Pubertal hormones alter circadian timekeeping: Evidence from two rodent models,
Rattus norvegicus* and *Octodon degus

by

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In loving memory of three vibrant souls who worked hard to make those around them feel comfortable and welcome:

Dorothy (Dot) Hagenauer
A smart, gregarious "old cookie" who weathered the Depression and cherished giving her children and grandchildren the opportunities she never had.

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A great listener whose quiet, thoughtful consideration was such a staple in my life that it never occurred to me how special it was.

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"To be continued…"

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Preface

This dissertation does not represent the majority of the work that I have completed during graduate school, but instead the research to which I am most attached. I desired to tell a coherent story that focused on research that I personally designed, implemented, and analyzed. I also was interested in receiving intensive feedback on this research, as most of it is not published yet and I will be building upon it in the near future. To create a more holistic picture of my experience in the lab of Dr. Theresa Lee, I have alluded to some of my other projects in each of the chapters, and included figures from several publications in the introduction.

For your reference, I have included a comprehensive list of the publications represented by each of these chapters, including their respective co-authors, as well as a list of the other publications produced by my work in graduate school:

Publications Included in the Dissertation:

Introduction (Chapter 1) and Conclusion (Chapter 5): Hagenauer MH, Perryman JI, Lee TM, Carskadon MA. 2009. Adolescent Changes in the Homeostatic and Circadian Regulation of Sleep. Developmental Neuroscience 31(4): 276-284. (*Adapted*)

Chapter 2: Hagenauer MH, King AF, Possidente B, McGinnis MY, Lumia AR, Lee TM. *In preparation.* Changes in Circadian Rhythms during Puberty in *Rattus Norvegicus:* Developmental Time Course and Gonadal Dependency.

Chapter 3: Hagenauer MH, Ku JHY, Lee TM. *In preparation.* Circadian reorganization during puberty depends on gonadal hormones in the *Octodon Degus.*

Chapter 4: Hagenauer MH, Wang SS, Altshuler DB, Lee TM. *Preliminary Results.* Period1 rhythms in the suprachiasmatic nucleus are delayed during puberty in the degu (*Octodon degus).*

Publications Excluded from the Dissertation:

Lee TM, Hagenauer MH. *In preparation.* The Diurnal Phasing of Wheel-Running Activity Rhythms in the Degu (*Octodon degus)* Depends on Laboratory Housing Conditions.

Mahoney MM, Rossi BV, Hagenauer MH, Lee TM. *Submitted.* Characterization of the estrous cycle in Octodon degus. Biology of Reproduction.

Mahoney MM, Ramanathan C, Hagenauer MH, Thompson RC, Smale L, Lee TM. 2009. Daily rhythms and sex differences in vasoactive intestinal polypeptide, VIPR2 receptor, and arginine vasopressin mRNA in the suprachiasmatic nucleus of a diurnal rodent, *Arvicanthis niloticus.* European Journal of Neuroscience 30(8): 1537-43.

Koch JM, Hagenauer MH, Lee TM. 2009. Response of *Per1* to Light in the Suprachiasmatic Nucleus of the Diurnal Degu (*Octodon degus*). Chronobiology International 26(6): 1263-71.

*Vosko AM, *Hagenauer MH, Hummer DL, Lee TM. 2009. Period gene expression in the diurnal degu (*Octodon degus)* differs from the nocturnal laboratory rat (*Rattus norvegicus).* American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology 296: 353-361. *Equal contribution of authors*

Hagenauer MH, Lee TM. 2008. Circadian Organization of the Diurnal Caviomorph Rodent, *Octodon degus.* Biological Rhythm Research 39(3): 269-289.

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Abstract

There is overwhelming evidence that the circadian timekeeping system is sensitive to gonadal hormones during perinatal development and adulthood. However, there is a noticeable lack of research in animal models focusing on circadian rhythms during puberty, a developmental window of dramatic hormonal change. This dissertation addresses this research gap using experiments in both fast-developing and slow-developing rodent species (*Rattus norvegicus* and *Octodon degus*).

The results indicate that the circadian system continues to develop across the post-weaning and pubertal periods in both species in a manner that exhibits strong sex differences. In males, pubertal changes in activity rhythms were robust, involving a switch from bimodal to unimodal activity patterns as well as a 3-5 hr magnitude phase-advance of activity rhythms relative to the environmental light-dark cycle. Pre-pubertal gonadectomy diminished these changes in both species, indicating that pubertal hormones were involved in producing the changes. Overall, females showed smaller circadian phase changes than males during puberty. In the degu, these sex differences were extreme, with female degus almost completely lacking phase changes during puberty.

Results suggest that pubertal hormones could act on multiple components of the circadian system. For example, preliminary data from the degu suggest that a photosensitive rhythmic component of the central circadian oscillator (*Per1*) exhibits phase changes during puberty that parallel phase changes in behavioral rhythms. However, pubertal rats exhibited a reorganization of activity rhythms under constant conditions, independent of photic entrainment. Thus, both the photic entrainment pathway and downstream circadian elements may be altered during puberty.

Taken in tandem with growing evidence from multiple species, as well as sleep electrophysiological studies from our own lab, it appears that the processes governing daily sleep and activity rhythms continue to develop far into the pubertal period in many

mammals. This conclusion is discussed in the context of the developmental ecology of these rodent species. The ramifications of these results for the wide-spread use of young animals by the scientific community for patch-clamp experiments of the circadian system are also discussed. Finally, this evidence can inform the national debate regarding teenage sleep patterns and high school start times.

Chapter 1

Introduction

Overview of Adolescent Sleep Patterns in Humans

Sleep deprivation amongst adolescents is epidemic. Recent studies show that many American adolescents maintain schedules during the school year that result in insufficient and ill-timed sleep. A poll by the National Sleep Foundation found that over 45% of adolescents in the United States report that they obtain inadequate sleep, defined as 8 hours on school nights (National Sleep Foundation 2000). Similar trends have been observed in other modern societies, including Korea, Brazil, and Italy (Andrade and Menna Barreto 2002; Giannotti and Cortesi 2002; Yang, Kim, Patel, and Lee 2005). In Iceland over 70% of individuals between the ages of 16-21 reported frequent daytime sleepiness (Thorleifsdottir et al. 2002). Not surprisingly, the prevalence of the phenomenon may cause problems in identifying sleep disorders and appropriate treatments in the adolescent age group since symptom criteria were generated from clinical studies of adult patients with such instruments as the Epworth Sleepiness Scale (Johns 1991). Such findings also suggest a novel etiology for the stereotypical "teenage" traits of mood lability, impulsivity, and irritability.

At the root of this chronic sleep deprivation is the adolescent tendency to stay up late. Many studies indicate that teenagers maintain later bedtimes than younger adolescents, even when wake times are constrained by school or work (**Figure 1.1**; Crowley, Acebo and Carskadon 2007; Thorleifsdottir et al. 2002). The delayed timing of sleep has been attributed to many external influences, ranging from evening work schedules and increased academic responsibilities to late night television and social opportunities (Carskadon, Mancuso, and Rosekind 1989; Manber et al. 1995; Van Den Bulk 2004). Current evidence demonstrates, however, that social factors do not completely account

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for the adolescent shift towards a "night-owl" behavior, otherwise known as an evening chronotype.

The developmental timing of the adolescent transition into a more evening chronotype suggests physiological underpinnings. Girls begin to show a delay in the timing of sleep one year earlier than boys, paralleling their younger pubertal onset. Similarly, girls show maximum delay at the age of 19.5 years, and boys show maximum delay later at 20.9 years (Roenneberg et al. 2004). The magnitude of this delay also exhibits sex differences (**Figure 1.2**; Roenneberg et al. 2004). In other cultures, similar developmental timing is observed, although the peak delay may occur as early as 15-16 years of age (Crowley, Acebo and Carskadon 2007; Russo et al. 2007; Thorleifsdottir et al. 2002; Yang, Kim, Patel, and Lee 2005). Most important, a delay in the timing of sleep during the second decade of life has been observed in over 16 countries on 6 continents, in cultures ranging from pre-industrial to modern (as reviewed in Carskadon 2008). Although most studies have been cross-sectional, retrospective longitudinal measures also indicate that the timing of sleep is delayed during adolescent development (Roenneberg et al. 2004).

Adolescents continue to show a delayed circadian (or "internal clock") phase as indicated by daily endocrine rhythms even after several weeks of regulated schedules that allow for sufficient sleep. This delay is maintained under controlled laboratory conditions in which there is limited possibility for social influence (Carskadon, Acebo, and Jenni 2004; Carskadon et al. 1997). Moreover, both home-based and laboratory studies of adolescents show that delayed circadian phase correlates with secondary-sex development (Carskadon, Acebo, and Jenni 2004; Carskadon et al. 1997; Carskadon, Viera, and Acebo 1993; Sadeh et al. 2009). This correlation holds true for subjective ratings of chronotype and puberty even when grade level in school is held constant (Carskadon, Viera, and Acebo 1993). If we assume that teenagers attending the same grade in school are exposed to a similar social environment, this evidence suggests that a biological component drives adolescent changes in sleep patterns.

Physiological Determinants of the Timing of Sleep and Activity

Traditionally, the timing of sleep is thought to derive from three primary components: an endogenous circadian timing system, a homeostatic drive, and other external constraints (this is often referred to as masking). The homeostatic drive for sleep, or "sleep pressure," increases with the duration of waking and dissipates during sleep. In humans, the circadian timing system promotes wakefulness in the evening, and promotes sleep in the early morning (Achermann and Borbely 2003). These circadian rhythms are generated endogenously by a pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph et al. 1990). Under normal conditions, the endogenous circadian rhythm must be regularly entrained by external time cues (such as sunlight) to maintain a stable phase relationship with the outside world (Roenneberg, Daan, and Merrow 2003).

The Carskadon laboratory developed a model of delayed sleep phase during adolescence that incorporates developmental changes in homeostatic drive and circadian timing (Carskadon 2008). According to this model, human adolescents develop a resistance to sleep pressure that permits them to stay up later. At the same time, their circadian phase becomes relatively delayed, which provides them with a drive to stay awake later in the evening and to sleep later in the morning (Carskadon 2008). There is evidence from human adolescents supporting both components of this model. Sleep electrophysiology studies indicate that more mature adolescents are able to tolerate somewhat longer waking episodes than pre-pubertal adolescents (Jenni, Achermann, and Carskadon 2005; Taylor et al. 2005), though the similarity in dissipation rates indicates that the sleep recovery process is developmentally stable (Jenni, Van Reen, and Carskadon 2005). The circadian timekeeping system also appears to have a delayed phase in human adolescents, as measured indirectly by using daily rhythms in the hormone melatonin (Carskadon, Acebo, and Jenni 2004; Crowley et al. 2006). The circadian pacemaker closely times the nightly onset of pineal melatonin production, and this onset is relatively unaffected by activity levels or sleep deprivation (Dewy and Sack 1989). Thus, the phasing of plasma or saliva melatonin rhythms can be used as a proxy for the phasing of the circadian pacemaker.

These results are provocative, but their interpretation is limited by several factors. First, most of the data come from cross-sectional studies, due to the difficult and intensive nature of conducting longitudinal studies in humans. Therefore, there remains a

possibility for cohort effects. Also, there is a limited degree of experimental control that can be ethically exerted over adolescents. This is important, because any age-related differences in the behavior of the adolescent subjects regarding daily light exposure before they arrive in the laboratory (*e.g,*. regular evening exposure to bright florescent lights while studying or working a part-time job) could have long-lasting effects on circadian data collection because the phase of the endogenous circadian pacemaker is slowly entrained by regular exposure to environmental time cues. Finally, these studies necessarily relied on peripheral indicators of circadian pacemaker phasing in the brain.

The experiments presented in this dissertation were originally conceived to complement the human literature and to address some of its limitations by using animal models. Specifically, we focused on pubertal changes in the circadian timekeeping system because of its already well-known sensitivity to gonadal hormones. Presented elsewhere are animal studies on pubertal changes in sleep homeostasis that were conducted simultaneously in our laboratory (for more detail, see Perryman 2010).

Introduction to Circadian Rhythms

Evolutionarily, circadian timekeeping is very old. Some of the very first organisms on earth, the cyanobacteria, are thought to have developed circadian rhythms in order to protect their DNA from UV light during replication and to allow efficient photosynthesis. As life diversified and multiplied, there was a constant competition for existence. Species made ever more sophisticated use of their time-keeping capabilities to adapt to a specific temporal niche (or chronotype). The *timing* of hunting, breeding, and hatching became just as important as their location. Plants timed their blooms to match the presence of pollinators, and pollinators adapted activity so as to seek out particular flower-types (DeCoursey 2004).

As organisms became more complex, the body's internal clock also became a mechanism that could coordinate activity in tissues in disparate locations, and regulate such essential functions as cell division, hormone release, growth, metabolism, and reproduction (Kennaway 2005; Sahar and Sassone-Corsi 2009; Turek and Van Cauter 1994). Even on the cellular level, circadian rhythms now coordinate and organize a remarkable variety of rhythmic processes. For example, within one microarray study of

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the mouse liver, over 8% of the transcriptome was found to be rhythmically expressed. Similar percentages have been found in other mammalian tissues, ranging from connective tissue to the liver (Akhtar et al. 2002). Within the experiments of this dissertation, we will focus primarily on behavioral rhythms, but it should be noted that any characterization of circadian changes during puberty will remain incomplete until we determine their generalizability to the wide assortment of rhythms present in mammalian physiology.

Characteristics of Circadian Rhythms

In the laboratory, circadian rhythms are often modeled as sinusoidal oscillations (**Figure 1.3**). As mentioned earlier, these rhythms are endogenously-generated. Thus, under conditions in which there are no time cues from the outside world (also referred to as constant or "free-running" conditions), the circadian system will continue to generate daily rhythms. These rhythms will appear to "drift" a little each day, because the period (or day length, τ) of the rhythms only approximates 24 hours (ranging from 23–25 hrs, varying by species and previous light cycle). Under normal conditions, the endogenous rhythm must be entrained by external time cues (or "zeitgebers," such as light, food availability, or daily arousal) to maintain a periodicity that matches environmental rhythms (called a T-cycle, *e.g,.* the 24-hr light-dark cycle in a laboratory, Moore-Ede, Sulzman, and Fuller 1982; Roenneberg, Daan, and Merrow 2003)

After a rhythm is entrained, there is a stable phase relationship between phase markers for the rhythm (*e.g.,* rhythm onset, peak, offset, and trough) and the zeitgeber time cue. Light is the dominant environmental zeitgeber, and under laboratory conditions the phase of biological and physiological rhythms is traditionally characterized by the relationship of phase markers to the laboratory light-dark (LD) cycle. Therefore, instead of referencing clock time, discussions of circadian rhythms frequently reference zietgeber time (ZT), which is the hours of the day relative to zeitgeber exposure (*e.g,.* lights-on = ZT0). If the phase of the rhythm shifts so that phase markers are occurring at a relatively earlier ZT, this is known as a phase-advance, whereas a shift that causes phase-markers to occur at a later ZT is known as a phase-delay (Moore-Ede, Sulzman, and Fuller 1982). Phase can also be compared between individuals or groups, for example the sleep

rhythms of human adolescents are described as being delayed in phase relative to those of pre-pubertal children or adults (Roenneberg et al. 2004).

Under constant conditions, when there are no zietgebers, time is referenced relative to the subjects' own activity rhythm (circadian time, CT). For diurnal (day-active) animals, CT0 is frequently defined as the time of activity onset, whereas for nocturnal animals the time of activity onset is defined as CT12. Circadian hours are then defined in respect to the subject's own endogenous period ($\tau/24$; Johnson 1992).

Despite the traditional modeling of circadian rhythms as sinusoidal waves, most rhythms also contain components (or harmonics) with shorter periodicities referred to as ultradian rhythms. The most common ultradian rhythm is bimodal (a rhythm with two peaks, or 12-hr harmonic). This is because most organisms, whether diurnal or nocturnal, increase activity around the transition times of sunrise and sunset (or lights-on and lights-off in the laboratory; Aschoff 1966). Animals that exhibit activity predominantly at these transition times are referred to as having a crepuscular chronotype (DeCoursey 2004).

Entrainment Mechanisms

Circadian entrainment is thought to occur by two different mechanisms: discrete (or non-parametric) entrainment and continuous (or parametric) entrainment (Roenneberg, Daan, and Merrow 2003). Discrete entrainment corrects for the difference between the period (τ) of the circadian pacemaker and the 24-hr day-length of the external world by daily phase-resetting (Moore-Ede, Sulzman, and Fuller 1982; Pittendrigh and Daan 1976). To measure the circadian system's sensitivity to the discrete effects of light, researchers experimentally produce a photic behavioral phase response curve (PRC). A photic PRC illustrates the magnitude of circadian phase-shift in response to a brief light exposure (e.g., a 20 min pulse) that is presented at any particular circadian time (CT) under conditions of constant darkness (Johnson 1992). **Figure 1.4** illustrates the photic PRC for adult male and female degus, a diurnal rodent species that we used as one of the model organisms in this dissertation (Lee, unpublished data). Light exposure during the subjective morning (when the degu begins its daily active period) produces a phase advance of circadian rhythms, whereas light exposure during the subjective evening produces a phase delay (Kas and Edgar 2000).

This general phenomenon is central to photic entrainment and common across species: morning light produces phase advances and evening light, phase delays. For example, in order for the circadian pacemaker of an adult male degu (period = 23.3 h) to entrain to a typical 24-hr day, light needs to reset the clock mechanism daily by causing a phase delay of 0.7 h. Entrainment to a LD cycle occurs when circadian rhythms shift until light exposure encompasses the end of the degu's active period to produce a daily phase delay (Goel and Lee 1997). This mechanism is thought to account for most of the entrainment of adult mammals (Moore-Ede, Sulzman, and Fuller 1982; Pittendrigh and Daan 1976a).

Continuous (or parametric) entrainment occurs when light exposure modifies τ so that it approaches the 24-hr day-length (T) without daily resetting (Roenneberg, Daan, and Merrow 2003). Although continuous mechanisms of entrainment are frequently ignored in many circadian studies, they are found in most species and hypothesized to be especially important for the entrainment of diurnal mammals (Hut, van Oort, and Daan 1999). The circadian system's sensitivity to the continuous effects of light can be measured in several ways. Since these effects are long-lasting, the aftereffects of a light-dark (LD) cycle on τ can often be observed for days after transferring an animal into constant conditions. The continuous effects of light on τ can also be observed by measuring τ under constant conditions with different lighting intensities. Finally, during a protocol for determining the light-induced PRC, changes in τ are sometimes observed as well as a phase shift following the light pulse (Pittendrigh and Daan 1976b).

The Physiology of the Circadian System

Circadian rhythms in mammals are generated within individual cells in the SCN by a transcriptional-translational feedback loop involving a group of genes commonly referred to as "Clock Genes" (Hastings and Herzog 2004; **Figure 1.5**). The core feedback loop consists of a positive arm, which contains proteins (BMAL1 and CLOCK) that drive transcription, and a negative arm, which contains proteins (PER, CRY, REVERBα) that inhibit transcription. To initiate the cycle, a heterodimer of BMAL1 and CLOCK proteins drives the transcription of *Per, Cry,* and *Reverbα.* The protein REVERBα then feeds back to inhibit the transcription of *Bmal1.* The PER and CRY proteins (PER1, PER2,

PER3, CRY1, CRY2) form heterodimers that inhibit their own transcription and the transcription of *Reverbα* (Bae et al. 2001; Zheng et al. 2001; for review see Hastings and Herzog 2004).

These oscillations are cell-autonomous, with cell-specific periodicities. Thus, in order for a high-amplitude, coherent rhythm to emerge from the SCN, cellular oscillations need to be coupled together (Yamaguchi et al. 2003). To do this, molecular oscillations are translated into oscillations of membrane potential and firing rate. Coupling then occurs via electrical gap junctions between SCN cells and synaptic communication (especially involving the neurotransmitters GABA and vasoactive intestinal polypeptide or VIP; Aton and Herzog 2005; Kuhlman 2010).

Light can influence oscillations in the SCN via three known pathways: a direct pathway between the retina and ventrolateral SCN (vlSCN) called the retinohypothalamic tract and two indirect pathways via the intergeniculate leaflet and raphe nuclei (Morin and Allen 2006). The retinohypothalamic tract is the primary pathway mediating photic entrainment. Light exposure on the retina causes a rapid release of glutamate in the vlSCN. This glutamate binds to NMDA receptors, producing an intracellular cascade of second messenger signaling. These signaling pathways then induce the transcription of immediate early genes, such as *cFos* (Meijer and Schwartz 2003), as well as two components of the clock gene feedback loop, *Per1* and *Per2* (Miyake et al. 2000; Shigeyoshi et al. 1997).

 If this light exposure occurs during the evening and early morning hours, when *Per1* and *Per2* transcript levels are low, induction of *Per1* and *Per2* causes an overall phase-shift of the molecular feedback loop (Shigeyoshi et al. 1997; **Figure 1.6**) as well as rhythms in membrane potential (Kuhlman 2010). This phase-shift propogates to the endogenously-rhythmic dorsomedial (dm) SCN (Antle and Silver, 2005; Nakamura et al. 2005; Yan and Silver 2002) and eventually to circadian output pathways. Consequently, phase-shifts in the molecular feedback loop in the SCN are thought to underlie the photic phase-shift of behavioral rhythms (Akiyama et al. 1999; Albrecht et al. 2001; Shigeyoshi et al. 1997; Tischkau et al. 2003). Thus, it has become common to examine the phasing of *Per1* and *Per2* rhythms within the SCN as a manner of characterizing the entrainment of

the central circadian pacemaker (e.*g.* Abe, Honma, and Honma 2007; Yamanaka, Honma, and Honma 2008).

However, it should be noted that the entrainment of the circadian pacemaker is not the only determinant of the final phase of circadian output. Substantial evidence now indicates that adult diurnal and nocturnal species have a similar phasing of many aspects of SCN physiology (Hagenauer and Lee 2008; Smale, Lee, and Nunez 2003). For example, the phase of the transcriptional-translational feedback loop in the SCN is very similar in the nocturnal rat, diurnal degu (Vosko et al. 2008; **Figure 1.7A**), and diurnal grass rat (Caldelas et al. 2003). Rhythms in deoxyglucose metabolism (Schwartz et al. 1983), photic sensitivity (**Figure 1.7B,** Mahoney, Bult, and Smale 2001; Slotten, Krekling, and Pevet 2005)**,** and neuropeptide expression (*e.g.,* VIP and arginine vasopressin (AVP); Mahoney et al. 2009) are also similar in diurnal and nocturnal species.

Therefore, the downstream coupling (or phase relationship) between the circadian pacemaker and central or peripheral systems plays an important role in determining the phase of behavioral and endocrine rhythms (Smale, Nunez, and Schwartz 2008). Recently it has been shown that many areas of the brain outside of the SCN (*e.g,.* cortex, striatum, hippocampus, amygdala) as well as other tissues in the body (*e.g.,* liver, heart, adrenals, ovaries) contain daily rhythms in clock gene expression. These semi-autonomous or slave oscillators are thought to be entrained by output from the SCN as well as by non-photic zeigebers such as food (Guilding and Piggins 2007). Previous work indicates that the phase relationship between the central SCN oscillator and slave oscillators in other regions of the brain closely relates to the phase of behavioral activity rhythms (Abe et al. 2001; Masubuchi et al. 2000; Mrosovsky et al. 2001; Vosko et al. 2009; Wakamatsu et al. 2001; **Figure 1.8**), suggesting that these regions may be important for expressed chronotype.

Circadian Rhythms Regulate the Reproductive Axis

Circadian rhythms regulate many aspects of reproduction, including the timing of hormone release, ovulation, mating, and parturition (Kennaway 2005; Turek and Van Cauter 1994). The circadian system also mediates the effects of photoperiod (day length)

on seasonal reproduction (Turek and Van Cauter 1994). In turn, reproductive hormones feed back on the circadian system (Karatsoreos and Silver 2007).

In mammals, the reproductive system is controlled by hormones within the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus releases pulsatile gonadotropin-releasing hormone (GnRH). GnRH then drives the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the anterior pituitary. LH and FSH stimulate the gonads to prepare gametes (spermatazoa, oocytes) as well as a hormonal environment conducive to reproduction (Johnson and Everitt 2000). The primary steroid hormones produced by the testes are androgens (*e.g.,* testosterone**,** dihydrotestosterone (DHT)), and the primary steroid hormones produced by the ovaries are estrogens (*e.g.,* estradiol), and progestins (*e.g.,* progesterone; Johnson and Everitt 2000). However, both sexes produce all three hormone classes and their steroidal precursors, as well as other non-steroidal hormones such as the inhibins (Nottelmann et al. 1987). These hormones feed back on the hypothalamus and pituitary to regulate their own production (Johnson and Everitt 2000).

In adult females, hormone production varies over the course of the reproductive cycle. In humans, this cycle lasts for around 28 days and contains two major phases: follicular and luteal. During the early follicular stage, estrogen and progesterone levels are low and the uterine lining is shed in preparation for a new cycle (menstruation). As follicular development proceeds, estrogen levels increase. When estrogen levels reach peak production, they cause positive feedback on the GnRH system, driving a surge of LH and, consequently, ovulation. Following ovulation, the follicle converts into a secretory luteal body and begins to produce progesterone as well as estrogen. Progesterone levels remain high for most of the luteal phase (Johnson and Everitt 2000; McCarthy and Becker 2002).

In laboratory animals, the female reproductive cycle follows a different progression. With the exception of primates, menstruation does not occur, and therefore the cycles are referred to as estrous cycles instead of menstrual cycles. For some species (*e.g.,* the degu), the estrous cycle contains both a follicular and luteal phase (Mahoney et al. *submitted*). However, in most traditional laboratory rodents (rats and mice), estrous cycles are short (4-5 days) and do not contain a true luteal phase, except following

vaginocervical stimulation (pseudopregnancy). In these species, estrogen peaks on the day of proestrus around 12 hours prior to ovulation, and is followed several hours later by a peak in progesterone. Ovulation occurs that night, and is followed by a period of sexual receptivity (estrus), despite a concurrent drop in steroid hormone levels (McCarthy and Becker 2002).

In traditional laboratory rodents (rats, mice, hamsters), circadian regulation of the HPG axis has received particular attention because lesions of the SCN eliminate ovulation and produce a state of persistent estrus (Raisman and Brown-Grant 1977; Chappell 2005). Further investigation revealed that the SCN sends a signal daily to GnRH neurons. When estrogen levels are high, this signal helps drive the GnRH/ LH surge that leads to ovulation. The nature of the SCN signal is currently unknown, but two prime suspects are the neurotransmitters VIP and AVP (Chappell 2005). Therefore, arguably in these species the SCN is actually an integrated component of the HPG axis.

Circadian Rhythms are Sensitive to Gonadal Hormones

Gonadal hormones can influence the brain in several ways. The activational effects of hormones are direct and transient. If a hormone is functioning activationally, then at any time an experimenter should be able to remove the hormone from the system or block its receptor and the effect will disappear. Activational effects can occur due to hormones binding to their traditional receptors (McCarthy and Crews 2002), or due to hormonal modulation of the efficacy of neurotransmitter receptors (*e.g.,* progesterone metabolites can alter the function of GABA or NMDA receptors; Lambert et al. 2003; Rupprecht 2002). Organizational (long term/permanent) effects can also occur in response to steroid hormones. These effects are due to steroid hormones binding at nuclear receptors to produce long-term changes in the transcription of particular genes (McCarthy and Crews 2002). Organizational effects typically occur during a sensitive period of development, known as a critical period. Although some organizational changes cause direct functional effects, others may alter hormone receptor distribution and sensitivity. In this case, the organizational effects will not be observed unless hormones are present (Sisk and Zehr 2005).

Due to the circadian regulation of ovulation in traditional laboratory rodents, there has been substantial interest in the influence of gonadal hormones on the circadian system. This research shows that gonadal hormones can affect the circadian system both activationally and organizationally. One of the best-known activational effects is on activity rhythms during the female reproductive cycle in rodents. On the day of estrus, following elevated estrogen, female rodents exhibit increased activity and begin their activity earlier in the day. The next day, after hormone levels drop, the females phase-delay activity onset and decrease overall activity levels (**Figure 1.9A,** Axelson, Gerall, and Albers 1981). During the human menstrual cycle, circadian rhythms in cortisol and sleep onset also shift their phasing (Manber and Bootzin 1997; Parry et al. 1994; Parry et al. 2000). Indeed, in adult laboratory rodents a wide variety of gonadal hormones can affect the phase of circadian rhythms, including estrogens, progestins, androgens (**Figure 1.9B**), and non-traditional neuroactive steroids (*e.g.,* rats: Albers, Gerall, and Axelson 1981; Axelson, Gerall, and Albers 1981; hamsters: Davis, Darrow, and Menaker 1983; Morin, Fitzgerald, and Zucker 1977; de Tezanos Pinto and Golombek 1999; mice: Daan, Damassa, Pittendrigh, and Smith 1975; Iwahana et al. 2008; Karatsoreos et al. 2007; degus: Jechura, Walsh, and Lee 2000; Labyak and Lee 1995).

Gonadal hormones can influence other circadian parameters as well, including τ (Albers 1981; Daan et al. 1975; Davis, Darrow, and Menaker 1983; Karatsoreos et al. 2009; Morin, Fitzergald, and Zucker 1977; Zucker, Fitzgerald, and Morin 1980), rhythm amplitude (Labyak and Lee 1995), range of entrainment (Davis, Darrow, and Menaker 1983), zeitgeber sensitivity (de Tezanos Pinto and Golombek 1999; Jechura and Lee 2004; Jechura, Walsh, and Lee 2003) and oscillator coupling (Thomas and Armstrong 1989). Many of these parameters also exhibit sex differences. For example, both hamsters and degus show sex differences in their phase-response to light (Davis, Darrow, and Menaker 1983, *unpublished data* **Figure 1.4**). Although some of these sex differences arise activationally from the contrasting hormonal milieu of adult males and females, the sensitivity of circadian parameters to steroidal hormones also exhibits sex differences. These sex differences are determined in some species by the organizational effects of gonadal hormones during the perinatal period (rat: Albers 1981; hamster: Zucker, Fitzgerald and Morin 1980).

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SCN Physiology is Altered by Gonadal Hormones

Some of the effects of gonadal hormones on circadian rhythms are due to modulation of the circadian pacemaker in the SCN. Gonadal hormones can alter key aspects of SCN physiology, including those necessary for circadian rhythm generation, entrainment, and coupling (**Figure 1.10**). For example, both androgens and estrogens can activationally increase photic sensitivity within the entrainment pathway, as measured by the induction of immediate early gene expression (Abizaid, Mezei, and Horvath 2004; Karatsoreos et al. 2007). The phasing and amplitude of rhythmic components in the molecular feedback loop (*Per2, Cry2*) are sensitive to estrogen in females (Nakamura et al. 2001; Nakamura et al. 2005). Estrogen can also alter the phasing of neurotransmitter rhythms in the SCN (**Figure 1.11**, Cohen and Wise 1988; Krajnak et al. 1998; Mahoney et al. 2009) as well as increase electrical intercellular coupling (Shinohara et al. 2000; Shinohara et al. 2001) in a manner that is opposed by progesterone (Shinohara et al. 2001). Gonadal hormones alter SCN neuron excitability in both sexes. In females, estrogen increases the sensitivity of SCN neurons to the neurotransmitters serotonin (5-HT) and acetylcholine (ACh; Kow and Pfaff 1984). In males, estrogen alters the excitability of SCN neurons by depolarizing resting membrane potential and increasing spontaneous firing rate (Fatehi and Fatehi-Hassanabad 2008). Hormonal manipulations in males (castration, excessive testosterone treatment) also increase the number of astrocytes in the dmSCN (Satriotoma et al. 2004), although the functional relevance of these results is unknown.

Puberty and Adolescence

Although colloquially the terms "Puberty" and "Adolescence" are used interchangeably, scientifically they refer to separate concepts (Sisk and Zehr 2005). Traditionally, puberty is defined as the process leading to the attainment of sexual maturation (Spear 2000), beginning with the activation of the HPG axis, and ending with reproductive competency (Plant 1994; Sisk and Foster 2004). Reproductive competency is rarely used as a developmental marker in neuroscience research, however, because it is unreasonable to run mating and pregnancy tests in the middle of an experimental procedure. Therefore, most researchers measure the physiological correlates of sexual

maturation – the development of the hypothalamic-pituitary-gonadal (HPG) axis, increases in circulating gonadal hormones (*e.g.,* testosterone, estrogen), and the maturation of the testes, ovaries, uterus, and external genitalia (Richardson and Tate 2002).

Adolescence, on the other hand, is defined as the period of social, emotional and cognitive transition between childhood and adulthood (Sisk and Foster 2004; Sisk and Zehr 2005). Adolescence encompasses puberty, and typically human neuroscience studies will discuss the period of "adolescence" instead of "puberty," since human subjects remain embedded in their social environment. In animal studies, the term "adolescence" is traditionally used specifically to refer to research focusing on the neural and behavioral changes accompanying the transition from juvenile dependence into the relative independence of adulthood (Spear 2000). This transition includes both the hormone-dependent and hormone-independent remodeling of cortical and limbic circuitry necessary for adult decision-making, cognition and social interaction (Sisk and Zehr 2005). As this dissertation focuses primarily on interactions between pubertal hormones and the circadian system, a regulator of the HPG axis, we will only use the term "adolescent" when referring to human studies.

It should be noted that the progression of puberty in laboratory species is not necessarily analogous to that of humans. To begin with, human puberty is commonly preceded by eight or more years of gonadal "quiescence" following infancy. During this time, gonadotropin releasing hormone (GnRH) pulsatility is suppressed and gonadal steroidogenesis is nearly absent (Plant 1994). Puberty is initiated when the HPG axis is released from juvenile inhibition (Plant 1994). Some mammalian species, such as the rhesus macaque, show a similar developmental pattern (Plant 1994), but rodents typically do not (Ojeda and Urbanski 1994). Most rodent species show low levels of steroidogenesis and secondary sex development throughout the juvenile period that then accelerates near the time that reproductive competence develops (Ojeda and Urbanski 1994).

Another key difference between the progression of puberty in humans and other species is the role of seasonality. Season plays a crucial role for determining the timing

and rapidity of secondary sex development in many species. Photoperiod is typically the environmental signal that indicates season physiologically (Gorman and Lee 2004).

