cord where they synapse with preganglionic SCG neurons. Pre-ganglionic SCG fibers synapse with post-ganglionic adrenergic SCG fibers and these adrenergic fibers travel to the pineal gland where they stimulate the production of melatonin. Picture courtesy of (Brainard 2000).

While the characteristic profile of melatonin secretion differs in amplitude among species, the pattern of increased melatonin in the dark phase and suppressed melatonin in the light phase remains constant across all mammals studied (Simonneaux and Ribelayga 2003; Goldman, Gwinner et al. 2004). Therefore, melatonin not only provides a daily signal of light and dark, but also provides a seasonal signal because melatonin secretion directly reflects the length of the dark phase (Goldman, Darrow et al. 1984; Gower, Nagy et al. 1996; Pevet, Agez et al. 2006). The rise of melatonin secretion during the night is likely controlled by the circadian release of norepinephrine into the pineal gland and subsequent transcription of $Aa$-nat (Ribelayga, Pevet et al. 2000; Garidou, Vivien-Roels et al. 2003). The duration of the melatonin signal is important in initiating and controlling photoperiodic influence on reproductive and metabolic responses (Bartness, Powers et al. 1993; Gower, Nagy et al. 1996). Short duration exogenous melatonin infusion into pinealectomized Siberian hamsters and sheep resulted in responses associated with longs days in both species (Bartness, Powers et al. 1993).
Furthermore, pinealectomy results in the loss of seasonal adaptations to long and short days (Hoffman and Reiter 1965; Vitale, Darrow et al. 1985).

When the light:dark cycle ceases or an animal enters hibernation, the role of melatonin becomes less clear. For animals living in high latitudes exposed to polar days and nights, an absence of melatonin rhythmicity has been reported (Florant, Rivera et al. 1984; Vaněcek, Janský et al. 1984; Vanecek, Jansky et al. 1985; Darrow, Tamarkin et al. 1986; Eloranta, Timisjärvi et al. 1995; Reierth, Van’t Hof et al. 1999; van Oort, Tyler et al. 2005). In golden-mantled and Richardson’s ground squirrels, pinealectomy did not disrupt the hibernation (Harlow, Phillips et al. 1980). However, removal of the pineal gland occurred just prior to entrance into hibernation and the authors concede that earlier removal may have negatively affected hibernation since the pineal is likely important for adaptations to changing photoperiods (Harlow, Phillips et al. 1980). During arousal from torpor bouts, increases in melatonin concentrations occur, presumably as a result of the increased sympathetic nervous system activation (Lyman, Willis et al. 1982; Florant, Rivera et al. 1984; Vanecek, Jansky et al. 1985). Furthermore, application of exogenous melatonin in Siberian hamsters during both natural and 2-deoxy-D-glucose-induced torpor produced arousal (Larkin, Yellon et al. 2003). There appears to be some circadian control of either, or both, entry and exit from torpor with the increase in melatonin during arousal most likely reflecting the changes in SCN activity and concomitant increase in sympathetic activation (Larkin, Yellon et al. 2003).

Cortisol

Similar to melatonin, the adrenal hormone cortisol (or corticosterone) exhibits a daily rhythm requiring the SCN (Moore and Eichler 1972; Raisman and Brown-Grant 1977; Kaneko,
Kaneko et al. 1981; Buijs, Wortel et al. 1999). The SCN has direct connections to corticotropic releasing hormone (CRH) producing neurons within the PVT of the hypothalamus and is connected multisynaptically to the cortisol producing adrenal glands (Vrang, Larsen et al. 1995; Buijs, Wortel et al. 1999). Cortisol is secreted by the cortex of the adrenal gland in response to the circadian dependent release of adrenocorticotropic hormone (ACTH) from the pituitary (Cauter and Buxton 2001). Upstream, the PVT of the hypothalamus produces (CRH) which drives ACTH release (Goodman 2009). Although regulated by the SCN, cortisol is not suppressed by light and the daily cortisol rhythm is characterized by a daily peak at or near waking, followed by a gradual decrease to nadir at roughly the middle of the night phase (Selmaoui and Touitou 2003). However, daily profiles must be smoothed because of the pulsatile secretory rhythm of cortisol in response to the activation and inhibition of the hypothalamic-pituitary-adrenal (HPA) axis (Veldhuis, Iranmanesh et al. 1989; Lightman 2008).

Several factors affect shape and amplitude of the cortisol waveform, but not the rhythmicity. Transient elevations in peripheral cortisol are noted at meal times, stress events and exercise (Brandenberger, Follenius et al. 1982; Van Cauter and Honinckx 1985; Luger, Deuster et al. 1987). Additionally, cortisol levels briefly drop at the onset of sleep (Born, Muth et al. 1988) and briefly rise on the offset of sleep (Spath-Schwalbe, Gofferje et al. 1991). Amplitude of the cortisol rhythm has also been linked to age (Linder, Esteban et al. 1990; Kiess, Meidert et al. 1995; Boily 1996), gender (Weiss and Richards 1971; Fowler 1988; Harlow and Beck 1990), and metabolic status (Saboureau, Laurent et al. 1979; Bubenik, Bubenik et al. 1983; Palumbo, Wellik et al. 1983; Harlow and Beck 1990; Gower, Nagy et al. 1996). In humans, high salivary levels of cortisol were measured during the first year of life (Kiess, Meidert et al. 1995).
and plays a role in gluconeogenesis, protein, fat, and carbohydrate metabolism, and suppression of the immune system (Goodman, 2009). Cortisol is reported to be present in greater concentrations in adolescents compared to prepubertal children (Linder, Esteban et al. 1990). However, another study found that neither age nor gender was correlated with cortisol levels in humans (Knutsson, Dahlgren et al. 1997). For black bears (Harlow and Beck 1990), Tasmanian devils (Weiss and Richards 1971), eastern grey kangaroo (Coghlan and Scoggins 1967), and European hedgehogs (Fowler 1988) females had greater serum cortisol than males. Conversely, in red deer (Huber, Palme et al. 2003), desert bighorn sheep (Turner 1984), and harbor seals (Gardiner and Hall 1997) there were no differences between the sexes.

Similar to melatonin, cortisol is seasonally regulated (Saltz and White 1991; Boswell, Woods et al. 1994; Gower, Nagy et al. 1996; Breuner and Orchinik 2001) in a proposed mechanism for mediating seasonal adaptations of physiology, mood, and behavior, which are all largely affected by adrenal hormones (Lemos, Downs et al. 2009). In humans, data are conflicting for seasonal variation of cortisol with some reports finding differences among seasons (Van Cauter, Virasoro et al. 1981; Levine, Milliron et al. 1994; Walker, Best et al. 1997) while others report no differences (Agrimonti, Angeli et al. 1982; Van Dongen, Kerkhof et al. 1998). The literature for non-human mammals, however, is less variable (Fowler 1988; Harlow and Beck 1990; Schiml, Mendoza et al. 1999; Lynch, Ziegler et al. 2002; Huber, Palme et al. 2003; McKenzie and Deane 2003). Few reports find no differences in cortisol levels among seasons (Bubenik, Smith et al. 1986). Even in studies where photoperiod did not appear to affect overall levels of cortisol, light did shift the rhythm indicating a responsiveness to photoperiodic changes (Lemos, Downs et al. 2009). Cortisol increases are oftentimes linked to
changes in reproductive status creating difficulties in examining the role of photoperiod on cortisol secretion (Schiml, Mendoza et al. 1996; Schiml, Mendoza et al. 1999; Lynch, Ziegler et al. 2002; McKenzie and Deane 2003). For example, in wild male tufted capuchin monkeys, *Cebus apella nigritus*, both testosterone and cortisol were elevated relative to baseline during the peak of female sexual activity (Lynch, Ziegler et al. 2002). Additionally, in socially housed squirrel monkeys *Saimiri sciureus*, gonadal and steroidal hormones elevated concurrent with the breeding season. However, when housed individually, the increase in cortisol was out of phase with the breeding season indicating that cortisol exhibits a clear seasonal variation in the absence of socially driven reproductive cues (Schiml, Mendoza et al. 1999).

Reproductive status is not the only factor influencing seasonal changes in cortisol levels. Decreased nutritional intake is correlated with increased levels of cortisol in several species (Harlow and Beck 1990; Saltz and White 1991; DelGiudice, Mech et al. 1992). In black bears, for example, during the hibernation period when food is not present, cortisol levels are greatest (Harlow and Beck 1990; Donahue, Vaughan et al. 2003). Cortisol plays an important role in stimulating gluconeogenesis and lipolysis along with causing the mobilization of amino acids to regulate energy balance (Goodman 2009). With 88% of maintenance energy demands coming from lipid stores in hibernating brown bears, higher concentrations of cortisol during this time are thought to be important for the lipolysis necessary to maintain a basal metabolic rate (Palumbo, Wellik et al. 1983; Hilderbrand, Schwartz et al. 2000). Though not hibernators, red deer, mule deer, and white-tailed deer all exhibited higher concentrations of cortisol during the food scarce winter compared to other seasons (Bubenik, Bubenik et al. 1983; Saltz and White 1991; Huber, Palme et al. 2003).
Physiology of the grizzly bear (*Ursus arctos horribilis*)

**Overview**

The bear family, Ursidae is comprised of eight species of widely distributed large carnivorous mammals. Within this group of eight are two evolutionarily distinct offshoots, the giant panda (*Ailuropoda melanoleuca*) and the spectacled bear (*Tremarctos ornatus*) (O’Brien, Seuanez et al. 1988). The remaining six species belong to the subfamily, Ursinae, and include the Asiatic black bear (*Ursus thibetanus*), polar bear (*U. maritimus*), brown bear (*U. arctos*), black bear (*U. americanus*), sun bear (*Helarctos malayanus*), and sloth bear (*Melursus ursinus*). The six species of Ursine bears have nearly identical karotypes with 74 chromosomes while the spectacled bear and giant panda have 52, and 42 chromosomes, respectively (O’Brien, Seuanez et al. 1988).

Historically the brown bear (*Ursus arctos*) clade, to which the grizzly bear (*Ursus arctos horribilis*) belongs, was one of the most widely distributed large carnivores with a range spanning from Mexico to northern Canada and Alaska and across Europe, Asia, Japan, and even Iran (Waits, Paetkau et al. 1999). However, habitat fragmentation due to human presence and encroaching development have reduced the occupied space of the North American brown bear (common nomenclature referring to *Ursus arctos* on the North American subcontinent) to less than 2% of the original range in the lower 48 states (Figure 1.4) (Waits, Talbot et al. 1998; Mattson and Merrill 2002). Furthermore, the grizzly bear population has been extirpated from an estimated 100,000 in the 19th century to less than 1,000 effectively creating pockets of geographically isolated subpopulations (Allendorf and Servheen 1986; Proctor, McLellan et al. 2005; Proctor, Paetkau et al. 2012). Mitochondrial DNA of 317 brown bears in varying localities...
was used to evaluate genetic variation, natural female gene flow, and phylogeographic organization of the North American brown bear (Waits, Talbot et al. 1998). From sequence analyses, four genetically distinct geographic clusters were observed (Waits, Talbot et al. 1998). Cluster, or clade, I includes the southeastern Alaskan islands of Admiralty, Baranof, and Chichagof. Clade II includes mainland Alaska and Kodiak Island. Clade III represents the regions in far eastern Alaska and the Yukon and Northwest Territories. Finally, Clade IV represents bears in the southern regions of British Columbia and Alberta along with the Idaho, Montana, and Wyoming regions (Waits, Talbot et al. 1998). However, morphological studies using cranial features find many more subspecies than the classifications reported from the mitochondrial DNA data (Kurten 1973; Hall 1984). Many of the taxonomic classifications include overlapping genetic clades making group designation increasingly difficult (Rausch 1963; Kurten 1973; Hall 1984; Waits, Talbot et al. 1998). Changes in phenotypic characteristics may be representative of environmental adaptations rather than true genetic variability as a result of geographic isolation (Wayne, Lehman et al. 1992). Regardless, these findings of diverse genetic groups in a species with large dispersal ranges are perplexing. Waits and coworkers propose that these isolated groups are resultant from glacial separation during the Pleistocene and the low rates of female genetic dispersion (Waits, Talbot et al. 1998). A more recent, large meta-analysis of genetic data from over 3,000 bears in the northwestern United States, Alaska, and southern Canada corroborate low female dispersion and small, isolated populations, but conclude the population fragmentation is a result of more recent human pressures and development (Proctor, Paetkau et al. 2012).
North American brown bear populations are increasing slightly alongside rapidly increasing human populations (MacHutchon, Boulanger et al. 2008; Kendall, Stetz et al. 2009; Eberhardt and Breiwick 2010; Mace, Carney et al. 2012). Mortality of brown bears is directly related to human presence and development (Mattson and Merrill 2002; Schwartz, Haroldson et al. 2010; Chamberlain, Rutherford et al. 2012). Grizzly bears remain listed as a threatened species under the Endangered Species Act and conservation efforts concentrate heavily on creating management polices based on mortality risk analyses that incorporate effects of management factors, level of human presence, and food availability (Schwartz, Haroldson et al. 2010).
Though geographically separated, nearly all grizzly bears (excluding those with constant food availability) pass through four yearly physiological stages of normal activity, hyperphagia, hibernation, and a hypometabolic transitional period following hibernation (Nelson, Folk et al. 1983). During normal activity, which ranges from late spring (April/May) to autumn (September), grizzly bears are active, mainly engaging in breeding and foraging activities (Nelson, Folk et al. 1983; Craighead 1995). North American grizzlies are omnivores that harvest seasonally available foods from fruit to salmon (Welch, Keay et al. 1997). Captive black bears consume roughly 5-8,000 kcal/day during this period (Nelson, Folk et al. 1983). Furthermore, during this time, attempts to artificially induce a hibernating state by reducing food intake fail with bears showing dehydration and muscle wasting (Nelson, Jones et al. 1975). For lactating females, the active period is when milk intake and production are the greatest with levels four times as high as compared to winter (Farley and Robbins 1995). While with cubs, females are not sexually receptive. Females without cubs engage in breeding from May until roughly July (Craighead 1969; Spady, Lindburg et al. 2007). Photoperiod has been proposed to be the principle zeitgeber regulating the switch to breeding readiness for males (Palmer, Nelson et al. 1988; Garshelis and Hellgren 1994; Spady, Lindburg et al. 2007).