Pubertal Hormones Influence the Circadian System

There is growing evidence that puberty is not only a time for the onset of the activational effects of hormones, but also a critical period for organizational changes (Sisk and Zehr 2005). Although there has been little work done examining the influence of pubertal hormones on the circadian system, there is some evidence for both types of effects. One study from the degu observed phase changes during puberty that were eliminated by prepubertal gonadectomy. They also observed a permanent sexual differentiation of τ that occurred in response to estrogen exposure during a critical period in young adulthood (degu: Hummer et al. 2007). An earlier study in hamsters also hinted that sex differences in the range of entrainment of the circadian system occurred due to gonadal hormone exposure during puberty (Davis, Darrow, and Menaker 1983). Little work has examined pubertal hormone effects on the SCN, but there are indications of anatomical changes (growth in nuclear size and nucleoli size) around mid-puberty in rats (Anderson, 1981; Morishita et al. 1978; Morishita et al. 1974), as well as an increased number of cells expressing a neuropeptide that is important for photic entrainment (VIP) and oscillator coupling during late adolescence in humans (Swaab et al. 1994).

Do Animal Studies Demonstrate Delayed Circadian Rhythms During Puberty?

In response to the overwhelming evidence that the circadian system is sensitive to gonadal hormones, we hypothesized that the delayed sleep patterns observed in humans might represent the influence of pubertal hormones on circadian phase. In this case, a similar phenomenon might be observed in other mammalian species.

As a preliminary test of this hypothesis, we reviewed data from existing studies that examined daily rhythms of animals during pubertal development. To compare appropriately across species, we had to address several challenges. The first regarded the definition of puberty. During circadian behavioral studies, most invasive measurements are problematic. Thus, in the studies that we reviewed, sexual development was typically

examined using only external genitalia development and growth (although one study, Hummer et al. 2007, ran spermatogenesis assessment and blood hormone sampling in a parallel group of animals). In most cases, reproductive development was not monitored at all. For the purposes of this review we have inferred the timing of puberty by referencing previously published reproductive studies for each species (Nelson et al. 1990; Ojeda and Urbanski 1994; Plant 1994; Safranski et al. 1993; Windmill et al. 2007). We defined "puberty" as the age interval between the first indication of secondary-sex development and the achievement of reproductive competency.

However, comparisons to previously published reproductive studies are only a rough estimate of pubertal timing. Since photoperiod is typically the environmental signal that indicates season physiologically (Gorman and Lee 2004), within a laboratory situation, the lighting conditions (daily light duration and intensity) of a circadian experiment can alter pubertal progression (*e.g.*, Gunduz and Stetson, 1994). Other conditions of a circadian experiment (*e.g.,* stress caused by social isolation or invasive blood draw) can also alter the timing of puberty (Drickamer 1990).

Second, in fast-developing rodent species (*e.g,.* rats, mice) weaning and puberty occur in close proximity. This fact is important because these species are altricial (born blind, hairless, and helpless) and thus the timing of nursing, not light, is the primary influence on the phase of activity rhythms in young pups (Shimoda et al. 1985; Sugishita et al. 1991; Thiels, Alberts, and Cramer 1990). Female rats and mice nurse their pups primarily during the species' rest period, so young pups are active at the opposite phase of adults. At the age of weaning, when the pups begin to consume solid food and engage in independent activity, they temporarily develop more ultradian rhythms, and then transition into an overall phasing of activity rhythms that resembles that of adults (Levin and Stern 1975; Thiels, Alberts, and Cramer 1990; Weinert 2005). Thus, circadian changes observed during the time of puberty in these species may represent the influence of pubertal hormones or the gradual transition from maternal to photic entrainment mechanisms (for full review see Weinert 2005).

Preliminary Conclusions from a Cross-Species Comparison. We found evidence for delayed circadian phase during puberty in all studies that we reviewed, which

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included five mammalian species: *Macaca mulatta* (rhesus macaque), *Octodon degus* (degu), *Rattus norvegicus* (laboratory rat), *Mus musculus* (laboratory mouse), and *Psammomys obesus* (fat sand rat). Overall, this evidence indicated that the delayed timing of sleep during human adolescence is likely to represent a developmental change common across mammalian species **(Table 1.1).**

Several themes emerged while comparing different species. The first concerned the magnitude of the phase-delay: with the exception of fat sand rats maintained under short, winter-like photoperiods (Neuman et al. 2005), all other species and all experimental conditions provided evidence for a 1-4 hr change in circadian phase during puberty. The phenomenon was robust. The daily rhythms measured were diverse and included such behavioral rhythms as sleep and activity, and physiological rhythms, such as endocrine (corticosterone, melatonin) and metabolic (temperature, oxygen consumption) rhythms. This delay appeared regardless of the chronotype of the species: both diurnal (human, macaque, degu, sand rat) and nocturnal species (rat, mouse) showed a delayed phase of daily rhythms during puberty.

In several species delayed phase during puberty was accompanied by increased crepuscularity, or activity around the transition times of dawn and dusk. In well-rested human subjects, the Carskadon laboratory found that adolescents showed a decreased propensity to fall asleep in the evening hours (Taylor et al. 2005), as well as an increase in midday sleepiness as measured by the multiple-sleep latency test (Carskadon et al. 1980). These results indicated that well-rested adolescents were most awake during the morning and evening hours. Pubertal rats and mice also showed more crepuscular or ultradian rhythms than adults (Cambras and Diez-Noguera 1988; Castro and Andrade 2005; Diez-Noguera and Cambras 1990; Ibuka 1984; Joutsinemi et al. 1991; Kittrell and Satinoff 1986), but it is unclear whether this age-related consolidation of activity is related to pubertal hormones or the shift from maternal entrainment to full photic entrainment (Weinert 2005).

Finally, and most intriguing, is the issue of the relationship between the timing of delayed phase and pubertal development. In the human literature, both home-based and laboratory studies of adolescents show that delayed circadian phase correlates with secondary-sex development (Carskadon, Acebo, and Jenni 2004; Carskadon et al. 1997;

Carskadon, Viera, and Acebo 1993; Sadeh et al. 2009), even after controlling for social environment (Carskadon, Viera, and Acebo 1993). Similar correlations are present in the animal literature. For example, a study comparing the activity rhythms of rhesus macaques with normal or disturbed pubertal development reported a strong correlation between pubertal timing and circadian phase delay (Golub, Takeuchi, and Hoban-Higgins 2002). Another study in degus found that delayed phase during puberty was eliminated by pre-pubertal gonadectomy (Hummer et al. 2007).

The developmental timing and structure of pubertal phase change seemed to differ by species (**Table 1.1**). In humans, peak phase delay occurs between 15-21 years of age (Crowley, Acebo and Carskadon 2007; Roenneberg et al. 2004; Russo et al. 2007; Thorleifsdottir et al. 2002; Yang, Kim, Patel, and Lee 2005), whereas regular overt cyclicity is usually established in girls and spermatogenesis in boys before 16 years of age (Plant 1994). Thus, the human peak phase delay occurs either during or following the final stages of gonadal development, although before the completion of the development of other neural systems (Giedd 1999). In several other species (macaques, degus, fat sand rats), peak phase delay appeared to occur in the middle of secondary sex development. Thus peak delay occurred in females between the first overt indications of cyclicity and the establishment of regular ovulation and in males during the establishment of spermatogenesis (**Table 1.1**). For the fast-developing, altricial laboratory rodents (rats, mice), chronotype changes related to the transition from maternal to photic entrainment were well-documented (for review see Weinert 2005) but detailed measurements of circadian phase in independent pre-pubertal animals was lacking. One study showed a phase-delay between weaning and puberty (Alfoldi, Tobler, and Borbely 1990), but it was unclear if these changes were just due to recovery from surgery and a change in the LD cycle on the day of weaning. Another study commented that phase stabilized between the pubertal ages of P31-36, but did not discuss the nature of the phase changes before that point. The lack of quantification of phase in that study may have been due to the presence of strong ultradian rhythms following weaning (Kitrell and Satinoff 1985). Thus, we can really only infer that circadian phase in these rodents is relatively more delayed during puberty and advanced during adulthood (McGinnis et al. 2007; Weinert 1994; Weinert and Waterhouse 1999).

Adding to a Cross-Species Comparison: Examining the Gonadal Dependence of Circadian Changes During Puberty (A Preview of Chapters 2&3). This evidence provides support for the hypothesis that delayed circadian phase around the time of puberty is a common phenomenon across mammalian species. However, several weaknesses are present that make it difficult to concretely compare animal data to the human literature. First, many of the studies were not intentionally designed to observe this phenomenon, and therefore they only measured circadian phase at 1 or 2 pubertal timepoints (Alfoldi, Tobler, and Borbely 1990; Hummer et al. 2007; McGinnis et al. 2007; Neuman et al. 2005; Weinert et al. 1994; Weinert and Waterhouse 1999). This inconsistency makes it difficult to compare the time course or magnitude of the developmental change across species or to verify that rhythms that did not show a significant developmental change were not missed by the sampling window (*e.g.*, temperature rhythms in Weinert and Waterhouse 1999; rhythms in melatonin synthesis and metabolism in Neuman et al. 2005; or activity rhythms in gonadectomized degus in Hummer et al. 2007). Third, many of these studies were only performed on one sex and did not simultaneously monitor pubertal development (McGinnis et al. 2007; Neuman et al. 2005; Weinert et al. 1994; Weinert and Waterhouse 1999), which is problematic because developmental time course can vary by laboratory and experimental condition (Ojeda and Urbanski 1994). Finally, it is unclear whether any of the changes occurring during puberty in fast-developing altricial rodents (rats, mice) are specifically due to puberty instead of the transition from maternal to photic entrainment (McGinnis et al. 2007; Neuman et al. 2005; Weinert et al. 1994; Weinert and Waterhouse 1999).

To address these weaknesses, during the first two experiments of this dissertation (Chapters 2&3) we followed the within-subjects development of activity rhythms during puberty while simultaneously measuring secondary sex development in two species: the fast developing, altricial rat, and slow-developing, precocial degu. Both species had previously demonstrated changes in circadian phase during puberty (Hummer et al. 2007; McGinnis et al. 2007; Tate et al. 2002), but the developmental time course and sex differences had not been explored in detail. By comparing both species, we were able to not only develop animal models that were conducive to both time-intensive circadian

experimentation and rapid physiological measures, but also compare pubertal changes in two species with different natural histories and developmental trajectories (precocial vs. altricial). To determine which changes were specifically related to pubertal hormones, we simultaneously followed rhythm development in a group of rats and degus that were gonadectomized prior to puberty.

The Mechanism Underlying Pubertal Changes in the Circadian Timekeeping: Altered Photic Entrainment of the Circadian Pacemaker?

The second goal of this dissertation research was to take advantage of animal models to gain a better understanding of the mechanism driving pubertal changes in the phase of circadian rhythms. Growing evidence supports the conjecture that the endogenous circadian period and photic sensitivity of the circadian system are altered during puberty in humans and animals. Such changes could explain the development of delayed sleep phase observed during puberty in human adolescents. We propose:

An Elongated τ: An elongated τ would cause the circadian pacemaker and its rhythmic output to delay relative to the light cycle (Roenneberg, Daan, and Merrow 2003; **Figure 1.12A**). In support of this hypothesis, the Carskadon lab found that human adolescents have a τ of 24.27 hr. This period length is significantly longer than that found in adults using similar protocols (24.12 hr), even after taking into account the variability present in both samples (Carskadon, Acebo and Jenni 2004; Carskadon et al. 1999). Of course, longitudinal data would provide stronger support for this hypothesis, and such research, though difficult in humans due to the long pubertal phase, is currently underway in the Carskadon laboratory (Hagenauer et al. 2009). One recent rodent study, in which the shorter maturational course facilitates such research, found that τ in pubertal male rats was longer than in adults (McGinnis et al. 2007). However, a careful developmental analysis of the degu did not find changes in τ during puberty at the age of the delay in entrained phase (Hummer et al. 2007).

Enhanced Light-Elongation of τ: This discrepancy between the results of human, rat, and degu studies might be explained by the presence or absence of light exposure during the free-running protocols. The pubertal rodents were exposed to constant

darkness (***DD,*** Hummer et al. 2007) or very dim red light (0.1 lux ***RR,*** McGinnis et al. 2007) while their τ was measured. On the other hand, humans were exposed to a light (LD; 20 lux vs. 0 lux) cycle with a day-length (T) that was outside of the range of entrainment for the circadian system (T=28 hrs). This procedure allowed the melatonin rhythm to free-run so that τ could be measured (Carskadon et al. 1999). Previous experiments have shown that light exposure can lengthen free-running period in four species that have delayed phase during puberty (degu: Lee and Labyak 1997; macaque: Martinez 1972; rat: Summer, Ferraro, McCormack 1984; human: Tokura and Aschoff 1978). Therefore, τ may not be longer during puberty, but the circadian pacemaker may be more sensitive to the continuous effects of light. In support of this hypothesis, one study found that the aftereffects of photoperiod on τ are more prolonged in pubertal than adult rodents (Hummer et al. 2007).

Increased Sensitivity to the Phase Delaying Effects of Light: An increase in the circadian pacemaker's relative sensitivity to the phase-delaying effects of light could also produce a pacemaker delay relative to the light cycle **(**Roenneberg, Daan, and Merrow 2003; **Figure 1.12B**). To provide a preliminary test for this hypothesis, the Carskadon laboratory examined the suppression of melatonin secretion by 1-h light-pulses in early pubertal and late pubertal human adolescents. Melatonin is secreted by the pineal gland at night and its suppression by light is mediated by the same photic pathways in the SCN as the discrete resetting of circadian rhythms. They found that late adolescents were significantly less sensitive to dim light exposure (15 lux) in the morning (03:00-04:00) than early adolescents, suggesting that a change in the phase-dependent photic sensitivity of the circadian system may have occurred during puberty (Carskadon, Acebo, and Arnedt 2002). Furthermore, additional data were recently acquired in adolescent humans (ages 15-17 years) who were asked to maintain a schedule that permitted a 2-hr later bedtime on weekends. Under these conditions, the adolescents showed a significant acute phase delay (0.6-0.75 hrs) of melatonin rhythms that persisted even when rise time was delayed by only one hour (sleep recovered with a mid-day nap) or when morning bright light was administered to anchor circadian phase (Crowley 2009). These studies indicate that pubertal humans may have a blunted ability to phase advance in response to morning light and an exaggerated ability to phase delay in response to evening light.

In the animal literature, one previous study tangentially characterized the phase-response curve of pubertal female mice (49 days of age) as well as late pubertal (63 days of age) and adult mice (Weinert and Kompauerova 1998). If we separate out the data specific to the 49-day old (P49) pubertal mice and compare it to the late pubertal and adult mice (**Figure 1.13**) an interesting trend emerges. First, the phase-delay portion of the phase-response curve has greater amplitude in the P49 pubertal mice. The phase advance and daytime portions of the phase response curve also appear to have a different shape, but it is difficult to draw conclusions due to the few sample points taken during these times. An earlier study by the same research group showed that pubertal mice (42 days of age) adjust to a phase delay of the light-dark cycle (similar to jet lag from a plane flight headed West) much faster than adult mice (Weinert et al. 1994). These results indicate that pubertal animals may be relatively more sensitive to the circadian phase-delaying properties of light.

Elucidating Mechanism: Determining Whether the Central Circadian Oscillator is Phase-Delayed During Puberty and the Role of Photic Entrainment in Developmental Changes (A Preview of Chapters 2&4). To test the hypothesis that circadian phase changes during puberty are due to differential photic entrainment of the circadian pacemaker we performed three initial analyses. During the first analysis, we analyzed τ under constant conditions in both sexes in the rat to determine whether it was indeed longer in pubertal animals. Simultaneously, we examined developmental changes in the phasing and organization of activity rhythms under constant conditions. We reasoned that if the developmental changes in the phasing and organization of activity rhythms occurred under both entrained and constant conditions, then these changes are not due to a developmental effects on the photic entrainment of rhythms. Finally, in the degu we examined whether a photo-responsive component of the molecular feedback loop in the SCN (*Per1*) was delayed during puberty in a manner that resembled the delay in behavioral rhythms.

Species	Human (Homo sapiens)	Rhesus Monkey (Macaca mulatta)	Degu (Octodon degus)	Laboratory Rat (Rattus norvegicus)	Laboratory Mouse (Mus musculus)	Fat Sand Rat (Psammomys obesus)
Magnitude of Delay	1-3 hrs	2 hrs	3-5 hrs	1-4 hrs	1 hr?	0-3 hrs under a long photoperiod*, 10-14 hrs under a short photoperiod
Sex Difference	Males > Females	Only females examined	No Sex Difference?	Only males examined	Only females examined	Sex unspecified
Rhythms Delayed	Sleep, Melatonin	Activity	Activity	Activity, Sleep?	Activity, Corticosterone, Temperature?*	Oxygen Consumption, Temperature
# of Experiments	>20	1	3	2	2	1
Age of Peak Delay	15-21 yrs	39 months	80-100 days	30-40 days?	Unknown, but delay evident at 35-45 days	Unknown, but delay evident at 35-42 days
Age of Establishing Overt Cyclicity in Females	Menarche: 12-13 yrs; Regular ovulation: 13-16 yrs (Plant 1994)	Menarche: 30-33 m; First ovulation: 42-45 months (Golub, Takeuchi, and Hoban-Higgins 2002)	Cycles in vaginal opening: 35-150 days (Hummer et al. 2007)	First ovulation: 35-45 days (Ojeda and Urbanski 1994)	First ovulation: 27-40 days; Regular ovulation: 30-80 days (Nelson et al. 1990; Safranski, Lamberson, and Keisler 1993)	Unknown
Age of Establishing Spermatogenesis	12-16 yrs (Plant 1994)	N/A	60-120 days (Hummer et al. 2007)	45-65 days (Ojeda and Urbanski 1994)	N/A	28-56 days (Windmill et al. 2007)
Gonadal Dependent	Maybe	Unknown	Maybe	Maybe	Unknown	Unknown
Circadian Citations	Reviewed in Crowley, Acebo and Carskadon 2007, Carskadon 2008	(Golub, Takeuchi, and Hoban-Higgins 2002)	(Hummer et al. 2007, Tate, Richardson, and Carskadon 2002)	(Alfoldi, Tobler, and Borbely 1990; McGinnis et al. 2007)	(Weinert et al. 1994; Weinert and Waterhouse 1999)	(Neuman et al. 2005)

* = not statistically significant

Table 1. **A delay in circadian phase has been observed around the time of puberty in six mammalian species. (Adapted from Hagenauer et al. 2009)**
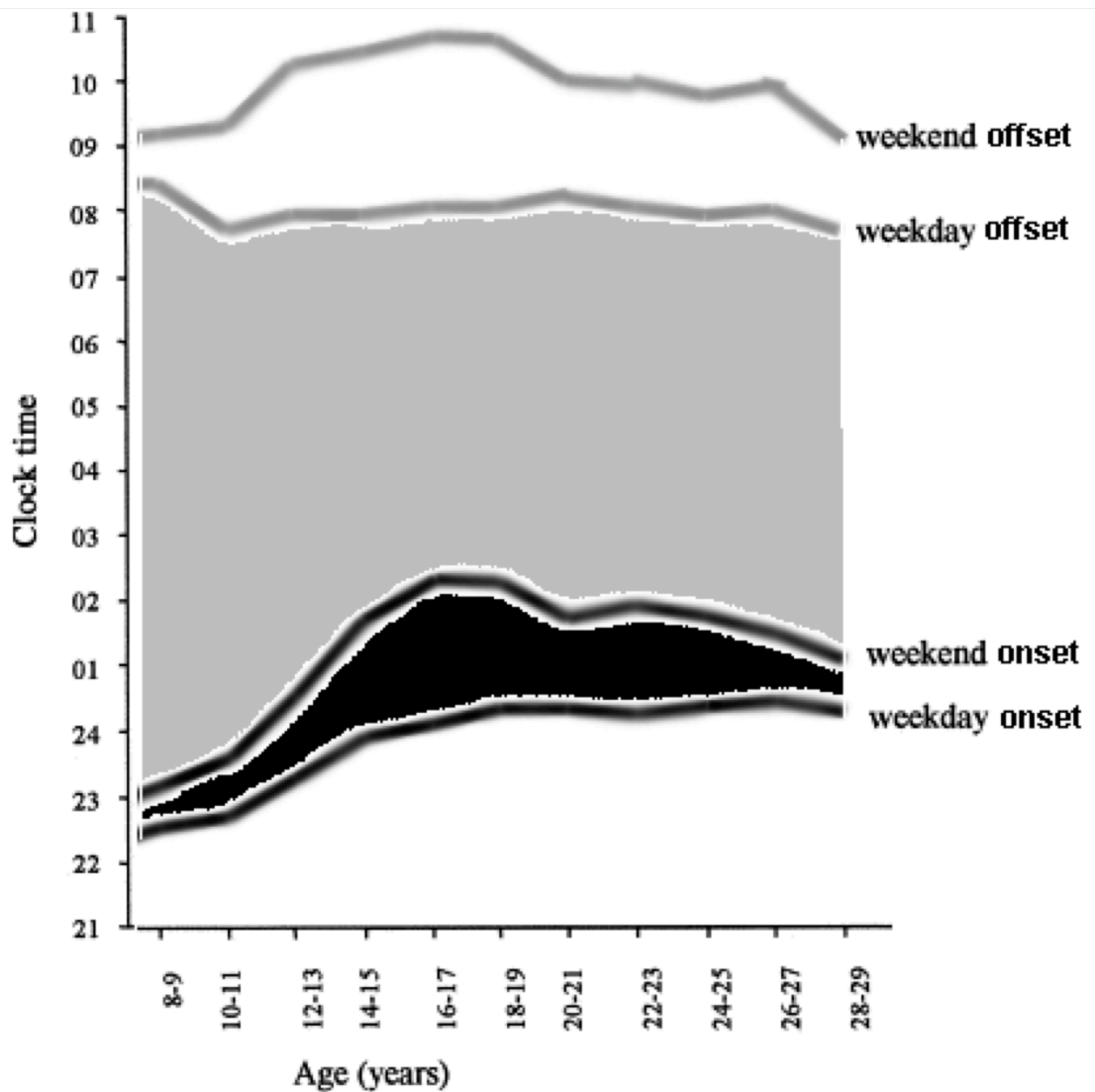
Figure 1.1. The timing of adolescent sleep is delayed at ages 16-19 years. Bedtimes are plotted in black and rise times are plotted in grey. Note that bedtimes continue to delay on weekdays despite regulated wake times. This leads to an overall shorter sleep duration during adolescence. (This figure was adapted from Thorleifsdottir et al. 2002, a longitudinal survey of 950 Icelandic children and adults.)
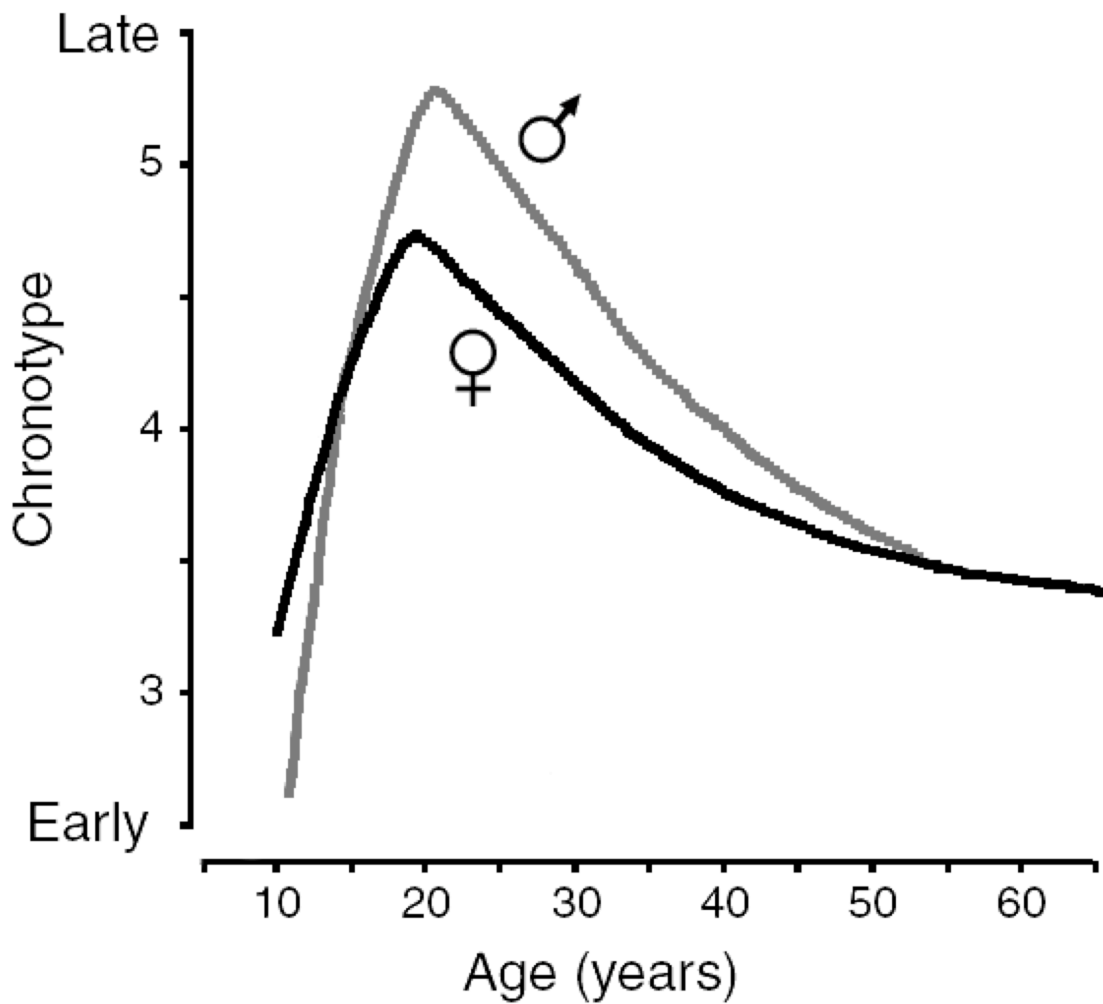
Figure 1.2. Chronotype changes with age. Individuals showed a transition into a more evening chronotype during adolescence (ages 10-20 yrs), as determined by self-reported mid-sleep phase. This trend then reversed for the rest of adult life. Males were more evening-type than females from the age of late puberty through menopause. (This figure was adapted from Roenneberg et al. 2004, a cross-sectional survey of 25,000 individuals in Germany and Switzerland).
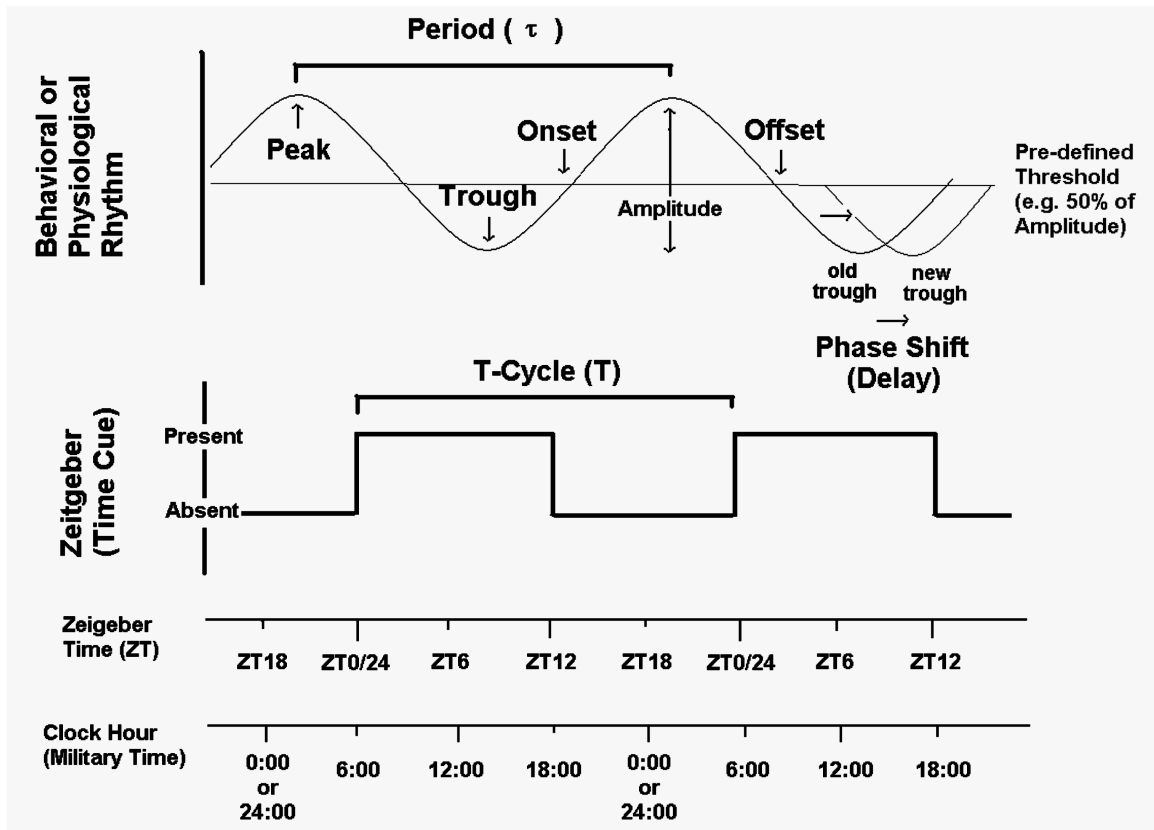
Figure 1.3. An illustration of commonly-used circadian terminology. Depicted on top is a simple sinusoidal model of a subject's behavioral or physiological rhythm (e.g. locomotor activity), and depicted below is the environmental cycle to which the subject was exposed (e.g. a light-dark cycle).
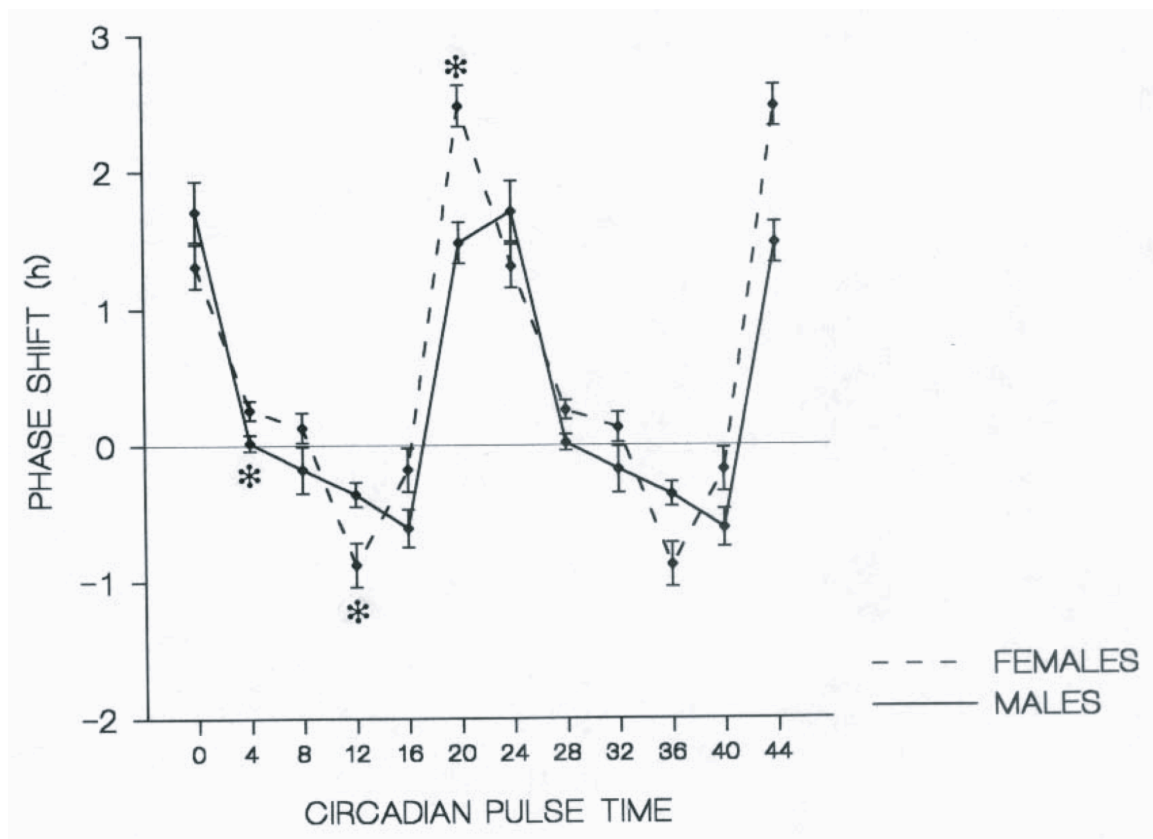
Figure 1.4. A PRC representing the circadian phase response of male and female degus to a light pulse. Degus (n=8 per sex) were housed in constant darkness (DD) for a day preceding the pulse. Pulse times were defined in reference to the former light cycle (previously, lights were on during hrs 0-12, Aschoff II methodology). The magnitude of circadian phase shift in response to a 30-min light pulse was determined by comparing activity rhythms following the light-pulse to those under control conditions (DD). Phase shift magnitude was measured in hours, with positive values indicating phase-advance, and negative values indicating phase delay. Degus were allowed several weeks to re-entrain to a light-cycle between measurements (Lee TM, unpublished data).
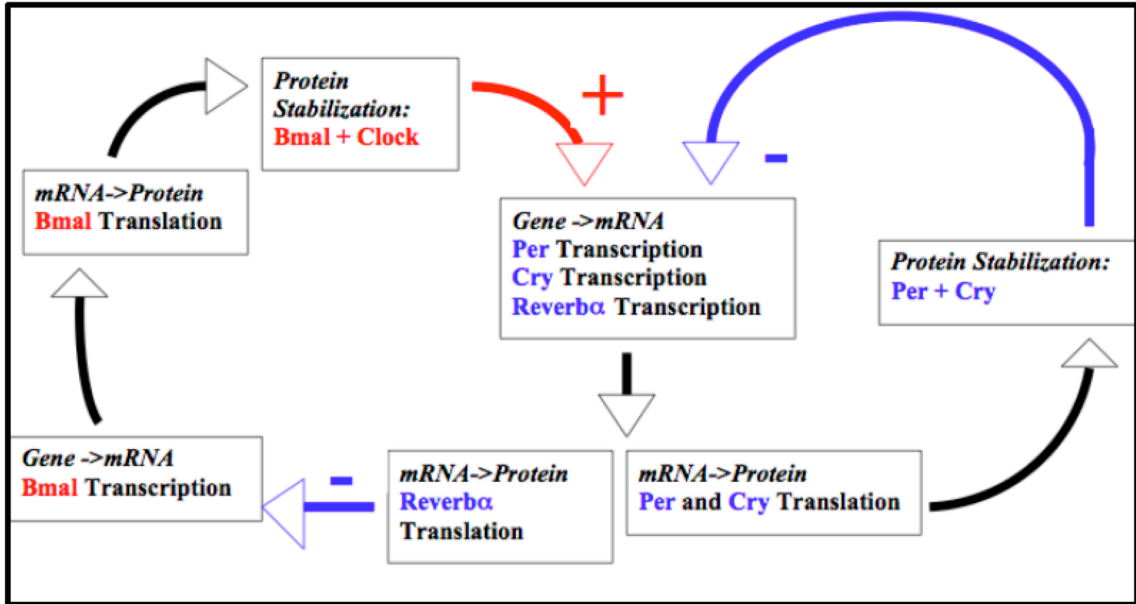
Figure 1.5. An illustration of the transcriptional-translational feedback loop that generates circadian rhythms in the SCN. Arrows with a plus sign (+) indicate that an element drives the transcription of another element, whereas arrows with a negative sign (-) indicate transcriptional inhibition.
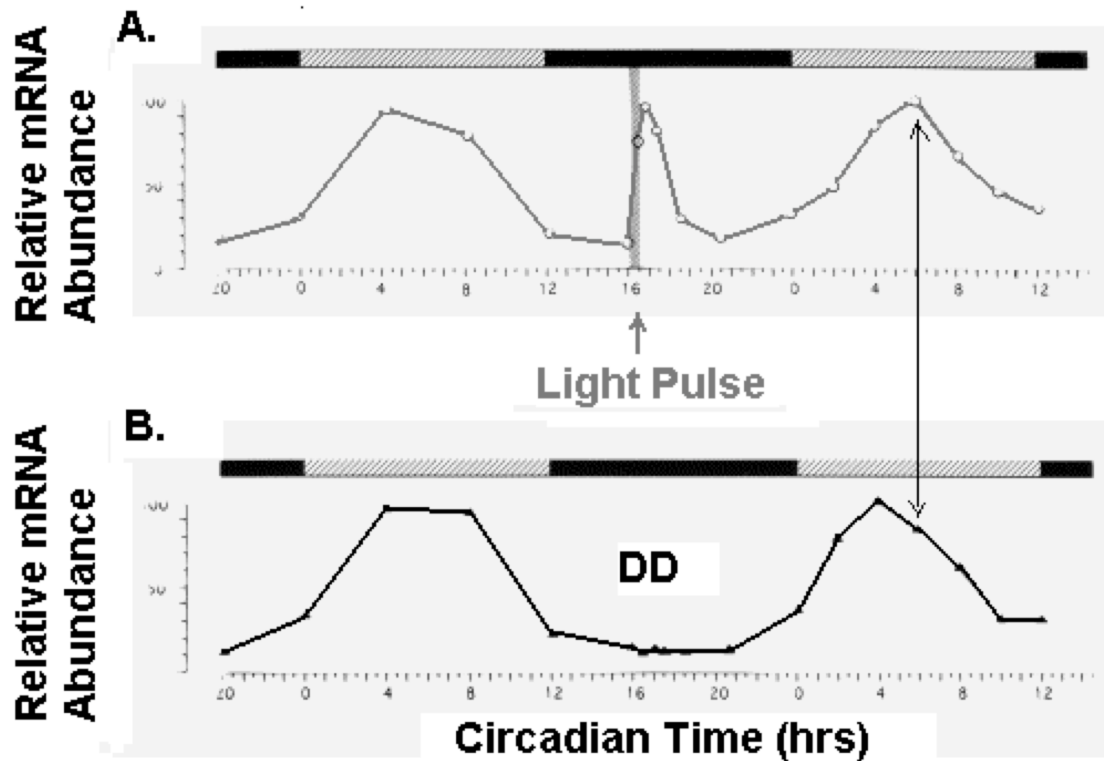
Figure 1.6. A light pulse increases *Per1* transcript and causes a phase shift in its daily rhythm in the SCN. *Per1* mRNA was quantified using in situ hybridization in mice. Each point represents the mean of two animals. Brains were collected for two days, with a 30-min light pulse presented in the subjective evening (CT16) on the first day (**A**). Control animals remained in constant darkness for both days (**B**). The bidirectional arrow indicates that the peak in *Per1* transcript is delayed on the second day following the light pulse relative to control conditions. (adapted from Shigeyoshi et al. 1997)

Figure 1.7. **A comparison of the daily rhythms and photic-sensitivity of period genes in the SCN of the diurnal degu and nocturnal rat.** **A-B.** Relative *Period* gene mRNA levels in the SCN of the degu (black) and rat (grey) at each time point were compared using in situ hybridization. The mean hybridization signal for each time point (*n=*3–5, *+/-*SE) is normalized as %peak, with the trough given the value of 0. ZT0 is double-plotted as ZT24. A black and white bar at the bottom illustrates the light-dark cycle. Both species demonstrate daily fluctuation (degu: *P<*0.001, rat: *P <*0.001) in *Per1* (*A*) and *Per2* (*B*) mRNA levels in the SCN that differed between the two species (*P<*0.05) and varied by time point (*P<*0.001). In the rat *Per2* dataset, ZT16 is absent, and ZT18 is not shown due to only having data from one animal. (Adapted from Vosko,* **Hagenauer**,* Hummer, and Lee 2009). **C**) Light exposure increases *Per1* transcript in the degu during the subjective night (ZT12 – ZT24). Grey bars illustrate *Per1* mRNA levels in the SCN of degus kept in constant darkness (mean +/-SE), whereas white bars show those of degus kept in constant darkness and then exposed to a 30-min light pulse 1.5-2 hrs prior to sacrifice. *Per1* was measured by in situ hybridization. The x-axis indicates the timepoint when the animal was sacrificed (extended from the previous LD cycle). (Adapted from Koch, **Hagenauer**, and Lee 2009). This rhythm in photic sensitivity resembles that already published for the rat (Miyake et al. 2000).
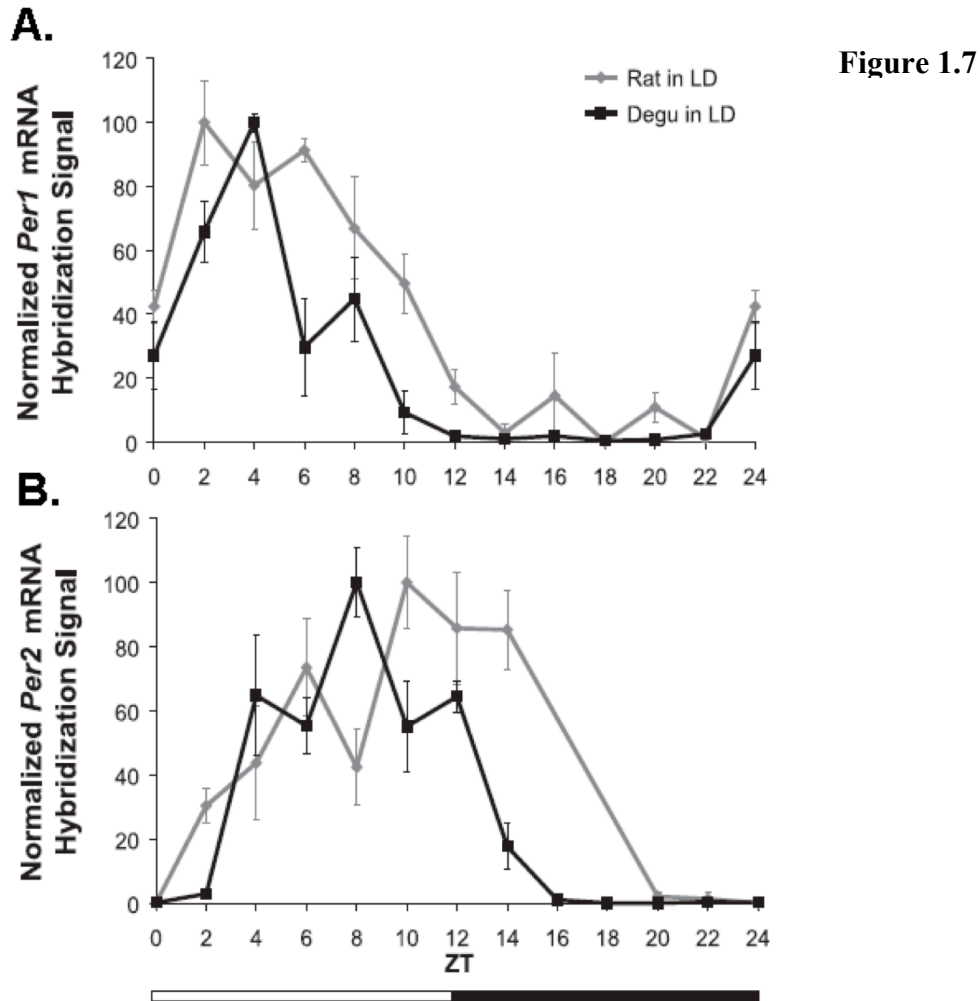
Figure 1.7
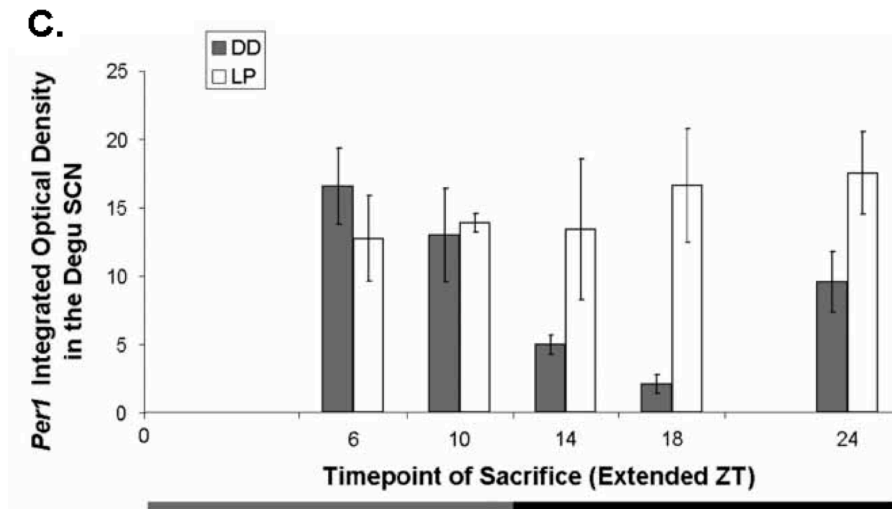
Figure 1.8

Figure 1.8. Period mRNA rhythms in brain regions outside of the SCN show dramatically different phasing in the diurnal degu and nocturnal rat. A) Representative autoradiographs showing *Per2* mRNA levels outside of the SCN in the diurnal degu and nocturnal rat. *Per2* mRNA levels in the putative striatum and cingulate and parietal cortices at each time point were visualized using in situ hybridization and autoradiography. The circles illustrate the specific locations on coronal slices that were used to sample hybridization signal from each brain region. **B-C)** Relative *Per2* mRNA levels at each time point were compared using in situ hybridization. The mean hybridization signal for each time point ($n =3$–5, +/-SE, LD 12:12) is normalized as %peak, with trough given the value of 0 (*A* and *B*). **B)** The degu demonstrated daily fluctuation of *Per2* mRNA levels in the putative parietal cortex and cingulate cortex ($P < 0.05$). **C)** The rat demonstrated daily fluctuation of *Per2* in the parietal cortex and striatum ($P < 0.05$). (Adapted from Vosko,* **Hagenauer**,* Hummer, and Lee 2009).
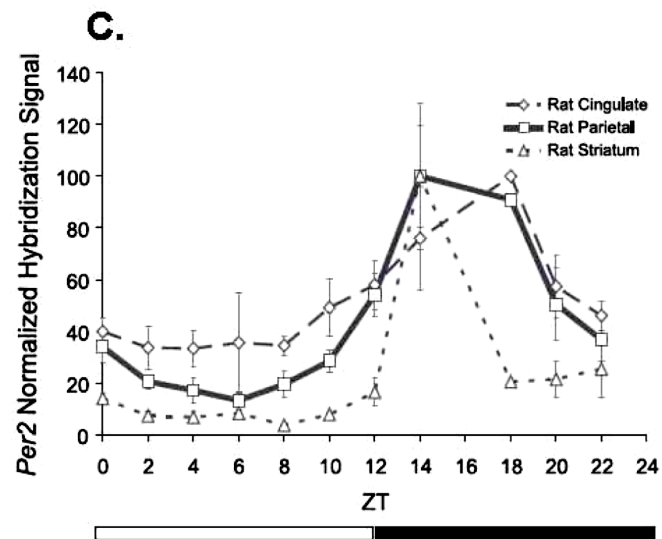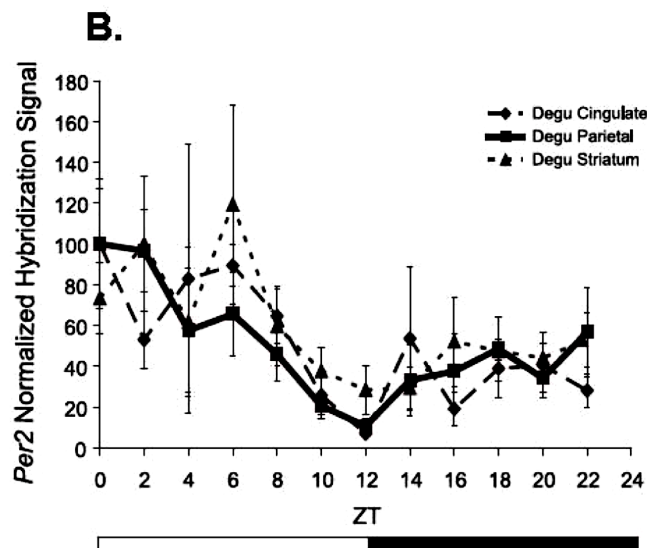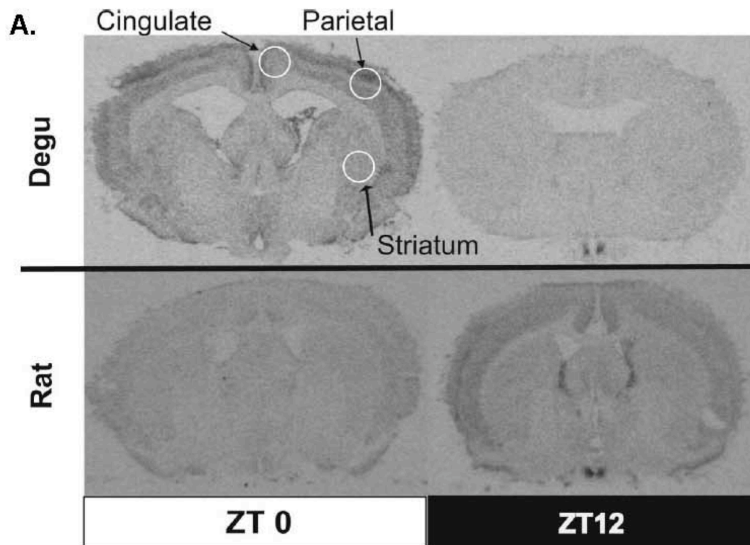
Figure 1.8

Figure 1.9. Two prominent examples of gonadal hormones influencing activity rhythms. A) In many rodent species, the day of estrus is accompanied by elevated wheel-running that begins earlier in the day (phase-advanced). Shown are two examples of estrus-typical wheel-running activity from a degu, with the alleged day of estrus marked with an "E." The activity is graphed as a double-plotted actogram, with each horizontal line sequentially representing 2 days of activity (# wheel turns/10 min bin) and the light-dark cycles for both days indicated by bars at the top of the figure. The grey line in the middle of the actogram represents a one-week break in recording, and the estrous cycle length for each female is noted at the bottom. **B**) Castration delays activity rhythm phase in male mice and androgen treatment restores normal rhythms. (top) Double-plotted actograms show the free running locomotor activity of male mice housed in constant darkness. The horizontal axis is 48 h and the vertical axis is consecutive days. Each box shows the rhythms of a mouse before and after hormonal manipulation. The bar graphs below show the distribution of activity across the day (+/-SE) for mice treated under several conditions: INT means intact, GDX means gonadectomized, +TP means treated with testosterone propionate, and DHT means treated with dihydrotestosterone. (Adapted from Iwahana et al. 2008).

Female

A.

E

1 week break

E

cycle length = 18 days

Figure 1.9

B.

Male

INT

GDX

GDX

+TP

Intact

GDX

GDX +DHT

35
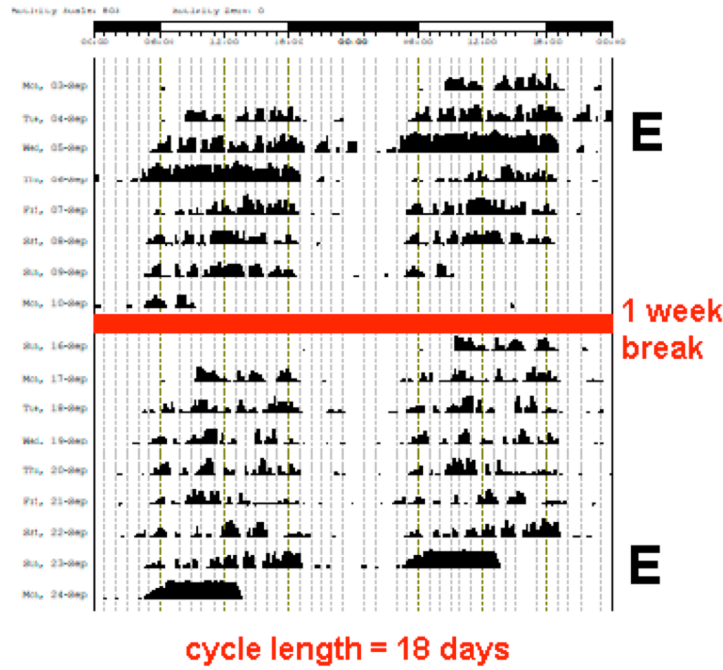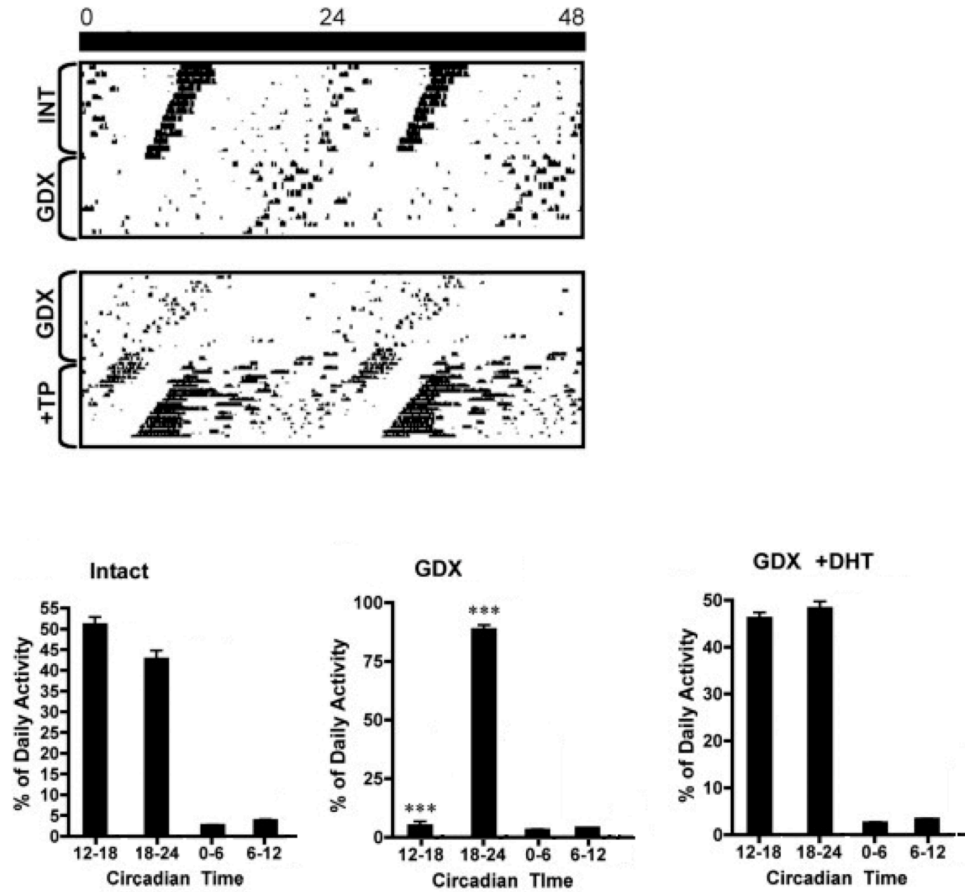
Figure 1.10. Gonadal hormones influence circadian physiology. Illustrated is a model SCN. Each component that is known to be hormone sensitive is labeled in grey, whereas if the hormone sensitivity is unknown the component is left black and white. The illustrated connections between input structures and the SCN represent particular pathways, whereas the connections between neurons do not specifically represent interactions between particular cell groups.
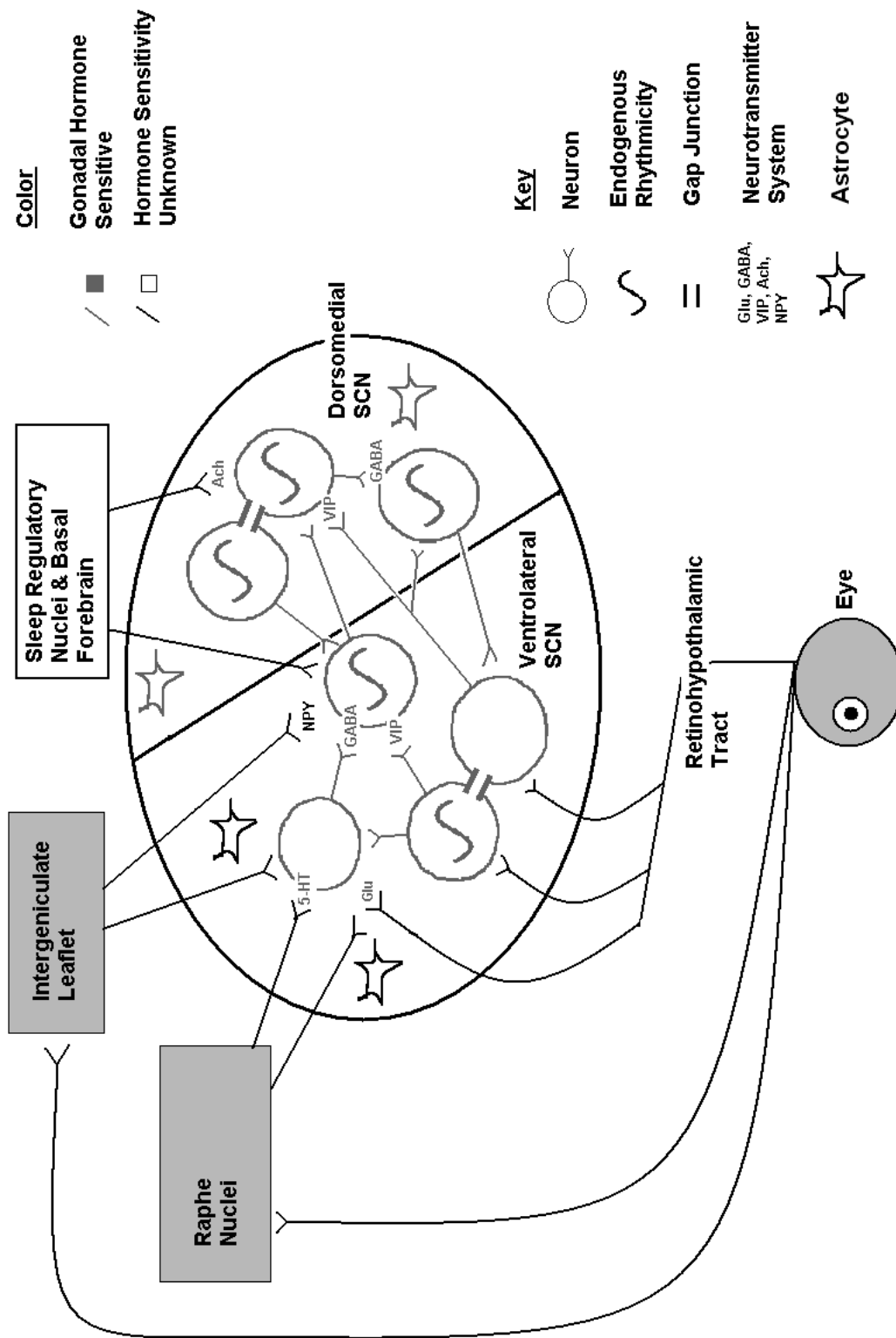
Figure 1.10
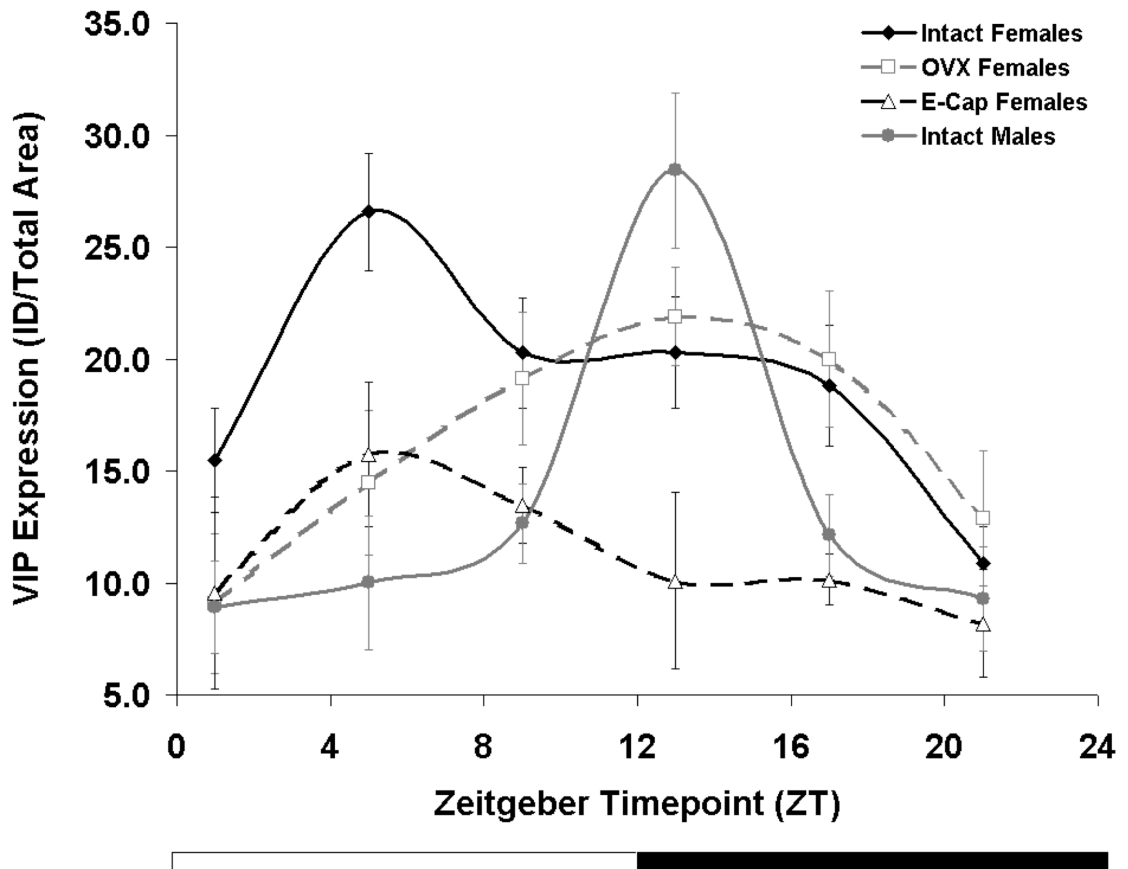
Figure 1.11. Ovariectomy causes an 8hr phase delay in the timing of peak VIP mRNA in the SCN of the diurnal grassrat (*Arvicanthis niloticus*). Illustrated are daily patterns in the VIP mRNA levels in the SCN of males, intact females, ovariectomized (OVX) females and OVX females given two estradiol capsules for 2 days. VIP mRNA was measured using in situ hybridization. Values are expressed as mean ± standard error. White and black bars indicate the time of lights-on and lights-off, respectively. (Adapted from Mahoney, Ramanathan, **Hagenauer**, Thompson, Smale, and Lee 2009). Note that these rhythms and hormonal dependencies resemble those already observed in the nocturnal rat (Krajnak et al. 1998).
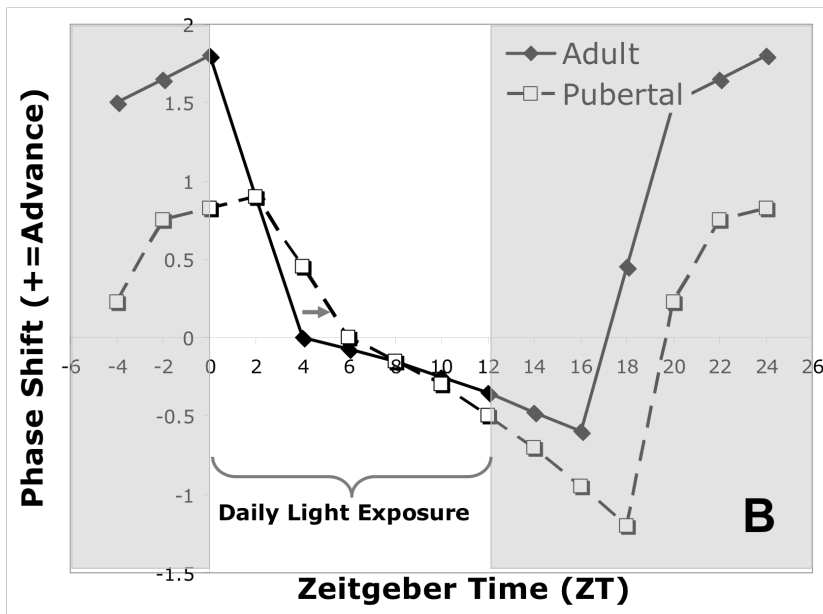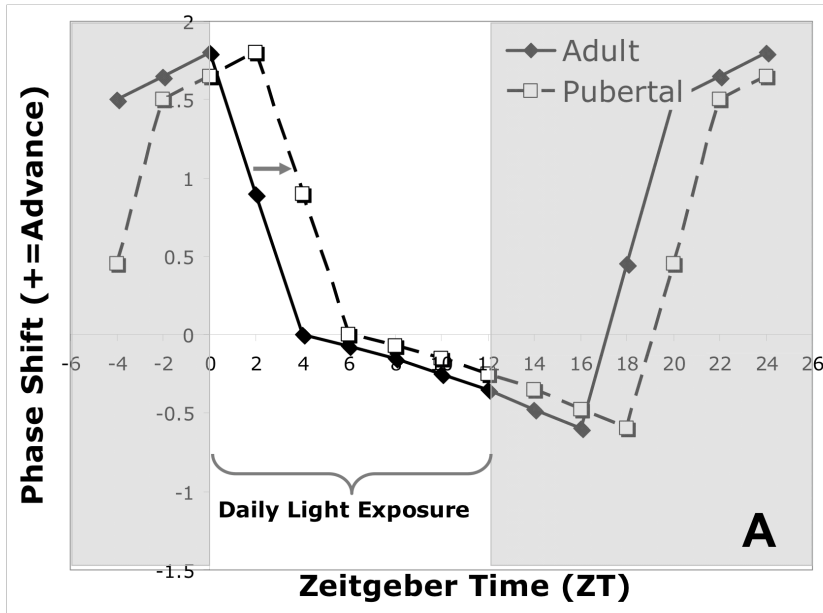
Figure 1.12. Two potential mechanisms underlying delayed circadian phase during puberty. A. An elongatation of τ during puberty would cause the circadian pacemaker and its rhythmic output to phase delay relative to the light cycle. The delay would provide more light exposure at a phase when the pacemaker is sensitive to advancing phase shift (represented above using a degu PRC), and thus allow entrainment (because τ - 24 hrs = ϕ, with ϕ, representing the magnitude of necessary daily phase-resetting). **B.** An increase in the circadian pacemaker's relative sensitivity to the phase-delaying effects of light would also cause the pacemaker to delay relative to the light cycle. This delay would result because the pacemaker would need more light exposure at a phase when it is sensitive to advancing phase shift. (Adapted from **Hagenauer** et al. 2009)
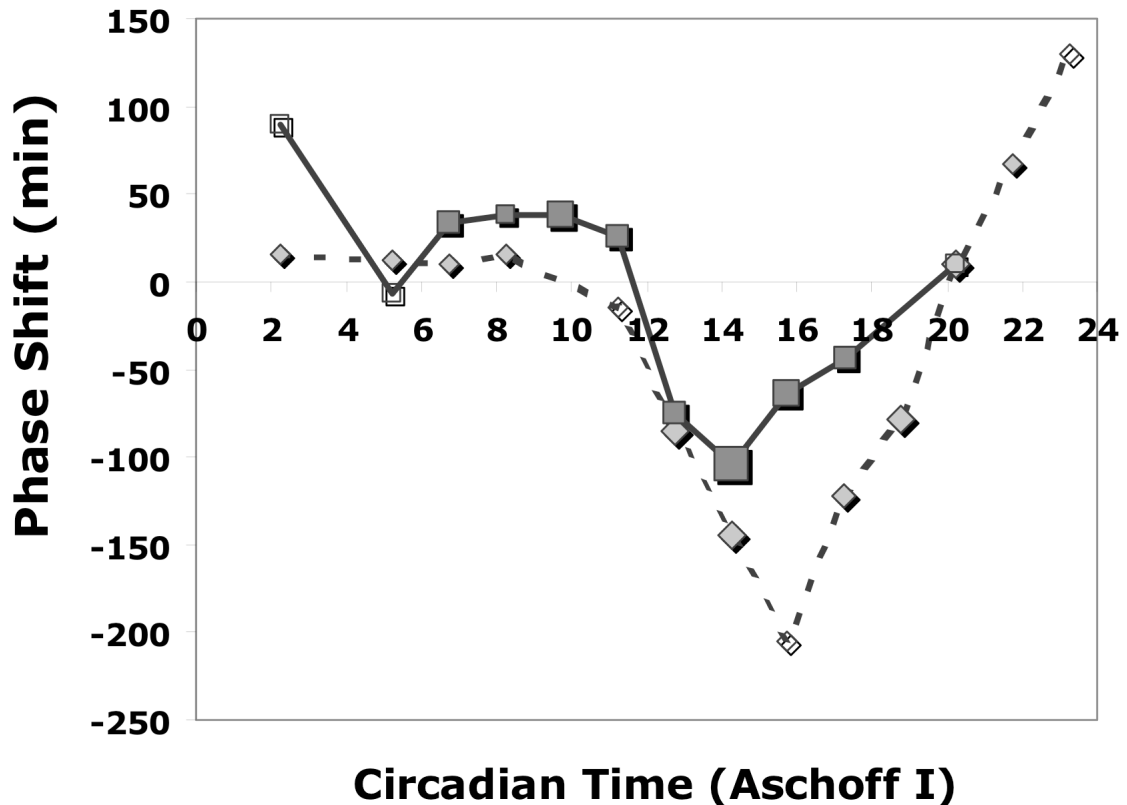
Figure 1.13. Pubertal mice exhibit an exaggerated delay in circadian phase in response to evening light. Female mice were placed into constant darkness (DD) for two weeks and then exposed to a 15 min light pulse (150 lux). Circadian phase shift in response to the pulse was calculated in reference to sham (no pulse) conditions, and circadian time was defined in reference to the activity rhythms of the individual mice (activity onset = CT12, Aschoff I methodology). The dotted line represents the phase response of mice that were likely to be pubertal at the time of the light-pulse (P49, n=34) and the dark line represents the phase response of adults (P140, n=34). Each point represents the average phase shift produced by light presented during a 1.5 hr bin. The sample size for each bin is represented by the size of the data point (n=1-8). (**Hagenauer** et al. 2009, Adapted from Weinert and Kompauerova 1998).