Following the normal active period, bears begin a period of preparation for hibernation where physiology and behavior change in order to accumulate large stores of energy. This hyperphagic period is spent almost exclusively foraging and hunting for food, spending up to 20 h per day on this task (Nelson 1980). Caloric intake more than doubles compared to normal active period with the bears consuming on the order of 20,000 kcal/day and, depending on body size, gaining 4 kg/day (Nelson 1980; Hilderbrand, Jenkins et al. 1999). These large
increases in energy intake are seen in other hibernating species as well. For example, in ground squirrels and marmots, food intake and body mass nearly double over the summer period with 60-80% of the increase in the form of white adipose tissue (Galster and Morrison 1976; Florant, Nuttle et al. 1990). The mechanisms driving this shift toward hyperphagia remain obscure but it is very likely that central sites of control such as the hypothalamus and pituitary are intimately involved in these annual cycles.

After a period of hyperphagia, through unknown mechanisms, the bear enters hibernation. Because hibernation in Ursids is a broad, foundational element in this review, it will be discussed in greater detail below.

At emergence from hibernation, the bears enter a transitional stage called “walking hibernation” (Nelson, Folk et al. 1983). This state is characterized by hypophagia (although food may be present), low water intake, low daily urine, and low excretion rates of metabolites such as calcium, phosphorous, and magnesium (Nelson 1979). Normal food and water intake resume after approximately three weeks in an observed grizzly bear (Nelson 1979).

**Hibernation**

**Overview**

Hibernation is a phenomenon that is described as an evolutionarily favorable adaptation for survival during periods of food scarcity or famine (Melvin and Andrews 2009). It is considered to be an ancestral trait of mammals (Carey, Andrews et al. 2003) with evidence for this conclusion found in the fact that species of hibernators exist in all three of the oldest lineages within the class Mammalia (Srere, Wang et al. 1992; Geiser and Ruf 1995).
Interestingly, hibernating species range in mass from ~5g to 80,000 g with the majority weighing between 10 and 1000 g (Geiser and Ruf 1995). Thus, it appears that hibernation is more prevalent and much more common in smaller mammals, but it is not known why only some species employ hibernation (Carey, Andrews et al. 2003).

**Characteristics of hibernation**

For the grizzly bear, hibernation is in many respects quite similar to hibernation in smaller mammals. However, bears in general exhibit unique physiological features during hibernation that are not shared by any other known hibernator. Common among mammals that hibernate is a reduced metabolic rate, reduced heart rate and bouts of torpor that can range in duration from a day to 5 wk in some species (Carey, Andrews et al. 2003). Torpor has been defined as the time in which body temperature drops below 30° C and a concomitant drop in metabolic rate of ~25% (Hudson and Scott 1979; Geiser 2004). Most hibernators employ a sharp decrease in body temperature as a mechanism to decrease energy utilization; this is not true for bears. Bears remain at near normal body temperature, dropping only 3-7° C, for the duration of their dormancy which can last up to 7 months (Nelson 1973; Folk, Larson et al. 1976; Watts, Oritsland et al. 1981; Hissa, Siekkinen et al. 1994). Furthermore, bears are not considered to be in a deep state of torpor because they can be easily aroused into an active state (Nelson 1973). In contrast, other hibernators exhibit body temperatures that reflect near ambient to as low as -2° C in arctic hibernators (Boyer and Barnes 1999). This deep torpor appears to be restricted to species weighing less than 10 kg (Geiser 2004). Also unique to the bears is the complete lack of digestive activity; they do not eat, urinate, defecate, and rarely, if ever drink, for the entirety of their dormancy (Tøien, Blake et al. 2011). Bears are able to
recycle toxic waste products, such as urea, through adaptations of the neuroendocrine system (Nelson 1973; Nelson, Folk et al. 1983; Barboza, Farley et al. 1997). Furthermore, the bear does not undergo appreciable muscle or bone loss during the months of dormancy (Floyd, Nelson et al. 1990; Donahue, Galley et al. 2006; Hershey, Robbins et al. 2008). Smaller hibernators arouse periodically to feed, urinate, and defecate (Pengelley and Fisher 1961; Folk 1974) and undergo bone loss (Steinberg, Singh et al. 1986; Kwiecinski, Krook et al. 1987).

Similar to their smaller hibernating counterparts, bears undergo drastically reduced metabolic rates and heart rates. Metabolic rates range from 33-73% of predicted basal metabolic rate during hibernation for brown, black and polar bears (Hock 1960; Kleiber 1975; Watts, Oritsland et al. 1987; Watts and Cuyler 1988; Farley and Robbins 1995). These rates are much greater than those of other hibernators whose metabolic rates average 5% of the basal metabolic rate (Geiser 2004). Heart rates drop from 40-80 beats per minute (bpm) in the active period to 8-20 bpm during hibernation with significantly decreased cardiac output in hibernation (117.7 vs 33.6 ml/min/kg) (Folk, Larson et al. 1976; Nelson, Robbins et al. 2008).

During hibernation female bears will undergo parturition around January or February and then proceed to lactate (Alt 1983). Reproductive hormones steadily rise through the pre-hibernation period of October and November prior to implantation (Tsubota, Takahashi et al. 1987; Hellgren 1998; Tsubota, Howell-Skalla et al. 1998). Approximately 60 days pre-partum, progesterone rises sharply; however, this rise does not correspond to entrance into hibernation suggesting that hibernation metabolism is not directly affected by reproductive physiology (Palmer, Nelson et al. 1988; Hellgren, Vaughan et al. 1991). Males are in a state of reproductive quiescence during the beginning of hibernation, but are at or near full reproductive
functionality at the end of hibernation indicating that testicular recrudescence is actively occurring during hibernation (Garshelis and Hellgren 1994; Tsubota, Howell-Skalla et al. 1997).

Control of hibernation

While extensive research has focused on the physiological details of hibernation, less is known about the timing control of hibernation. In 1969, the first report of a “hibernation induction trigger” (HIT) was described in which the blood from hibernating ground squirrels induced hibernation-like activity in summer in active ground squirrels (Dawe and Spurrier 1969). However, following that report, numerous attempts to identify and purify the HIT have been unsuccessful. Furthermore, varied tissue and fluid extracts from hibernating species that were injected into non-hibernating animals have elicited conflicting results (Wang, Belke et al. 1988; Hisa 1997; Vybiral and Jansky 1997). Rather, it appears that induction and termination of hibernation is derived by a vast interplay of physiological systems under the control of the neuroendocrine system (Suomalainen and Nyholm 1956; Boyer and Barnes 1999).

A large number of investigations have been conducted to examine individual subsystems within the neuroendocrine system. For example, within the HPA axis, CRF immunoreactivity was diminished in hibernating hamsters compared with euthermic controls (Nurnberger 1995). In hypothermic rats, secretion of ACTH is reduced (Gibbs 1985). Additionally, decreases in growth-hormone releasing factor immunoreactivity and low levels of thyrotropin-releasing factor in the brain have been reported (Stanton, Winokur et al. 1982; Nurnberger and Heinrichs 1989). Taken together, these results indicate an overall reduction in neuroendocrine activity during hibernation which is not unexpected as brain activity decreases in a step-wise manner with decreasing body temperature (Shtark 1972). However, these
experiments were conducted in hypothermic animals while bears remain more euthermic during hibernation. Therefore, the general conclusion that the neuroendocrine system is downregulated and non-functioning must be made with caution.

One region that appears to remain active in low body temperatures is the SCN. However, action potentials within the SCN cease at around 16°C (Miller, Cao et al. 1994). Whether SCN function continues with smaller, lower amplitude spikes not detectable by extracellular recording has yet to be demonstrated. SCN input systems, including vasopressin, substance-P, and serotonin show increased immunoreactivity to staining (Nurnberger and Heinrichs 1989). Although 2-deoxyglucose uptake was decreased markedly in the whole brain of hibernating ground squirrels, the SCN showed significantly less reduction than other brain regions (Kilduff, Sharp et al. 1982). Furthermore, ablation or lesions of the SCN effectively destroy the ability to generate synchronized hibernation rhythms in several rodent hibernators (Ruby, Ibuka et al. 1989; Dark, Kilduff et al. 1990; Zucker, Lee et al. 1991; Ruby and Zucker 1992). Hibernation duration increased by ~1 month in 50% of SCN lesioned animals and hibernation never ended for the other half of SCN lesioned animals (Ruby, Dark et al. 2002). On a molecular level, there are overall decreases in gene transcription of mRNA and protein synthesis during hibernation (Bocharova, Gordon et al. 1992; Frerichs, Smith et al. 1998; Knight, Narus et al. 2000; van Breukelen and Martin 2002). A recent investigation into the gene profile of the SCN during hibernation in the European hamster revealed similar results (Revel, Herwig et al. 2007). The clock genes Per1, Per2, and Bmal1 failed to show differences in day/night expression in hibernating hamsters (Revel, Herwig et al. 2007). Recently, work has emerged tying cellular nutrient status to the molecular clock machinery within the SCN (Melvin and
Andrews 2009; Nakahata, Sahar et al. 2009; Ramsey, Yoshino et al. 2009). Central to this theory are the ratios of intracellular concentrations of NAD$^+$ to NADH; during fasting and prior to hibernation NAD$^+$ levels are increased (Rodgers, Lerin et al. 2005; Serkova, Rose et al. 2007). Elevated NAD$^+$ activates the silent mating type information regulation 2-homolog 1 (SIRT1) which inactivates the clock protein BMAL1. BMAL1 inactivation prevents Per and Cry expression, thus disrupting the circadian molecular clock and causing it to slow or stop altogether. Non-functioning or dampened circadian rhythms have been widely reported during hibernation (Hut, Van der Zee et al. 2002; Heller and Ruby 2004; Revel, Herwig et al. 2007; Tøien, Blake et al. 2011).

Evidence supporting the SCN’s role is reinforced by the large amount of data illustrating circadian and circannual entrainment of hibernation and torpor. Golden-mantle ground squirrels exhibit light-dark entrainment with arousals from torpor during hibernation (Ruby, Dark et al. 2002). Entry into torpor precedes lights-on in nocturnal species and torpor bouts can re-entrain to photoperiod following phase shifts (Daan 1973; French 1977; Geiser and Baudinette 1985; Kirsch, Ouarour et al. 1991; Ruby and Zucker 1992). Furthermore, the timed entry and entrainment to the light-dark cycle appears to be representative of the underlying endogenous circadian rhythm. In constant darkness, multiple species including bats, mice, pygmy possums, and Siberian hamsters, will exhibit free-running bouts of torpor (Menaker 1961; French 1977; Thomas, Jewett et al. 1993; Kortner, Song et al. 1998). In contrast, several species show no apparent circadian regulation with regard to timing arousals from torpor (Kristoffersson and Soivio 1964; Pohl 1987; Thomas, Jewett et al. 1993; Wassmer and Wollnik 1997). However, the implications of circadian control on timing of arousal bouts may be less
important in the bear because they do not exhibit deep torpor. Körtner and Geiser surmised that the persistence of a circadian rhythm during hibernation is most advantageous for those species that must arouse periodically to engage in foraging in order to maintain energy homeostasis (Körtner and Geiser 2000).

However, the mechanisms driving circannual rhythms are not well described and no anatomical substrate has been identified (Zucker and Prendergast 1999). Nevertheless, some species exhibit continued annual cycles of physiological events such as reproduction, activity, and torpor when held in constant conditions for multiple years (Pengelley and Fisher 1957; Kenagy 1980; Ward and Armitage 1981). Furthermore, lengthening of bout duration during the course of hibernation persists even in the absence of light or temperature indicating the presence of an endogenous annual oscillator (Pengelley and Fisher 1961; French 1985; Geiser, Hiebert et al. 1990). While the SCN is integral to the expression of circadian rhythms, ablation does not destroy circannual rhythms, but the rhythms are altered suggesting that the circannual and circadian systems are most likely linked (Zucker, Boshes et al. 1983; Ruby, Dark et al. 1996; Ruby, Dark et al. 1998).
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CHAPTER 2

Temporal organization of activity in the brown bear (*Ursus arctos*): Roles of circadian rhythms, light and food entrainment

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ABSTRACT

Seasonal cycles of reproduction, migration and hibernation are often synchronized to changes in daylength (photoperiod). Ecological and evolutionary pressures have resulted in physiological specializations enabling animals to occupy a particular temporal niche within the diel cycle leading to characteristic activity patterns. In this study, we characterized the annual locomotor activity of captive brown bears (*Ursus arctos*). Locomotor activity was observed in 18 bears of varying ages and sexes during the active (Mar-Oct) and hibernating (Nov-Feb) seasons. All bears exhibited either crepuscular or diurnal activity patterns. Estimates of activity duration (alpha) and synchronization to the daily light:dark cycle (phase angles) indirectly measured photoresponsiveness. Alpha increased as daylength increased but diverged near the autumnal equinox. Phase angles varied widely between active and hibernating seasons and exhibited a clear annual rhythm. To directly test the role of photoperiod, bears were exposed to controlled photoperiod alterations. Bears failed to alter their daily activity patterns (entrain) to experimental photoperiods during the active season. In contrast, photic entrainment was evident during hibernation when the daily photocycle was shifted and when bears were exposed to a skeleton (11:1:11:1) photoperiod. To test whether entrainment to non-photonic cues superseded photic entrainment during the active season, bears were exposed to a reversed feeding regimen (dark-fed) under a natural photocycle. Activity shifted entirely to a nocturnal pattern. Thus, daily activity in brown bears is highly modifiable by photoperiod and food availability in a stereotypic seasonal fashion.

**Keywords:** entrainment, food-entrainable oscillator, hibernation, phase shift, brown bear
INTRODUCTION

Activity patterns are a useful behavioral measure to inform ecological and conservation studies. By examining these patterns, the impact of habitat destruction, food availability and conspecific interactions can be revealed. Furthermore, a large body of literature has revealed that rather than merely being stimulated or suppressed by light and dark, many activity patterns are generated by a clock-like mechanism (17, 60). This biological clock is highly conserved because it enables animals to accurately and reliably perform functions integral to survival due to the predictable nature of the earth’s rotation and seasonal changes in daylength (5). The biological clock thus serves as both a timekeeper and calendar.