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Chapter 2

**Changes in Circadian Rhythms During Puberty in *Rattus Norvegicus:*
Developmental Time Course and Gonadal Dependency**

Abstract

During puberty, human adolescents begin to show a delayed timing of sleep and other daily physiological rhythms. To explain this phenomenon, we hypothesized that pubertal increases in gonadal hormones affect the phasing of the circadian timekeeping system. To test this hypothesis, we tracked the phasing and distribution of wheel-running activity rhythms during post-weaning development in rats that were gonadectomized before puberty or left intact. We found that intact peripubertal rats had activity rhythms that were phase-delayed relative to adults. Young rats also exhibited a bimodal nocturnal activity distribution. As puberty progressed, bimodality diminished and late-night activity phase-advanced until it consolidated with early-night activity. By late puberty, intact rats showed a strong, unimodal rhythm that peaked at the beginning of the night. These pubertal changes in circadian phase were more pronounced in males than females. Gonadal hormone increases during puberty partially accounted for these changes, as rats that were gonadectomized before puberty demonstrated smaller phase changes than intact rats and maintained ultradian rhythms into adulthood. We then investigated whether these developmental changes were due to altered photic entrainment. We compared circadian development in intact animals under constant and entrained conditions. We found that the period (τ) of free-running activity rhythms developed sex differences during puberty. However, these changes in τ did not account for pubertal changes in circadian phase, as the consolidation of activity at the beginning of the subjective night persisted under constant conditions in both sexes. We conclude that the circadian system remains highly plastic and hormone-sensitive during puberty.

Introduction

Human adolescents exhibit dramatically different sleep patterns than adults. Most notable is their propensity towards night-owl behavior, or "evening chronotype" (*e.g.* Crowley et al., 2007; Roenneberg et al., 2004; Thorleifsdottir et al., 2002; Yang et al. 2005). As puberty progresses, the timing of sleep grows later, such that the latest, or most delayed, sleep patterns occur around the time of achieving sexual maturity (between the ages of 15-21; Crowley et al., 2007; Roenneberg et al., 2004; Thorleifsdottir et al., 2002; Yang et al. 2005). Although adolescent sleep patterns are influenced by social factors, changes in the timing of sleep are at least partially rooted in hormonal influences on the body's circadian timekeeping system. In support of this assertion, a delayed phasing of both sleep and endocrine rhythms relative to daily light exposure persists in adolescents housed under controlled, laboratory conditions (Carskadon et al., 2004; Carskadon et al., 1997). Later sleep times also correlate with secondary-sex development, even after taking into account age and related social influences (Carskadon et al., 1993; Sadeh et al. 2009). Moreover, similar to other pubertal changes, girls begin to show delayed chronotype earlier than boys. The magnitude of these developmental changes exhibits sex differences as well, and by late adolescence boys are more evening-type than girls (Roenneberg et al., 2004). This evidence strongly implicates a role for gonadal hormones in adolescent changes in sleep patterns.

The sensitivity of the circadian timekeeping system to gonadal hormones during early development and adulthood is already well-documented. In humans, daily rhythms in cortisol and sleep onset shift their timing during different stages of the menstrual cycle (Manber and Bootzin, 1997; Parry et al., 1994; Parry et al., 2000). In adult laboratory rodents, a wide variety of gonadal hormones affect the phase of circadian rhythms, including estrogens, progestins, androgens, and non-traditional neuroactive steroids (*rats:* Albers et al., 1981; Axelson et al., 1981; Kent et al., 1991; Li and Satinoff, 1996; *hamsters:* Davis et al., 1983; Morin et. Al, 1977; de Tezanos Pinto and Golombek, 1999*; mice:* Daan et al., 1975; Iwahana et al., 2008; Karatsoreos et al., 2007; *degus:* Jechura et al., 2000; Labyak and Lee 1995). The sensitivity of the adult circadian system to these steroidal hormones exhibits sex differences that are determined by the organizational

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effects of gonadal hormones during the perinatal period (*rat:* Albers, 1981; *hamster:* Zucker et al., 1980). The influence of gonadal hormones on the circadian system during puberty is less understood, although it is reported that pubertal hormones can alter circadian phase (*degu:* Hummer et al., 2007) as well as produce organizational effects on the circadian system (*hamster:* Davis et al., 1983). Indeed, in some species, there is a critical window of sensitivity to the organizational effects of gonadal hormones as late as young adulthood (*degu:* Hummer et al. 2006).

Despite these indications that adolescent sleep patterns are likely to be partially due to pubertal increases in gonadal hormones, there has been little attempt to determine how common these developmental changes are across mammalian species or to elucidate their hormonal or neural bases using animal models (Hagenauer et al., 2009). Evidence from five species suggests that pubertal changes in circadian phase are not uniquely human (*rhesus macaque:* Golub and Takeuchi, 2002; *laboratory mouse*: Weinert et al., 1994; Weinert and Waterhouse, 1999; *laboratory rat:* McGinnis et al., 2007; Kittrell and Satinoff, 1986; *Octodon degus* (degu): Hummer et al., 2007; Tate et al., 2002; *Psammomys obsesus*: Neuman et al., 2005). However, only three of the studies (using the slow-developing, diurnal species of the macaque and degu) have attempted to thoroughly characterize the full developmental progression of circadian phase change in relation to secondary-sex development (Golub & Takeuchi 2002, Tate, Richardson, and Carskadon 2002, Hummer et al. 2007), and only one study directly examined the role of pubertal hormones (Hummer et al. 2007). Similar to humans, the macaque and degu show a delayed circadian phase during puberty (around the time of first menarche in the rhesus macaque, and first vaginal or prepucial opening in the degu) that reverses by adulthood. These developmental changes do not occur following pre-pubertal gonadectomy (Hummer et al., 2007). Therefore, pubertal elevations in sex hormones are likely to drive circadian phase changes.

To produce phase changes during puberty, these hormones may act on several different components of the circadian system. Circadian rhythms in mammals are generated by an endogenous pacemaker (Ralph et al., 1990). Therefore, under conditions in which there are no time cues from the outside world (also referred to as constant or "free-running" conditions), the circadian system continues to generate daily rhythms.

These endogenously-generated rhythms have a period (or day length, τ) that only approximates 24 hours (ranging from 23–25 hrs). Consequently, under normal conditions, the endogenous rhythm must be entrained by external time cues (or "zeitgebers," such as light) to maintain a stable phase relationship with the outside world (Moore-Ede et al., 1982).

If this entrainment mechanism were altered during puberty, it could produce a change in the phasing of daily rhythms in a manner that resembles the delayed sleep of human teenagers. Research indicates that everything from the circadian system's responsiveness to time cues (Hummer et al., 2007; Weinert et al., 1994; Weinert and Kompauerova, 1998) to the coupling of oscillators within the pacemaker (Cambras and Diez-Noguera, 1988; Diez-Noguera and Cambras, 1990) may be altered during pubertal development. One popular theory is that the endogenous period of the circadian pacemaker (τ) elongates during puberty, producing a delay in circadian phase (Carskadon et al., 2004). In support of this theory, τ appears to elongate during puberty in humans and shorten during adulthood in a manner that parallels changes in the timing of sleep (Carskadon, et al., 1999; Carskadon et al., 2004). Male rats in one study also showed a longer τ during late puberty (postnatal age P47-59 days, τ=23.89 hrs) than during adulthood (age P105-P115, τ=23.75 hrs; McGinnis et al., 2007). However, this study only examined one sex and sampled at one late-pubertal time point. These features are important because pubertal changes in circadian phase in the degu are not dependent on changes in τ. In degus, τ remained stable until a critical period in young adulthood when it underwent a final sexual differentiation in which the males developed a shorter τ but the females did not (Hummer et al., 2007). Thus, it is desirable to replicate the original findings in the rat (McGinnis et al., 2007) using both sexes and a larger number of sampling time points during puberty before concluding that pubertal phase change in the rat is caused by changes in τ.

The rat is a useful animal model for examining the mechanism underlying circadian phase change during puberty because of the wide-assortment of anatomical and physiological tools available for this species, as well as previous indication that male rats undergo a 3 hr magnitude phase change during puberty (McGinnis et al., 2007). However, it should be noted that the progression of puberty in this fast-developing

species exhibits several major differences from that of humans. Human puberty is commonly preceded by eight or more years of gonadal "quiescence" following infancy (Plant, 1994). The rat does not show this period of quiescence and continues to show a low level of steroidogenesis and secondary-sex development throughout the juvenile period that accelerates at the time of puberty (Ojeda and Urbanski, 1994).

Also, due to the short life-span of this species, puberty closely follows weaning. Under the gentle conditions of the laboratory, secondary-sex characteristics typically first appear around the postnatal age (P) of P30 in females and P45 in males, with mature sexual characteristics evident by around P60 (Ojeda and Urbanski, 1994; the specific age depends on the strain of rat and the health of the laboratory colony). This close proximity between weaning and puberty means that our data from the rat illustrates both the effects of pubertal hormones, as well as the already well-documented changes in circadian rhythms that occur as young rats develop independence and switch from maternal to photic entrainment mechanisms (for review see Weinert, 2005). In order to distinguish between these two events, we have also extensively examined the influence of pubertal hormones on circadian phase in the slow-developing diurnal species, the degu (Chapter 3).

Both experiments initially characterize the developmental time course and sex differences present in changes in circadian phase under entrained conditions across pubertal development. In *Experiment 1*, we further evaluated the correlation between phase change and sexual maturation in both sexes, and then determined the dependency of phase change on pubertal exposure to gonadal hormones. In *Experiment 2,* we examined the relationship between developmental changes in circadian phase and changes in the endogenous period of the circadian pacemaker under constant conditions (τ) in both sexes. Additionally, as several previous studies have commented on the prevalence of ultradian components in the circadian rhythms of rats around the age of puberty (Cambras and Diez-Noguera, 1988; Castro and Andrade, 2005; Diez-Noguera and Cambras, 1990; Ibuka, 1984, Joutsinemi et al., 1991; Kittrell and Satinoff, 1986), in both studies we also characterized the changing contribution of these components to circadian phase measurements across development.

We hypothesized that pubertal rats would show a delay in circadian phase that peaks around mid-puberty, similar to what is observed in slow-developing mammalian species (the degu and macaque; Golub & Takeuchi, 2002; Hummer et al., 2007). We further hypothesized that, as in the degu, these changes in phase would be dependent on gonadal hormones but independent of pubertal changes in τ. Finally, we expected that the development of delayed phase during puberty might be accompanied by a decrease in the ultradian components prevalent in the rhythms of newly-weaned young rats.

Methods

All procedures specified below were conducted in accordance with the guidelines established for the care and use of laboratory animals by the National Institute of Health and under approved by local animal use and care committees (IACUC).

Experiment 1: Pubertal Changes in Circadian Rhythms under Entrained Conditions

Animals: Four iterations of the same experiment were conducted with a total sample size of 62 Sprague-Dawley rats. These rats were obtained from breeding colonies at the University of Michigan, comprising eight litters from eight dams and sires. The dams and sires were purchased from Charles-River Laboratories (Wilmington, MA). Litters were reduced to 8 rats by postnatal day 3 (P3) with roughly balanced sex ratios. The pups used in iteration 1 were raised under a 14:10 light-dark (LD) cycle (lights on 05:00-19:00) until the age of P8-P14, when they were moved to the testing environment (12:12 LD, lights on 06:00-18:00). All other rats were raised and tested on a 12:12 LD cycle (lights on 06:00-18:00).

Within the testing environment, cages were kept on tables to standardize light exposure. During the lighted part of the LD cycle, the testing environment was dimly lit (40 lux measured at cage level, provided by fluorescent house light) to reduce photic masking. The rats were placed in the testing environment before weaning, and a subset of the rats were provided with running wheels during this time as well. At weaning (age P19-P22), rats were placed in individual opaque plastic cages (42.5 x 46 x 19.5 cm) with Nalgene running wheels (9 x 34.5 cm, Mini Mitter, Bend, OR) and free access to food (5001 Rodent Diet, PMI Nutrition) and water. They remained there for the duration of the experiment. Wheel-running data from the first two days after weaning was not used to

avoid artifacts due to the adjustment of the pups to the running wheel and social isolation. All rats had been exposed to the testing environment for 5-16 days before their activity data was used for analysis (beginning P22-P24).

Surgery: Approximately half of the animals in a litter underwent gonadectomy (GDX) surgery prior to puberty at age P12-P15. The rest of the animals in the litter underwent SHAM surgery. A second control included females and males that underwent no surgery. Any GDX animals that developed secondary-sex characteristics (*e.g.,* vaginal openings) were removed from the analysis (n=3), as were two females (no surgery) that exhibited low wheel-running counts, leaving a final sample size of 58 rats. (8 SHAM males, 8 SHAM females, 12 GDX males, 11 GDX females, 7 no-surgery males, and 15 no-surgery females).

During surgery, rats were anesthetized with 4% isoflurane. Castrations and ovariectomies were performed using procedures similar to those previously described in Hu and Becker (2003). Due to the young age of the rats, several additional measures were performed to ensure health. For ovariectomies, a 1-1.5 cm dorsal incision was made bilaterally below the ribs. For castrations, a single incision was made in the scrotal sac and the testes were visualized via palpation. The major blood vessels of the testes and ovaries were cauterized prior to organ removal. Then, the skin and the abdominal wall (in the case of the ovariectomies) were sutured closed. Post-operative care included subcutaneous saline injection and the application of Nolvasan antiseptic ointment (Fort Dodge Labs, Madison, NJ) on the incision. Sham operations included incisions and suturing similar to gonadectomy, but no organ removal. Animals were placed back in their cage with mother and siblings for at least 5 days of recovery before weaning.

Circadian Data Collection: Running wheel activity data was collected in 10-minute bins using VitalView software (Minimitter, Bend, OR) from an age just prior to the onset of puberty (P19-P23) until animals were post-pubertal (P60-P70). To prevent circadian rhythm disruptions, routine procedures involving handling of the animals occurred at random times during the lighted period of the LD cycle. For iterations 2 and 3, a plastic rod was inserted in the wheel during routine procedures to prevent handling-elicited wheel-running.

Monitoring Pubertal Development: Two secondary-sex characteristics, the development of a vaginal opening in female rats and a prepucial opening in male rats, were monitored daily around the typical age of puberty (around P27 to P40 for females, P33 to P65 for males). The androgen-dependent development of the prepucial opening is marked by the separation of the sheath from the glans of the penis (Ojeda and Urbanski, 1994). The development of vaginal opening is estrogen-dependent and one of the first indications of puberty in female rats (Ojeda and Urbanski, 1994). Testicular volume was measured in male rats twice weekly (around P32 to P65) and was calculated by multiplying the testicular length and width in millimeters as defined by existing parameters (Hummer, et al., 2006). Weight was also recorded twice weekly for all rats during the testing period (around P25 to P65).

Analysis: Circadian parameters were measured via wheel running activity and analyzed using ActiView software (Minimitter, Bend, OR). Automated analysis was used to determine daily mean activity (average wheel turns/10 min bin). The timing of peak activity was also automatically generated via cosinor analysis. Other indicators of circadian phase (initial activity (1°) onset, onset of the second (2°) major activity bout, and final offset) were scored by researchers blinded to the sex and gonadal status of the animals. The "threshold" for all analyses of circadian phase was defined as each day's mean activity so as to avoid the confounding effects of developmental and estrus-related changes in activity level. 1°activity onset and final activity offset were defined respectively as the first and last three consecutive bins of activity exceeding this threshold around the time of lights off (hours ZT12-18 in zeitgeber time, which is defined as hours relative to lights on), and lights on (between ZT18-24). Visual inspection revealed that young rats exhibited a bimodal activity pattern with a short, initial (1°) activity bout that begins almost immediately following the time of lights off and rarely lasts longer than 2 hours. Thus, to track changes in the second (2°) major activity bout, we also tracked the onset of this bout (2°onset), which was defined as three consecutive bins of activity exceeding this threshold following two hours of lights off (ZT14). On days where three consecutive bins of activity did not occur during the designated time periods of interest a circadian parameter could not be calculated. These null values were replaced by the latest 1°onset or earliest offset allowable (ZT18), or by the mean 2°onset

for ages P24-P60. Null values accounted for 10% of daily activity 1°onset and final offset scores, and 2.4% of 2°onset scores.

To better visualize changes in bimodality, data were plotted as a percent of nightly activity so as to control for age-related changes in activity levels. To produce these figures, raw vitalview activity data from the dark period (ZT12-ZT24) was averaged by hour. Activity data from the light period was ignored because the activity counts were so minimal that it provided little information about bimodal activity distribution. The hourly bins were then divided by the overall average activity for the night to produce a percent of nightly activity. These hourly percentages were averaged over five four-day sampling periods that evenly spanned the developmental period (P25-28, P33-36, P41-44, P49-52, P57-60).

Statistics: Initially, all intact animals (SHAM and no-surgery) were pooled and circadian parameters were analyzed for normal developmental changes over time and sex differences using a repeated measures ANOVA. Subsequently, all data were analyzed for the effects of gonadectomy surgery and sex on change over time using a 3x2 repeated measures ANOVA (GDX x Sex). A Huynh-Feldt correction was used for most analyses because the assumption of sphericity was violated. For *post-hoc* analyses of the effect of surgery type (no surgery, SHAM, GDX) on age-related changes in circadian parameters, individual repeated measures comparisons were run by group followed by Bonferonni correction. Between-subjects effects were also examined using 3x2 ANOVA, and significant effects were followed-up using Dunnett's T3 *post-hoc* comparisons. Two sets of analyses were run: one using data averaged bi-daily, to detect fast developmental changes, and one using weekly data averaged weekly to decrease variance. In the end, both analyses illustrated similar developmental changes and group effects, but these effects were stronger in the weekly data set, as expected. Therefore, the results section only presents analyses from the weekly data analysis, although the bi-daily data is presented graphically for comparison.

Experiment 2: A Comparison of Pubertal Changes in Circadian Rhythms Under Entrained and Constant Conditions

Animals: Thirty-two juvenile Sprague-Dawley rats (16 male, 16 female, 45-50 g) were purchased from Charles River laboratories (Wilmington, MA). Upon arrival, the rats were immediately placed in individual, transparent plastic cages ($47 \times 27 \times 20$ cm) with a 34 cm diameter running wheel (age P23). The rats were maintained under a 12:12 LD cycle (lights on at 08:00-20:00). During the lighted period, the light intensity at the bottom center of the cages averaged 59 lux and was provided by overhead fluorescent lights. Cages were arranged on open racks in the testing environment so that they alternated by sex. Food and water were provided *ad libitum*.

Procedure: After four days of baseline 12:12 LD recording, half of the rats (8 males, 8 females) were placed in constant conditions (constant red light or *RR*, < 1 lux at cage level) for six weeks (P29-P70). The red light was provided by three safelights in a light-tight room, each with a number one Kodak red monochromatic filter and a 15 W bulb. The other 16 rats were kept under the 12:12 LD condition for the same duration of time. The rats were weighed at the end of the experiment to ensure normal growth.

Analyses: Running wheel activity data was collected and analyzed in a manner identical to Experiment 1 with the following exceptions. First, the phase variables of 1°onset, 2°onset, and final offset were not monitored, but another phase variable, light-dark activity (LD) ratio, was evaluated. A lower LD Ratio score indicated greater nocturnality. Second, period (τ) was analyzed under both entrained and constant (RR) conditions using a periodogram analysis that considered a window of 22-26 hrs while sampling successive 1-min intervals. Third, for initial analyses, data from the initial entrainment period (P24-P28) were binned; other data were averaged in two week bins: P29-P42, P43-56, P57-P70.

In order to better compare developmental changes in the distribution of activity under entrained and constant conditions, the percent of daily activity occurring during each 10 min bin was quantified for several ages: P27-P28 (when all animals were still entrained to a 12:12 LD cycle), P29-P30 (the first two days in RR, useful for determining the influences of photic masking), P35-36, P43-P44, P51-P52, P67-68. To subtract out the influence of free-run, only two days were included in each sample and the onset of the active period was aligned for all RR animals, such that the average onset of the active period for a sample (e.g. P67-68) was made equivalent to that found under entrained

conditions (P27-P28). The onset of the active period (CT12) was defined as the beginning of the 12 hrs during the day when the most activity occurred as identified by moving window analysis. The time of peak activity was also measured relative to the onset of the active period under both RR and LD conditions.

Statistics: Circadian parameters specific to entrained conditions (time of peak activity, LD ratio) were analyzed for age-related change and sex differences using repeated measures ANOVA. General circadian parameters characterizing entrained and constant conditions (mean activity and τ) were analyzed for age-related change and effects of sex and condition using a 2x2 repeated measures ANOVA. For *post-hoc* analyses, if there was no main effect of condition but a main effect of sex, then individual comparisons were run by sex for each time point using independent samples T-tests followed by a Bonferonni correction. If there was a main effect of both condition and sex, then individual 2x2 ANOVAs (sex x condition) were performed by time point followed by Bonferonni correction. A Huynh-Feldt correction was used for most repeated measures analyses because the assumption of sphericity was violated.

A separate analysis was run to analyze the effect of masking on the distribution of pre-pubertal activity in both sexes. This analysis examined the within-subjects change in the timing of peak activity relative to the onset of the active period (CT12) during the last two days in LD conditions (P27-P28) and the first two days in RR (P29-30). These changes were compared to the control animals that remained in LD conditions at both ages using a 2x2 repeated measures ANOVA (sex x condition at 2 ages).

The progression of developmental changes in the distribution of activity under entrained (LD) or constant (RR) conditions was also further analyzed statistically by comparing the timing of peak activity relative to the onset of the active period in both sexes at 5 ages (P29-P30, P35-36, P43-P44, P51-P52, P67-68) using a 2x2 repeated measures ANOVA (sex x condition at 5 ages).

Results

Experiment 1: Pubertal Changes in Circadian Rhythms under Entrained Conditions

Secondary-sex development and circadian rhythms in wheel-running activity were monitored in rats of both sexes that received gonadectomy (GDX) or SHAM surgery

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prior to puberty as well as rats that had not received surgery ("no-surgery"). These data were collected from an age just prior to the typical onset of puberty (P19-P23) to maturation (P60-P70)

Developmental Changes in Circadian Phase in Intact Animals: Both male and female intact animals (no-surgery and SHAM) demonstrated a bimodal activity pattern during early puberty, with increased activity around both times of transitioning light. This bimodality disappeared by late to post-puberty (**Figure 2.1**, **Figure 2.2**) due to the later (2°) activity bout phase-advancing until it merged with the initial (1°) activity bout. This change was reflected in a phase advance in the 2°onset, final activity offset, and the time of peak activity as indicated using repeated measures ANOVA (*2°onset:* $F(2.367, 78.098) = 52.542$, *p<0.001*; *the timing of peak activity:* $F(3.327, 123.099) = 6.417$, *p<0.001*; *activity offset:* $F(3.331, 123.243) = 10.642$, *p<0.001*). These changes were particularly pronounced in no-surgery intact males, which showed a 4.03 hour phase advance in 2°onset across the pubertal period. In all animals, the 1°onset at the beginning of the dark period held constant across the developmental period ($F(4, 148) = 1.228$, *p=0.302*). Consequently, by the time of maturation the rats showed a unimodal activity rhythm that peaked during the early portion of the dark period (**Figure 2.1E&F, Figure 2.2E&F**).

Sex Differences in Circadian Phase in Intact Animals: Similar to human adolescents, male rats demonstrated greater within-subjects change in circadian phase over development than females (**Figure 2.3**). This sex difference in the magnitude of age-related change reached significance for 2°onset (**Figure 2.3B**, $F(3.392, 125.491) = 3.021$, *p=0.027*) and final activity offset (**Figure 2.3D**, $F(3.331, 123.243) = 4.593$, *p=0.003*) but not for the time of peak activity ($F(3.327, 123.099) = 1.772$, *p=0.150*) or 1°onset ($F(4, 148) = 1.925$, *p=0.109*). There was also a between-subjects main effect of sex on 2°onset ($F(1, 37) = 4.835$, *p=0.034*), with males showing an overall more delayed phase than females (**Figure 2.3B**). Main effects of sex were not seen for any of the other phase variables (*p>0.10*).

There were also sex differences in the developmental *timing* of circadian phase changes in intact animals that paralleled sex differences in the developmental timing of secondary-sex development. Delayed phase in males was most prominent between the

ages of P31-P37, as indicated by 2°onset, the time of peak activity, and final activity offset, whereas delayed phase in females was most prominent at ages P24-P30, as measured by 2°onset. Similarly, females showed 50% of their age-related change in 2°onset by P37, whereas males showed 50% of their age-related change in 2°onset by P41 (**Figure 2.3**).

The Effect of Gonadectomy on Circadian Phase: The GDX animals exhibited a similar bimodal activity pattern as intact animals around the typical age of pre-puberty and early puberty. This bimodality lessened over time, but remnants still persisted into adulthood (**Figure 2.4, Figure 2.5**). GDX animals also demonstrated a phase advance in some of their circadian markers by late- to post- puberty, but again to a lesser extent than the intact animals (*e.g.* **Figure 2.6C-D**). Thus, GDX surgery had a significant effect on within-subject change in 2°onset (F(5.860, 149.420)=4.451, p<0.001) and final offset (F(6.926, 179.615)=2.682, *p<0.001*). Overall, GDX animals exhibited a later 2°onset than intact animals, as indicated by a main effect of GDX between-subjects (F(1, 51)=5.619, p=0.022).

The manner with which gonadectomy altered age-related change was different between the sexes: in males, gonadectomy consistently decreased the amount of age-related change in circadian phase parameters (**Figure 2.6D, F, H**), whereas in females gonadectomy had variable effects. The effects of gonadectomy in the females included: a) a decrease in age-related change (2°onset, **Figure 2.6C**), b) little effect where change in intact animals was already minimal (final offset and time of peak activity, **Figure 2.6G&E**), c) an increase in age-related change (1°onset, **Figure 2.6A**). Therefore, several of the circadian parameters showed a significant interaction (or a trend towards an interaction) between the influence of sex and gonadectomy on within-subjects change (*offset:* F(6.926, 176.615)=3.086, *p=0.004*; *time of peak activity:* F(5.961, 145.132)=1.856, *p=0.096*). These sex differences were particularly striking for age-related change in 1°onset (F(3.944, 201.157)=3.027, *p=0.019*). Whereas males and no-surgery females exhibited almost no change in 1°onset, SHAM and GDX female rats were relatively phase-advanced during pre-puberty, phase delayed during puberty, and finally phase-advanced again during late- and post-puberty (effect of gonadectomy: F(11.542, 201.157)=2.384, p=0.018; **Figure 2.6A**). Overall, GDX females also exhibited

an earlier offset than intact females whereas GDX males had a later offset than intact males, statistically producing a significant between-subjects interaction between the influences of sex and GDX (F(2, 51)=3.308, p=0.045).

These data suggest that the effects of gonadectomy may be partially accounted for by the surgery itself, in addition to the hormonal changes produced by removing the gonads. The developmental changes that occurred in intact animals appeared dampened in those with SHAM surgery (e.g. **Figure 2.4 C, D, F, H**). When comparing no-surgery and SHAM animals using individual *post-hoc* comparisons there were no circadian phase parameters that were significantly affected by surgery. However, the amount of age-related change exhibited by SHAM's appeared intermediate between that of no-surgery and GDX animals. There was a significant difference between GDX and no-surgery animals in within-subjects change in 1°onset, 2°onset, and final offset (*p*<0.05), as well as sexually diergic effects of gonadectomy on the time of peak activity, final offset (both *p*<0.05), and 2°onset (*p*=0.054*trend). However, SHAM and GDX groups only showed differences in within-subjects change in final activity offset, and this variable was influenced in a sex-specific manner (*p*<0.05, overall main effect of GDX: *p*=0.069*trend).

Mean Activity: There were significant age-related changes in mean activity (F(1.848, 94.239)=64.708, p<0.001; **Figure 2.7 A&B**). These age-related changes were affected by sex (F(1.848, 94.239)=11.779, *p*<0.001), with females showing much greater increases in wheel running activity over development than males. These age-related changes were also affected by gonadectomy (F(3.696, 94.239)=10.359, *p*<0.001). GDX animals maintained a relatively constant mean daily activity throughout development. Individual *post-hoc* comparisons revealed that SHAM and no-surgery animals demonstrated a greater age-related increase in mean daily activity than GDX animals (*p*<0.05). The manner with which gonadectomy affected age-related change depended on sex (F(3.696, 94.239)=3.060, *p*=0.023), with females showing a much larger effect of GDX than males (**Figure 2.7 A&B**).

As previously shown in other studies, between-subjects comparisons revealed overall sex differences in mean activity levels (F(1, 51)=8.891, *p*=0.004), with females demonstrating a greater mean activity than males. There was also an overall effect of

gonadectomy ($F_{(2, 51)}=7.943$, $p=0.001$). Individual *post-hoc* comparisons indicated that GDX rats were different from SHAM or no-surgery animals ($p<0.05$), showing much lower overall mean activity levels (**Figure 2.7 A&B**). This between-subjects effect varied by sex ($F_{(2,51)}=3.537$, $p=0.036$), with females showing much larger effects of GDX on overall mean activity levels (**Figure 2.7 A&B**).

Pubertal Development: The development of vaginal and prepucial openings around the time of mid-puberty provides us with a developmental marker for comparison with the circadian data. Similar to humans, female rats showed overt signs of puberty at an earlier age than males (**Figure 2.7E**). The average day of first vaginal openings was P33.1 (+/-0.38 SE), with all females exhibiting vaginal openings by P36. The average day of first prepucial openings was P43.1 (+/-1.42 SE), with all males exhibiting prepucial openings by P59. There was no significant difference in the timing of pubertal onset between SHAM and no-surgery animals, although SHAM males showed a trend towards developing prepucial openings later than no-surgery males ($T_{(8.121)}=-1.872$, $p=0.098$). Testicular volume showed a linear increase across pubertal development ($r^2=0.487$, $p<0.001$) in a manner that was similar in SHAM and no-surgery males ($F_{(1, 126)}=0.418$, $p=0.519$, **Figure 2.7F**).

The timing of development was also monitored via weight gain (**Figure 2.7C, D**). Measures of body weight for both males and females showed a linear within-subjects increase throughout development with little variability ($F_{(2.173, 112.991)}=1378.033$, $p<0.001$). Males showed a greater age-related increase in weight than females ($F_{(2.173, 112.991)}=36.773$, $p<0.001$). There was significant effect of gonadectomy on within-subjects change in weight ($F_{(4.346, 112.991)}=5.683$, $p<0.001$), with no-surgery animals showing a slower weight gain than SHAM or GDX animals. This effect of gonadectomy on weight gain differed by sex ($F_{(4.346, 112.991)}=5.590$, $p<0.001$), with males showing more effect of surgery than females. Males were also heavier overall than females ($F_{(1, 52)}=21.552$, $p<0.001$), and GDX and SHAM animals were heavier than no-surgery animals ($F_{(2, 52)}=6.082$, $p=0.004$), leading to a trend towards an interaction between the influences of gonadectomy and sex on weight ($F_{(2, 52)}=2.973$, $p=0.060$).

Experiment 2: A Comparison of Pubertal Changes in Circadian Rhythms Under Entrained and Constant Conditions

Rhythms in wheel-running activity were monitored in pre-pubertal rats of both sexes under a standard light cycle (12:12 LD; P23-28). Then half of the animals were placed in constant conditions (dim RR) and half were kept under entrained conditions until maturation (P70).

Circadian parameters under entrained conditions: The rats' entrained activity rhythms showed a similar pattern of developmental change across puberty as those observed during *Experiment 1*. Activity rhythms showed a pronounced bimodal activity distribution during early/mid-puberty that disappeared around the age of maturation (**Figure 2.8A, Figure 2.9B-D**). This bimodality dissolved as the later activity bout phase-advanced until it merged with activity occurring earlier in the evening. Thus, we found that the daily timing of peak activity phase-advanced by approximately 3 hrs between ages P24-P70 (**Figure 2.9A**, *within-subjects change:* $F(1.735, 24.294)=33.890$, $p<0.001$). These changes in circadian phase were accompanied by a relative decrease in daytime activity, as reflected by within-subjects change in the light/dark (LD) activity ratio during ages P24-P70 (**Figure 2.9B**, $F(1.599, 22.385)=6.693$, $p=0.008$). Neither of these age-related changes differed by sex (*time of peak activity*: $F(1.735, 24.294)=0.800$, $p=0.445$; *LD Ratio:* $F(1.599, 22.385)=1.387$, $p=0.266$), nor was there an overall sex difference for either variable (*time of peak activity*: $F(1, 14)=2.946$, $p=0.108$; *LD Ratio:* $(F(1,14)=2.265$, $p=0.155$).