Considerable research has characterized activity patterns of wild brown (Ursus arctos) and black bears (Ursus americanus), although much less is known about the physiological mechanisms that generate them. Both brown and black bears are capable of exhibiting a wide variety of activity patterns depending on season, geographic location, food availability, and human influences, just to name a few (22, 27, 34, 41, 49, 54, 77). Bears exhibit distinct seasonal timing of activities such as reproduction and hyperphagia prior to entrance into hibernation (8, 9). For example, brown bears feeding at salmon streams in Alaska exhibit both diurnal and crepuscular patterns (54) whereas brown bears in Europe exhibit predominantly nocturnal activity (34). Similar to brown bears, black bears in various locations in North America are diurnal and crepuscular (2, 22). Sympatric black and brown bears also exhibit widely different activity patterns, presumably as a means for the smaller black bear to avoid brown bear encounters (77). Anthropogenic influences on bear activity patterns vary on both a daily and an annual basis, often in relation to food accessibility (26, 43, 50, 54, 55, 84). While these studies
have contributed greatly to our understanding of the putative influences on bear activity patterns, a major gap remains in our knowledge of the basic physiological mechanisms responsible for the generation of daily and seasonal activity cycles in bears.

Annual changes in daylength (photoperiod) associated with the tilt of earth’s axis are reliable geophysical phenomena that are often anticipated by animals to synchronize important biological events, such as reproduction, migration and hibernation, with specific times of the sidereal year (5, 6). Moreover, there are accompanying changes in the daily light:dark cycle that are transformed into a photoperiod-dependent hormonal signal in the form of melatonin secretion by the pineal gland (25). In mammals, this photoperiod transduction requires, in part, the integration of photic information from the retina with an endogenous pacemaker, or circadian clock, in the brain to organize the daily cycle of melatonin (31, 56).

This pacemaker, or circadian (about 24 hr) clock, resides in the suprachiasmatic nucleus (SCN) of the hypothalamus and generates an endogenous oscillation with a period slightly different from 24 hr via a molecular transcription-translation feedback loop (17, 64). The rhythm of such an oscillation can be described by its period, amplitude, and phase. Period (or tau) refers to the duration of each rhythm or distance between peaks in the rhythm (64). Amplitude is defined as the “extent of an oscillatory movement, measured from mean to extreme value” (15). Finally, the phase, or phase-angle, of a rhythm can be envisioned as the relationship between a periodic quantity, such as locomotor activity, and a reference point of the environmental cycle, such as dawn (15, 64).

Environmental light serves as the primary effector of synchronization between the biological clock and the external photo-cycle; this function is mediated by the SCN via a diverse
set of SCN neural projections (31, 64). The endogenous clock synchronizes to the external clock, by being reset (or phase-shifted) each day to match the 24 h photo-cycle. This process of synchronization, referred to as entrainment, is integral to an organism’s optimum functioning, as it allows reliable predictions for behaviors such as feeding, migrating, and sleeping-waking based on the 24 hr daily rhythm (1, 12). Entrainment of the circadian clock combined with the direct masking effects of light and dark are therefore thought to be major determinants of activity patterns in animals (10, 65, 71). Together, these effects are thought to produce an optimal temporal niche occupied by an organism and one that is considered a relatively fixed feature of a species via selection (38). Unfortunately, most research examining entrainment has used prey species (primarily rodents) housed in artificial laboratory conditions. Much less is known about the physiological mechanisms determining activity patterns of predators, although a high degree of flexibility is evident.

The endogenous nature of the biological clock can be observed in the absence of external light:dark cycles (e.g., constant light or dark), where it free-runs with a period (tau) significantly different from 24 hr (33, 64). Intriguingly, these constant conditions are experienced normally by some species living at extreme latitudes and by those hibernating in burrows. Yet, the role of the circadian clock under these conditions is controversial. For example, arctic dwellers, including reindeer (Rangifer sp.) and Svalbard ptarmigans (Lagopus mutus hyperboreus) exhibit a complete absence of circadian rhythmicity during polar summers and winters (66, 82). In hibernating species, including those that show torpor, a role for the SCN is supported by lesion studies in golden-mantled ground squirrels (Citellus lateralis), Djungarian hamsters (Phodopus sungorus sungorus), and Syrian hamsters (Mesocricetus
auratus) (13, 73-75, 87), but this may not be true for all species (68). In addition, entry into torpor is rhythmic while arousal from torpor does not exhibit circadian rhythmicity (72, 85). Bears enter a state of hibernation during the colder months when food is scarce. Bear hibernation is characterized, and distinct from smaller rodent hibernators, in that body temperature decreases by only 3-5°C and bears do not undergo periodic arousals (19, 53). However, similar to smaller hibernators, there is a large decrease in metabolism, heart rate, digestive and locomotor activity (19, 53). In black bears, multi-day cycles of body temperature have been recorded, but circadian rhythmicity may be absent (29, 81). Collectively, it appears that the role of the circadian system is more important during hibernation in those species that arouse periodically compared to those that do not undergo periodic arousal and torpor bouts (37), but this remains to be determined.

Considering the stereotypical seasonal behaviors of brown bears, including profound annual changes in body weight (52), annual cycles of hibernation (19) and reproduction (8, 18), together with a wide geographic distribution in northern latitudes (44), we hypothesized that photoperiod would serve as an important proximal cue requiring a functional circadian timing system. Photoperiodic time measurement is dependent on the circadian system (25, 56, 58). Furthermore, given the absolute necessity of bears to gain sufficient body fat to survive a prolonged fast of several months (18), we hypothesized that other non-photic cues could serve as ultimate factors to entrain the bears’ daily activity patterns. This prediction is based on the evidence that bears must engage in feeding activities for extended periods (53) in order to increase their total energy uptake despite exposure to decreasing daylengths.
METHODS

Animals

Male (n = 6) and female (n = 12) brown bears (1-21 yr old) were housed at the Washington State University Bear Education, Conservation and Research Center (WSU Bear Center, 46° 43' 53" N / 117° 10' 43" W). The animals were maintained according to the Bear Care and Colony Health Standard Operating Procedures with all procedures approved by the Washington State University (WSU) Institutional Animal Care and Use Committee (IACUC). Bears were housed in pairs in dens (3m x 3m x 2.5m) with access to an adjacent outdoor pen (3m x 5m x 5m) and an adjacent 0.56 ha outdoor enclosure. For characterization of seasonal and daily behavioral rhythms, animals were exposed to natural photoperiod and temperature fluctuations. During all photoperiod experiments (excluding hibernation) and the nighttime feeding experiment, bears were allowed daily access to the outdoor enclosures. Normal feedings during the active time of the year occurred twice daily at 0700 ± 1 hr and 1600 ± 1 hr (standard time). Bears were fed at, or slightly above, maintenance levels from April to early August. Then, because of the bears' increased appetite between August until mid-October, feeding amounts were significantly increased to well above maintenance levels. During the active time of the year, bears are also expected to forage for grasses and clover in the irrigated 0.56-ha exercise yard to supplement commercial foods fed twice daily (70). The commercial foods include a dry chow (25.3% protein, 16.2% fat, 51.7% carbohydrate, and 2.0% crude fiber; Hill’s Pet Nutrition, Topeka, KS), apples, and small amounts of meat and pastries. In the fall, based on the bears’ appetite, food was gradually reduced in early to mid-October until completely withdrawn in late October. Hibernation began when all food was withdrawn (October 24 ± 7d). Water was available ad libitum during all seasons. Hibernation ended based
on subjective evaluation of general increases in activity at which time feeding was restored (March 1st ± 16 d).

**Activity determinations**

For baseline activity, monitored bears were housed as described above and hibernated in pairs in their indoor and outdoor dens. For studies directly examining photic responsiveness during hibernation, bears (n=5) were moved to an offsite-location consisting of a light- and temperature-controlled building. For these studies the bears were housed individually in culvert-type bear enclosures (2.44m x 1.22m x 1.22m, Teton Welding, Choteau, MT) fitted with automatic waterers. Lighting was supplied by overhead fluorescent lights and supplemented with halogen lamps to increase light intensity when necessary.

Activity measurements were made (n=18) during the winters of 2003-2006, June to December of 2008, entire year of 2009-2010, excluding times when experiments were being conducted. Behavior was characterized using digital video recording (OpenEye Digital Video Security Solutions, Spokane, WA). Indoor and outdoor bear pens were fitted with infrared-sensitive cameras and infrared light sources to facilitate the monitoring of activity during the day and night. Subsequent studies used Actical activity physical activity monitors (Minimitter Corp., Bend, OR) housed in protective aluminum cases that were glued to bears’ fur on the neck just cranial to the shoulder. Acticals were programmed to collect incidence of movement and movement velocity data via an omnidirectional accelerometer in 1 min epochs for a maximum recording duration of 45 d. Application and removal of the acticals was performed on non-anesthetized bears (n=4) that had been trained to remain still for a food reward. For non-trained bears (n=6), application and removal of Acticals was facilitated by sedation with 1-1.5
mg/kg of tiletamine HCL and zolazepam HCl (Telazol, Pfizer Animal Health, New York, NY) and
0.04-0.08 mg/kg of medetomidine HCl (Dormosedan, Pfizer Animal Health, New York, NY).
Intravenous or intramuscular administration of atipamezole HCL (Antisedan, Pfizer Animal
Health, New York, NY) was used to reverse the anesthesia. Activity data were downloaded into
Actical 2.12 software (Respironics Inc., Murraysville, PA) and subsequently exported for further
analysis (see below).

For both video and Actical-based measurements, activity onset and offset was recorded
each day. Activity onset based on video records was defined as the time when the bear was
standing, sitting, or exhibiting alert head movements (looking side to side or up and down) for
at least 5 consecutive min. Activity offset was defined as the time when animals failed to
exhibit any bouts of standing, sitting, or head movement activity for greater than 5 consecutive
min. Activity onsets and offsets derived from Actical records were determined using Clocklab
software (Actimetrics, Wilmette, IL). Comparison of the two methods confirmed a high
concordance (Pearson r = 0.93, data not shown). We also determined the time difference
between activity onset/offset and sunrise/sunset (phase angle) for each day of video recording
in addition to the duration of activity (alpha) by subtracting the time of activity onset from
activity offset. Lastly, when possible, estimates of rhythm period (tau) were made using
ClockLab software.

*Photoperiod experiments*

Photoperiod was manipulated for ≥ 2 wk at 4 times of year: spring (April-May, n=7; avg.
ambient photoperiod = 14.50 :9.5), summer (June, n=8; avg. ambient photoperiod =
15.33:8.67), fall (October, n=6; avg. ambient photoperiod = 10.76:13.24), and winter
(November-December, n=4; avg. ambient photoperiod = 8.68:15.32). During the times of year when the bears were active, the photoperiod was extended by adding additional lighting to the indoor and outdoor dens. Photoperiod contractions were not possible during the active season because ambient lighting could not be sufficiently reduced to mimic nighttime conditions. We therefore performed photoperiod contraction only during the inactive period (winter) when bears were hibernated in individually in enclosures (65) (described above) in an environmentally controlled facility. The length of each photoperiod extension was ≥ 4 hr longer (+2 hr morning, +2 hr evening) than either the prevailing photoperiod or the animals’ baseline alpha during that time of year, whichever was longer. For photoperiod contractions, the daylength was reduced by 4 hr compared to the prevailing photoperiod. Thus, the following light:dark cycles were used, 18L:6D, 20L:4D, 18L:6D, and 4L:20D for spring, summer, fall, and winter photoperiod experiments, respectively.

For the spring, summer, and fall photoperiod extensions, 2-500 watt halogen lights (Cooper Lighting, Houston, TX) were positioned to face the indoor den and 3-500 watt halogen lights were placed 1.8 m above the outdoor den of each bear. Illumination levels at the approximate level of the bear’s head measured at 5 different locations within the indoor dens averaged 193.3 ± 24.8 lux during lights on, with no ambient light filtering in from their outdoor den. Outdoor light levels measured at 4 locations in the outdoor dens averaged 151.7 ± 24.3 lux after sunset. Daytime light levels were subject to normal daily fluctuations and weather conditions with noontime light levels on a sunny day measuring >10,000 lux. For the winter contraction photoperiod experiment lighting provided by 1-500 watt halogen light placed 0.61 m from the end of the enclosure produced an average interior illumination of 1146.7 ± 607.3
lux. During the hibernation photoperiod experiment, room temperature was maintained at 10 ± 1.2° C for the duration of the study.

For photoperiod experiments during the active season twice-daily feeding was randomized to occur at least 2 hr post sunrise and 2 hr pre sunset in order to remove any confounding influence of feeding cues with photic cues. Random feeding times within the specified interval (i.e. between 2 hr post sunrise and 2 h pre sunset) were obtained by using an online random number generator (www.random.org). However, because the online number generator output the random times in ascending temporal order, the order of the randomly generated feeding times had to be re-randomized as follows. Briefly, the randomly generated feeding times were exported into an Excel spreadsheet. Then the ‘rand’ function was applied to fill cells in the adjacent column to the feeding times. Finally, the two adjacent columns were sorted in ascending order according to the random numbers generated by Excel. The amount, placement, type, and approximate total energy content of food provided were similar to that during the twice-daily scheduled feeding. Bear center staff and visitors were not allowed in the facility prior to the first feeding of the day in order to reduce disturbance and possible entrainment by human presence. Bears were not eating during hibernation when photoperiod contraction was being performed.

Light entrainment and circadian rhythms

We also determined if activity: 1) could entrain to a 5 hour shift (delay) of the light:dark cycle, 2) could be entrained to a minimal (so-called skeleton) photoperiod, and 3) would exhibit an endogenous circadian rhythm when held in constant conditions. These manipulations were only possible during the hibernating season (winter) when the duration and application of
photoperiod could be closely controlled. Once in hibernation, bears (n = 4) were placed on a 4L:20D photoperiod for 17 d and then the photophase was delayed by 5 hr. Bears were exposed to the shift for 21 d and then animals were transferred into constant light conditions (LL, 24L:0D) for 42 days to determine if an endogenous circadian rhythm was expressed. Lastly, the bears were exposed to a skeleton photoperiod consisting of 1 hr dark pulses interposed between 11 hr of light (11:1:11:1) for 3 weeks. Dark pulses were applied at 1100 hr and 2300 hr. Further evaluations of circadian rhythmicity were made with 2 additional bears. One bear was held in constant dark for 33 days during hibernation while the other bear was held in constant dark during the active season for 20 days. Activity profiles based on Actical data were used to evaluate entrainment and circadian rhythmicity.