Circadian parameters under constant conditions: Immediately upon placement in constant conditions, the initial activity bout diminished slightly leading to a delay in the time of peak activity. These changes did not occur when the animals were left in an LD cycle (**Figure 2.8B & C, Figure 2.10**). This resulted in a significant main effect of lighting condition on within-subjects change in the time of peak activity relative to the onset of the active period (CT12, as defined by the beginning of the 12 hour period during the day which contained the highest mean activity, $F(1, 28)=7.431$, $p=0.011$). Then, under both entrained and constant conditions, the later (2°) activity bout phase advanced and consolidated into a unimodal rhythm, such that the time of peak activity grew closer to the onset of the active period (**Figure 2.10H**, $F(4, 112)=36.351$, $p<0.001$).

This phase advance occurred faster under constant conditions, leading to an overall effect of lighting condition (RR vs. LD) on within-subjects change in the time of peak activity relative to the onset of the active period ($F(4, 112)= 7.630$, $p<0.001$). This phase advance was also slightly smaller in females ($F(4, 112)=3.372$, $p=0.017$), in a manner that depended on lighting condition ($F(4, 112)=2.672$, $p=0.036$). Females in general showed an earlier time of peak activity relative to the onset of the active period than males ($F(1, 28)=9.835$, $p=0.004$) and this sex difference was exaggerated under entrained conditions ($F(1, 28)=5.879$, $p=0.022$).

Under constant conditions, free running-period (τ) changed over development in a manner that depended on sex (**Figure 2.9C**). We also measured τ under entrained conditions to make sure that age-related phase changes were biasing periodogram analyses, and τ was shown to hold steady around 24 hrs (*data not shown*). Therefore, within-subjects change in τ was affected by an interaction between sex and lighting condition ($F(2, 56)=3.549$, $p=0.035$, but no main effect of sex or condition $p<0.05$). Around the age of mid-puberty (P29-P42) both males and females had a τ that was close to 24 hrs in duration (24.07 +/- 0.15 SE and 23.911+/- 0.16 SE, respectively). *Post-hoc* comparisons indicated that τ at this age did not differ in either sex from τ under entrained conditions (sex x condition: $p>0.05$). As puberty progressed, females developed shorter τ's, eventually plateauing at 23.75 (+/- 0.08 SE) and males developed longer τ's, eventually plateauing at 24.21 (+/- 0.08 SE). *Post-hoc* comparisons indicated that a sex difference developed under constant conditions by ages P43-P56, and continued into ages P57-P70 (sex x condition: $p<0.05$). Overall, females had a shorter τ than males, as indicated by a main effect of sex ($F(1, 28)=5.101$, $p=0.032$; *sex x condition(LDvs.RR)*: $F(1, 28)=3.989$, $p=0.056$*trend).

Activity level: Under both entrained and constant conditions activity levels increased over development (**Figure 2.9D**; *within-subjects change:* $F(2.212, 61.946)=71.928$, $p<0.001$). This within-subjects change varied by sex ($F(2.212, 61.946)=9.940$, $p<0.001$), with females showing greater age-related increases in mean activity than males. There was no main effect of lighting condition on age-related change (LD vs. RR, $F(2.212, 61.946)=1.065$, $p=0.356$) or interaction between sex and condition ($F(2.212, 61.946)=1.631$, $p=0.202$). *Post-hoc* comparisons indicated that this sex

difference only reached significance at age P43-56 ($p<0.05$). Overall, females were more active than males, as indicated by a main effect of sex (F(1, 28)=9.360, $p=0.005$).

Weight: Predictably, there was a main effect of sex on final body weight, with females weighing less than males (F(1, 28)=125.629, $p<0.001$). There was a non-significant trend towards rats under constant conditions weighing more than those under entrained conditions (F(1, 28)=3.743, $p=0.063$, *data not shown*) in a manner that did not vary by sex (F(1,28)=1.090, $p<0.305$).

Discussion

These experiments indicate that circadian activity remains highly plastic during post-weaning and pubertal development in the fast-developing, nocturnal rat. Under both entrained and free-running conditions, we observed a crepuscular distribution of activity during early post-weaning, with the majority of activity occuring near the end of the active phase. As male rats progressed through puberty, this later bout of activity phase-advanced 4 hrs until it consolidated into a strong, unimodal rhythm that peaked near the beginning of the animal's active phase. These pubertal changes in circadian phase were smaller in females, with female rats showing (at most) a 3.5 hr magnitude phase advance during puberty.

These results complement previous developmental sleep and activity data in post-weaning rats. For example, a small sleep electrophysiology study that monitored male rats across post-weaning development (n= 1 rat per age group, Ibuka, 1984) found that total sleep followed a mostly ultradian pattern in juveniles (3-4 weeks of age). Between 4-5 weeks of age these ultradian rhythms developed two pronounced crepuscular periods of decreased sleep around the beginning and end of the dark period. These two periods of decreased sleep eventually consolidated into a unimodal rhythm in adults, with very little sleep occurring at the beginning of the dark period. Slow-wave sleep followed a similar trend (Ibuka, 1984). Behavioral studies of sleep and activity in young rats similarly indicated that rats grow more nocturnal across the post-weaning period (Norton, 1975; Thiels et al.,1990), although one low-resolution study of activity using group recordings showed that rats were fully nocturnal even during the fourth week of life (P21-27, Joutsiniemi et al., 1991). Our previous developmental research on wheel-running rhythms

in rats also suggested a more bimodal activity pattern in the pubertal rats, which consolidated by adulthood, producing an overall 3hr phase advance in the timing of peak activity (McGinnis et al., 2007). Taken collectively, this evidence indicates that processes governing the daily sleep and activity cycles of rats mature during the pubertal period.

Previous research in the slow-developing degu suggested that changes in circadian phase during puberty are dependent on gonadal hormones (Hummer et al., 2007). Our experiments in the rat support this conclusion on two counts. First, we found that the reorganization of activity rhythms during puberty in the rat exhibited sex differences in timing and magnitude in a manner that strongly indicated a role for gonadal hormones in circadian development. Our data from rats gonadectomized prior to puberty also supported this hypothesis. Gonadectomized (GDX) animals maintained a more dispersed activity distribution and delayed phase into adulthood. The organization of circadian rhythms in the castrates as well as in pre-pubertal intact animals strongly resembled that of castrated adult rodents in previous studies (*mice:* Daan et al., 1975; Iwanahana et al., 2008; Karatsoreos et al., 2007; *hamsters:* Davis et al., 1983; Morin et al., 1981; although not the *degu:* see Jechura et al., 2000). In these studies, castrated adult males showed an altered distribution of activity under entrained and free-running conditions such that activity was more dispersed across the active period, leading to less cohesive activity at the onset of the active period (Morin et al., 1981), or such that the initial activity bout was diminished, lost, or delayed (Karatsoreos et al., 2007; Iwanahana et al. 2008, Daan et al. 1975; Davis et al. 1983). The administration of testosterone or di-hydrotestosterone was able to restore adult castrates to their original circadian activity patterns (Karatsoreos et al. 2007; Iwanahana et al. 2008, Daan et al. 1975; hamsters: Morin et al. 1981), indicating a role for the androgen receptor in maintaining vigorous activity at the beginning of the active period (Iwanahana et al. 2008; although the role of aromatized testosterone can certainly not be completely ruled out: Hummer et al. 2006). Thus, our observation that as rats mature they develop increasingly unimodal rhythms that peak at the beginning of the active period may reflect the activational effects of increasing testosterone during puberty.

We also observed a similar, although smaller magnitude, circadian reorganization during puberty in female rats. Previous results have been divided as to the effect of adult

ovariectomy on the organization of female circadian rhythms. All studies acknowledge the disappearance of cyclic estrus-related elevations of activity (a pattern described as "scalloping" by Morin et al. 1977) following ovariectomy (Morin et al. 1977, Davis, Darrow, and Menaker 1983; Labyak and Lee 1997; Iwanahana et al. 2008). However the effect of ovariectomy on overall circadian phase or activity distribution appears to be species specific. In the mouse and degu, ovariectomy had little overall effect on activity phase or the distribution of activity across the active period (Iwanahana et al. 2008, Labyak and Lee 1997), whereas ovariectomy in hamsters led to an overall delay in activity onset (Davis et al. 1983). In hamsters, this delay was reversed by treatment with estradiol, which consolidated activity within the early hours of the subjective night (Morin et al. 1977), similar to our observations across puberty in intact female rats. Two studies in female rats also noted an increase in the ultradian components of activity rhythms following ovariectomy, as well as a reconsolidation of rhythms following estrogen treatment (Wollnik and Döhler 1986; Thomas and Armstrong 1989). Therefore, the circadian changes we observe during puberty in female rats may depend on the activational effects of increasing estrogen during puberty, but such changes may be species-specific. This interpretation coincides with data from the slow-developing degu (Hagenauer, Ku, and Lee, *in preparation*). In this species, females do not show the significant changes in activity phase or distribution during puberty seen in males, but adult ovariectomy also does not affect the overall distribution of activity (Labyak and Lee, 1997).

Interestingly, GDX rats of both sexes still continued to show some circadian change at the typical age of puberty. This raised the possibility that a similar magnitude of change might still be occurring in GDX rats, but with a delayed developmental time course, perhaps due to the stress of surgery. To address this question, we examined the onset of the later (2°) activity bout in a subset of intact and GDX animals that we recorded for an additional ten days (up to P68). We found that in GDX animals the developmental changes in 2° activity bout stabilized by P49, and the circadian phase from P49-P68 was reliably more delayed than that of intact animals (**Supplemental Figure 2.11**). This indicated that the developmental changes in GDX animals were

indeed smaller in magnitude than those found in intact animals, and that overall phase differences between the two groups were long-lasting.

The presence of some circadian change in GDX rats around the typical age of puberty contrasts with what we found in the slow-developing, precocial degu. In the degu, GDX males did not show any changes in circadian organization or phase that resembled intact males (Chapter 3). One possibility is that the hormone-independent circadian changes that we observe in the fast-developing rat are due to the gradual transition from maternal to photic entrainment mechanisms that accompanies weaning in altricial species (for review see Weinert, 2005). In fast-developing species, weaning and puberty occur in close proximity. In most laboratories (including our own) weaning is performed artificially at age P21 by moving the pups into a separate cage from the dam. However, rats may continue to show nursing behavior until around ages P28-P40 when allowed to remain with the dam (Calhoun 1962; Cramer, Thiels, and Alberts 1990).

Therefore, at the time of weaning in the laboratory (P21) the juvenile rat is at an age when it would still be heavily dependent on the dam in the wild. As an altricial species, this dependence extends into the realm of circadian entrainment. Although rat pups can respond to photic time cues soon after birth in the lab (Weaver and Reppert 1995), in the wild the nursing dam seals off burrow entrances with leaves and mud (Calhoun 1962). Thus, pups are unlikely to be exposed to regular photic cues until they venture from the burrow during the third week of life (Calhoun 1962; Weinert 2005). Consequently, the primary influence on the phasing of activity rhythms in young rats is the diurnal nursing pattern of the dam (Shimoda et al. 1985; Sugishita et al. 1991; Thiels, Alberts, and Cramer 1990). If pups are kept in the presence of the dam, they exhibit this diurnal activity pattern until around age P18, at which point they begin to consume solid food (Bolles and Wood 1964) and develop a more ultradian rhythm (Thiels, Alberts, and Cramer 1990). When isolated from the dam, the pups can exhibit nocturnal rhythms as early as P15-19 (Anderson and Smith 1986; Levin and Stern 1975; Teicher and Flaum 1979), but the light-dark cycle doesn't trump the influence of the dam until age P22 (Levin and Stern 1975). Even after this point, the dam serves as an effective zeitgeber until the 4th or 5th week of life (approximately P28-P35; Levin and Stern 1975; Takahashi, Hayafuji, and Murakami 1982). Thus, the earliest part of our sample (P21-

P30) overlaps with the typical time of transition from maternal to photic entrainment, as well as from dependent, diurnal activity to independent, exploratory nocturnal activity. These changes would occur regardless of the hormonal condition of the animal. Similarly, the high metabolic demands on young, fast-growing animals would be likely to promote ultradian rhythms of sleep and activity that would diminish with age regardless of hormonal environment (Alfoldi et al. 1975).

Animals that received SHAM surgery showed significant pubertal circadian changes, but they were dampened compared to those of no-surgery animals. Males also showed a trend towards delayed secondary-sex development. It seems unlikely that these differences between SHAM and no-surgery rats were directly due to continued stress from the surgery, as both groups showed healthy weight gain throughout the recording period and almost identical activity levels. Perhaps instead there was a secondary effect of surgery on maternal care (Barnett & Burn 1967), or a sexually-diergic long-term effect of isoflurane anesthesia (Siegal and Dow-Edwards, 2009; McCann et al. 2009).

It is unclear what aspect of the circadian mechanism is affected by pubertal hormones to produce changes in circadian phase. Originally, we hypothesized that hormones altered the entrainment of the circadian system to the LD cycle to produce pubertal changes in daily activity rhythm phase and distribution. This altered entrainment could be produced by changes in the photic sensitivity of the circadian pacemaker or via an elongation of the endogenous free-running period (τ). Indeed, under conditions of constant darkness we observed developmental changes in τ: Male rats developed a longer τ and female rats developed a shorter τ. However, these changes seem unlikely to explain pubertal changes in the phasing and distribution of activity under entrained conditions for several reasons. First, both males and females showed similar changes in the phase of rhythms during puberty despite showing divergent developmental changes in τ. Second, the changes that we observed in τ during puberty in males in this study are actually the opposite of what we found in our earlier study (McGinnis et al. 2007). In the earlier study, males showed a shortening of τ such that, when first placed in constant conditions during mid-puberty (P47-59), they showed a τ that was close to 24 hrs. Later, when placed in constant conditions again during adulthood (P105-115), their τ dropped to 23.75 hrs (McGinnis et al. 2007). In the current study, τ again is near 24 hrs (24.07) when

the male rats are first placed in constant conditions at P29-P42, but then lengthens to 24.21 hrs by P43-P70. One possible explanation for the discrepancy between results in the two studies is the duration of time the rats were placed in constant conditions. In an earlier study in pubertal degus, younger animals were found to maintain a free-running period close to 24 hrs for almost 2 weeks after placement in DD. These pronounced aftereffects disappeared as the animals matured (Hummer et al. 2007). Thus, it may be that the τ of 24 hrs that we observed in male and female rats at the earliest samples in both studies also reflects aftereffects of the photoperiod.

Either way, these changes in the entrainment mechanism are unlikely to fully explain pubertal changes in activity rhythm phasing because our data also indicate that rats undergo a redistribution of activity rhythms under constant conditions. Pre-pubertal rats continued to exhibit a strongly bimodal activity rhythm with most activity at the end of their active period even after they entered constant conditions. Placement in constant conditions actually produced a small immediate decrease in the proportion of activity occurring during the activity bout at the beginning of the subjective night, suggesting that the light-dark cycle was masking a more delayed phase of pre-pubertal activity. This activity then consolidated into a unimodal rhythm that peaked near the beginning of the subjective night in a manner that resembled circadian development under entrained conditions. These results resemble those of an earlier report that used a periodogram analysis of the activity rhythms of rats born and raised under conditions of constant light (Diez-Noguera and Cambras, 1990). Within that study, a primary 24-hr circadian harmonic didn't fully emerge until 1-3 weeks post-weaning. Our data also strongly resemble data from previous studies that showed an affect of gonadectomy and hormone replacement on the distribution of activity rhythms under constant conditions (*e.g.* Daan et al. 1975; Iwahana et al. 2008; Karatsoreos et al. 2007). These studies showed that gonadal hormones could restore vigorous activity to the beginning of the subjective night. Thus, despite evidence that pubertal hormones might affect the τ and photic sensitivity of the circadian pacemaker (Carskadon et al. 2004; Hummer et al., 2007; Weinert et al., 1994; Weinert and Kompauerova, 1998), our data suggest that pubertal hormones in this species affect the phasing and distribution of activity rhythms in a manner that is independent from τ and the photic entrainment mechanism. Likewise, our data strongly

indicate that changes in activity rhythms during the pubertal period are not due to the passive masking of rhythms by direct behavioral responses to the light-dark cycle; instead, masking may dampen the expression of these changes.

Our results add to growing evidence that circadian plasticity during puberty is common across the mammalian kingdom. It is well-known that human teenagers show dramatic changes in the phasing of sleep and activity (*e.g.* Crowley, Acebo, and Carskadon 2007; Roenneberg et al. 2004; Thorleifsdottir et al. 2002; Yang et al. 2005), and that these changes exhibit sex differences, with males showing larger changes than females (Roenneberg et al. 2004). Less well-known is the fact that circadian phase-changes during the pubertal period have also been observed during studies of mice, degus, fat sand rats, and rhesus macaques ((*rhesus macaque:* Golub & Takeuchi 2002, *laboratory mouse*: Weinert et al. 1994, Weinert and Waterhouse 1999, *laboratory rat:* Kittrell & Satinoff 1986; McGinnis et al. 2007; *Octodon degus* (degu): Hummer et al. 2007; Tate, Richardson, and Carskadon 2002; *Psammomys obsesus*: Neuman et al. 2005; for full review see Hagenauer et al. 2009).

The structure of these changes differs somewhat by species. Diurnal primates (humans and rhesus macaques) show a relatively advanced phase of activity during pre-puberty, phase-delay during puberty, and then phase advance again during adulthood (Thorleifsdottir, Roenneberg, Golub et al. 2002). Our previous study in the degu also reported a more advanced phase during pre-puberty (Hummer et al. 2007), as did preliminary data in the degu and rat (Hagenauer et al. 2009). However, in the current study we discovered that a more advanced, unimodal rhythm was occasionally observed during adjustment to the experimental conditions regardless of what age the activity recording was initiated. Once the initial days of recording were removed from the analysis, this advanced phase during pre-puberty statistically disappeared. Thus, from our current study in the fast-developing rat, it seems clear that during the post-weaning period in this species, rhythms progress simply from a more phased-delayed, bimodal distribution to one that is relatively phase-advanced and unimodal.

These species differences suggest that a relatively advanced phase of activity during pre-puberty is characteristic of species that develop slowly and progress through a pre-pubertal quiescent period, such as humans and macaques. However, since the species

diversity in reproductive behaviors and sex differences is already well-acknowledged (Crews, 2002) it should not be surprising that the structure of pubertal changes in circadian rhythms is similarly diverse. More interesting then might be questions regarding the evolutionary pressures driving a widespread sensitivity of activity rhythms to gonadal hormones. It seems paradoxical that such precise timekeeping capabilities would be so sensitive to an element of physiology that fluctuates in response to a wide variety of social and environmental stimuli. In a previous review of hormonal affects on the circadian system, Karatsoreos and Silver argued that this sensitivity reflects a feedback loop from circadian modulation of the hypothalamic-pituitary-gonadal axis (2007).

In the context of our developmental data in the rat, we suggest an additional purpose for hormonal influences on circadian rhythms. In the wild, rats of lower social-status exhibit activity rhythms that differ from those of dominant individuals, presumably as a manner of gaining access to resources during a time when there is less personal risk (Calhoun 1962). Similar within-species temporal partitioning has been observed in bellbirds, toads, and trout (Alanara et al. 2001; Craig & Douglas 1984; Freeland and Kerin, 1991). In all of these species, the dominant individuals claim the hours when the greatest rewards are available, which frequently corresponds to the beginning of the active period. Pre-pubertal animals in most species will be lower-ranking than adults. Thus, pre-pubertal rats exhibit an activity distribution typical of lower-status animals: active at times of the night when there is less reward. As they progress through puberty, their rhythms develop characteristics similar to those found in more dominant individuals. Since gonadal hormones are already well-known to signal competitive status (Hirschenhauser and Oliveira, 2006), it makes evolutionary and ecological sense that gonadal hormones would play a role in eliciting these changes in activity distribution. Future pubertal circadian studies that include the social housing (such as those performed by Paul and Schwartz, 2007) of developing rats could help distinguish between these two possibilities.

Acknowledgements

Figure 2.1. Intact males demonstrate a bimodal activity pattern during pre-puberty that disappears by late puberty due to a phase advance of the later activity bout. (A) Average percent of nightly activity (+/- SE) for animals during pre puberty P25-P28, (B) pre/early puberty P33-P36, (C) early/mid puberty P41-P44, (D) mid/late puberty P49-P52 and (E) late/post-puberty P57-P60 from zeitgeber time (ZT) 12 to 24 (the dark period). (F) Example actogram of wheel-running activity across pubertal development. The x-axis shows the clock time for one full day, with the light-dark bar illustrating the time of lights off and lights on. Each line on the y-axis represents one day's worth of activity as measured by wheel-running and has an upper threshold set at 200 wheel turns per 10-minute bin. The arrow indicates first day of prepucial opening, an androgen-dependent secondary-sex characteristic. The solid dark bar within the actogram on the 5th day indicates a power surge.

Figure 2.1
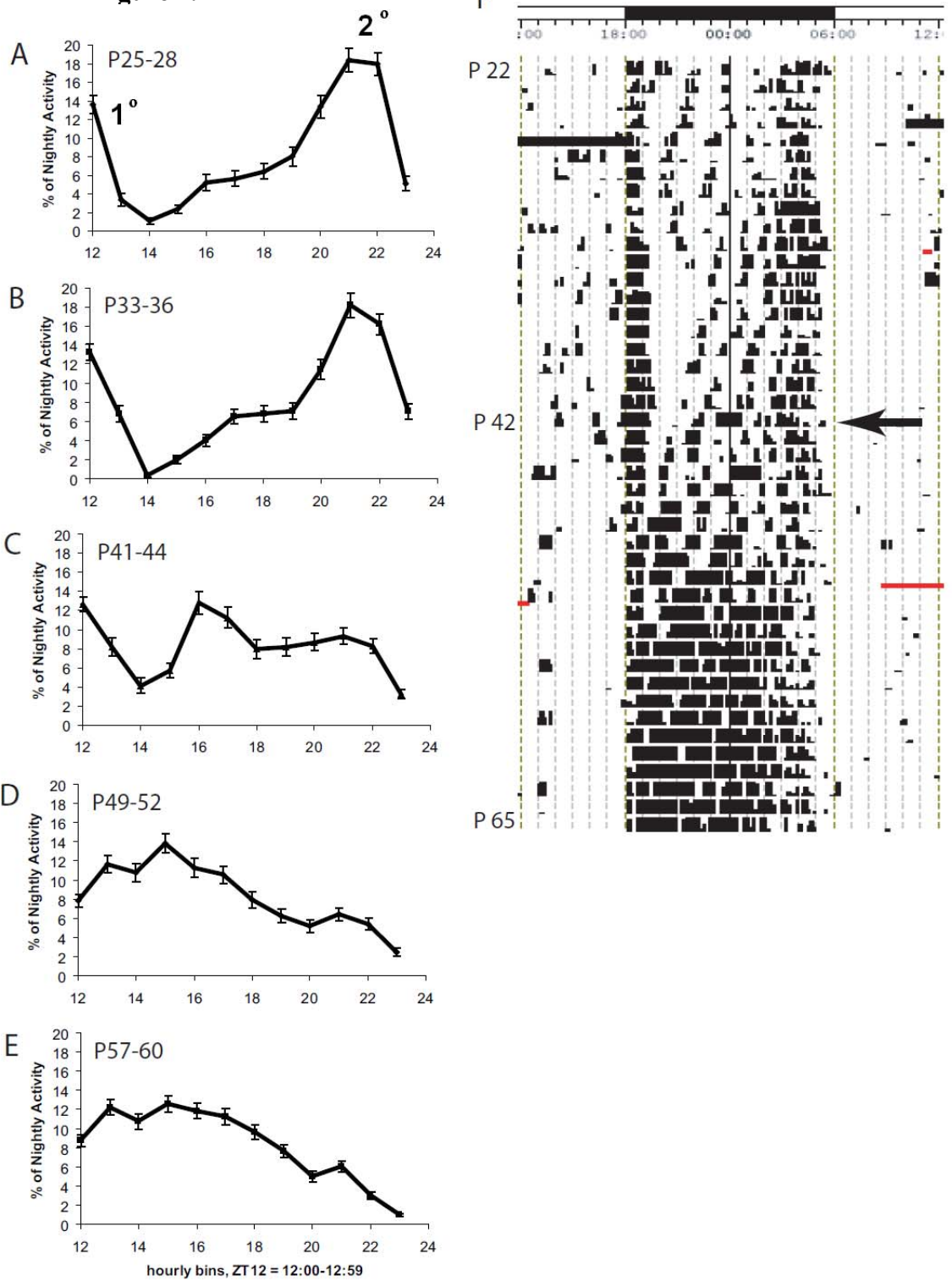
Figure 2.2. Intact females demonstrate a bimodal activity pattern during pre-puberty that disappears by late puberty due to a phase advance of the later activity bout and increase in early night activity. (A) Average percent of nightly activity (+/-SE) for animals during pre/early puberty P25-P28, (B) early/mid puberty P33-P36, (C) mid/late puberty P41-P44, (D) late/post-puberty P49-P52 and (E) post-puberty P57-P60 from zeitgeber time (ZT) 12 to 24 (the dark period). (F) Example actogram of wheel-running activity across pubertal development. The x-axis shows the clock time for one full day, with the light-dark bar illustrating the time of lights off and lights on. Each line on the y-axis represents one day's worth of activity as measured by wheel-running and has an upper threshold set at 200 wheel turns per 10-minute bin. Intact females begin to show an increase in activity approximately every 4 days during puberty due to the initiation of estrous cycles. The arrow indicates first day of vaginal opening, an estrogen-dependent secondary-sex characteristic. The solid dark bar within the actogram on the 5th day indicates a power surge.

Figure 2.2

A P25-28

B P33-36

C P41-44

D P49-52

E P57-60

hourly bins, ZT12 = 12:00-12:59

F

P22

P35

P65
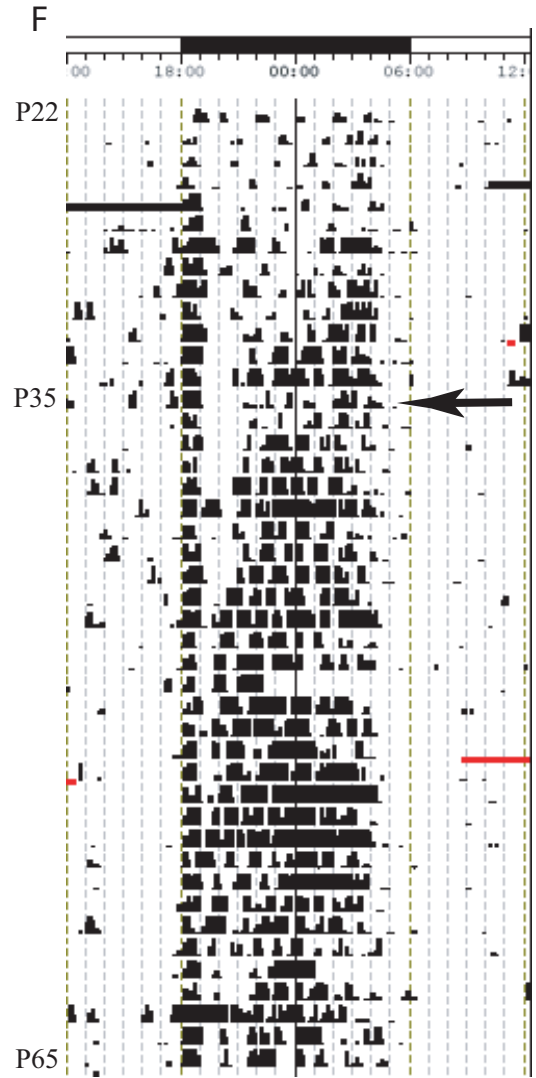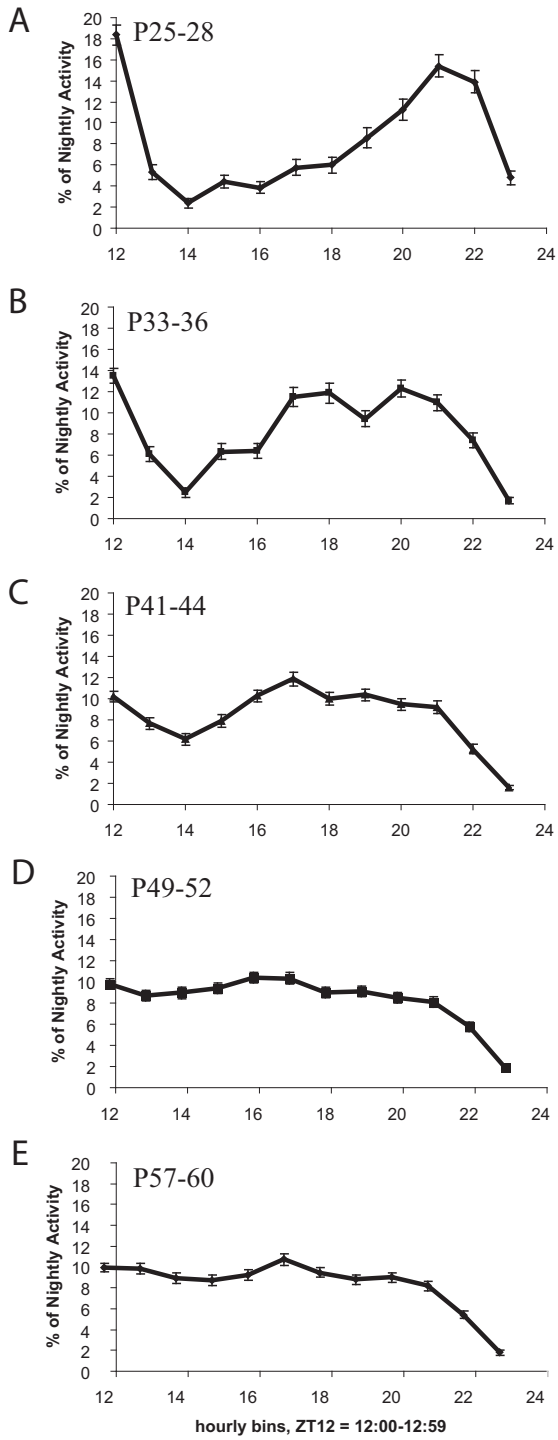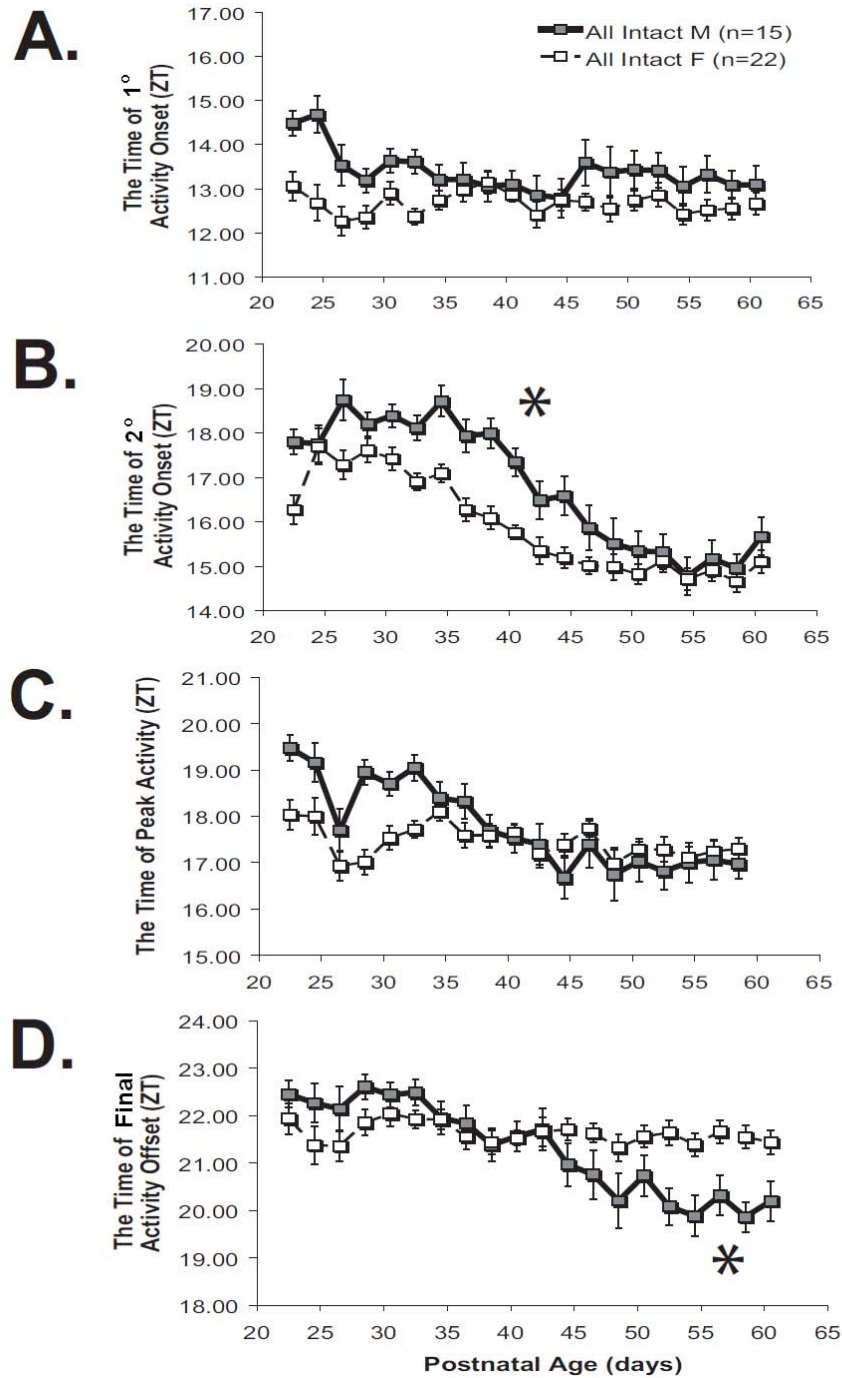
Figure 2.3. Sex differences in circadian phase changes during puberty. Graphs depict bi-daily mean values +/- SE for intact males (grey squares) and females (open squares). Values are given in zeitgeber time (ZT, lights off at ZT12) for the daily (A) initial (1°) activity onset, (B) onset of the second (2°) major activity bout, (C) time of peak activity, and (D) final activity offset. All variables except 1°onset show significant age-related change ($p<0.001$). Asterisks indicate that age-related changes in 2°onset and final offset have significant sex differences ($p<0.05$).