**Food entrainment**

The role of food to entrain activity patterns was examined as follows. Following a period of standard twice-daily feeding, bears (n=8) were exposed to a reversed feeding schedule for 18 days in the fall (September-October, 2011, average natural daylength during experiment 11.63 h). Average ambient temperatures during the experiment were 19.7° and 5.7°C for maximum and minimum, respectively. In this case, the bears were fed twice nightly at 2100 hr and 0300 hr. Nighttime feeding and cleaning of the dens was facilitated with the aid of a red lamp producing <5 lux illumination in the dens. Following feeding and cleaning, the red lamps were turned off leaving the animals exposed only to ambient moonlight. To further limit any disturbances and possible entrainment, daytime visitation to the WSU bear center was tightly restricted. During this time the bears were exposed to natural photoperiod and temperature conditions. As noted above, the location of the food, amount and type of food,
and energy content was kept identical to the twice-daytime scheduled feeding protocol described above. Under these conditions bears were exhibiting an annual cycle of body weight gain with an average weight gain of 4.8 ± 0.93 kg during the experiment. Annually, the bears at the WSU center fluctuate an average of 72.7 kg in body mass.

As for the photoperiod experiments, activity onsets and offsets were calculated. To confirm that a temporal niche shift occurred, both phase angles and midpoint of the daily rhythm (acrophase) were also estimated. To provide additional evidence of true entrainment by food, we also quantified anticipatory activity occurring prior to feeding onset by dividing each bears’ hourly activity count by its total daily activity count to produce a normalized activity ratio. Activity ratios were computed for all bears during twice-daily timed daytime feeding, twice-daily random daytime feeding, and nighttime feeding experiments. Means for activity ratios were calculated and compared between feeding conditions.

Statistical analysis

Data were analyzed using Graphpad Prism5 (La Jolla, CA) and SAS v9.0 statistical software (Cary, N.C.). One- and two-way ANOVAs were used for estimating seasonal variation in activity counts, phase angles, and activity profiles. Bonferroni post-tests were used to evaluate differences among means for treatment groups.

Linear regression was used to evaluate activity onset/offsets against time, monthly alpha averages against daylength, and activity onsets/offsets against time during photoperiod extension experiments. Statistical comparisons of regression slopes were made using a Student’s t-test. Ability to phase shift and entrain to a skeleton photoperiod were tested by t-
test comparing activity counts in the light and dark period for each experimental condition. Period (tau) determinations were made using Lomb-Scargle periodograms in Clocklab software. Nighttime feeding was evaluated by analyzing acrophases on the last day of the reversed feeding using circular statistics (Oriana, RockWare Inc., Golden, CO). Differences between acrophases were evaluated using Watson’s $U^2$ test (42). Effects were considered significant at $p < 0.05$.

**RESULTS**

**Baseline activity**

There were dramatic differences in amount and amplitude of activity among seasons, with a clear 24 hr periodicity detected for all animals (Figure 2.1A; Lomb-Scargle periodogram analysis, tau $P < 0.05$). Activity differed among seasons (1-way ANOVA, $P < 0.0001$, Bonferroni post-tests $P < 0.05$) with hibernation having the lowest activity (25567.3 ± 1914.0 mean cnts/day) and spring and summer the highest activity (415550 ± 27177.0 and 447580.0 ± 20379.0 mean cnts/day, respectively) (Figure 2.1B). Activity counts increased during the spring transition season and decreased during the fall transition season (287319.0 ± 14712.0 and 106381 ± 9241.0 mean cnts/day, respectively). All bears were diurnal with a strong crepuscular component to activity during the active season (Figure 2.1). During hibernation while fasting, all bears remained diurnal when exposed to natural photoperiod and ambient temperatures, although the amplitude of their daily activity rhythm was diminished (Figure 2.1).

Despite the predominately diurnal niche, phase angles varied considerably throughout the year (Figure 2.2A). Phase angle means varied among seasons (Figure 2.2B-C; 1-way ANOVA,
Phase angles were associated with sunrise and sunset in the spring, post-emergence (i.e., near-zero values). After the summer solstice (late July), the phase angles shifted with the animals initiating activity before sunrise (positive values) and ceasing activity after sunset (negative values). This pattern continued until late September to mid October when the phase angles spontaneously began to compress as the bears became anorectic in preparation for hibernation. During hibernation, the phase angles were completely inverted compared to the late summer/fall period with animals initiating activity well after sunrise (negative) and ceasing activity well before sunset (positive, Figure 2.2A). Changes in phase angles observed during late summer and fall did not appear to relate to changes in daylength (Figure 2.2A). However, activity duration (alpha) was strongly related to daylength ($r^2 = 0.80; P < 0.0001$, $F = 475.6$ (1 122); Figure 2.3A). Alpha varied significantly with season even when daylengths were similar ($P < 0.001$, unpaired t-test, $t = 1.65$, df = 14; Figure 2.3B).

**Photoperiod experiments**

During the active season, bears were exposed to photoperiod extensions at three times: spring (18L:6D, April), summer (20L:4D, June), and fall (18L:6D, October). Activity did not expand with the extended daylength (Figure 2.4A,B). In both spring and summer, regression analysis failed to detect any differences in locomotor activity onsets or offsets when compared to control periods ($P > 0.05$; Figure 2.5A). By contrast, extension of the photoperiod in the fall caused activity to *contract* when compared to control periods (Figure 2.5B).

During hibernation (December), a shortened photoperiod (4L:20D) applied for 17 d caused activity onsets and offsets to quickly shift in apparent synchrony with the new light:dark
cycle. This apparent entrainment however was not stable and soon began to dissociate from the light:dark cycle after about 1 wk (Figure 2.5B). Although nonlinear regression fits of activity onsets and offsets against time revealed significant differences between control and experimental periods ($P < 0.001$) during hibernation, alpha (estimated from last 5 days) did not differ significantly from the control period (Figure 2.4B; Bonferroni posttest, $t = 3.16, P > 0.05$).

**Light entrainment and circadian rhythms**

Following the 17 d exposure to 4L:20D photoperiod during hibernation, the photocycle was shifted (delayed) by 5 hr (Figure 2.6A). Examination of the last day of the initial photocycle revealed a clear aggregation of activity in the photophase compared with the scotophase (Figure 2.6B1). When the photocycle was shifted by 5 hr, a gradual shifting of activity with transients occurred with a corresponding accumulation of activity (on the last day) within the photophase (Figure 2.6B2). Comparison of light vs. dark counts following the shift revealed a significant difference (one-sample t-test, $P < 0.01$). Interestingly, little evidence of anticipatory activity was observed under either 4L:20D photocycle (compare Figure 2.6B1 and 2.6B2).

Exposure to a skeleton photoperiod consisting of 22L:2D (11:1:11:1) resulted in activity concentrating between the later dark pulses (approximately 1700 hr; Figure 2.6A,B). Locomotor activity was significantly greater in the photophase between 1200 and 2300 hr compared to the period between 2400 and 1100 hr (one-sample t-test, $P < 0.001$).

To explore whether or not an endogenous circadian rhythm could be expressed during hibernation and active seasons, we maintained animals in either constant darkness (DD) or in constant light (LL) in the active and hibernating seasons for approximately 4 weeks. Only one
animal remained on trial for the active season DD experiment with $\tau = 24.05$ hr (Figure 2.7A). The effects of hibernation coupled with the darkness, suppressed locomotor activity to such an extent that only one bear remained sufficiently active to allow an accurate assessment of the free-running period to be made (24.5 hr, Figure 2.7B). In constant light during hibernation, the free-running circadian rhythm ($n=4$) was $24.3 \pm 0.17$ hr (Figure 2.7C). While exposed to LL and constant temperature, all bears exhibited a spontaneous increase in activity towards the end of hibernation (Figure 2.7D).

**Food entrainment**

The general lack of responsiveness to photic manipulations during the active season led us to test food entrainment as a potentially more important modulator of bear activity patterns. Following a period of standard twice-daily feeding, bears were exposed to a reversed feeding schedule with feedings occurring during the scotophase at 2100 and 0300, for 18 days in the fall. Normally, when bears were fed twice daily at 0700 ± 1 hr and 1600 ± 1 hr (standard time), a crepuscular pattern of activity was evident (Figure 2.8A). Coincident with this feeding pattern was clear anticipation of both feeding times evident as increases in activity prior to feeding. When feeding times were randomized during daylight hours, activity profiles changed to reveal two daily peaks in activity, with one occurring near the time of the previous feeding time of 0700 and the second peak occurring between 1200-1400, approximately 4 hr earlier than the previous 1600 feeding time. Comparison of the two feeding schedules by two-way ANOVA revealed a significant interaction between time of feeding and type of feeding schedule, $F = 7.54$, df = 69, $P < 0.0001$, Bonferroni posttests $P < 0.01$ at 0700, 1200, 1300, 1400,
and 1700). While there was a main effect of time on activity, there was no main effect of feeding type ($F = 11.44, df = 23, P < 0.001$ and $F = 0.05, df = 3, P > 0.05$, respectively). Activity in both cases was constrained to the light portion of the photocycle.

When feeding was changed from scheduled daytime feeding to night-time feeding, pattern of activity aligned with the nighttime feedings (Figure 2.8B). Actograms reveal how bears quickly became nocturnal within several days of transients (Figure 2.9A) suggesting true entrainment rather than a simple masking effect of food. Once nighttime feeding ended, the bears returned to their diurnal niche coincident with twice daily feedings (Figure 2.9A). This was reflected in adjustments of the phase angles until activity was stably entrained to the feeding schedule but not the photocycle (Figure 2.9B). By the last day of nighttime feeding, clear anticipation of the evening meals was exhibited by all bears (Figure 2.8B; 2-way ANOVA time by feeding schedule interaction, $F = 6.31, df = 23, P < 0.0001$, Bonferroni posttests $P < 0.05$ at 0200, 0300, 0400, 0800, 0900, 1600, 1700, and 2000) further suggesting entrainment.

Regression fits for the last 5 days of phase angle onsets and offsets under reversed feeding revealed no significant differences from zero, indicating stable entrainment (Figure 2.9B (bottom); one-sample t-test, $P = 0.73$ and $P = 0.16$ for onset and offset phase angle regressions, respectively). Acrophases aligned near the midpoints of the respective daily feeding schedules (i.e., around mid-day for daytime feeding (1254 hr) and nighttime feeding (0240 hr) (Watson’s $U^2$ test $P < 0.001$, Figure 2.9C). Despite the complete inversion of activity patterns, nighttime feeding did not significantly affect total activity ($P = 0.26$) although the percentage of activity occurring in the light phase was significantly lower when animals were fed during the night (Figure 2.9D-E).
DISCUSSION

Activity patterns during both active and hibernating seasons in the captive bears varied significantly as a function of daylength and food availability in a time-of-year-dependent fashion. Manipulations of the light:dark and feeding schedule revealed that daily rhythms in bears are jointly regulated by light-entrainable and food-entrainable oscillators, permitting adaptive flexibility of activity patterns. Entrainment, to light, and perhaps food, is likely to be mediated by a circadian clock that is expressed in brown bears during hibernation. We hypothesize that the inherent behavioral flexibility exhibited by brown bears is likely to have conferred a selective advantage to this apex predator.

Estimation of the activity phase angles and alpha allowed us to indirectly examine the influence of photoperiod on activity. One would expect if bears were entrained to environmental photoperiod that stable (statistically revealed as linear) relationships with those light cues would be evident. Contrary to our initial expectation the bears in this study exhibited quite variable, yet predictable, changes in phase angles. Unstable phase angles have been observed in animals residing at high latitudes when daylengths shorten to less than 5 hr or increase to more than 19 hr (11) likely due to the limits of entrainment being reached. In two extreme cases occurring at polar latitudes, reindeer (Rangifer sp.) and Svalbard ptarmigans (Lagopus mutus hyperboreus) exhibit a complete loss of circadian rhythmicity (i.e., activity becomes arrhythmic) during the continuous days and nights of the polar summer and winter, respectively (66, 82). Although the animals in our study never experienced such extreme daylengths except in one experiment (see below), the phase angles of entrainment varied. Reproductive hormones have previously been shown to influence phase angles of entrainment
(11, 28, 86). Although we did not directly test the effect of reproductive hormones, male bears exhibit a clear breeding season with annual changes in circulating testosterone and testicular recrudescence beginning in hibernation with regression beginning in summer ((8), Jansen, unpublished). In other hibernators, reproductive hormones were not responsible for circannual variation in entrainment of activity but it should be cautioned that although other species that hibernate or exhibit torpor also undergo changes in activity duration and phase angles (20, 36, 39, 83) it may not be appropriate to compare these species with larger hibernators such as bears that do not undergo periodic torpor and arousal bouts.

Alpha varied seasonally, but in a less-than-straightforward way. This was clearly evident when comparing months in which daylengths are similar but seasons differed (e.g., near the equinoxes). The circadian system can respond to photoperiods differently depending on prior photoperiod history. Specifically, although animals are exposed to the same photoperiods twice each year, the changes in daylength preceding these differ dramatically – in one the daylengths are increasing while in the other they are decreasing. Prior photoperiod history is deterministic to reproductive outcome in sheep (69) and birds and mammals living at high latitudes (11). A second possibility is that animals in the wild are acutely influenced by multiple environmental cues such as food availability and temperature, either of which can alter activity patterns (58). For providing predictive value, the first mechanism would have a clear long-term advantage compared to second.