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Figure 2.4. Males gonadectomized (GDX) prior to puberty demonstrate an activity pattern that is bimodal during post-weaning and that remains dispersed into adulthood. The later activity bout diminishes and phase advances, but never fully consolidates with early evening activity. (A) Average percent of nightly activity (+/- SE) for animals during age of pre puberty P25-P28, (B) age of pre/early puberty P33-P36, (C) age of early/mid puberty P41-P44, (D) age of mid/late puberty P49-P52, (E) age of and late/post-puberty P57-P60 from zeitgeber time (ZT) 12 to 24 (the dark period). (F) Example actogram of wheel-running during post-weaning development. The x-axis shows the clock time for one full day, with the light-dark bar illustrating the time of lights off and lights on. Each line on the y-axis represents one day's worth of activity as measured by wheel-running and has an upper threshold set at 200 wheel turns per 10-minute bin.

Figure 2.4

A P25-28

B P33-36

C P41-44

D P49-52

E P57-60

hourly bins, ZT12 = 12:00-12:59

F P21

P41

P62

83
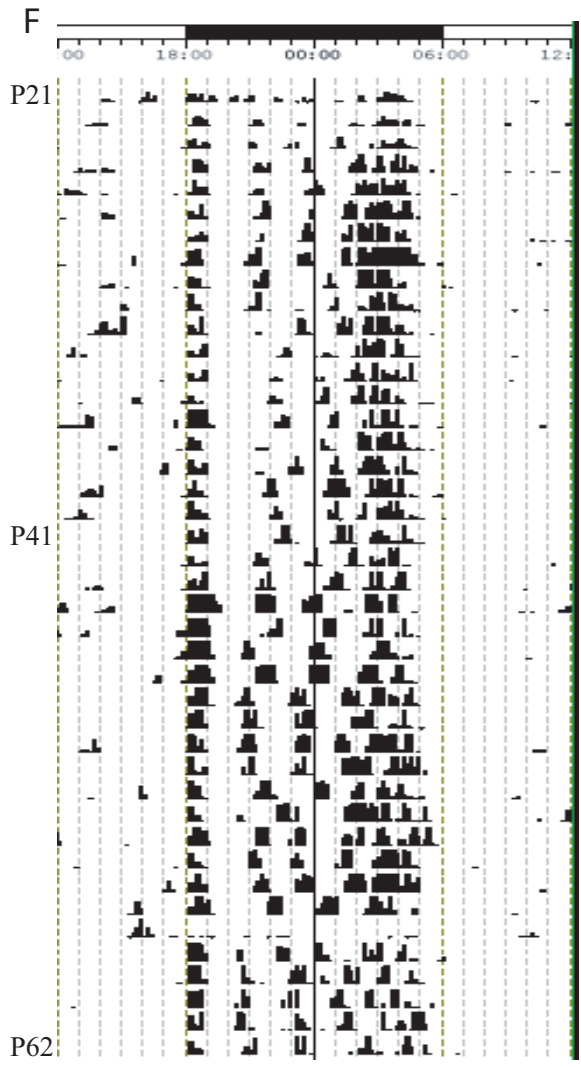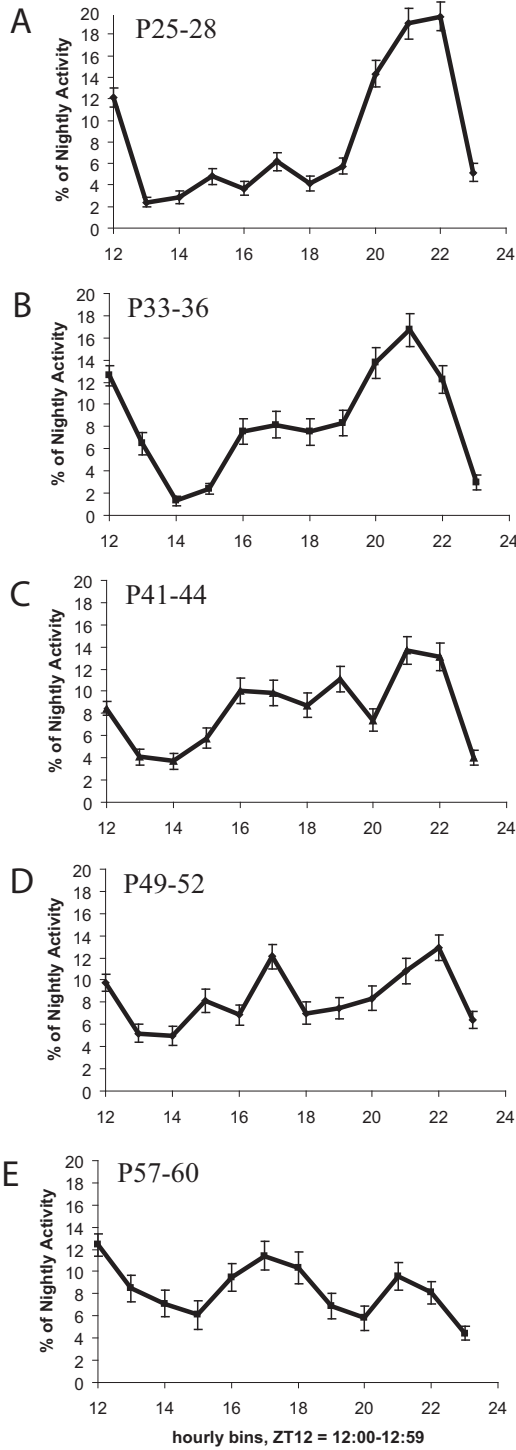
Figure 2.5. Females gonadectomized (GDX) prior to puberty demonstrate an activity pattern that is bimodal during post-weaning and that remains dispersed into adulthood. The later activity bout diminishes and phase advances, but never fully consolidates with early evening activity. (A) Average percent of nightly activity (+/- SE) for animals during the age of pre/early puberty P25-P28, (B) age of early/mid puberty P33-P36, (C) age of mid/late puberty P41-P44, (D) age of late/post-puberty P49-P52, (E) age of post-puberty P57-P60 from zeitgeber time (ZT) 12 to 24 (the dark period). (F) Example actogram of wheel-running during post-weaning development. The x-axis shows the clock time for one full day, with the light-dark bar illustrating the time of lights off and lights on. Each line on the y-axis represents one day's worth of activity as measured by wheel-running and has an upper threshold set at 150 wheel turns per 10-minute bin.

Figure 2.5
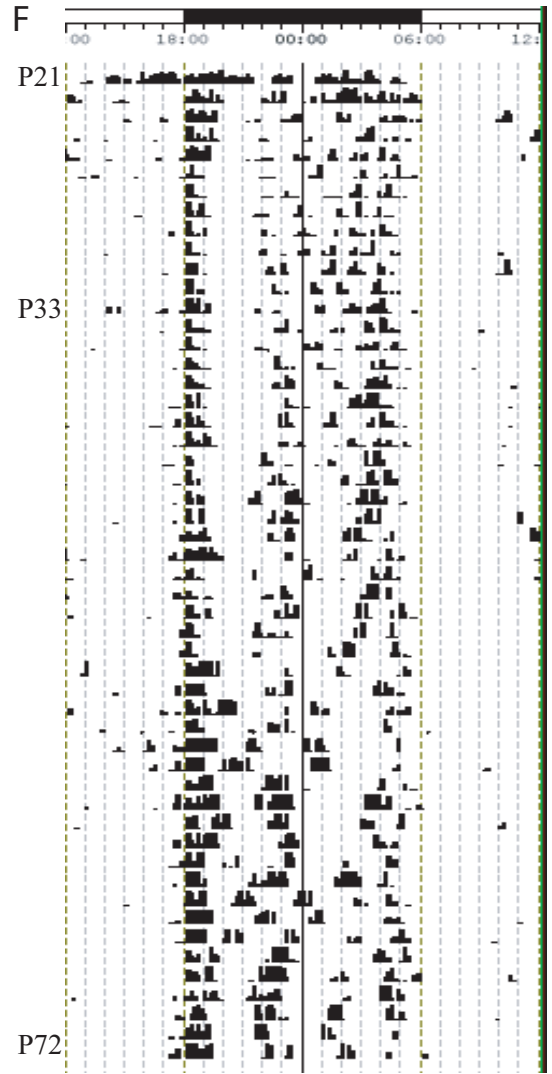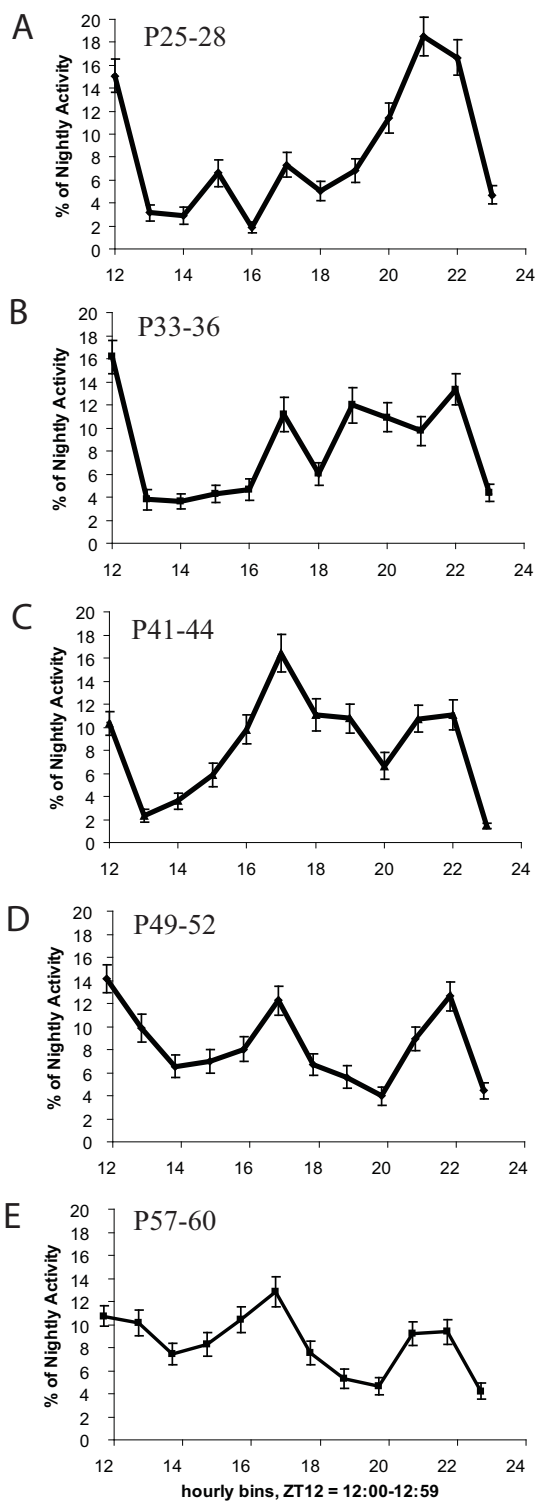
Figure 2.6. Effect of pre-pubertal gonadectomy surgery on developmental changes in circadian phase parameters. The left column of graphs illustrates age-related changes in circadian phase parameters in females, the right column of graphs illustrates males. The points on all graphs represent the mean for a six-day sample (+/- SE), with the age-range for the sample presented on the x-axis in postnatal-days. The line colors indicate gonadal status: Gonadectomized (GDX, light grey), SHAM gonadectomized (dark grey), or no-surgery (black). The y-axis for all graphs is given in zeitgeber time (ZT, lights off at ZT12). Asterisks indicates significant effects ($p > 0.05$), whereas # indicates a non-significant trend ($p < 0.10$). (A&B) The time of initial (1°) activity onset shows changes in SHAM and GDX females but not males or no-surgery females. (C&D) The onset of the second (2°) major activity bout phase advances across development in both males and females. These changes are larger in males, and smaller following gonadectomy surgery. (E&F) The time of peak activity phase advances across development in no-surgery and SHAM males but not in females or GDX males. (G&H) The time of final activity offset phase advances across development in no-surgery and SHAM males but not in females or GDX males.
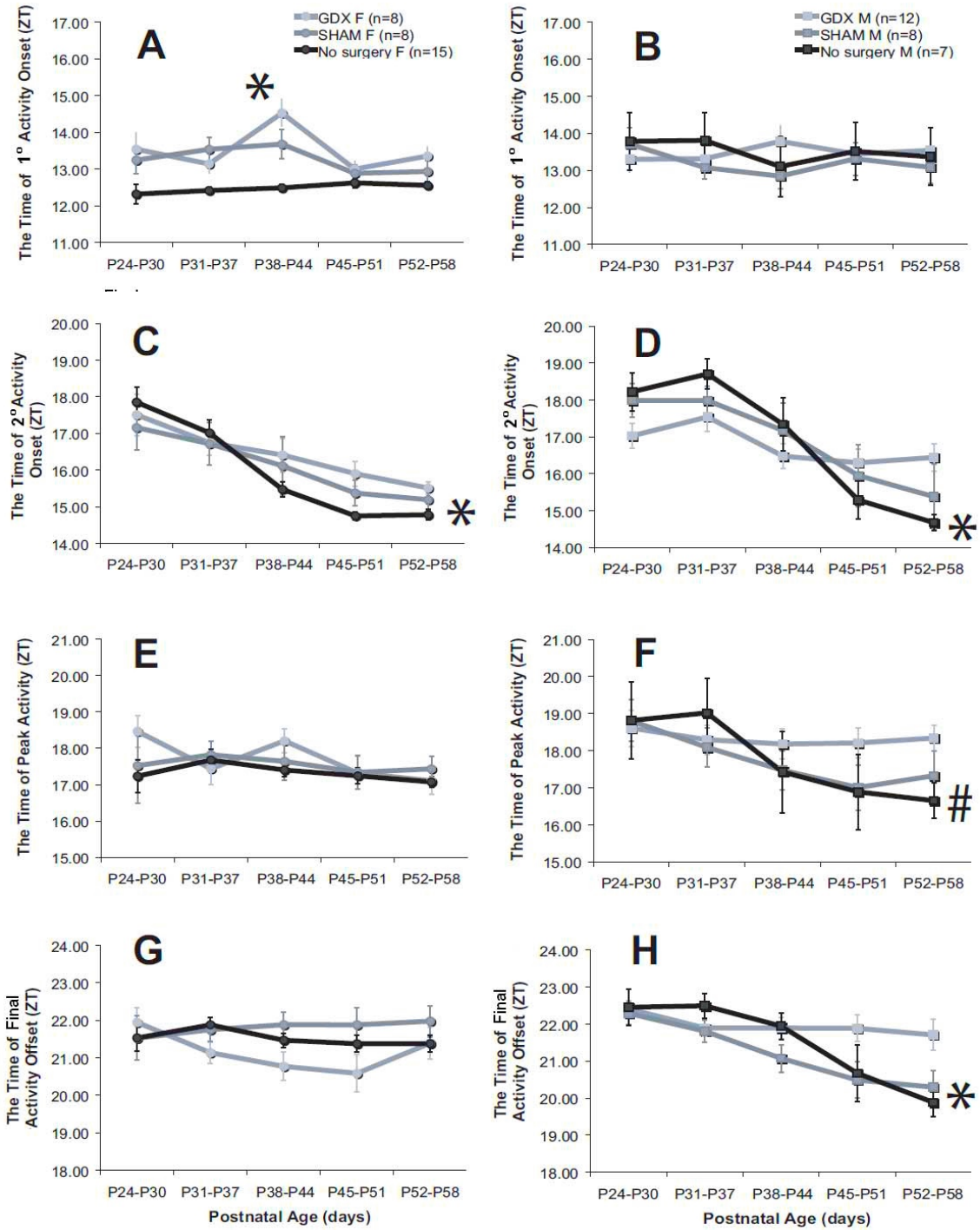
Figure 2.6

Figure 2.7. Indicators of health and maturation in *Experiment 1*. Asterisks indicates significant effects (*p*>0.05). (A&B) Daily mean activity increases across development for females (A) and males (B). Females are more active than males, and no-surgery and SHAM animals are more active than GDX. The points on both graphs represent the mean daily wheel turns/10 min bin for a six-day sample (+/- SE), with the age-range for the sample presented on the x-axis in postnatal-days. The line colors indicate gonadal status: Gonadectomized (GDX, light grey), SHAM gonadectomized (dark grey), or no-surgery (black). (C&D) Weight increases linearly over development for females (C) and males (D). Males weighed more than females after early-mid puberty. No-surgery animals showed the lowest weights. The x-axis shows postnatal age in days (+/- SE) and the y-axis shows weight in grams (+/- SE). (E) The timing of puberty as determined by the age of first prepucial opening for intact males (solid line) and by first vaginal opening for intact females (dashed line). The x-axis shows age in postnatal days and the y-axis shows percent of total animals showing opening. No-surgery rats are depicted in black and SHAM rats are in grey. (F) Testes volume (cm2) increases in a linear fashion across postnatal age (x-axis) in both no-surgery (black) and SHAM males. Each point represents a measurement from a single animal.
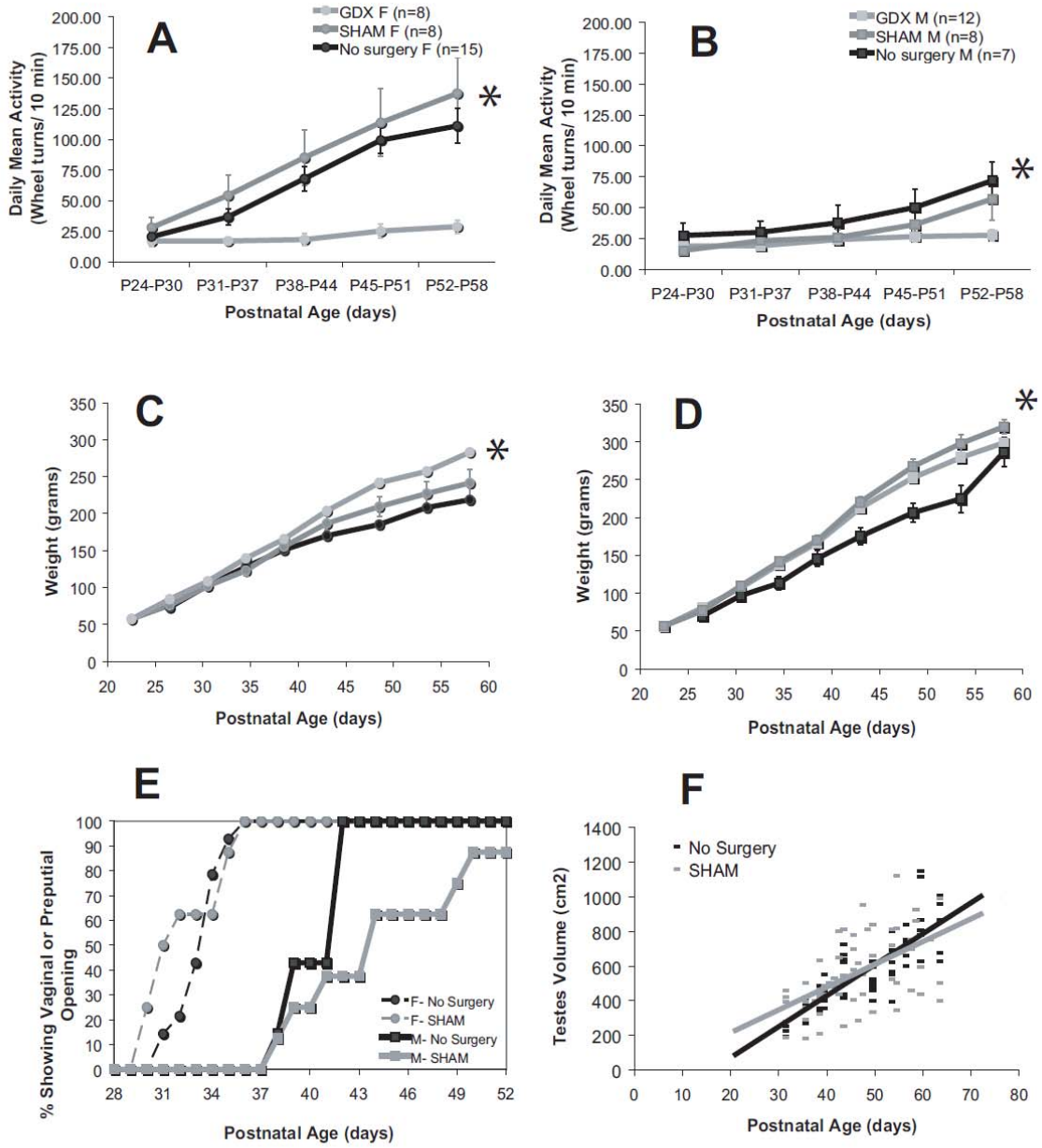
Figure 2.7

Figure 2.8. Representative actograms comparing circadian rhythms in wheel-running activity across pubertal development (ages P24-P70) under entrained and constant conditions during *Experiment 2*. The x-axis shows the clock time for either one or two full days (B and C are double plotted). The light-dark bar at the top of each panel indicates the time of lights off and lights on. Each line on the y-axis represents activity as measured by the number of wheel turns per 10 min bin. The solid dark bar on Day 7 (Actograms A & B) was caused by an equipment failure. **A.** A representative actogram for a male rat under entrained conditions (12:12 LD). Note that the activity rhythm shows a pronounced bimodal pattern during puberty, which diminishes and phase-advances as development progresses. Mean daily activity also increases with age. The upper threshold for the y-axis each day is set at 416 wheel turns per 10-minute bin. **B.** A representative actogram for a female rat under entrained conditions (ages P29-P70) followed by placement in constant conditions (RR, ages P29-P70). The transition into constant conditions is denoted by a grey background. The bimodal activity pattern is still evident following the transition into RR and diminishes with age. The small daily leftward shift in the onset of activity indicates that the rat's free-running period is shorter than 24 hrs. The upper threshold for the y-axis each day is set at 500 wheel turns per 10-minute bin. **C.** A representative actogram for a male rat under entrained (ages P24-28) and constant conditions (RR, ages P29-P70). The small daily rightward shift in the onset of activity indicates that the rat's free-running period is longer than 24 hrs. The upper threshold for the y-axis each day is set at 303 wheel turns per 10-minute bin.

Figure 2.8

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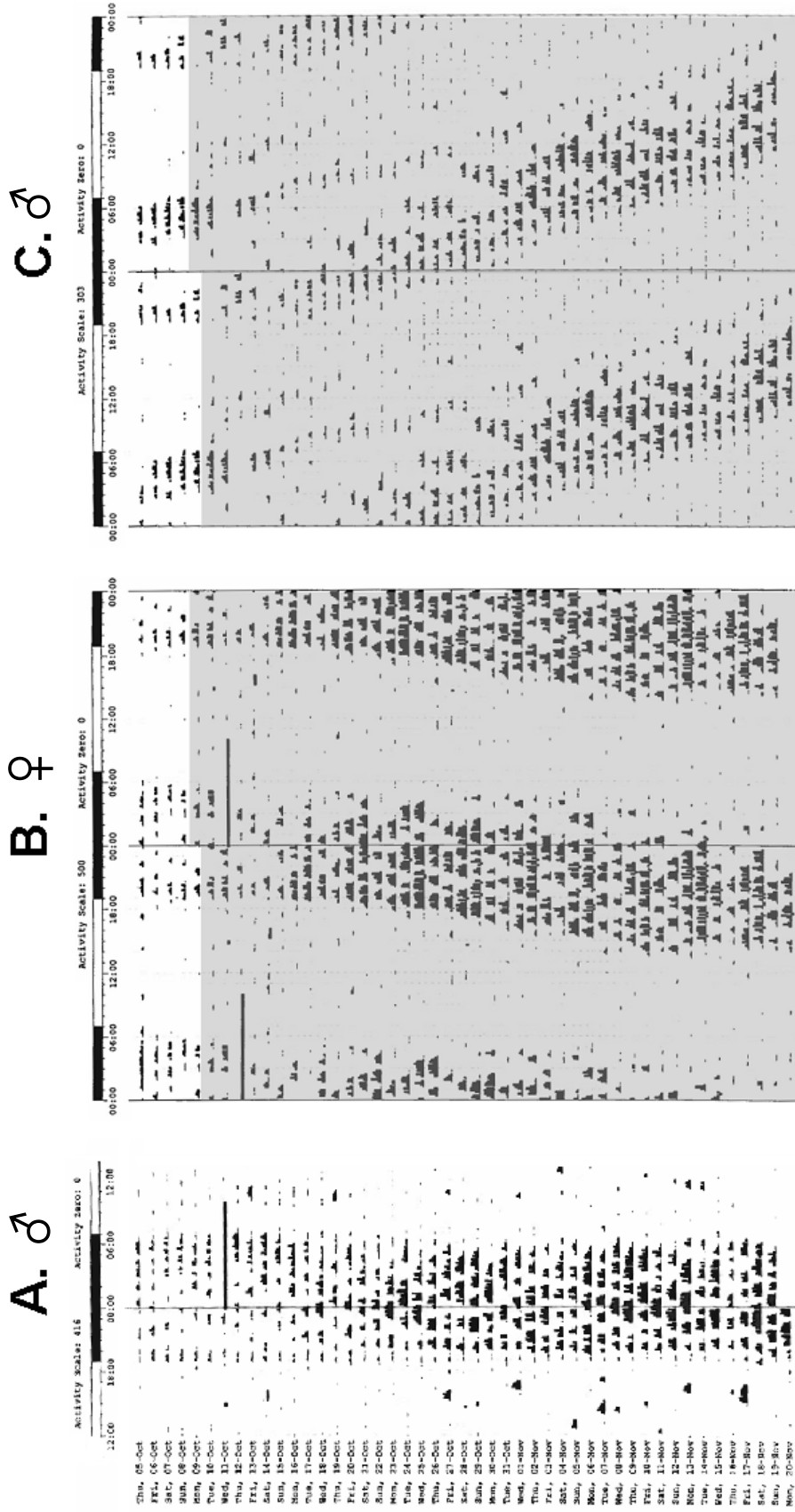
Figure 2.9. Developmental changes in circadian parameters are compared in parallel under entrained and constant conditions. Error bars represent +/-SE. **A.** The timing of peak activity phase-advances across pubertal development (P24-P70) under entrained conditions (12:12 LD, $p<0.001$). This change occurs similarly in both sexes (males = filled squares, females = open squares). The timing of peak activity is represented on the y-axis in zeitgeber time (hrs, lights-off=ZT12). **B.** Nocturnality intensifies across pubertal development ($p=0.008$) as indicated by light-dark activity ratio. A lower score indicates greater nocturnality. Differences between the sexes did not reach significance. **C.** Under constant conditions (RR), free-running period changes in a sex-specific manner across pubertal development (males = filled triangles, females = open triangles, $p=0.035$). Asterisks indicate specific ages at which this sex difference reaches statistical significance ($p<0.05$). **D.** Daily mean activity increases across pubertal development under both entrained (squares) and constant (triangles) conditions ($p<0.001$). Overall activity levels differed by sex ($p<0.001$; the asterisk indicates the specific age at which the difference reached significance ($p<0.05$).

Figure 2.9

Legend:
- ■ LD Males, n=8
- □ LD Females, n=8
- ▲ RR Males, n=8
- △ RR Females, n=8

A. The Time of Peak Activity (ZT)

B. LD Activity Ratio (Light/Dark)

C. Free Running Period (Hrs)

D. Daily Mean Activity (Wheel turns/10 min)

Developmental Stage: P24-P28, P29-P42, P43-P56, P57-P70
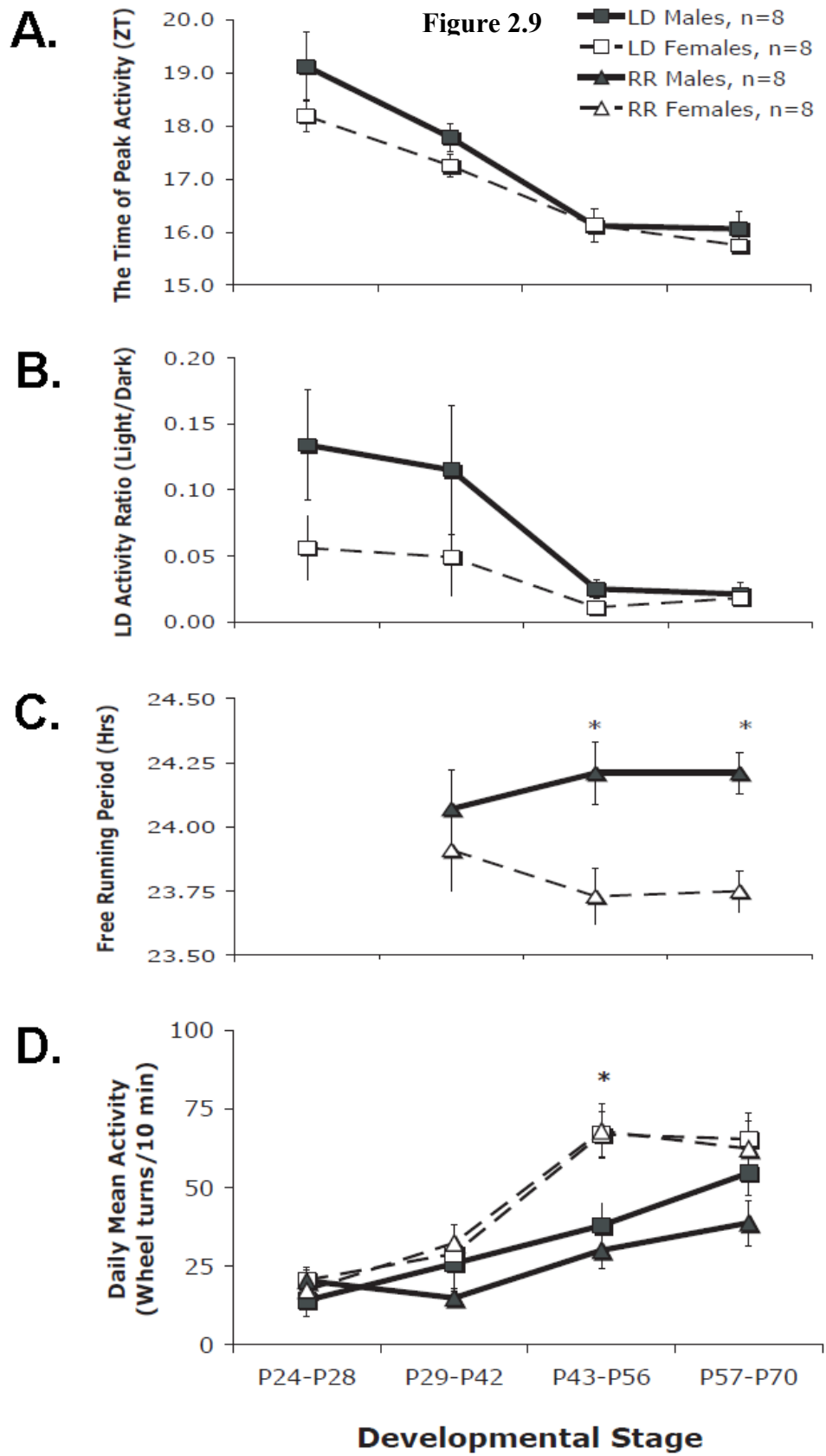
Figure 2.10. Pubertal changes in the distribution of activity across the subjective night persists under constant lighting conditions. A-C. The daily distribution of activity for male rats (n=8) kept under entrained conditions at four ages. Activity is plotted relative to zeitgeber time (ZT, ZT0=lights on). The light cycle is illustrated by the light-dark bar at the bottom of the column of graphs. Each point represents the average activity per hour (+/- SE) from a two-day sample (*i.e.* the point at ZT12= the average activity for 12:00-12.59). The lines illustrate four representative ages: B. pre-pubertal: P27-P28, P29-P30, C. early pubertal: P35-36, D. late/post-pubertal: P67-68. ZT0/24 are double-plotted. Arrows indicate the time of peak activity as measured via cosinor analysis. **D-F.** The daily distribution of activity for male rats (n=8) placed into constant conditions at age P29. Activity is plotted relative to circadian time (CT, CT12-24=subjective night) so that the onset of the active period is aligned with its previous timing under entrained conditions. In the first graph (E) the grey line illustrates the activity of pre-pubertal animals under entrained conditions (P27-P28), and the black line shows activity under constant conditions (P29-30). All other graphs illustrate activity distribution under constant conditions. Note that a bimodal activity distribution is present during pre-puberty and when the rats are placed in constant conditions activity shifts from the initial (1°) activity bout to the later (2°) activity bout (*p*=0.011), demonstrating the influence of photic masking. Then activity phase-advances relative to the onset of the active period by P35-36 (F) and P67-68 (G). **G.** The time of peak activity phase-advances relative to the onset of the active period (CT12) under entrained and constant conditions (*p*<0.001). This advance occurs faster under constant conditions (*p*<0.001) and shows a sex difference (*p*=0.017). Each point represents the average of a two day sample (+/-SE). The first time point occurs under entrained conditions for all groups.