Consistent with the variable phase angles expressed by bears in our first experiment, extending the photoperiod at three different times during the active season resulted in dramatic changes in phase angles but without significant effects on alpha. Thus, although there
appears to be a relative insensitivity to changes in photoperiod during the active season, an
influence of light *per se* cannot be ruled out. Specifically, changes in light intensity, also known
as continuous or parametric effects of light (3) or discrete effects of dawn and dusk (i.e., non-
parametric effects), may be involved. Continuous (parametric) effects of light refer to the
relationship between light intensity and speed, or period, of the endogenous clock. Thus the
increases and subsequent decreases in light intensity across the day serve as entraining signals
for organisms (16). For example, diurnal European ground squirrels (*Spermophilus citellus*) that
emerge from their burrows after dawn and return before dusk never are exposed to discrete
dawn and dusk cues yet remain stably entrained to the ambient photocycle indicating that the
subtle changes in light intensity during the period in which the animal is out of the burrow are
sufficient to entrain the circadian clock (10). Increases in light intensity have also been shown
to affect tau and alpha in degus (*Octodon degus*) housed in constant conditions (40). Thus,
despite the additional lighting to extend the photoperiod in the current study, our bears still
experienced sufficient natural changes in light intensity to potentially overcome the
photoperiod extension. Alternatively, discrete effects of light on the circadian clock may have
occurred but were masked by another entraining signal such as food (discussed below). These
discrete (nonparametric) effects of light cause phase shifts of the endogenous clock sufficient
to advance or delay the free-running period to match the external light cycle; under natural
conditions, these light cues are the sunrise and sunset transitions (16). Nonparametric effects
have been shown to be effective at entraining seasonal rhythms of reproduction in the
European hamster, but only at specific times of the year (summer and winter solstice) (48, 76).
These findings are important in light of our finding that during the period surrounding the
summer solstice bears were unresponsive to increases in photoperiod. Because of our limitations for performing photoperiod contractions, we cannot rule out the possibility that bears may have been photosensitive to short days at that time. However, in the face of decreasing photoperiods, bears still remained unresponsive to photoperiod manipulations suggesting that nonparametric effects of light do not exert powerful effects on activity patterns during the active season. Regardless of the precise reason(s), our observations are similar to those in ptarmigan where alpha increases while the birds are foraging and exposed to rapidly shortening daylengths (67).

The apparent lack of responsiveness to photoperiod manipulations raised the possibility that the light entrainable circadian clock in bears is non-functional or was masked by another circadian oscillator. Arguing against this, bear activity rhythms began to free-run with a tau differing significantly from 24 hr when housed in constant light or darkness. These results provide strong support of a circadian clock in the brown bear operating during both active and inactive (hibernating) periods. Although temperature measurements indicating level of torpor were not made in this study, activity and temperature are related. Furthermore, evaluations of decreased heart rate and metabolism have been characterized previously at this facility during hibernation suggesting bears at our location were indeed in hibernation torpor during this study (51). In addition, the spontaneous changes in activity that occurred prior to entering hibernation combined with the spontaneous increases in activity towards the end of hibernation suggest an even longer duration endogenous cycle, i.e., a circannual rhythm in brown bears, although this remains to be determined directly. Similar spontaneous changes in
length and depth of torpor bouts and seasonal sleep rhythms have been observed in other hibernators and species that exhibit bouts of torpor (21, 23, 59, 83).

In contrast to what was observed during the active season, hibernating bears responded to a daylength compression quickly (but not permanently). Two features of this experiment are worth noting: 1) Bears were not exposed to natural light cues and 2) light intensity was approximately 10 times greater than what we could achieve in the active season. These results suggest that both parametric and nonparametric effects are involved. Nonparametric effects of light have been well characterized in many species and are manifested as large, predictable, changes in the activity phase after application of discrete light pulses (61). Although a definitive conclusion regarding entrainment mechanism is not possible, based on the effects observed using a photoperiod contraction, clear evidence to support a nonparametric effect of light to entrain the circadian clock comes from the phase shift study. A 5 hr delay of the photocycle resulted in all bears shifting their activity to the new light:dark cycle after several days of transients. However, a masking effect of light to stimulate activity (or suppress, in a nocturnal animal) is also possible (63, 65). Indeed, there was very little evidence of anticipatory activity occurring prior to light onset on the last day of the shifted photophase. Nevertheless, a more likely explanation is that the duration of exposure was simply not long enough to allow full entrainment of the circadian clock to occur. Also arguing against simple masking effects is the clear daily adjustment (delay) in activity that was observed for each bear following the phase shift.

The third approach used to examine light entrainment in the brown bear consisted of applying a minimal (skeleton) photoperiod comprising two 1 hr dark pulses spaced 12 hr apart
on a background of bright light during hibernation. Skeleton photoperiods can supplant a complete photoperiod to maintain entrainment (62). Dark pulses rather than light pulses were used in the current study because the diurnal phenotype of our bears resulted (with rare exception, See Fig. 8B) in exceedingly low levels of activity when housed in constant darkness, thus making interpretation of endogenous rhythmicity or entrainment difficult. However, the application of discrete dark cues resulted in well-organized activity patterns, suggesting true entrainment had occurred. Interestingly, the consolidation of activity was greatest during the second portion (1200-2300 hr) of the diel cycle compared to the first portion. We hypothesize that this was due to the relationship between the dark pulses, the individual bear’s circadian phase, and position on the phase response curve. The general form of the response curves is very similar between nocturnal and diurnal animals (62) and appeared opposite to that of light in our study, supporting similar findings in rodents (4, 80). In addition, responses to dark pulses appear to differ between nocturnal and diurnal species with nocturnal species generally being most responsive during the day and diurnal species during the night (7, 14, 24, 30, 32, 40, 45). Indeed, for half the bears, the first dark pulse appeared to fall on the presumed dead zone of the phase response curve (i.e. no effect), but phase advances or delays occurring in response to the second dark pulse, depending on whether the pulse fell late in the subjective day or early in the subjective night, respectively. The opposite was true for the other bears. These results indicate that the activity consolidation that occurred within the second photophase of the symmetrical light:dark skeleton photoperiod was interpreted by the bears as subjective day rather than subjective night.
The sensitivity to photic manipulations during hibernation and apparent lack thereof during the active season suggested that other non-photic cues could be responsible for entrainment during the active season. Food is a powerful entraining signal sufficient to override the effects of light under certain experimental conditions (47). In support of the importance of food entrainment in bears, we observed robust activity changes in response to shifted feeding schedules. Thus, even with exposure to natural photoperiod and bright ambient light when bears were fed twice daily their activity patterns changed within 4 days to a completely nocturnal activity pattern. Similar observations have been made in other species (35, 79). Much evidence suggests that an endogenous food entrainable oscillator (FEO) operating entirely independently of the light entrainable oscillator is involved (46). Much like light entrainment, that a true food oscillator was involved in this behavioral shift, rather than simple masking, is supported by three observations: 1) clear transients were expressed following the shift to nocturnal feeding, 2) a shift in anticipatory increases in activity corresponding to the new feeding times on the last day of the experiment was observed and 3) a stable phase angle relationship between anticipation and feeding was established during the last week of the experiment.

In summary light entrainable and food entrainable oscillators in bears may be operating in a mutually exclusive or mutually suppressive fashion to modulate seasonal activity patterns. Indeed, such temporal flexibility would serve bears well because restricted food availability is common to virtually all bears in the wild (57). In addition, the temporal niche switching we observed is also known to occur in some animals and in wild bears potentially as a mechanism to cope with food availability, predation, and temperature fluctuations (35, 65, 78), Fortin and
Robbins, unpublished). Thus, although considered rare (38), our findings suggest it may be a relatively common in brown bears perhaps as a result of segregation between circadian light entrained and food entrained oscillators.

PERSPECTIVES AND SIGNIFICANCE

Activity patterns in captive brown bears are modulated by a seasonally-dependent shift in Zeitgeber strength with food entrainment predominating during the active season whereas sensitivity to light:dark cues is uncovered during hibernation. The ability of brown bears to respond to non-photic and photic cues in a seasonal manner may explain the extensive behavioral flexibility exhibited by these animals. This flexibility may be required for the massive gains in body mass necessary to survive a prolonged fast while also retaining the ability to emerge from hibernation at an advantageous time of year or prior to starvation. Implicit in this hypothesis is the existence of an underlying circannual rhythm entrained by these two cues. Regardless of the precise mechanisms involved, these adaptations may allow brown bears to successfully navigate a human influenced landscape.
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Figure 2.1. A. Representative double-plotted actogram for a bear housed in natural photoperiodic conditions illustrating changes in activity levels from the active to hibernation and back to active season. Shaded areas indicate approximate dark phase. Blank area is result of missing data. Tau significant at $p < 0.05$. B. Mean (± SEM) activity counts for each season. Seasons defined as: Hibernation (Dec 1 to Feb 14), Spring Transition (Feb 15 to Mar 30), Spring (Apr 1 to May 15), Summer (May 16 to Aug 31), Fall (Sept 1 to Oct 24), and Transition (Oct 25-26 to Nov 30). Different letters indicate differences $P < 0.05$, 1-way ANOVA).
Figure 2.2. A. Mean (± SEM) weekly phase angles of activity onset and offset relative to sunrise and sunset, respectively, across the year. Dashed line represents hours of daylight. Shaded areas indicate hibernation (winter) season. B. Comparison of mean weekly (± SEM) phase angles among seasons. Different letters indicated differences $P < 0.01$. 
Figure 2.3.  A. Mean (± SEM) monthly alpha. Shaded area represents hibernation period. B. Comparison of alpha for months with similar hours of daylight. * = $P < 0.001$. 
A

B

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Figure 2.4. A. Double-plotted actograms of a representative bear exposed to natural and extended photoperiod conditions in different seasons. Shaded areas indicate darkness. B. Comparison of mean alpha (± SEM) for natural and extended photoperiods.
Figure 2.5. Regression fits of activity onsets and offsets during both control and photoperiod experimental periods in different seasons. P-values represent linear regression slope comparisons between control and experiment onsets and control and experimental offsets during photoperiod extensions (A) and photoperiod contraction (B). (18L:6D, March; 20L:4D, June; 18L:6D, October; 4L:20D, December for panels respectively).
Figure 2.6. A. Actograms of 4 bears during hibernation illustrating responses to a photic shift, constant light conditions, and skeleton photoperiod (11:1:11:1). B. Daily activity profiles over the last two days of entrainment during 4L:20D photoperiod (1), following a 5h phase shift (2) and during a skeleton photoperiod. Comparisons between light and dark periods from last two days of each photoperiodic condition are also presented. * = p< 0.05.
A  

Active DD

B  

Hibernation DD

C  

Hibernation LL

d  

LL (n=4)  DD (n=1)

Hibernation phase
Figure 2.7. Representative actograms, Lomb-Scargle periodograms, and activity profiles for bears in constant dark during the active season (A), hibernation (B), and constant light during hibernation (C). Mean activity counts from the first 8 day and last 8 days of constant conditions are plotted in D. Activity profile axes have been adjusted to reflect significant taus with time ‘0’ reflecting 00:00 clock time ($p < 0.05$).
Figure 2.8. A. Daily activity profiles during twice daily scheduled feeding and randomized daytime feeding and B. during twice nightly scheduled feeding with twice daily scheduled feeding replotted for comparison. Light gray bars indicate twice daily scheduled feeding times and dark gray shaded bars represent twice nightly scheduled feeding times. * = p < 0.05.
Phase Angle (mean ± SEM)

Days after initiation of nighttime feeding

Watson’s U Test = p < 0.001

Acrophase Vector: 1254

Acrophase Vector: 0240

% Activity counts during light
Figure 2.9. A. Representative actograms during daytime and nighttime scheduled feeding. B. Phase angles of activity onsets/offsets to sunrise/sunset, respectively during daytime and nighttime scheduled feeding for entire experiment with the last 5 days replotted in insert (boxed area indicates replotted days) to illustrating stable entrainment occurring by end of the reversed (night) feeding experiment. C. Acrophases of activity on the last two days of nighttime feeding compared to daytime feeding. D. Total activity counts and E. Percentage of activity occurring during the light period during the last two days of daytime and nighttime feeding schedules. * = $p < 0.05$.
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CHAPTER 3

Seasonal and daily variation of melatonin and cortisol in the brown bear (Ursus arctos): living at the limits?

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ABSTRACT

Many temperate zone animals anticipate annual changes in seasons by altering their physiology. This is mediated in large part by biological clocks and hormonal signals that code prevailing daylengths, regulate energy balance and influence metabolism. The objectives of this study were to determine if the daily patterns of two important hormones, pineal melatonin and adrenal cortisol, varied with daylength in captive brown bears (*Ursus arctos*). Both melatonin and cortisol varied with time of day and season, but concentrations of melatonin were exceedingly low (between 1-4 pg/ml during darkness, n=7). There was no effect of season on mean melatonin concentrations (non-anesthetized; \( P = 0.27 \)), but winter concentrations were over 7.5 times greater than summer (4.57 vs 0.60 pg/ml, respectively) in anesthetized bears (\( P < 0.001 \)). In contrast to melatonin, cortisol concentrations varied seasonally in non-anesthetized bears (\( P < 0.05 \)), but winter did not differ from summer in anesthetized bears. Notably, cortisol was undetectable in adult, non-anesthetized bears in mid-summer. The duration of melatonin elevation above the mean was correlated (\( P < 0.01 \)) with daylength; however, cortisol duration was not (\( P = 0.20 \)). Functional assessment of the pineal revealed significant reduction of melatonin by light, but no response to beta-adrenergic stimulation. Cortisol was suppressed with a synthetic glucocorticoid. The relatively weak seasonal modulation of melatonin and cortisol may help explain the behavioral flexibility of the brown bear, likely enabling it to cope with both extreme variations in food availability and expanding human influence.
INTRODUCTION

Photoperiodic time measurement is an important trait that has evolved to enable species to reliably and repeatedly perform such seasonal functions as migration, reproduction, and hibernation (10). Brown bears represent a highly adaptable species that inhabit various temperate climes, experience widely changing food availability, and are exposed to an increasing human influence (35, 42, 50, 63, 69, 71, 74, 75, 92). We have previously shown that captive brown bears use light and food to synchronize (entrain) daily locomotor rhythms in a seasonally dependent manner (98). However, the underlying mechanisms used to entrain these rhythms and facilitate the behavioral flexibility remain unknown.

To be functional, seasonal rhythms in physiology depend heavily on the integration of environmental cues with a neuroendocrine system that can be organized in a temporal fashion (6, 17). The pineal hormone melatonin is secreted into the bloodstream at night and thereby provides a daily endocrine signal of the light:dark cycle (4, 5). Importantly, the duration of nightly melatonin secretion varies with daylength to create a seasonally specific endocrine signal (5, 36, 37, 78). The daily and annual changes in melatonin secretion are mediated by a series of neural pathways beginning in the retina where light:dark information is transferred by the retinohypothalamic tract to the suprachiasmatic nucleus of the hypothalamus (SCN) (80). The SCN in turn projects via a multisynaptic pathway to the pineal gland to stimulate melatonin secretion (80). Once produced, melatonin binds to g-protein coupled receptors (MT1, MT2) found in a variety of hypothalamic regions (89). In this way, melatonin is thought to facilitate adaptations to the annual photoperiodic cycle (46, 89). It is therefore widely accepted that
melatonin serves a vital role in temperate zone species to time the onset and cessation of many biological events (102).

Similar to melatonin, the adrenal hormone cortisol (or corticosterone) also exhibits a daily rhythm requiring the SCN (14, 51, 68, 79). The influence of light on cortisol is also indirect, involving both the hypothalamus and pituitary (59). Cortisol plays an important role in stimulating gluconeogenesis and lipolysis along with causing the mobilization of amino acids to regulate energy balance (38). The amplitude of the daily cortisol rhythm varies seasonally in many species, including those that exhibit annual body mass and hibernation cycles (12, 40, 45, 76, 87). Higher concentrations of cortisol during hibernation are thought to be important to increase lipolysis necessary to maintain a basal metabolic rate, a critical function for hibernating bears (76). The effects of cortisol on energy homeostasis are therefore important mediators of seasonal adaptations (57).

Considering the profound annual changes in body weight (73), cycles of hibernation (32) and reproduction (21, 30), together with a wide geographic distribution in northern latitudes (66), we hypothesized that the daily rhythms of cortisol and melatonin in brown bears would vary in response to changes in daylength and, thereby, be important triggers of seasonal metabolism. We tested these ideas in both anesthetized and non-anesthetized bears during daily and yearly cycles.

**METHODS**

*Animals*

Male (n = 3) and female grizzly bears (n = 4) housed at the Washington State University (WSU)
Bear Education, Conservation and Research Center (WSU Bear Center, 46° 43' 53" N / 117° 10' 43" W) were used. Bears were maintained according to the *Bear Care and Colony Health Standard Operating Procedures* with all procedures approved by the Washington State University Institutional Animal Care and Use Committee. Bears were housed in pairs in dens (3m x 3m x 2.5m) with continuous access to an adjacent outdoor run (3m x 5m x 5m). During the active season, bears were released daily for 6 to 12 hrs into an adjacent 0.56 ha outdoor enclosure. During the active season (March-November), the bears were exposed to natural photoperiod and temperature fluctuations. Feedings occurred twice daily at 0700 ± 1 hr and 1600 ± 1 hr (standard time). Bears were fed at, or slightly above, maintenance levels from April to early August. Then, because of the bears’ increased appetite (hyperphagia) between August and mid-October, feeding amounts were increased to well above maintenance to provide adequate energy for fat accumulation necessary for hibernation. Bears were also allowed to forage for grasses and clover in the irrigated 0.56-ha exercise yard to supplement their commercial food diet (84). The commercial foods included dry chow (25.3% protein, 16.2% fat, 51.7% carbohydrate, and 2.0% crude fiber; Hill’s Pet Nutrition, Topeka, KS), apples, and small amounts of meat and pastries. In the fall (early to mid-October) and based on the bears’ appetite, food amounts were gradually reduced until completely withdrawn in late October. Hibernation began when all food was withdrawn (October 24 ± 7d). Water was available *ad libitum* at all times. Hibernation ended based on subjective evaluation of general increases in activity at which time feeding was restored (March 1st ± 16 d).

**Endocrine Measurements**

Blood samples were obtained from 4 trained female (non-anesthetized) bears between 2008
and 2011 during the Spring (March; 12 hr light:12 hr dark (L:D)), Summer (June; 16:8 L:D), Late Summer (August; 14:10 L:D) and Fall (October; 11:13 L:D). Bears previously trained in a positive reward paradigm offered their rear limb voluntarily for blood sampling from the dorsal metatarsal or lateral saphenous vein in exchange for a food reward. Blood samples could not be collected from this cohort of bears during hibernation because food could no longer be used as a reward given their anorexic state.

To collect blood samples during hibernation (2008-09 hibernation season: late Dec-early Jan; 8:16 L:D), we sampled three anesthetized adult male bears via jugular venipuncture. Two of these male bears were also anesthetized and used to collect blood samples during the active season (June; 16:8 L:D) for direct comparison with hibernation samples. Bears were anesthetized with 1.5 mg/kg of tiletamine HCL and zolazepam HCl (Telazol, Pfizer Animal Health, New York, NY) and 0.08 mg/kg of medetomidine HCl (Dormosedan, Pfizer Animal Health, New York, NY). Following sampling, 3-5 ml atipamezole HCL (Antisedan, Pfizer Animal Health, New York, NY) was administered I.V. as a reversal agent. Apexa artificial tears eye lubricant (Bausch and Lomb, Madison, NJ) was placed in the bears’ eyes.

Twenty-four hour endocrine profiles were compiled from samples collected over a 7 ± 5 day period. Blood samples were collected into heparinized tubes, centrifuged (1750 x g) for 20 min. and the plasma stored at -80°C until assayed.

**Radioimmunoassay**

Plasma was assayed for melatonin and cortisol by commercially available radioimmunoassay kits (ALPCO Diagnostics, Salem, NH; MP Biomedicals, Solon, OH, respectively). The assay
detection limits were 0.3-0.84 pg/ml for melatonin and 1.7 ng/ml for cortisol. For melatonin, 1 ml of sample was thawed and either subjected to enzymatic pretreatment or C$_{18}$ reverse-phase column extraction. No differences were observed between the recovery of added melatonin using the pretreated and extracted methods (data not shown). Duplicate 400µl samples were then incubated with G280 anti-melanin antibody (96) and $^{125}$I-melatonin tracer for approximately 20 h at 4°C. Next, 100 µl of solid phase anti-donkey secondary antibody was added and the tubes were incubated for 15 min. One ml of double distilled and deionized water was then added and the tubes were centrifuged at 2000 x g. The supernatant was decanted and the precipitates were retained for gamma counting (Apex 10/880 plus gamma counter, MP Biomedical, Solon, OH). Extraction efficiency was 99% (determined by spiking a bear sample with $^{125}$I melatonin and counting pre- and post-eluting). Chi-square tests of parallelism for serially diluted samples indicated no difference between expected and measured values. Low, medium, and high internal controls were used to confirm consistent results between assay runs. Intraassay variation was 10.6% and interassay variation was 15.6%.

For cortisol, 25 µl of non-extracted plasma was assayed in duplicate in a competitive binding assay with $^{125}$I-cortisol tracer in rabbit anti-cortisol antibody-coated polypropylene tubes. Reagents were incubated for 45 min in water bath at 37 ± 1° C. Liquids were decanted and tubes drained before counting in a gamma counter. Cortisol serial dilution curves confirmed parallelism using the Chi-square test for independence ($P > 0.05$). Intra- and interassay variation was 10.0% and 17.7%, respectively.
Pineal gland

Examination of brown bear and sheep pineal glands were initially performed on necropsy specimens. For a more in depth examination of the pineal *in situ*, we also performed a series of MRI studies at the WSU Veterinary Teaching Hospital. These studies used a Phillips 1.0T MRI on post-mortem bear and sheep tissue *in situ*. For comparison, the same brains were then analyzed following harvest and fixation in 10% formalin. The size of the pineal gland relative to brain size was determined for both bear and sheep by measuring the width of the pineal gland and brain. Both brain and pineal measurements were taken at the same coronal plane.

Neuroendocrine function

Pineal gland responsiveness was determined by: 1) application of bright light to test for melatonin suppression, and 2) administration of the beta-adrenergic agonist isoproterenol to test for melatonin stimulation. For melatonin suppression, animals were anesthetized in the dark phase of the light:dark cycle (i.e., when plasma melatonin is elevated). Just before light application, a baseline blood sample was obtained under dim red light illumination (<5 lux). Then, approximately 1500 lux of light was applied at eye level for 30 min with a blood sample taken at the end of the light application. Eyelids were blinked manually every 10 min. Pupillary response was recorded via camcorder to confirm effectiveness of light placement. For melatonin stimulation, isoproterenol (ISO; 0.005 mg/bear) was administered intravenously during the middle of the light phase to four non-anesthetized bears housed under natural photoperiod in the active season. Blood samples were taken immediately before administration and 3 h following drug treatment and stored until analyzed for melatonin. This
was repeated during hibernation using two anesthetized bears at the end of their light phase. Given the preliminary findings of unresponsiveness to ISO as well as previous findings in rodents concerning timing of ISO treatment (34, 52, 94) the timing of ISO injection was delayed to occur at the end of the light phase. To further increase potential effects of ISO while limiting stress on the cardiovascular system, we administered ISO twice, approximately 1 hr after initial injection at twice the concentration (0.01 mg/bear per injection) of the active season dose. Blood samples were collected immediately before ISO administration (baseline) and then 5 min, 50 min, 90 min and 120 min post initial ISO dose.

The responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis was evaluated in both active (n=5) and hibernation (n=2) seasons. Only cortisol suppression was determined using the negative feedback of exogenous glucocorticoids on cortisol secretion (38). To this end, betamethasone valerate (BETA; Celestone Soluspan, Schering-Plough, Kenilworth, NJ), a synthetic glucocorticoid, was administered (0.1mg/kg) orally in a 50% honey water mixture during the active season or injected intramuscularly (0.05mg/kg) during hibernation. Blood samples were collected 24 and 48 hr post-BETA administration and blood samples collected for cortisol determination as described above.

**Statistical analysis**

Data were analyzed using Graphpad Prism5 (La Jolla, CA) and SAS v9.0 statistical software (Cary, N.C.). Because samples were taken at slightly different times of the day across seasons the values were binned into one of six periods (0100-0300, 0400-0600, 0700-1000, 1100-1500, 1600-1900, and 2000-2400) to create a complete 24 h profile. Assay values that fell
beyond the linear portion of the assay standard curve were replaced with the assay detection limit value. The duration of elevated hormone concentrations was defined as the time between the upward and downward crossing of the daily mean value for each individual (25). Non-anesthetized and anesthetized samples were analyzed separately. One- and two-way ANOVAs or t-tests were used to estimate daily and seasonal variation in hormone profiles, variation in duration of hormone secretion, and effectiveness of stimulation or suppression tests. The duration of elevated hormone concentrations was also correlated with ambient daylength for each season. Effects were considered statistically significant at p < 0.05.

RESULTS

**Melatonin-Non-anesthetized bears:** Peripheral melatonin concentrations exhibited significant daily variation in all seasons (2-way ANOVA, main effect of time of day, F = 12.55, df = 5, P < 0.0001; Figure 3.1) with higher concentrations occurring, as expected, during the scotophase of the light:dark cycle (Figure 3.2; 1.57 ± 0.17 and 0.51 ± 0.06, for dark and light, respectively; 1-way ANOVA, F = 47.07, df 1, P < 0.001). No main effect of season was observed (Spring, Summer, Late Summer, and Fall (P = 0.33)) (Figure 3.3). However, an interaction between season and time of day was observed (Figure 3.1; F = 2.53, df = 15, P < 0.01). Primarily, this interaction was the result of the significantly higher (P < 0.01) concentrations of melatonin during 0400-0600 time period in the Fall (2.88 ± 0.46 pg/ml) compared to Spring, Summer, and Late Summer (0.64 ± 0.04) (Figure 3.1). Additionally, Summer values at 2000-2400 (2.17 ± 0.23 pg/ml) were higher (P < 0.01) than both Spring and Late Summer (avg. 0.80 ± 0.42 pg/ml).

Duration of elevated melatonin did not differ between Spring, Summer, Late Summer, and Fall
seasons (Figure 3.4; 1-way ANOVA, F = 2.09, df = 3, P = 0.15) but duration was inversely correlated with daylength (P < 0.01, Pearson r = -0.99) (Figure 3.5).

**Melatonin-anesthetized bears:** There was no effect of time of day on peripheral melatonin concentrations (1-way ANOVA, P = 0.84), but there was a large seasonal difference between Summer and Winter (Figure 3.3; F = 22.82, df = 1, P < 0.001; 4.19 ± 0.59 vs 0.55 ± 0.05 pg/ml). Melatonin amplitude remained largely stable regardless of whether samples were taken in the dark or light phase (Figure 3.2). Summer anesthetized melatonin levels were just above assay detection limit (assay detection limit = 0.5 pg/ml, summer avg. = 0.55 pg/ml) while Winter concentrations were tonically elevated (Figure 3.1). Additionally, duration of melatonin concentrations above the mean was much longer in Winter than Summer (Figure 3.4; unpaired t-test, P < 0.01; 15.5 ± 0.66 vs 1.75 ± 1.75 hr for Winter and Summer, respectively).

**Cortisol-non-anesthetized bears:** Peripheral plasma cortisol concentrations varied with time of day and season (Figure 3.6; 2-way ANOVA, main effect of time of day, F = 10.22, df = 5, P < 0.0001 and main effect of season, F = 7.28, df = 3, P < 0.0001). Like melatonin, cortisol was higher during the scotophase (Figure 3.2; 1-way ANOVA, F = 29.87, df = 1, P < 0.0001; 34.76 ± 3.68 and 11.91 ± 1.74 ng/ml for dark and light periods, respectively). There was no interaction between season and time of day for cortisol concentrations (P = 0.13). However, the duration of cortisol elevation varied between seasons (Figure 3.4; 1-way ANOVA; F = 7.19, df = 3, P < 0.01). Specifically, Summer had a shorter duration than both Spring and Fall (Bonferroni post-test, P < 0.05; 2.59 ± 1.09 for Summer vs avg. Spring and Fall 10.37 ± 1.63 hr). Although
duration of cortisol elevation varied between seasons, this seasonal variation only tended \((P = 0.07, \text{ Pearson } r = -0.93, r^2 = 0.86)\) to correlate with daylength (Figure 3.5).