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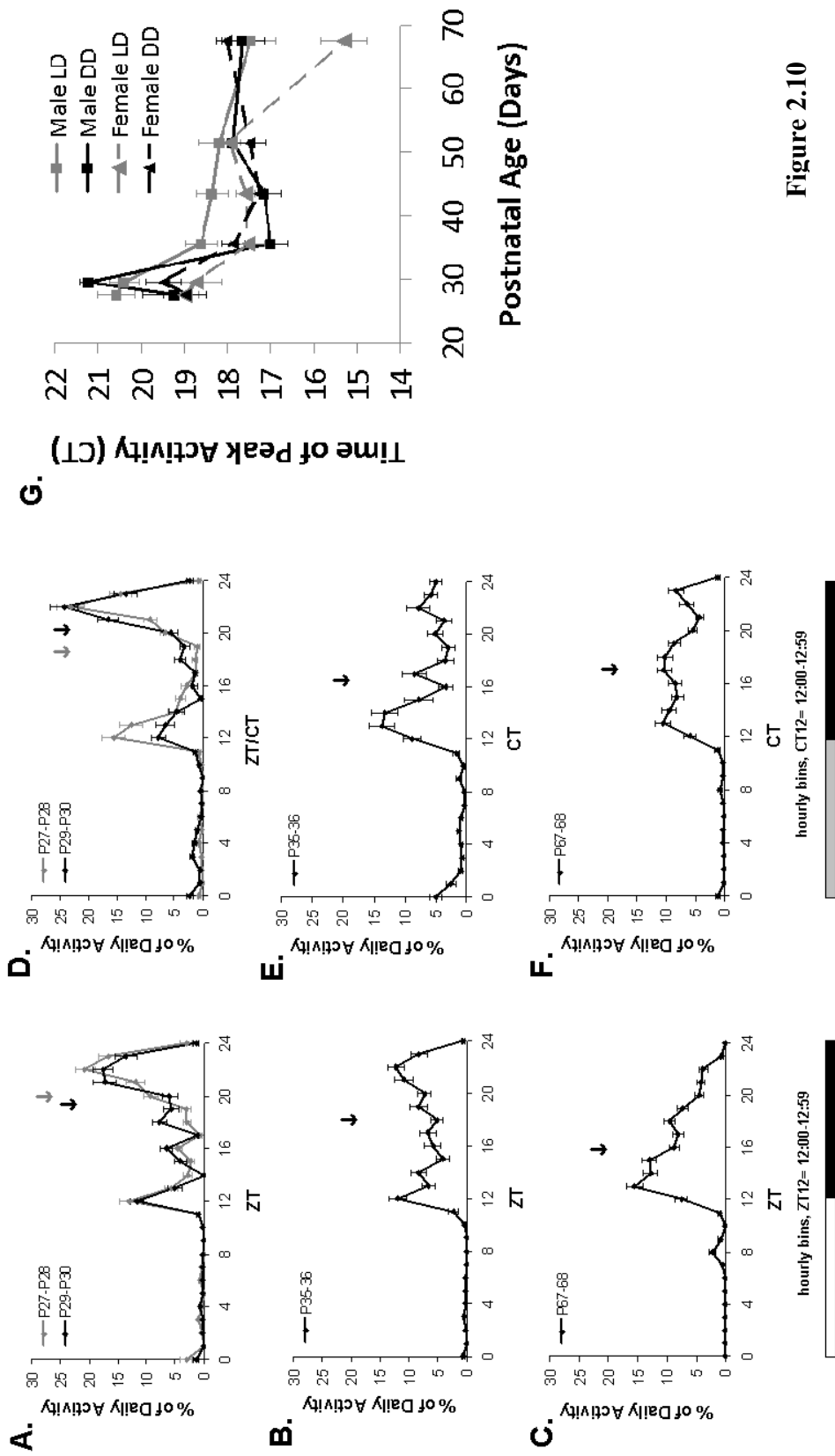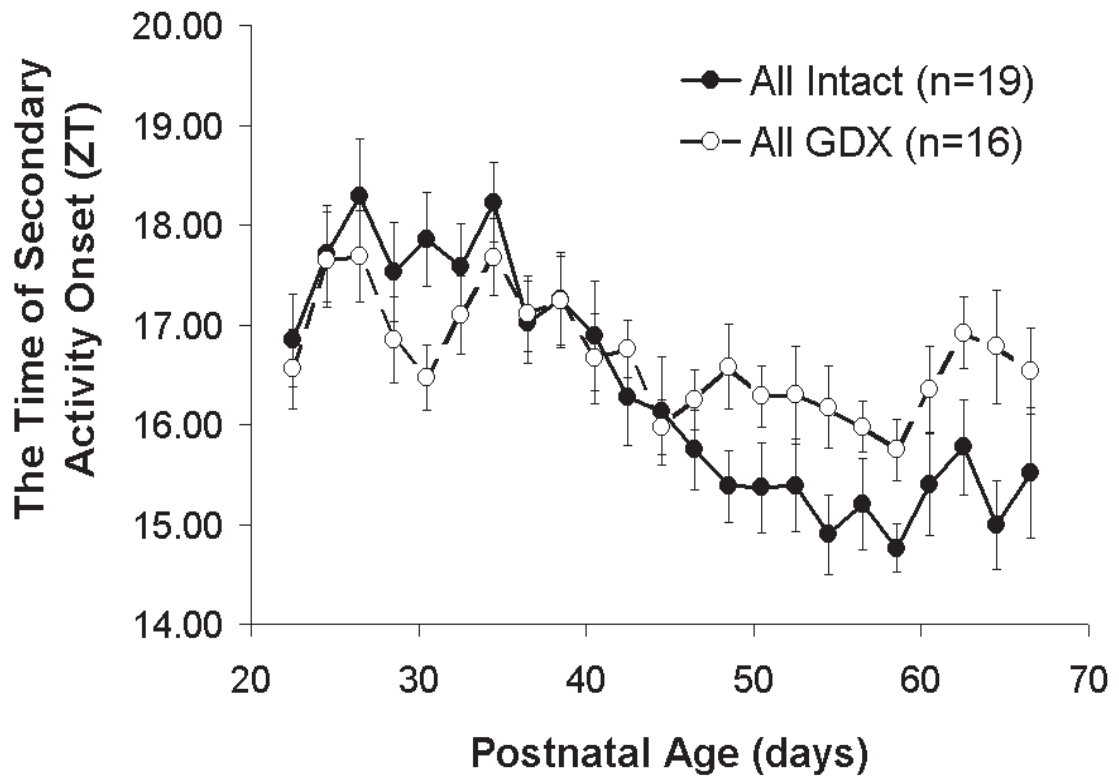
Figure 2.10

Figure 2.11. When considering additional data collected at later ages, gonadectomized animals (GDX: open circles, dashed lines) continue to lack typical age-related changes in the timing of the onset of the second (2°) major activity bout. Any males and females that had data extending out to P68 were averaged to produce this figure. The intact group (closed circles, black line) includes both no-surgery and SHAM rats. Graphs depict mean 2°onset (+/- SE) for every two days across ages P22-P68 in terms of zeitgeber time (ZT, lights off at ZT12).

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Chapter 3

Circadian Reorganization during Puberty Depends on Gonadal Hormones in the Slow-Developing Rodent, *Octodon degus*

Abstract

Human adolescents exhibit dramatically different sleep patterns than adults. Growing evidence suggests that these differences may be related to pubertal changes in the circadian timekeeping system. To test this hypothesis, we carefully tracked daily activity rhythms across puberty in the slow-developing rodent *Octodon degus*. We confirmed that male degus showed a dramatic reorganization of activity rhythms that correlated with secondary sex development during puberty, including a loss of bimodality and 3-5 hr phase-advance. Similar to humans, this circadian reorganization showed distinct sex differences, with females showing little change during puberty in two separate experiments. Prepubertal gonadectomy (GDX) eliminated the changes, whereas SHAM gonadectomy had little impact. Therefore, sex hormones likely play an essential role in pubertal circadian reorganization in this rodent species. Using evidence from a variety of species, including our recent studies in the rat, we conclude that circadian plasticity during puberty is a well-demonstrated phenomenon across the mammalian kingdom. Therefore the pronounced phase-delay exhibited by human teenagers is unlikely to be exclusively of social origin.

Introduction

Recent studies show that most American adolescents maintain schedules during the school year that result in insufficient and ill-timed sleep (National Sleep Foundation 2000). At the root of this chronic sleep deprivation is the delayed sleep onset characteristic of adolescents. Teenagers maintain later bedtimes than younger adolescents, even when wake times are constrained by school or work (Thorleifsdottir et al., 2002; Crowley, Acebo, and Carskadon, 2007). The timing of sleep grows later as puberty progresses, such that the latest, or most delayed, sleep patterns occur around the time of achieving sexual maturity (between the ages of 15-21; Roenneberg et al., 2004; Thorleifsdottir et al., 2002; Yang et al., 2005; Crowley, Acebo, and Carskadon, 2007).

The delayed timing of sleep has been attributed to many external influences, including increased academic responsibilities and late night social opportunities (*e.g.,* Yang et al., 2005; Carskadon, 2002), but there is strong reason to believe that these changes in the timing of sleep are partially rooted in pubertal influences on the body's circadian timekeeping system. Late adolescents continue to exhibit a more delayed (later) phasing of both sleep and endocrine rhythms than early adolescents even under controlled, laboratory conditions (Carskadon, Acebo, and Jenni, 2004; Carskadon et al., 1997). These delayed rhythms of sleep correlate with secondary-sex development, even after taking into account social influences (Carskadon, Viera, and Acebo, 1993; Sadeh et al., 2009). Moreover, the *timing* of circadian changes during adolescence exhibits sex differences in the same manner as the timing of other pubertal events: girls begin to show delayed chronotype earlier than boys. The *magnitude* of these developmental changes shows sex differences as well, and by late adolescence boys are more evening-type than girls (Roenneberg et al., 2004).

This evidence strongly implicates a role for gonadal hormones in adolescent changes in sleep patterns. Indeed, the sensitivity of the circadian timekeeping system to gonadal hormones is already well-documented. In humans, daily rhythms in cortisol and sleep onset shift their timing during different stages of the menstrual cycle (Manber and Bootzin, 1997; Parry et al., 1994; Parry et al., 2000). In adult laboratory rodents, a wide variety of gonadal hormones affect the phase of circadian rhythms, including estrogens,

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progestins, androgens, and non-traditional neuroactive steroids (*e.g.,* rats: Albers, Gerall, and Axelson 1981; Axelson, Gerall, and Albers, 1981; hamsters: Davis, Darrow, and Menaker, 1983; Morin, Fitzgerald, and Zucker, 1977; de Tezanos Pinto and Golombek, 1999; mice: Daan, Damassa, Pittendrigh, and Smith, 1975; Karatsoreos, Wang, Sasanian, Silver, 2007; Iwahana, Karatsoreos, Shibata, and Silver, 2008; degus: Labyak and Lee, 1995; Jechura, Walsh, and Lee, 2000). The sensitivity of the circadian system to these steroidal hormones exhibits sex differences. These sex differences are determined in some species by the organizational effects of gonadal hormones during the perinatal period (rat: Albers 1981; hamster: Zucker, Fitzgerald and Morin, 1980).

The influence of gonadal hormones on the circadian system during puberty is less understood, although it is known that pubertal hormones can alter circadian phase (degu: Hummer et al., 2007; Hummer and Lee, *unpublished data*) as well as produce organizational effects on the circadian system (hamster: Davis, Darrow, and Menaker, 1983). Indeed, in the degu, there is even a critical window of sensitivity to the organizational effects of gonadal hormones as late as young adulthood (Hummer et al., 2006).

Despite these indications that adolescent sleep patterns are likely to be influenced by a circadian sensitivity to pubertal increases in gonadal hormones, there has been little attempt to determine how common these developmental changes are across mammalian species or to elucidate their hormonal or neural bases using animal models (Hagenauer et al., 2009). Evidence from five species suggests that pubertal changes in circadian phase are not uniquely human (*rhesus macaque:* Golub and Takeuchi 2002; *laboratory mouse*: Weinert et al., 1994; Weinert and Waterhouse, 1999; *laboratory rat:* McGinnis et al., 2007; Kittrell and Satinoff, 1986; *Octodon degus* (degu): Tate, Richardson, and Carskadon, 2002; Hummer et al., 2007; *Psammomys obsesus*: Neuman et al., 2005). However, only three of the studies (using the slow-developing, diurnal species of the macaque and degu) have attempted to thoroughly characterize the full developmental progression of circadian phase change in relation to secondary-sex development (Golub and Takeuchi, 2002; Tate, Richardson, and Carskadon, 2002; Hummer et al., 2007), and only one study directly examined the role of pubertal hormones (Hummer et al., 2007). Similar to humans, the macaque and degu show a delayed circadian phase during puberty

(around the time of first menarche in the rhesus macaque, and first vaginal or prepucial opening in the degu) that reverses by adulthood. This developmental change does not occur following pre-pubertal gonadectomy (Hummer et al., 2007). Therefore, pubertal elevations in sex hormones are likely to drive circadian phase changes.

This current study is designed to follow-up on previous work in the degu with more complete measurements of circadian phase and activity distribution across the pubertal period and additional experimental controls of phase-influencing environmental variables (*e.g.,* lighting intensity). The previous study regarding hormonal dependency was provocative but only examined circadian phase at one mid-pubertal time point (Hummer et al., 2007). To gain a better understanding of how well pubertal changes in circadian phase occur and compare to other well-characterized species (*human;* Roenneberg et al., 2004; Thorleifsdottir et al., 2002; *macaque:* Golub and Takeuchi, 2002) it was necessary to sample more frequently across the pre-pubertal and pubertal periods. This more frequent sampling also allowed for a careful examination of any sex differences present in the timing or magnitude of the phenomenon, such as those seen in humans (Roenneberg et al., 2004). Finally, we wished to confirm that the lack of developmental change observed in animals gonadectomized prior to puberty in the previous study (Hummer et al., 2007) was not merely due to a developmental delay, placing circadian changes outside of the sampling window.

The degu may be a particularly useful animal model for researching the role of pubertal hormones in circadian development for several reasons. Unlike other commonly-used laboratory rodents, degu females exhibit spontaneous, long reproductive cycles containing both follicular and luteal phases (Mahoney et al., *submitted*). Thus, the hormonal environment of the degu is perhaps a better approximation to that of human females. The degu's longer reproductive cycles also provides a less-interrupted record of circadian activity, as many rodents show elevated activity levels during estrus (*e.g.,* Axelson, Gerall, and Albers, 1981; Labyak and Lee, 1995). The degu is slower-developing than most laboratory rodent species. In both the wild and the laboratory, weaning does not take place until ~4-7 weeks after birth (Reynolds and Wright, 1979; Kenagy, Place and Veloso, 1999). The pubertal period follows weaning, and by 2-3m of age in the wild, 60% of male juveniles have testosterone levels that resemble those of

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adults (Kenagy, Place, and Veloso, 1999), and by 6m of age, male degus in the wild exhibit high testosterone (Soto-Gamboa, 2004, as cited in Soto-Gamboa, 2005). In the lab, males and females begin to exhibit secondary sex characteristics between 2-3.5m of age, with full maturity reached by 5m (Hummer et al., 2007). This long developmental period allows for a more complete analysis of pubertal circadian development. Additionally, unlike most laboratory rodents, degus are precocial. On the day of birth their eyes are already open and they are fully furred and ambulatory. They can consume solid food after 6 days of life (Reynolds and Wright, 1979). Thus, juvenile degus are relatively independent and by 2-3 weeks of age in the wild they begin to emerge from their burrows (Jesseau, 2004). Thus, the circadian changes that occur during puberty in this species are dissociated from other well-characterized developmental changes in the circadian system related to juvenile rodents gaining independence from the dam (Weinert et al., 2005).

Finally, wild degus are day-active (diurnal) similar to humans, although during the hot summer months they exhibit a more crepuscular pattern of activity when living in conditions that lack sufficient shade (Fulk, 1976; Kenagy et al., 2002; Bacigalupe et al., 2003). More dramatic chronotype flexibility is reported in the laboratory (*e.g,*. Kas and Edgar, 1999; Ocampo-Garces, 2005). This chronotype flexibility in the laboratory appears to be related to thermoregulatory constraints due to running wheel access (Kas and Edgar, 1999; Ocampo-Garces, 2005) and housing temperatures (Hagenauer and Lee, 2008). In this experiment we also found an effect of lighting intensity on degu activity distribution when the degus were housed directly under the light source (*i.e.,* exposed conditions that lacked the cover of shelving).

Methods

General Methods

Subjects: The degus were obtained from a breeding colony at the University of Michigan. All animal handlings including cage changes were performed at random times during the lighted period of the day. All procedures for the housing and handling of the degus were approved by the University Committee on Use and Care of Animals at the University of Michigan.

In the colony, breeding degus were kept in large (42.5 x 46 x 19.5 cm) transparent acrylic cages containing one sire, two dams, and their respective young. One side of each cage was kept on a heating pad at low heat in a room maintained at 20 +/- 1°C with a 12:12-h light-dark (LD) cycle (light intensity 250 lx). During breeding, pregnancy, and early development (0-90 days of age) the breeding animals and pups were provided *ad libitum* with Prolab Laboratory Animal Diet Product 5P06 and acidified water (2.5 x 10^{-5}% HCl) to prevent infections. They were also given generous handfuls of dried alfalfa 2 times weekly. This was reduced to once weekly for pups after weaning, which took place between ages P35-P64. At the age of 3 months (90 days), pups began receiving adult chow (5001 Rodent Diet, PMI Nutrition) and tap water.

Testing environment: The testing environment consisted of two light-tight white wooden environmental chambers. These chambers were maintained on a 12:12 LD cycle (at 21 +/- 1°C). The location of the cages was exposed (no shelving, cages maintained within 1m of the light source), so as to maintain even light exposure. After weaning, the degus were moved to smaller opaque plastic cages (42.5 x 22 x 19 cm) equipped with Nalgene running wheels (9 x 34.5 cm) for individual daily activity recordings. In order to prevent abnormal development due to social isolation (*e.g.,.* Ovtscharoff and Braun, 2001), on alternating weeks the degus were housed with a same-sex sibling. During these weeks, the animals were still kept in the testing environment but activity data were not recorded. During Experiments 1 and 2, the degus were allowed access to a running wheel during this time. This change in protocol was intended to eliminate drifts in circadian phase that were observed during the weekly recording sessions during the pilot experiment (Suppl. Experiment 1), as wheel exposure can exert fast influence on degu chronotype under laboratory conditions (Kas and Edgar, 1999).

Monitoring activity rhythms: Activity rhythms were quantified using the number of running wheel turns per 10 min bin and stored using Vitalview Software (Mini mitter, Bend OR). These data were visualized as double-plotted actograms using Actiview Software (Mini mitter, Bend OR). Days with disruptions in data collection, while rare, were discarded from analysis.

The distribution of daily wheel-running activity was characterized for each degu's weekly recording session by averaging the activity counts for each of the ten minute bins

(i.e., data from the 06:10-06:20 bin would be averaged across 7 days of recording). To control for between-subjects variation in overall activity level, these average bins were converted to a percentage of daily activity for the weekly recording session. In order to examine pubertal changes in the distribution of daily activity, we averaged these distributions for animals in each of the experimental groups using two-week age bins across pubertal development.

We also quantified several parameters indicative of circadian phase relative to the LD cycle. These parameters varied a little depending on the experiment, but typically included activity onset, activity offset, the time of peak activity, and the ratio of activity during the light period to activity during the dark period (LD ratio). The time of peak activity, the LD ratio, and the mean activity level for each degu's weekly recording session were calculated automatically using Actiview Software (Mini mitter, Bend OR). Within this program, the time of peak activity is determined using cosinor analysis, with the peak defined as the acrophase of a cosine curve fit to the activity data.

The time of activity onset and activity offset were calculated either blindly by hand (Experiment 1, Supp. Experiment 1) or semi-automatically (Experiment 2, Supp. Experiment 2). In all experiments, if the time of either onset of offset was ambiguous (containing two times that met the definition presented below), then both potential values were averaged. When scored by hand, activity onset and offset were scored by two experimenters blind to experimental treatment. Morning activity onset was defined as the first three consecutive bins of data which contained activity levels that exceeded the weekly mean activity level and that followed at least two hours of early morning inactivity (generally falling between ZT20-ZT23). Evening activity offset was defined as the last three consecutive bins of data which contained activity levels that exceeded the weekly mean activity level and preceded at least two hours of early morning inactivity. If a degu was found to have a strongly nocturnal activity pattern (LD Ratio <0.5) then it was scored as if it were a nocturnal animal, with onset defined as the first three consecutive bins surpassing the weekly mean activity level around the time of lights-off, and offset defined similarly but in proximity to lights-on.

When activity onset and offset were scored semi-automatically the parameters were calculated using the activity distribution produced for each degu's weekly recording

session. Morning activity onset was defined as the first three consecutive bins of activity that surpassed the weekly mean activity level following a period of relative inactivity in the early morning. The time of evening activity offset was calculated similarly, and was defined as the end of the last three consecutive bins that surpassed the weekly mean activity level. The predominant active period was calculated using a moving-window analysis of the activity distribution produced for each degu's weekly recording session. The twelve-hour period that contained the highest average activity was defined as being the animal's predominant active period, and the first bin of this period was defined as the onset of that active period

Statistical Analyses: All analyses were reviewed by the Center for Statistical Consulting (CSCAR) at University of Michigan to ensure proper interpretation. These analyses were performed using SPSS 17.0 software (SPSS, Chicago IL) with an alpha of 0.05. To determine if there was a relationship between pubertal development and circadian parameters, two statistical models were used for each experimental data set:

1) *Linear model*: The variable of age was best treated as a continuous variable, because it contained individual variations in startpoint, endpoint, and full sampling duration (*e.g.,* **Supplementary Table 1**). Therefore, to examine age-related change in each of the circadian variables, as well as group differences in the circadian variables, we examined the slope and intercept of a linear model fit to the data. The linear fit was performed using a "Random Coefficients Model" so that each animal had its own random intercept and slope with unstructured covariance within the linear mixed model function of SPSS.

The individual intercept term takes into account each degu's individual variability in chronotype. It seemed most appropriate to control for this variability using circadian data from a relatively stable time period, thus the intercept was always centered around a post-pubertal age (*e.g,.* P150). Depending on the experiment, intercept and slope terms were also included to provide group-related comparisons. For any particular model, if there was not sufficient variability in a term to run the model (for example, the individual intercept), that term was removed from the model and the conclusions for that term were treated as being clearly non-significant.

2) *Marginal model:* The linear model makes full use of the variable of age, but relies on the assumption that age-related changes fit a linear form. To provide a second analysis of age-related variation in the circadian variables we also ran a marginal model that defined the circadian variables as repeated measures. For this model, the variable of age was treated as categorical and divided into two week sampling bins. Bins that contained data from less than 70% of the animals were deleted from the analysis. To properly control for the correlated error terms produced by a repeated measures protocol, the appropriate covariance structure for the marginal model was determined by comparing the Akaike Information Criterion (AIC) for several different model types that are commonly used for repeated measures data sets: Diagonal, First-Order Autoregressive (AR(1)), and Compound Symmetry.

Activity from the first week the animals had access to a wheel was removed when constructing figures in order to exclude any possible behavioral changes the animals might have exhibited while adjusting to the testing environment. Similarly, all statistical analyses were performed twice using data that included the first week of recording as well as data that excluded it.

Experiment 1

This experiment tracked activity rhythms of male degus across pubertal development. Initial pilot work (**Supp. Figures 3.6&3.7**) indicated that pubertal changes might be diminished when the light phase of the LD cycle was of high intensity, therefore this experiment also explored two lighting intensities. All variables that might alter the chronotype of the degus (housing temperature, proximity to light source, wheel exposure, handling times) were controlled.

Subjects and Housing: Eight male *Octodon degus* (P61-P66) were selected from two litters the first week following weaning. To examine the effects of lighting condition on pubertal changes in circadian rhythms, degus were divided evenly between two light-sealed boxes that were maintained at different light intensities within a 12:12 LD cycle. Four degus were exposed to dimmer lighting levels (10 lux as measured from cage bottom) and kept in opaque dark grey plastic cages. The other four degus were exposed

to brighter lighting levels (60 lux as measured from cage bottom) and kept in opaque white plastic cages. These lighting intensities were chosen because our pilot experiment (Suppl. Experiment 1) indicated that higher intensity (>250 lux) conditions could drive the wheel-running rhythms of young degus towards a nocturnal chronotype. To control for confounding genetic effects, we divided siblings from each litter in this experiment evenly between the two lighting conditions. The location of the degus within the chamber and in relationship to the lights was recorded, and great care was taken to make sure that all lighting and temperature conditions remained stable throughout the course of the experiment.

Monitoring pubertal development: Males were examined weekly for the growth of spikes on the glans of the penis. Penile spikes are an androgen-dependent sign of pubertal development of the male degu (Hummer et al., 2007). To measure penile spike growth, the prepuce was gently retracted. The number, color, and length of the spikes were recorded. Spike length was measured from the base to the tip, and if the spikes were multiple lengths, the shortest length was recorded, or, if the difference between the shortest and longest spikes was greater than 1.5 mm, an average length was recorded.

Statistical Analyses: After confirming that all males had undergone puberty during our recording period, we ran both a linear and marginal model analysis to determine if there was a relationship between postnatal age and circadian parameters, as well as to determine if this relationship varied with the intensity (lux) of the daily LD cycle. The marginal model was run using all age bins because there were circadian data for all animals in each bin. The linear equation used to fit these data was:

$$Y_{it} = \beta_0 + \beta_1 \text{Age} + \beta_2 \text{Lightlux} + \beta_3 (\text{Lightlux} * \text{Age}) + b_{0i} + b_{1i} \text{Age} + \varepsilon_{it}$$

In this linear equation, Y_{it} represents the value of a circadian parameter for any particular individual (i) during a sampling period at age (t): β_0 is the overall intercept, β_1 is the overall slope of within-subject age-related changes in the circadian parameter, Age is the average postnatal age for each of the week-long sampling periods (in days), β_2 is the main effect of lighting intensity (Lightlux: dim or dimmer), and β_3 is the effect of the interaction between lighting intensity and age. The final variables stand for the individual variation in the model fit: b_{0i} and b_{1i} represent the random deviations of an individual's (i) intercept and slope from the overall intercept and slope terms in the model,

respectively, and ε_{it} is the residual deviation of the subject's (i) data at each of the sampling points (t) from the subject's linear model fit. To have intercept terms that reflected group differences at the age of maturity, the variable of "Age" was centered around P150, which means that 150 was universally subtracted from all age values so that the linear fit would cross the y-axis at a meaningful location.

Experiment 2

This experiment examined sex differences in pubertal changes in circadian rhythms, as well as the role for pubertal gonadal hormones. In addition to the procedural controls used during Experiment 1, some of these animals were maintained in the testing environment with wheel exposure in a cage with dam and siblings before weaning in order to confirm that the changes observed during puberty did not just reflect an overall adjustment to a new housing environment (**Supplementary Table 1)**.

Subjects: This experiment examined the pubertal development and circadian rhythms of 23 male and 27 female *Octodon degus* (degus) from weaning (P39-50) until maturity (~P156-194) under different hormonal conditions. It was conducted in three waves (Jul-Oct, Jan-Apr, May-Sept), with each wave containing degus from 3-4 litters to ensure genetic diversity.

Gonadectomy surgery: To examine the influence of pubertal hormones on circadian parameters degus were either 1) gonadectomized (GDX) or 2) SHAM gonadectomized prior to weaning. These surgeries occurred before external evidence of pubertal onset (between ages P22-P47) during the light phase of the LD cycle. To control for genetic effects, same-sex siblings were evenly divided between GDX and SHAM groups when possible. During the procedure, degus were anesthetized with 4% isoflurane gas and a saline injection was given in order to prevent dehydration. In the females, two 1-2 cm long dorsal incisions were made. Then, if a female was assigned to the GDX group, the ovarian blood supply was cauterized and the ovaries were removed. In the males, one 1-2 cm long ventral incision was made. Then, if a male was assigned to the GDX group, the blood supply to the testes was cauterized and the testes and epididymis were removed. All internal incisions were sutured using dissolvable suture, and skin incisions were closed using surgical staples. External wounds were coated in Nolvasan

antiseptic (Fort Dodge Labs, Madison, NJ) and the animals were kept on a heating pad until regaining consciousness. During recovery, the degus were placed back in the home cage with siblings and dam. Staples were removed 8-11 days after surgery.

Testing environment: During recovery from surgery, the dam and litter were moved into the testing environment. The 12:12 LD cycle had a light intensity of ~100 lx at cage level. After at least six days of recovery from surgery, the degus were provided with running wheels. At weaning, the degus were moved to individual dark gray, opaque cages equipped with running wheels.

Monitoring Pubertal Development: The progress of puberty was monitored by examining secondary sex development. All animals were also weighed every week to monitor their growth.

Males: Males were examined weekly for the development of a prepucial opening, and, later, for the growth of spikes on the glans of the penis. The development of prepucial opening was defined as the first observation of a full separation between the prepuce (or sheath) and glans of the penis as determined by gently pulling on the skin external to the urethral cone. The development of a prepucial opening is androgen-dependent in degus (Jechura TJ and Lee TM, unpublished data) and one of the first visible signs of puberty in male rodents (Hummer et al., 2007; Korenbrot, Huhtaniemi, Weiner, 1977). The growth of penile spikes was recorded in a manner identical to Experiment 1.

Females: Intact female degus are sexually receptive for one day during their three-week estrous cycles. They maintain vaginal openings only during a few days of elevated estrogen. Therefore, pubertal development cannot be monitored using vaginal cytology as is typical in other rodents (Labyak and Lee, 1995). Instead, we monitored pubertal development by examining female degus three times weekly for the presence of vaginal opening beginning between ages P25-58. We also blindly scored running wheel activity records for dates that contained the altered activity rhythms typical of estrus: persistent or elevated activity occurring for 1-2 days with an elongated active period (α, Labyak and Lee, 1995). Typically these dates also contained activity at hours when the degu would normally be inactive, as well as a relative *decrease* in activity and delayed active phase during the following day. Circadian analyses were later run with data that

both included and excluded dates with estrus-typical wheel-running that was predictive of vaginal opening.

Statistical Analysis: To determine if there was a relationship between pubertal development and circadian parameters, we first ran separate linear and marginal model analyses for the SHAM male and females. Within the marginal model, due to limited sample size, data from the first and last age bins (before P55 and after P180, respectively) was removed from the analysis. The linear equation used to fit these data was:

$$Y_{it} = \beta_0 + \beta_1 Age + b_{0i} + b_{1i} Age + \varepsilon_{it}$$

In this linear equation, the variables are defined in an identical manner as in Experiment 1, except that depending on the analysis the variable of Age can be defined either as postnatal age or as pubertal stage (age relative to either first prepucial or vaginal opening).

After confirming that circadian variables exhibited developmental changes in the intact animals, we ran a full linear and marginal model analysis using all four groups (SHAM and GDX males and females) to determine sex and gonadal dependency. Within the marginal model, due to limited sample size, data from the first and last age bins (before P55 and after P180, respectively) was removed from the analysis. The linear equation used to fit these data was:

$$Y_{it} = \beta_0 + \beta_1 Age + \beta_2 Sex + \beta_3 GDX + \beta_4 (Sex*GDX) + \beta_5 (Sex*Age) + \beta_6 (GDX*Age) +$$
$$\beta_7 (Sex*GDX*Age) + b_{0i} + b_{1i} Age + \varepsilon_{it}$$

In this linear equation, many variables are defined in an identical manner as for Experiment 1 (β_0, β_1, Age, b_{0i}, b_{1i}, ε_{it}). In addition, there are several variables that characterize group effects on the circadian parameter: β_2 is the main effect of sex (male or female), β_3 is the main effect of gonadal status (GDX: GDX or SHAM), and β_4 is the effect of the interaction between Sex and GDX. The next set of variables represents the effect of group variables on the slope of within-subject age-related changes in the circadian parameter: β_5 is the effect of sex on age-related changes, β_6 is the effect of GDX on age-related changes, and β_7 represents the effect of the interaction of Sex and GDX on age-related changes. The intercept terms reflected group differences at the age of maturity by centering the variable of "Age" around P150, which means that 150 was

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universally subtracted from all age values so that the linear fit would cross the y-axis at a meaningful location.

Results

Pubertal Development

Weight: Both sexes gained weight rapidly until around age P80. At this point, weight gain slowed, and finally plateaued by P155. Males weighed more than females (an average of 10 grams across the pubertal period), but GDX and SHAM animals within each sex did not differ (**Figure 3.1A**).

Penile Maturation: All eight males without surgery (Experiment 1) and all eleven SHAM males (Experiment 2) developed both a full prepucial opening and complement of spikes on the glans of the penis. Eleven of the CAST males showed neither characteristic, although 10 temporarily showed a small or partial prepucial opening. One CAST male developed a full prepucial opening on the day of surgery and was subsequently removed from the data analysis.

All males without surgery (Experiment 1) had a full prepucial opening by the first time they were examined between age P61-66, and six of the eight already had their full complement of spikes. These spikes continued to grow at a linear rate until ~P90, at which point the growth plateaued, with final lengths reaching 2-3 mm (mean 2.6 mm +/- 0.14 SE, **Figure 3.1C**).

Full prepucial opening developed in the SHAM males between P38-65, with a median age of P58 (**Figure 3.1B**). Within a week of developing a full prepucial opening, most males showed small white nubs on the tip of the glans that were the beginning of spikes. These spikes grew at a linear rate (**Figure 3.1C**). For 45% of the males, the spikes also increased in number, reaching a full complement of 4-6 spikes between ages P52-P95 (median age P73). Spike length plateaued between ages P100-P135, with final lengths reaching 1.8-3 mm (mean: 2.55 mm +/-0.51 SE).

Estrous Cycles: Twelve SHAM degus showed clear signs of estrous cyclicity as evidenced by both the cyclical presence of vaginal opening and estrus-typical wheel-running activity. The median age of first vaginal opening was P74 (ranging from P33-P89, **Figure 3.1B**). Five animals with relatively early openings exhibited first opening less

than one cycle length from when we first started monitoring. In these cases, it is not clear that the first observed opening was truly the first opening that the female experienced, although it seems likely, since over 50% of the sample clearly demonstrated a first opening at an older age (>P74). Of the remaining two SHAM degus, one died following her first vaginal opening and the second showed two intermediate cycles (dimpling but no full opening), and then ceased to show any indications of cyclicity. Circadian data from these two degus were removed from the data set. Three of the ovariectomized females eventually developed small vaginal openings and were also removed from the data set. All other ovariectomized females (n=10) showed no sign of vaginal opening or cyclic patterns of estrus-typical wheel running.

Pubertal Changes in Circadian Rhythms

  Intact Males: There was a dramatic reorganization of activity rhythms during puberty in the males that never received surgery (*Experiment 1*). At the youngest age, males showed a strongly crepuscular activity distribution, with a large percentage of activity occurring during the evening hours. As the males grew older, this evening activity bout diminished and daytime activity increased (**Figure 3.2A, B).** Therefore, using a linear fit, three circadian phase parameters were found to advance with age: activity onset ($F_{(1, 9.763)}=4.889$, $p=0.052$), the time of peak activity ($F_{(1, 17.549)}=15.511$, $p=0.001$), and activity offset ($F_{(1, 10.470)}=12.558$, $p=0.005$). The light-dark activity (LD) ratio also increased with age ($F_{(1, 19.196)}=8.060$, $p=0.010$), confirming that older males were more diurnal (**Figure 3.2C**). Mean activity (mean # of wheel turns/day) peaked around mid-puberty and then decreased over the rest of the recording period. It did not show linear change with age ($F_{(1, 12.714)}=0.196$, $p=0.665$), but a marginal model found significant overall within-subject variability across age ($F_{(6, 35.461)}=5.664$, $p=0.000$). All other circadian variables similarly showed significant within-subject variability across age using a marginal model (*onset:* $F_{(6, 12.853)}=3.700$, $p=0.023$; *time of peak activity*: $F_{(6, 33.283)}=4.484$, $p=0.002$), $p<0.023$; *offset*: $F_{(6, 31.763)}=3.120$, $p=0.016$), with the exception of the LD ratio ($F_{(6, 33.761)}=1.740$, $p=0.142$). None of the circadian phase variables were significantly affected by lighting intensity ($p>0.10$ using both marginal and linear models), nor did lighting intensity alter

age-related changes in any of the variables ($p>0.10$ using both marginal and linear models, *data not shown*).