**Cortisol-anesthetized bears:** In samples taken from anesthetized bears in the Summer and Winter seasons, there was no effect of either season or time of day on cortisol concentrations (Figure 3.3, 3.6; 2-way ANOVA, \(F = 1.54, \text{ df} = 6, P = 0.22\)). The lack of time of day effect was further reflected in a lack of difference in cortisol concentrations between nighttime and daytime and duration of cortisol elevation between Summer and Winter (Figure 3.2, 3.4). In contrast to melatonin, a clear effect of anesthesia on cortisol was evident (i.e. Summer non-anesthetized versus Summer anesthetized values; Figure 3.3).

**Pineal gland anatomy:** The bear pineal gland was extremely small compared to the sheep. Measurement of brain and pineal size revealed that the ratio of brain width to pineal gland width of the brown bear greatly exceeded that of the sheep (Figure 3.7) 57.05 versus 9.39, respectively. Thus, the relative size of the brown bear pineal gland is approximately one-sixth that of the sheep.

**Melatonin stimulation and suppression tests:** Regardless of season or dosage, ISO failed to cause an increase in peripheral melatonin concentrations; values pre- and post-ISO treatment remained undetectable (< 1.0 pg/ml) during the light period (Figure 3.8). This occurred despite elevated heart rate during the active season where heart rates remained elevated at 3 h post-ISO administration (pre-ISO avg. heart rate = 56.5 ± 4.3 bpm vs post-ISO avg. heart rate = 137.0
± 18.4 bpm; Figure 3.8A). During the hibernation season, heart rate increased only transiently after each of the two ISO treatments but did not remain elevated (Figure 3.8B). The transient increase in heart rate did not significantly differ from baseline heart rate (unpaired t-tests, $P > 0.05$). In contrast to the stimulation tests, bright light caused a significant suppression of melatonin by 30 min after application (pre-light application mean = 4.33 ± 2.3 pg/ml and post-light = 3.75 ± 2.2 pg/ml; Figure 3.9, paired one-tailed t-test, $t = 2.59$, df = 3, $P < 0.05$).

**Cortisol suppression test:** Responsiveness of the HPA axis was examined in both the active and hibernation seasons. Betamethasone treatment, regardless of season and administration route, decreased cortisol levels to below baseline levels for up to 48 h post-administration (Figure 3.9, 2-way ANOVA, main effect of treatment $F = 12.96$, df = 1, $P < 0.01$, main effect of season $F = 0.58$, df = 1, $P = 0.46$).

**DISCUSSION**

Brown bears have evolved a highly seasonal physiology to coincide with annual environmental changes. The success of these adaptations relies on internalizing a predictable environmental cue (36, 77). The daily light:dark cycle and seasonal changes in daylength represent highly reliable cues of seasonal phase used to entrain (synchronize) an endogenous circannual clock (36, 43, 67, 103). We reasoned that brown bears would use endocrine signals such as melatonin and cortisol to entrain daily, seasonal, and metabolic rhythmicity (36, 38, 76, 102).
Both melatonin and cortisol concentrations varied in a predictable fashion during the day; however, the seasonal variability was less clear-cut. Although widely reported for other species (23, 24, 26, 36, 97, 102), there were no differences in average daily melatonin concentrations between March and October (active season). Given the strong modulation of melatonin by light, it would be predicted that there would be changes in concentrations of melatonin depending on daylength. Indeed, we found that the duration of melatonin elevation was strongly (inversely) related to daylength. We interpret these results to indicate that the pineal gland in brown bears is responsive to changes in daylength despite an exceedingly low amplitude melatonin rhythm in this species. Interestingly, in anesthetized bears, winter (hibernation) melatonin concentrations were approximately 7.5 times greater than Summer suggesting that a strong seasonal change in basal melatonin is also experienced. Because melatonin has somnogenic properties (58, 62, 102), the elevated concentrations during hibernation may provide a mechanism to decrease activity and increase the amount of sleep required for hibernation. Although melatonin appeared to vary during the day in hibernation, this did not reach statistical significance. This absence of a strong rhythm in hibernation would be consistent with arrhythmic melatonin patterns observed in other hibernating species (23, 31). Additionally, lack of daily melatonin rhythms has also been reported in animals living at extreme latitudes where environmental light cycles disappear during the polar summer and winter (27, 28, 81). These conditions could also be experienced by brown bears in their hibernaculum, but this remains to be confirmed. Because the bears in our study were exposed to a light:dark cycle, one possibility for the slight variation observed is light masking could have suppressed melatonin. However, given that melatonin was tonically elevated in hibernation
irrespective of the phase of the light:dark cycle it seems unlikely that light masking was a significant factor.

Even when considering the elevated concentrations of melatonin during hibernation in the current study, the concentrations measured in the bear are still exceptionally low compared to other mammals (5, 93). Although assay differences could account for some of the differences, comparison to studies using a similar assay (3) suggest this not entirely the case. Potentially, the extremely small pineal gland of the bear relative to its brain size may reflect an anatomical limitation to melatonin production and/or an underlying defect in melatonin biosynthesis, similar to what is observed in several mouse strains (39). Future studies will be required to differentiate between these possibilities.

A functioning pineal gland is necessary for seasonal responses to photoperiod (36) and based on the suppression with light application it appears the bear is able to respond to photic signals but perhaps less robustly based on the absence of melatonin stimulation with isoproterenol. With the exception of summer, brown bear melatonin profiles fit the ‘Type A’ pattern in which melatonin levels rise several hours after dark and show only a transient peak as seen in Syrian hamsters (*Mesocricetus auratus*) (83). In the Fall, melatonin remained elevated for longer after lights on compared to other seasons. This variable pattern of melatonin secretion relative to lights on and off times in different photoperiods (seasons) has been previously observed in lemmings (*Dicrostonyx groenlandicus*) (40). Taken together, the bear pineal melatonin response to light appears functional but of far smaller amplitude than other seasonal species.
Another possibility is that seasonal changes in pineal gland size are occurring as has been reported for the hare (Lepus europaeus) (61). Early reports of antigenadotropic effects of melatonin based on the inverse relationship with gonadal size were supported by findings in the rabbit (61). This would however be inconsistent with what is observed in the brown bear where testicular growth (recrudescence) occurs during hibernation, when we observed elevated melatonin. However, it should be cautioned that due to the low number of bears in which we have analyzed pineal gland size and combined with sampling of brains only during hibernation, it is not possible to draw a firm conclusion regarding an effect of season on pineal gland size in this species. Furthermore, additional analysis of other ursids will be required to determine if the small pineal gland is a feature common to bears.

Unlike melatonin, mean daily concentrations of cortisol varied significantly with season. Seasonal changes in cortisol are well documented (9, 33, 40, 87) with greater concentrations of cortisol associated with periods of food scarcity (12, 45, 48, 76, 88, 95). The effects of cortisol are generally to mobilize energy reserves via lipolytic action thereby causing the release of free fatty acids and glycerol during hibernation (41, 44). This action primarily on fat reserves protects valuable lean (muscle) tissue (41). Based on these earlier findings we predicted that hibernating bears have higher levels of cortisol. Unfortunately, the effects of anesthetics on basal cortisol, which were revealed by comparing hibernation and summer samples in anesthetized bears, makes it difficult to draw a firm conclusion. Previous reports of cortisol concentrations for brown bears only evaluated them during the active season and under anesthesia (11, 15). In those studies, cortisol concentrations ranged from 54 to 177 ng/ml (11, 15); very similar to our active season anesthetized average of 85 ng/ml. Black bears have been
more extensively studied and similar to our findings, cortisol levels were lowest in the summer (38 and 18 ng/ml, (47, 76), respectively).

Intriguingly, we observed that in non-anesthetized bears, there appeared to be an age effect between adults (5 yrs old) and subadults (3 yrs old) although the small sample size prevented us from statistically comparing these groups. Although all age groups were combined and analyzed, when visualized alone, adult bears had undetectable cortisol during Summer while subadults had detectable concentrations. Moreover, when the same cohort of bears was resampled in Late Summer all had detectable concentrations of cortisol. Age and reproductive status are reported to affect cortisol concentrations (8, 20, 45, 53, 65, 86, 90) though some reports have found no effects of age (18, 48). The very low cortisol levels in adult non-anesthetized bears during the summer were in sharp contrast to those measured in anesthetized bears during the same period suggesting that the anesthetics used in these studies caused the increases. Indeed, sympathomimetics, including the one used in the current studies have been well documented to increase cortisol (2, 13, 19, 60). However, there is also some evidence that the benzodiazepine, Zolazepam, included in Telazol, may mitigate some of the HPA stimulation resulting from tiletamine hydrochloride (7, 49, 85). In addition, medetomidine or dexmedetomidine were also included in the anesthetic cocktail for these studies – both have been reported to reduce sympathetic activation (1, 16, 54). Collectively, it appears that the administration of Telazol for continued sedation elevated cortisol. However, it is unclear why melatonin was not affected in the same way. Apparently, the inclusion of an α-2 adrenergic agonists (medetomidine and dexmedetomidine) may have counteracted any melatonin stimulation as has been previously reported in rats (70). A final explanation for the
differential effects on cortisol relate to gender. The non-anesthetized samples were exclusively from female bears while anesthetized samples were obtained from males. Generally, males of several species are found to have higher basal levels of circulating cortisol than females (86).

Glucocorticoids are critical for a variety daily functions, including locomotion, appetite, and food-seeking behavior during the wake period of the sleep:wake cycle (64). In our non-anesthetized bears there was a strong daily variation in cortisol during the active season. However, this daily rhythm did not change with season (i.e. there was no interaction between season and time of day), suggesting that cortisol was not strongly influenced by changes in photoperiod. This conclusion is further supported by the lack of strong correlation between daylength and duration of cortisol concentrations. In the anesthetized samples, no statistically significant daily variation was present, although a distinct rise in the dark phase was evident, similar what was observed for melatonin. The lack of daily variation during the Winter may be a function of requiring a consistent, constant increased level of cortisol for lipolysis and energy metabolism, as previously suggested for bears (45, 47, 76).

PERSPECTIVES AND SIGNIFICANCE

The suppression of melatonin by light, combined with the strong inverse correlation between daily melatonin elevation and daylength suggests that the bear pineal, despite its small relative size, is responsive to seasonal changes in daylength. However, the melatonin:pineal system appears to be less sensitive to stimulation compared to other species. Combined with seasonal changes in cortisol elevation, yet tenuous correlation to daylength, there appears to be a severely attenuated neuroendocrine output in the brown bear.
Nevertheless, these weak associations could be beneficial to brown bears by allowing them the flexibility to adapt to changing food resources and diverse temporal niches (98). Such flexibility has been observed in a variety of other temperate zone species (29, 55, 56, 72, 82, 91, 98-101). Taken together, it appears that the brown bear efficiently translates photoperiodic information into seasonally-relevant endocrine profiles. However, the extremely low amplitudes of these seasonal rhythms likely reflects the bear’s extraordinary ability to operate ‘at the limits’ of the physiological range.
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Figure 3.1. Double-plotted seasonal and daily profiles of peripheral melatonin concentrations (± SEM) under ambient photoperiods for non-anesthetized and anesthetized bears (n = 4, and n = 2-3, respectively). Melatonin varied with time of day in a seasonally dependent manner (P < 0.05). Shaded areas represent the dark phase of the L:D cycle. Spring = vernal equinox, March 16-24\textsuperscript{th}; Summer = summer solstice, June 11-27\textsuperscript{th}; Late Summer = August 11-15\textsuperscript{th}; Fall = October 7-16\textsuperscript{th}, Hibernation = Dec 20\textsuperscript{th}-Jan 2\textsuperscript{nd}.
Figure 3.2. Mean (± SEM) nighttime and daytime concentrations for all seasons of (A) melatonin and (B) cortisol. *** = \( P < 0.001 \). Nighttime was defined as 20:00 to 06:00 standard time during active seasons and from 17:00 to 07:00 during hibernation.
Figure 3.3. Mean (± SEM) seasonal concentrations of (A) melatonin (pg/ml) and (B) cortisol (ng/ml). Non-anesthetized and anesthetized groups analyzed separately. Spring = vernal equinox, March 16-24\textsuperscript{th}; Summer = summer solstice, June 11-27\textsuperscript{th}; Late Summer = August 11-15\textsuperscript{th}; Fall = October 7-16\textsuperscript{th}; Hibernation = Dec 20\textsuperscript{th}-Jan 2\textsuperscript{nd}. * = $P < 0.05$, *** = $P < 0.001$. 

* = $P < 0.05$, *** = $P < 0.001$. 

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Figure 3.4. Mean (± SEM) seasonal duration of (A) melatonin and (B) cortisol. Duration defined as the length of time (hr) between the upward and downward crossing of the daily mean concentration. Non-anesthetized and anesthetized groups analyzed separately. Spring = vernal equinox, March 16-24th; Summer = summer solstice, June 11-27th; Late Summer = August 11-15th; Fall = October 7-16th, Hibernation = Dec 20th-Jan 2nd. n.s. = non-significant * = P < 0.05, ** = P < 0.01.
Figure 3.5. Mean (± SEM) correlations of (A) melatonin and (B) cortisol duration to daylength for active season (non-anesthetized bears). Duration defined as the length of time (hr) between the upward and downward crossing of the daily mean concentration. Spring = vernal equinox, March 16-24th; Summer = summer solstice, June 11-27th; Late Summer = August 11-15th; Fall = October 7-16th.
Figure 3.6. Double-plotted seasonal and daily profiles of peripheral cortisol concentrations (± SEM) for non-anesthetized and anesthetized bears (n = 4, and n = 2-3, respectively). Cortisol varied with time of day in a seasonally dependent manner (P < 0.05). Shaded areas represent the dark phase of the LD cycle. Spring = vernal equinox, March 16-24th; Summer = summer solstice, June 11-27th; Late Summer = August 11-15th; Fall = October 7-16th, Hibernation = Dec 20th-Jan 2nd.
Figure 3.7. Images of the sheep (A,C,E) and bear (B,D,F) brains illustrating the differences in pineal gland size. A,B. T2 weighted MRI images of sheep (A) and bear (B) brains *in situ*. C,D. Photographs of the same brains shown in A and B after removal from the skull. E,F. Higher magnification views of the sheep (E) and bear (F) pineal gland and adjacent structures. Rostral is to the left in all figures. cc – corpus callosum; Cb – cerebellum; cq – copora quadrigemina; th – thalamus. Scale bar = 1 cm (A-D), 0.5 cm (E,F).
Figure 3.8. Effects of melatonin stimulation experiments on heart rate (indicated by line; bpm ± SEM) and melatonin (indicated by bars; pg/ml) in (A) active season, non-anesthetized bears (n = 4; 0.005 mg ISO/bear) and (B) inactive (hibernation) season, anesthetized bears (n = 2; 0.01 mg ISO/bear). There were no effects on melatonin for either season; bars indicate assay detection limit. Arrow indicates time of 2nd isoproterenol injection; times are relative to time of 1st injection. * = P < 0.05, *** = P < 0.001
Figure 3.9. Results of neuroendocrine suppression experiments on (A) melatonin via light application in the inactive season (n = 4) and on (B) cortisol via synthetic glucocorticoid (BETA) treatment in both active and inactive season (n = 5). * = P < 0.05, ** = P < 0.01.
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The Circadian Rhythm of Salivary Cortisol in Growing Pigs: Effects of Age, Gender, and Stress.


CHAPTER 4

’Split parturition’ observed in a captive North American brown bear (*Ursus arctos*)

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ABSTRACT

Reproductive physiology in North American ursids is characterized by mating during the spring to early summer, delayed implantation, and birth during hibernation. During spring 2008, a captive adult female brown bear was mated with two adult males. Pregnancy was determined by elevated progesterone concentrations during the late fall prior to hibernation. Two male cubs were born on December 31, 2009, and a third female cub was born 17 days later on January 16. All were successfully raised, and all were confirmed to have identical paternity. When normalized to age, cub growth rates did not differ. To our knowledge, this is the first documented case of markedly different birth dates in a single litter of brown bear cubs.

Key Words: Ursid, Pregnancy, Hibernation, Brown bears

INTRODUCTION

Reproductive physiology in North American ursids, such as the brown bear (Ursus arctos), is characterized by mating-induced ovulation in late spring to early summer and delayed implantation (Craighead, 1969; Tsubota et al., 1987). In both brown bears and American black bears (Ursus americanus), females can mate multiple times with one or more males (Craighead, 1992; Schenk and Kovacs, 1995). Implantation of blastocysts occurs in late fall to early winter followed by a ±60 day gestation (Pasitschniak-Arts, 1993). All embryos are assumed to undergo implantation at the same time to create a single litter (Spady et al., 2007). Birth of 1 to 4 cubs per litter typically occurs during January or February (Craighead, 1969).
It is not uncommon to observe offspring of different sizes within a single litter, although the precise causes may differ. For example, superfetation, i.e., multiple estrous cycles resulting in multiple fertilizations, superfecundation, i.e., a single estrous cycle resulting in multiple fertilizations and split parturition, have been implicated in a wide range of species, including cattle, sheep, pigs, dogs, humans, rabbits, and mice (Fredholm and Winterø, 1996; Roellig et al., 2011; Sparrow, 1977). Additionally, intrauterine growth restriction, is well documented and results in smaller than expected offspring (Dawes, 1976; Wu et al., 2006). In bears, observations of females emerging from hibernation with cubs of different sizes have been documented (McNamee, 1997). Furthermore, embryos from different fertilizations have been shown to result in a single parturition in the American black bear (Boone et al., 1999). One occurrence of potential split parturition in the American black bear has also been reported, although those cubs were either aborted or not viable at parturition (Scanlon et al., 1998). We now report a case of confirmed split parturition in a captive brown bear that resulted in a single litter of viable triplets with differing birth dates.

**METHODS**

Bears were housed and maintained according to the *Bear Care and Colony Health Standard Operating Procedures* at the Washington State University Bear Research, Education, and Conservation Center with all procedures approved by the Washington State University (WSU) Institutional Animal Care and Use Committee (IACUC). Females were observed to mate with both resident males during the month of May, 2008. Hibernation occurred from late October when feeding ceased to March when feeding resumed. For blood sampling, female
bears were sedated with 1.5 mg/kg of tiletamine HCL and zolazepam HCl (Telazol, Pfizer Animal Health, New York, NY) and 0.08 mg/kg of detomidine HCl (Dormosedan, Pfizer) on October 24; blood was collected by venipuncture. Blood samples were centrifuged and the plasma stored at -80°C until assayed for progesterone by radioimmunoassay (MP Biomedicals, Solon, OH).

All pregnant females were housed individually in unheated dens (3m x 3m x 2.5m) with a contiguous outdoor run (3m x 5m x 5m) from October through March. Bears had free access to water and were given straw for bedding. Dens and outdoor runs were illuminated with natural lighting. Activity within the pens was monitored continuously and recorded using video cameras and infrared illuminators. Human entry into the den area was restricted to minimize disturbance.

Cub genders, weights, and paternity were determined on April 29, 2009, approximately three months after birth. Parentage was determined by genotyping hair collected from the triplets, the dam, and two possible sires using 20 microsatellite loci found in the *Ursid* genome (G1A, G10B, G10C, G1D, G10H, G10J, G10L, G10M, G10P, G10U, G10X, UarMu23, UarMu50, UarMu51, UarMu59, CXX20, CXX110, 145P07, A06, CPH9) (Wildlife Genetics International, Nelson, B.C.) (Fredholm and Wintero, 1995; Ostrander et al., 1993; Paetkau et al., 1998; Taberlet et al., 1997).

**RESULTS**

Plasma progesterone levels (2.63 ng/ml) on October 24 indicated that one female was pregnant (non-pregnant standard of < 1 ng/ml). The female was 5 yrs old and weighed 137 kg at the onset of hibernation. She gave birth on December 31, 2008 to two cubs. Normal
maternal behavior and cub noises were observed for the next 17 days. On January 16th, 2009, the female stood and moved just outside the edge of her nest. A prominent flow of amniotic fluid was observed and within minutes, a third cub was born. The female immediately moved the small cub to the nest with the other two larger siblings, licked, and nursed. Based on video recordings, all cubs appeared to be approximately the same size and at the same level of immaturity at birth. Thus, the cubs born 17 days earlier were noticeably larger when the third cub was born. The difference in size between the cubs continued throughout hibernation. The two early born cubs were males and the later born cub was a female. When first weighed on April 29, the male cubs weighed 9.5 kg and 10.9 kg and the female 7.5 kg. These differences between the early and later born cubs (2.0 to 3.4 kg) are within the range expected (3.1 kg) solely due to their differences in age (Robbins et al., unpub.). Genotyping results revealed that all 3 cubs were sired by a single male (Table 4.1).

**DISCUSSION**

The present finding of split parturition in the brown bear could be attributed to at least two possible scenarios. First, implantation dates for the blastocysts may have differed by approximately 17 days for one of the three embryos fertilized. Given that brown bears are induced ovulators with relatively prolonged estrus that lasts from 1 to 18 days (reviewed by Spady 2007) with evidence of ‘split estruses’, it is possible that either superfetation or superfecundation occurred in this case (Craighead, 1995). Indeed, the female in the present report was observed to mate multiple times with two resident males. However, superfetation or superfecundation does not necessitate differential implantation dates of blastocysts. In fact,
in animals that undergo both embryonic diapause (delayed implantation) and superfetation or superfecundation, such as the European badger (*Meles meles*) and American mink (*Neovison vison*), blastocysts implant and produce term offspring in a single parturition (Renfree and Shaw, 2000; Roellig et al., 2011). In these cases, embryonic diapause allows congruent development and coalesced parturition of the offspring. By contrast, in the domestic pig and rat, where superfetation or superfecundation was ruled out, differential implantation of blastocysts was implicated as the cause of split parturition (Sparrow, 1977; Vandeplassche, 1969). To our knowledge, embryonic diapause has never been observed to manifest as natural split parturition in the brown bear.

A second possibility is that all embryos implanted at the same time but that differential growth rates *in utero* led to a delay in parturition of one embryo. Following a fertile mating, Ursid oocytes divide into a roughly 250-300 cell blastocyst before becoming dormant until implantation (Craighead, 1969; Pasitschniak-Arts, 1993; Wimsatt, 1963). Thus, in the present case, all embryos were likely at the same blastocyst stage at the onset of hibernation. The anecdotal reports of lactating females emerging from hibernation with cubs of different sizes in the wild (McNamee, 1997) may be explained by blastocysts that implanted simultaneously but exhibited differential growth rates during development leading to different size cubs. In the giant panda, (*Ailuropoda melanoleuca*), ultrasound was used to confirm that twin fetuses at different development stages were present during a gestation; however, that pregnancy only produced a single cub (Sutherland-Smith et al., 2004). In contrast, the cubs in the present case were born in two distinct parturition events and all were viable. Additional factors, such as intra-litter competition for a limited milk supply or exchanges of cubs between litters may be
other causes of markedly different cub sizes; however, none of these occurred in the current example as the female was isolated and the differences in cub sizes could be entirely accounted for by their different ages.

CONCLUSIONS

1. Brown bears are capable of producing temporally separated litters (up to 17 days based on this observation) within a single reproductive year.

2. Split parturition likely reflected either variable implantation dates of blastocysts or intrauterine growth restriction.

3. This is the first known confirmed observation of split parturition in the brown bear.

ACKNOWLEDGEMENTS

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Table 4.1. Genotypes (*Ursus arctos horribilis*) of the dam, her three offspring, and potential sires at 4 microsatellite loci (fragment lengths in base pairs) to confirm paternity of offspring.

<table>
<thead>
<tr>
<th>Microsatellite loci</th>
<th>G10B</th>
<th>G10J</th>
<th>G10P</th>
<th>G10U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam</td>
<td>144</td>
<td>160</td>
<td>186</td>
<td>186</td>
</tr>
<tr>
<td>Potential Sires</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 1</td>
<td>158*</td>
<td>160</td>
<td>186</td>
<td>196</td>
</tr>
<tr>
<td>Male 2</td>
<td>160</td>
<td>160</td>
<td>194</td>
<td>196</td>
</tr>
<tr>
<td>Offspring</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cub 1</td>
<td>158</td>
<td>160</td>
<td>186</td>
<td>196</td>
</tr>
<tr>
<td>Cub 2</td>
<td>160</td>
<td>160</td>
<td>186</td>
<td>186</td>
</tr>
<tr>
<td>Cub 3</td>
<td>158</td>
<td>144</td>
<td>186</td>
<td>196</td>
</tr>
</tbody>
</table>

* Loci bolded represent unique alleles to Male 1. The individual genotypes of all offspring confirm the parental cross of the known dam and Male 1.
REFERENCES


CHAPTER 5

GENERAL CONCLUSIONS

This work represents the first examination of daily and seasonal activity and endocrine patterns for their roles in shaping grizzly bear behavior and physiology. In Chapter 2, we showed that grizzly bears exhibit a desynchronized behavioral phenotype during the hyperphagic and hibernating seasons as they uncouple their activity from photic cues. Experimental manipulations of photoperiod confirmed that light was not a powerful enough entrainer to alter activity patterns during the active seasons. However, when we examined the role of feeding by altering the feeding regimen of the bears, clear entrainment was present, leading us to conclude that food is a proximal cue driving behaviors during the active season. During hibernation, when food is not present, light pulses and photoperiod changes elicited entrainment and phase shifts of activity. Because the bears’ activity free-ran in constant conditions regardless of season, we further concluded that the entrainment we had observed was mediated by an endogenous circadian oscillator. In Chapter 3, we examined the seasonal and daily rhythms of melatonin and cortisol, known circadian and seasonal phase markers. While both hormones exhibited daily and seasonal rhythmicity, the correlations to season appeared to be tenuous. Interestingly, absolute concentrations of melatonin were quite low and the pineal gland did not appear to respond to adrenergic stimulation. This potentially dampened neuroendocrine axis corroborates the seasonally flexible entrainment we showed in Chapter 2. These results highlight a remarkably flexible species that likely evolved to cope with variations in light and food. A final example of the bears’ apparent flexibility is our observation of split parturition. In Chapter 4, we describe the birthing event in which a female had two
separate parturitions within the same hibernation period. Based on this observation, we hypothesize that the female bear is able to regulate the number, and timing, of egg implantation based upon metabolic and energy status. Fat content of the female prior to hibernation has been shown to affect cub birth date and growth [1]. While these factors have not been directly linked, a potential candidate is leptin, a hormone marker of adiposity.

Taken together, the results of this work reveal potential mechanisms for the brown bear to exhibit such flexibility in behavior and physiology in response to widely fluctuating food availability, temperatures, and photoperiods.

**Future Directions**

Our results indicate that both light and food are involved with shaping the bears’ behavior and physiology. For light effects on behavior, the hormones melatonin and cortisol were examined. However, investigations into regulators involved with feeding and metabolism, such as leptin, which interacts with hypothalamic nuclei that also receive input from the SCN, are necessary to further elucidate the interplay among photoperiod, energy demands, and metabolic status. Preliminary results from our lab indicate that leptin exhibits a seasonal rhythm with a peak just prior to hibernation followed by a decrease to nadir around mid-hibernation. These results are in accordance with previous research of black and brown bears [2, 3]. Increased sampling times and number of bears are needed to improve resolution of these results. However, the role of leptin has not been directly investigated regarding hibernation initiation.
Our work provides insight into circadian and seasonal rhythmicity of the brown bear, but we recognize the limitation of using locomotor activity as our measure of clock output because it can be masked or altered by other entrainers such as food. When the feeding schedule was inverted to occur during the scotophase bears exhibited a divergence of activity from the light:dark cycle. However, it remains unclear as to whether the clock entrained to this regimen or activity output was positively masked by food availability. A future experiment determining the presence of peripheral clocks that keep time with feeding rhythmicity rather than the light:dark cycle would be revealing. We would predict that peripheral oscillators located within the digestive system, such as the liver, stomach, etc. would exhibit rhythms coinciding with feeding while central clock outputs of melatonin would remain entrained to the light:dark cycle. This would also be the first step in demonstrating a food-entrainable oscillator independent of the SCN.

Finally, translating this work into the wild bear model is necessary to confirm that our results are not a unique feature of either captive populations or brown bears alone. Preliminary activity data from the Greater Yellowstone Ecosystem has begun to address this question [4]. Grizzly bears from this population exhibit temporal niche switching, presumably as a response to food availability, while black bears remain exclusively diurnal, entrained to the light:dark cycle. We would like to expand these analyses to additional wild populations from varying latitudes, landscapes, and human presence.
REFERENCES