The reorganization of activity rhythms that occurred during puberty in SHAM males (*Experiment 2*) was identical to that found for males without surgery (*Experiment 1*). At the youngest ages, males showed a strongly crepuscular activity distribution. This crepuscularity disappeared across pubertal development as the evening activity bout diminished and daytime activity increased (**Figure 3.3A).** Therefore, using a linear fit, three circadian phase parameters were found to advance with age: the onset of the active period ($F(1, 10.937)=4.795$, $p=0.051$), the time of peak activity ($F(1, 10.028)=14.253$, $p=0.004$), and the offset of the evening oscillator ($F(1, 10.437)=4.873$, $p=0.051$, **Figure 3.3B**). The LD ratio also increased with age ($F(1, 19.063)=10.339$, $p=0.005$, **Figure 3.3B**), confirming that older males were more diurnal. The onset of the morning oscillator remained relatively stable across pubertal development ($F(1, 50.091)=1.788$, $p=0.187$). Mean activity (wheel turns/10 min) peaked around mid-puberty and then decreased over the rest of the recording period. It therefore did not show any linear change ($F(1, 10.527)=1.678$, $p=0.223$), but a marginal model did find significant overall within-subject variability across age ($F(8, 73.108)=2.982$, $p=0.006$). For all other circadian variables, the marginal model lacked sufficient power to detect age-related changes ($p>0.15$).

It was ambiguous whether these developmental changes were specifically related to pubertal timing. To test this hypothesis, we compared developmental timing by using age adjusted to first prepucial opening in our statistical models. Most of the circadian variables which exhibited significant relationships with age also showed significant relationships with developmental timing (*active period onset:* $F(1, 10.964)=4.880$, $p=0.049$; *peak activity:* $F(1, 10.010)=14.121$, $p=0.004$; *evening offset:* $F(1, 10.407)=4.999$, $p=0.048$), but LD ratio only exhibited a trend towards a relationship with developmental timing ($F(1, 11.222)=3.552$, $p=0.086$). However, developmental timing did not produce a better model fit than age using the criteria of p-value or model fit (as determined by Akaike's Information Criterion (*AIC*)).

Intact Females: Similar age-related changes were not observed in the circadian rhythms of SHAM Females, even after removing the day of estrus from the analyses (**Figure 3.3C**). If anything, there was a trend towards both the onset of the active period

(F(1, 6.195)=5.022, p=0.065) and the time of peak activity (F(1, 8.004)=3.784, p=0.088) drifting later as the animals grew older. All other variables showed no within-subject change (*morning onset*: F(1, 93.647)=0.120, p=0.730; *evening offset:* F(1, 9.579)=0.040, p=0.847, *LD ratio*: F(1, 16.661)=0.263, p=0.615, **Figure 3.3D**). When pubertal timing was included in the model using age in relationship to first vaginal opening, females continued to lack significant age-related changes in circadian phase (p>0.10). Similar to males, mean activity did not show a linear change during puberty when examined in relationship to age (F(1, 10.345)=1.479, p=0.251) nor to age in relationship to first vaginal opening (F(1, 9.347)=1.038, p=0.334), and the females showed no within-subject variability across age using the marginal model (F(8, 76.663)=1.626, p=0.131). For all circadian phase variables, the marginal model lacked sufficient power to detect age-related changes (p>0.35).

Hormonal Dependency

The activity rhythms of both males and females gonadectomized (GDX) prior to puberty followed developmental trends that resembled those of SHAM females. As the animals grew older, the rhythms were essentially stable, although slightly more crepuscular and evening type (**Figure 3.3E,G**). There was no significant within-subject change in the GDX males for any of the circadian phase variables using a linear model (**Figure 3.3F;** *active period onset*: F(1, 10.776)=1.576, p=0.236; *peak activity:* F(1, 10.430)=0.558, p=0.472; *evening offset:* F(1, 9.888)=1.623, p=0.232; *LD ratio*: F(1, 25.512)=0.925, p=0.345; *morning onset*: F(1, 21.351)=2.200, p=0.153). The marginal model indicated a trend towards GDX males showing a delay in the onset of the active period (F(8, 69.619)=2.036, p=0.055) but all other variables showed no change (p>0.18). In GDX females, there was a significant delay in evening offset as indicated using the linear model (F(1, 8.328)=5.191, p=0.051) but no change in any of the other phase variables (**Figure 3.3H;** *active period onset*: F(1, 8.736)=0.147, p=0.711; *peak activity:* F(1, 8.635)=0.089, p=0.773; *LD Ratio*: F(1, 31.309)=0.157, p=0.695; *morning onset*: (F(1, 35.838)=2.019, p=0.164). The marginal model confirmed the within-subject change in evening offset in the GDX females (F(8, 58.599)=2.293, p=0.033) and the lack of change in any of the other variables (p>0.36)

Therefore, a linear model including all four groups indicated that GDX significantly influenced age-related change in most circadian phase variables. There was a main effect of GDX on age-related changes in evening offset ($F(1, 39.409)=8.085$, $p=0.007$; but not *peak activity*: $F(1, 38.207)=2.580$, $p=0.116$; *active period onset*: $F(1, 38.414)=1.130$, $p=0.294$; *LD Ratio*: $F(1, 40.573)=2.346$, $p=0.133$; *morning onset*: $F(1, 36.081)=2.364$, $p=0.133$ **Figure 3.4**). There was also an interaction between the effects of GDX and sex on age-related changes in the timing of peak activity ($F(1, 38.207)=7.162$, $p=0.011$) and onset of the active period ($F(1, 38.414)=4.995$, $p=0.032$; but not *evening offset*: $F(1, 39.409)=0.941$, $p=0.338$; *morning onset:* $F(1, 36.081)=0.279$, $p=0.601$; or *LD Ratio*: $F(1, 40.573)=2.133$, $p=0.152$). Surprisingly, age-related changes in mean activity were not affected by GDX ($F(1, 39.617)=0.189$, $p=0.666$) even after accounting for sex ($F(1, 39.617)=0.301$, $p=0.586$). The marginal model lacked sufficient power to detect group differences, but it did identify overall within-subject change in the offset of evening activity ($F(8, 262.509)=1.977$, $p=0.050$) and the timing of peak activity ($F(8, 282.457)=1.924$, $p=0.056*trend$).

In addition to effects of GDX, a linear model fit including all groups (male and female, GDX and SHAM) also indicated that the effect of age on circadian phase was dependent on sex with males showing greater changes than females, as indicated by sex differences in the changes in the time of peak activity ($F(1, 38.207)=6.154$, $p=0.018$). Age-related changes in other circadian phase variables were not found to show sex differences within the linear model (*active period onset:* $F(1, 38.414)=2.953$, $p=0.094$; *evening offset*: $F(1, 39.409)=2.440$, $p=0.126$; *LD ratio*: $F(1, 40.573)=0.534$, $p=0.469$; *morning onset:* $F(1, 36.081)=0.338$, $p=0.564$). A marginal model again lacked sufficient power to detect sex-related effects on any of the circadian phase variables ($p>0.19$).

Sex differences were clear in overall mean activity. Mean activity peaked at mid-puberty and then decreased over the rest of the recording period in both sexes, but the duration of this pubertal period of elevated activity was longer in males, leading males to have overall higher activity during the recording period even though post-pubertal activity levels were almost identical between the sexes. Therefore, mean activity in all groups showed some overall linear age-related change (**Figure 3.5;** $F(1, 39.617)=4.524$, $p=0.040$), in a manner that did not differ by sex ($F(1, 39.617)=0.474$, $p=0.495$), but a

marginal model found that sex influenced within-subject variability across age in activity levels (F(8, 281.579)=2.921, *p*=0.004), as well as showed a trend towards influencing activity levels overall (F(1, 48.829)=3.569, *p*=0.065).

Controlling for wheel adaptation

To control for wheel adaptation, any recordings performed within the first week of wheel exposure were removed from the analysis. For Experiment 1, the conclusions were mostly unchanged when using a linear model, although the age-related effects in diurnality and activity onset were weakened and lost significance (*LD ratio:* F(1, 14.122)=3.003, *p*=0.105, *activity onset*: F(1, 8.182)=1.893, *p*=0.205). Conclusions using a marginal model were weakened regarding the age-related change in activity onset (F(5, 14.025)=2.124, *p*=0.123), activity offset: (F(5, 28.538)=1.924, *p*=0.121) and LD ratio (F(5, 29.093)=0.919, *p*=0.483).

For Experiment 2, removing the first week of wheel exposure from the data set had little overall effect on the developmental changes discussed above. In the SHAM males, age-related changes in two circadian variables became trends (*active period onset*: F(1, 10.339)=3.961, *p*=0.074); *evening offset:* F(1, 10.522)=3.868, *p*=0.076). For SHAM females, trends towards age-related change in the onset of the active period and the timing of peak activity dissolved (*time of peak activity*: F(1, 8492)=1.928, *p*=0.200; *onset of the active period:* F(1, 7.394)=2.459, *p*=0.159). The delay in offset in GDX females weakened to a trend using the linear model (F(1, 7.440)=3.895, *p*=0.087). All conclusions regarding the effects of GDX and sex on age-related change remained strong and unchanged.

Discussion

These experiments in the slow-developing degu indicate changes in daily activity distribution and circadian rhythm phase over the course of pubertal development that are dependent on sex. In four separate experiments (*Experiments 1&2, Supplemental Experiments 1&2*) we found that male degus exhibited a profoundly crepuscular distribution of activity between post-weaning and pubertal onset, with the majority of activity occurring at the beginning of the dark period. As the males progressed through

puberty, evening activity diminished and phase-advanced, and daytime activity increased. By the age of maturity (5 months), the activity rhythms of male degus were strongly diurnal. Females, in contrast, showed a crepuscular rhythm during the post-weaning period that remained stable into maturity. Male and female animals gonadectomized (GDX) prior to puberty maintained a more delayed, crepuscular activity distribution across development similar to intact females. These results indicate that pubertal hormones drive circadian changes in intact male degus.

These results closely parallel data regarding the development and sex differences in sleep in this species. Perryman (*in preparation*) examined sleep in mid- and late-pubertal male degus using electrophysiology. She found that mid-pubertal male degus spent less time in NREM sleep around the crepuscular transitions than their late-pubertal or adult counterparts, and spent significantly more time in NREM during the light phase. These data complement the current results indicating that pubertal male degus are more active at the crepuscular transitions and less active during the day. Perryman also found that adult females maintained a more crepuscular distribution of sleep than adult males. Collectively, these sleep data indicate that the sex differences in pubertal changes in activity distribution that we observed in these experiments are unlikely to represent artifacts due to running wheel exposure (such as seen in Kas and Edgar, 1999). These sex differences are also unlikely to be a side-effect of surgery, as they were present in both SHAM animals (*Experiment 2*) and animals that had not undergone surgery (*Experiment 1, Supplemental Experiment 2*). Sex differences were also not due to estrus-induced wheel-running, as circadian analysis including and excluding the days of estrus produced similar results (*data not shown*).

The pubertal changes in activity distribution and circadian phase exhibited by the degu also strongly resemble our recent data in the developing nocturnal rat (Chapter 2). In that study, we found that rats showed a phase-delayed, bimodal activity distribution during post-weaning that consolidated into a more phase-advanced, unimodal rhythm by maturity. These pubertal changes in the rat showed sex differences in magnitude, with males showing larger circadian phase changes than females. However, females still continued to exhibit some developmental changes in circadian phase. Pre-pubertal

gonadectomy diminished these developmental changes in both sexes, causing a more distributed and delayed activity distribution (Hagenauer et al. *in preparation*).

The similarity between the pubertal circadian development of these two species suggests that these changes are not limited to animals from a particular temporal niche or developmental trajectory. These data also suggest that circadian changes during puberty are not limited to animals that are known for exhibiting chronotype flexibility. Indeed, the circadian changes observed during puberty in both species are remarkably similar to those observed in other laboratory rodents in response to differing levels of testosterone (*mice:* Daan et al., 1975; Iwanahana et al., 2008; Karatsoreos et al., 2007; *hamsters:* Davis et al., 1983; Morin et al., 1981). In those studies, castrated adult males showed an altered distribution of activity under entrained and free-running conditions such that activity was dispersed across the active period and less cohesive at activity onset (Morin et al., 1981), or the initial activity bout was diminished, lost, or delayed (Karatsoreos et al., 2007; Iwanahana et al., 2008; Daan et al., 1975; Davis et al., 1983). The administration of testosterone or di-hydrotestosterone was able to restore the adult castrates to their original circadian activity patterns (Karatsoreos et al., 2007; Iwanahana et al., 2008; Daan et al. 1975; hamsters: Morin et al. 1981). Earlier studies with the adult male degu showed different effects of castration on circadian phase (Jechura et al., 2000), in which activity onset advanced instead of delayed. Thus, the pubertal effects of male gonadal hormones on circadian phase and activity distribution in degus may be specific to this developmental time period. Future hormone replacement studies during puberty are required to distinguish between these possibilities.

In should be noted that the female degus in the current experiment, as well as in the sleep studies, were more crepuscular than is typically observed in previous studies in our lab or in the wild (*e.g.,* for review see Hagenauer and Lee, 2008). The reason for this appears to be related to housing conditions. In the current study, the degus were housed directly under the light source in order to prevent variability in light exposure. In our recent sleep studies, degus were also housed in more exposed conditions (transparent cages) to allow the animals to be visually monitored. These housing conditions are likely to influence activity distribution, since we found in our pilot experiment that exposed conditions combined with relatively bright light (250 lux) drive degus towards a more

crepuscular/nocturnal chronotype (this was further confirmed in Lee and Hagenauer, *in preparation*). Thus, we dimmed the lights for Experiments 1 & 2, and the majority of male degus showed a highly diurnal activity pattern by maturity.

However, this leaves open the possibility that the more crepuscular activity distribution observed in the females may represent sex differences in the sensitivity of degu chronotype to environmental variables like housing and lighting conditions. This seems possible, as Hummer et al. (2007) observed a delayed phase during mid-puberty in *both* male and female degus, which disappeared upon reaching maturity. In that earlier study, the degus were housed on shelves which provided overhead cover (and therefore they were more diurnal overall) but lighting intensity was also less controlled, which could have produced artifacts in the phase-angle results. Similarly, in our recent study in the developing rat, we found that both males and females showed circadian changes during puberty, although these circadian changes exhibited sex differences in magnitude (Hagenauer et al., *in preparation*). As a preliminary test of the possibility that photic masking could account for the lack of developmental changes in the activity rhythms of female degus, we examined the activity records of nine female degus that were placed in DD during mid-puberty or late/post-puberty (*Supplementary Experiment 2*). The first two days after placement in DD, we observed an immediate increase in activity during the subjective day and a 1-3.5 hr phase-advance of circadian parameters, suggesting that rhythms were masked at both ages. However, a comparison of these "unmasked" rhythms gave little indication of pubertal change. Masking also seemed unlikely to account for the lack of circadian phase-advance that we observed during the development of GDX animals in the current study, as our previous studies in the degu under less exposed housing conditions (Hummer et al., 2007) as well data in the rat (Hagenauer et al. *in preparation*) similarly indicated that pre-pubertal gonadectomy inhibited circadian phase changes during puberty. Collectively, these results support our interpretation that the pubertal reorganization of activity rhythms arises from a gonadal-hormone sensitive circadian system.

Interestingly, earlier reports in the degu reported a more advanced phase during pre-puberty (Hummer et al., 2007), as did preliminary data in the degu and rat (Hagenauer et al., 2009), in a manner that better resembled the sleep patterns of children

(*e.g.,* Thorleifsdottir et al., 2002). However, in these current studies we discovered that a more advanced, unimodal rhythm was occasionally observed during adjustment to the experimental conditions regardless of what age the activity recording was initiated. Once the initial days (for the rat) or week (for the degu) of recording were removed from the analysis, this advanced phase during pre-puberty statistically disappeared (*e.g., Supplementary Pilot Experiment*). Early exposure to the testing environment also diminished this advanced phase (*e.g,. Experiment 2*). Whether this artifact can fully account for the discrepancies between our current data and that of Hummer et al. (2007) is unclear. During our current study puberty occurred almost a month earlier than in Hummer et al. (2007) due to the overall improved health and rapid growth of our colony. Thus, during these new studies the earliest data may have been collected when the animals were already undergoing the initial neuroendocrine changes associated with puberty. Indeed, in the wild newly-emerged juvenile degus (approximately 2-4 weeks old) exhibit rhythms of extra-burrow activity that are *more* unimodally diurnal and phase-advanced than those of their adult counterparts (Fulk, 1976). We should be able to address this issue in the future by tracking the development of degu rhythms prior to weaning using new technology that allows for the simultaneous recording of co-housed animals (Paul and Schwartz, 2007).

Our results add to growing evidence that circadian plasticity during puberty is common across the mammalian kingdom. Dramatic changes in the phasing of sleep and activity occur during puberty in humans, mice, rats, degus, fat sand rats, and rhesus macaques (*e.g.,* Roenneberg et al., 2004; Thorleifsdottir et al., 2002; Yang et al., 2005; Crowley, Acebo, and Carskadon, 2007; Golub and Takeuchi, 2002; Weinert et al., 1994; Weinert and Waterhouse, 1999; McGinnis et al., 2007; Kittrell and Satinoff 1986; Tate, Richardson, and Carskadon, 2002; Hummer et al., 2007; Neuman et al., 2005; for full review see Hagenauer et al., 2009). Thus, the tendency for teenagers to stay up late likely has both biological and social impetus. In this case, to reduce the epidemic of adolescent sleep deprivation (National Sleep Foundation, 2000), it may not be sufficient to recommend that teenagers go to bed earlier. Perhaps more useful would be public health efforts that acknowledge that the phenomena may have similarities to jet-lag, and that therefore encourage circadian interventions, such as increased exposure to morning

sunlight, dimmer light in the evening, and overall sleep hygiene (National Sleep Foundation, 2000).

Acknowledgements

Figure 3.1. Pubertal development in the degu. A. Both males (left) and females (right) showed the fastest weight gain before 100 days of age, although females overall weighed less than males. Gonadectomized male (CAST) and female (OVX) degus showed similar growth curves as intact animals (SHAM M and SHAM F). Error bars represent +/- SE. **B.** SHAM males and females both showed the first external indication of pubertal onset (first prepucial opening for males and first vaginal opening for females) between ages P33-P89. **C.** Spikes on the glans of the penis continued to grow (length in mm +/- SE) until age ~P100 for both naïve males (Exp. 1, black) and SHAM males (Exp. 2, dark grey).

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Figure 3.1

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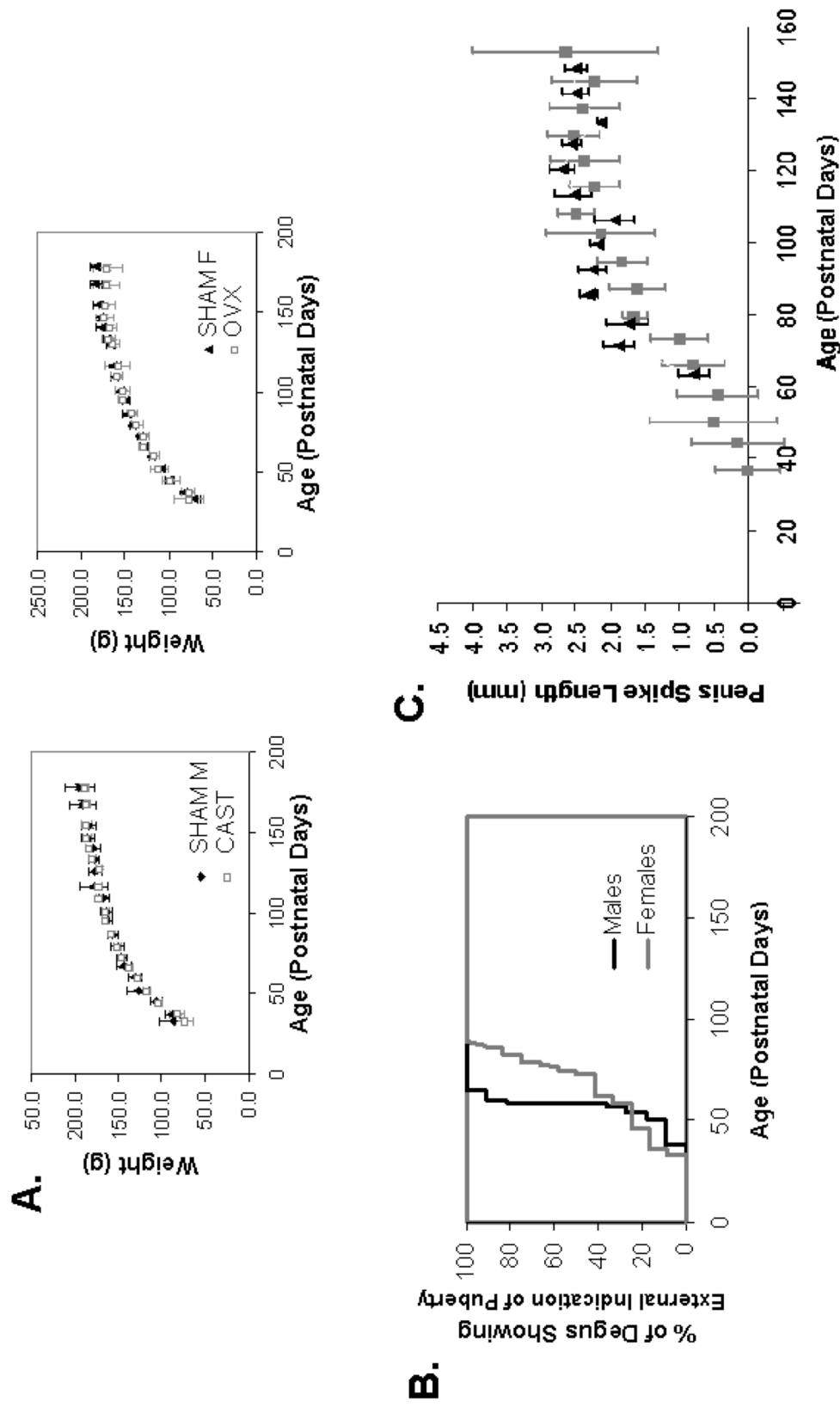
Figure 3.2. Male degus show a dramatic reorganization of circadian activity during puberty. A. A representative double-plotted actogram tracking wheel-running activity rhythms across pubertal development in one male. Each horizontal line on the graph sequentially represents 2 days of activity (# of wheel turns/ 10 min bin) and the LD cycles for both days are indicated by bars at the top of the figure. Grey bars indicate week-long gaps in the recording when the degu is living with his sibling. The average postnatal age of the animal during each recording session is listed on the left. Note that as the degu grew older his activity during the hours immediately following lights-off (evening) diminished and his daytime activity increased. **B.** The average percent of daily activity occurring during each 10 min bin across the day is presented for several ages during pubertal development (n=8). Cool colors represent data from the youngest recording sessions (early pubertal), and warm colors represent the oldest recording sessions (late-pubertal). The white and black bar at the top of the figure illustrates the time of lights on and lights off. **C.** All circadian phase parameters indicate that intact male degus phase-advance during pubertal development. The y-axis depicts the average within-subject change in each parameter, with positive values indicating that the parameter is more advanced than the individual's mean for the recording period (P75-P152). To avoid introducing possible artifacts, the first week in the wheel (mean age P65) is disregarded in all calculations and left off the graph, although its parameters followed the same developmental trends. In the case of the LD ratio, positive values indicate a relatively more diurnal activity distribution. X- and Y-error bars represent +/-SE
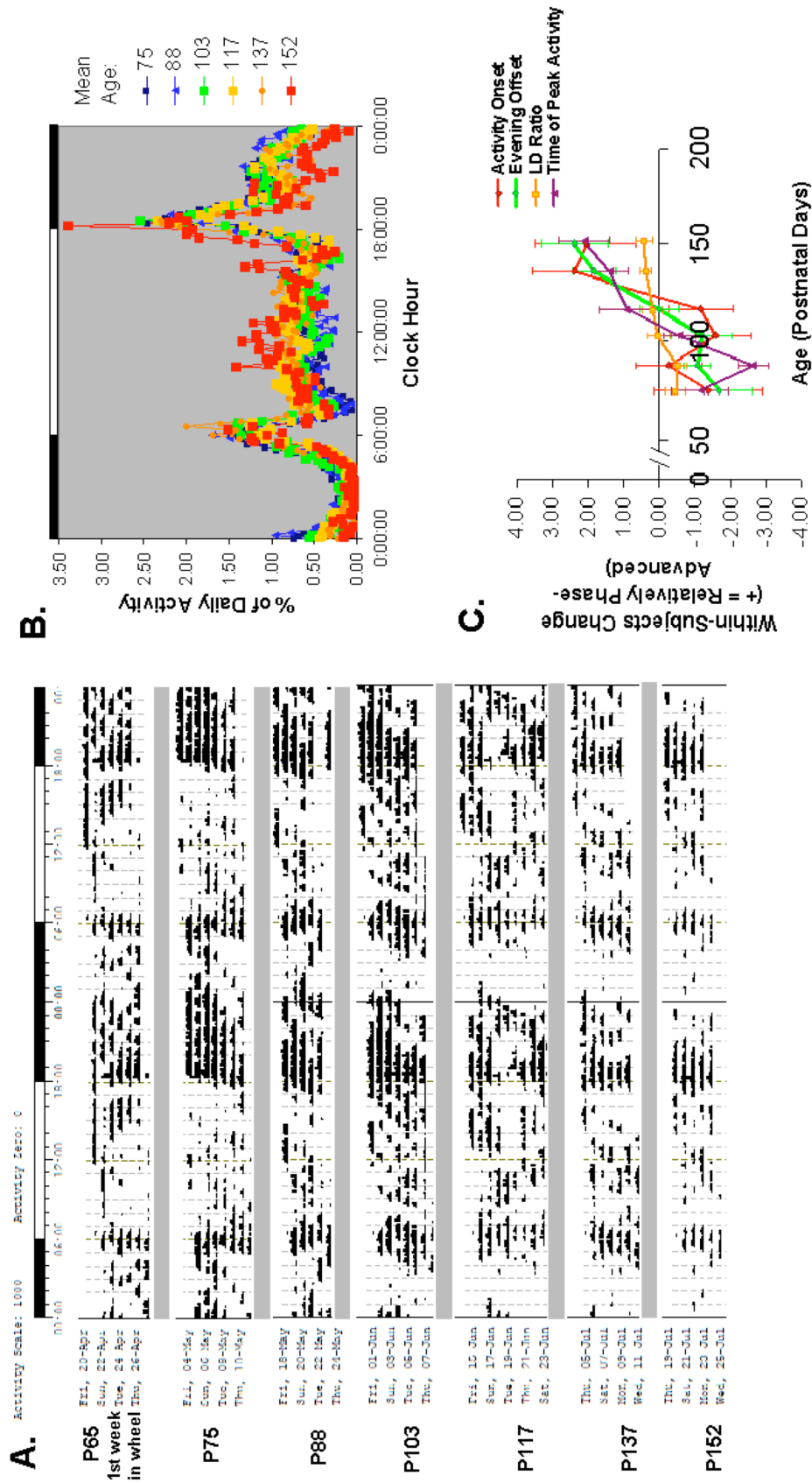
Figure 3.2

Figure 3.3. The reorganization of circadian activity during puberty is dependent on pubertal gonadal hormones. A. As the SHAM male degus (n=11) grew older their activity during the hours immediately following lights-off (evening) diminished and their daytime activity increased. Depicted is the average percent of daily activity occurring during each 10 min bin across the day is presented for several ages during pubertal development. Cool colors represent data from the youngest week-long recording sessions (early pubertal), and warm colors represent the oldest recording sessions (post-pubertal). The white and black bar at the top of the figure illustrates the time of lights on and lights off. **B.** These changes are reflected in the phase advance of several circadian parameters shown by SHAM males during pubertal development. Depicted is the average within-subject change in each circadian phase-parameter across development. Positive values on the y-axis indicate that the parameter is more advanced than the individual's mean for the recording period (P75-P152). To avoid potentially introducing artifacts, the first week in the wheel (before age P55) is disregarded in all calculations and left off the graph, although parameters followed the same developmental trends. In the case of the LD ratio, positive values indicate a relatively more diurnal activity distribution. X- and Y-error bars represent +/-SE. **C, E, G.** SHAM females (n=12), castrated (CAST) males (n=11), and ovariectomized (OVX) females (n=10) did not show the same developmental changes in activity distribution as SHAM males. In general, their evening activity increased and daytime activity decreased across pubertal development. **D, F, H.** Unlike SHAM males, degus from all other groups (SHAM females, Castrated (CAST) males, and Ovariectomized (OVX) females) showed either a small delay or no change in circadian phase parameters across development.
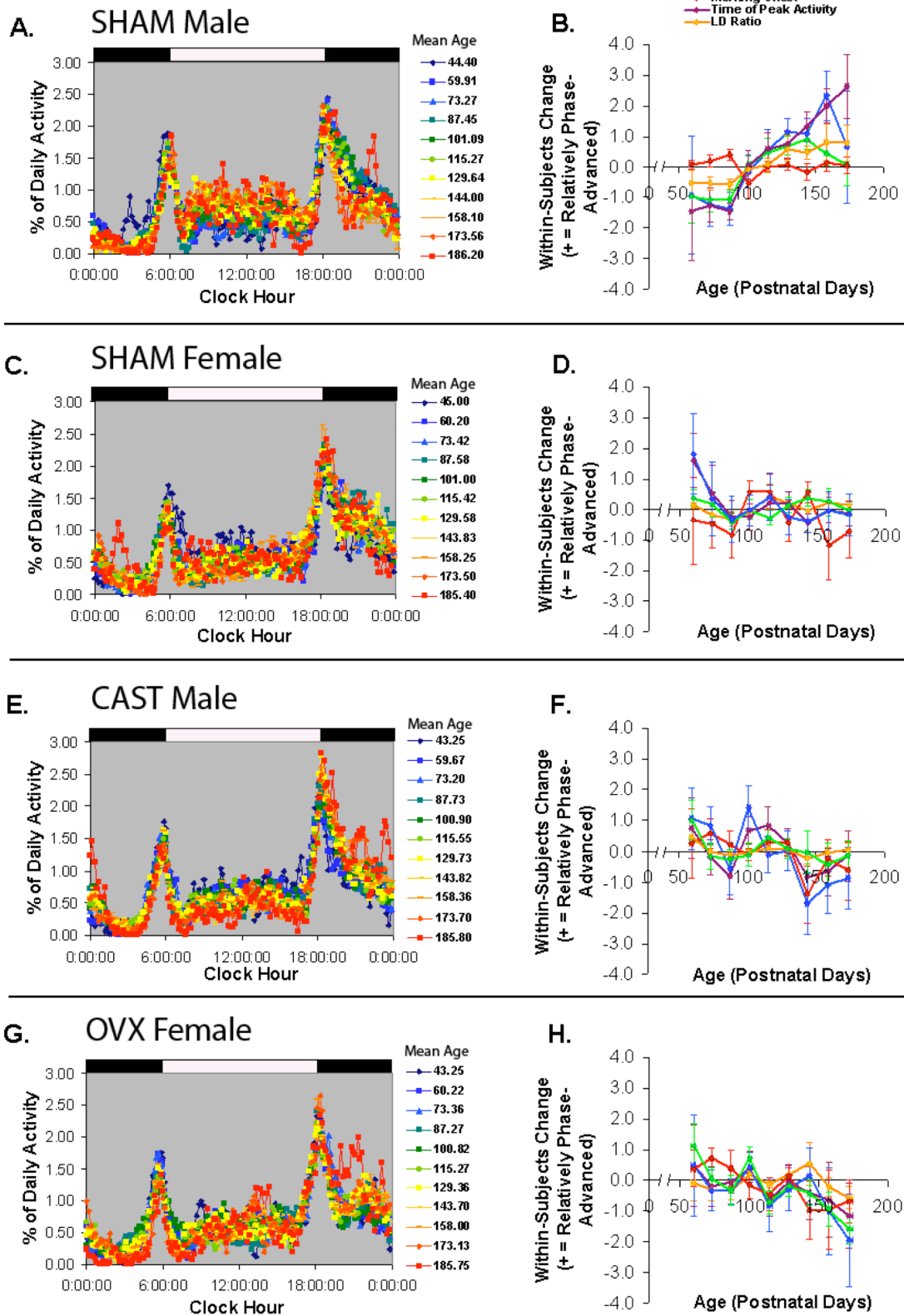
Figure 3.3

Figure 3.4. Circadian phase parameters exhibit sex differences during puberty that depend on gonadal hormones. Degus of both sexes that were gonadectomized prior to puberty (GDX) appear similar for almost all parameters. Circadian phase is presented in terms of zeitgeber time (ZT), with ZT12 equal to the time of lights-off and ZT0 or ZT24 equal to the time of lights on. X- and Y- error bars represent +/-SE. Asterisks indicate significant within-subject change ($p<0.05$). **A.** Pre-pubertal GDX prevented males from developing a phase-advanced onset to their active period. Their daily active period was defined as the 12 hrs of the day containing the highest mean activity. **B.** Neither SHAM nor GDX females showed significant changes in the onset of the active period. **C, D.** The onset of the morning activity showed little change over development in any of the groups studied. **E.** Pre-pubertal GDX prevented males from developing a phase-advanced time of peak activity. **F.** Neither SHAM nor GDX females showed significant changes in the time of peak activity. **G.** Pre-pubertal GDX prevented males from developing a phase-advanced offset of evening activity. **H.** SHAM females did not show age-related changes in offset, whereas GDX females showed a significant delay. **I.** Pre-pubertal gonadectomy prevented males from developing a more diurnal activity pattern as determined by LD ratio. **J.** Neither SHAM nor GDX females showed age-related changes in LD ratio.

Male Degus

Female Degus
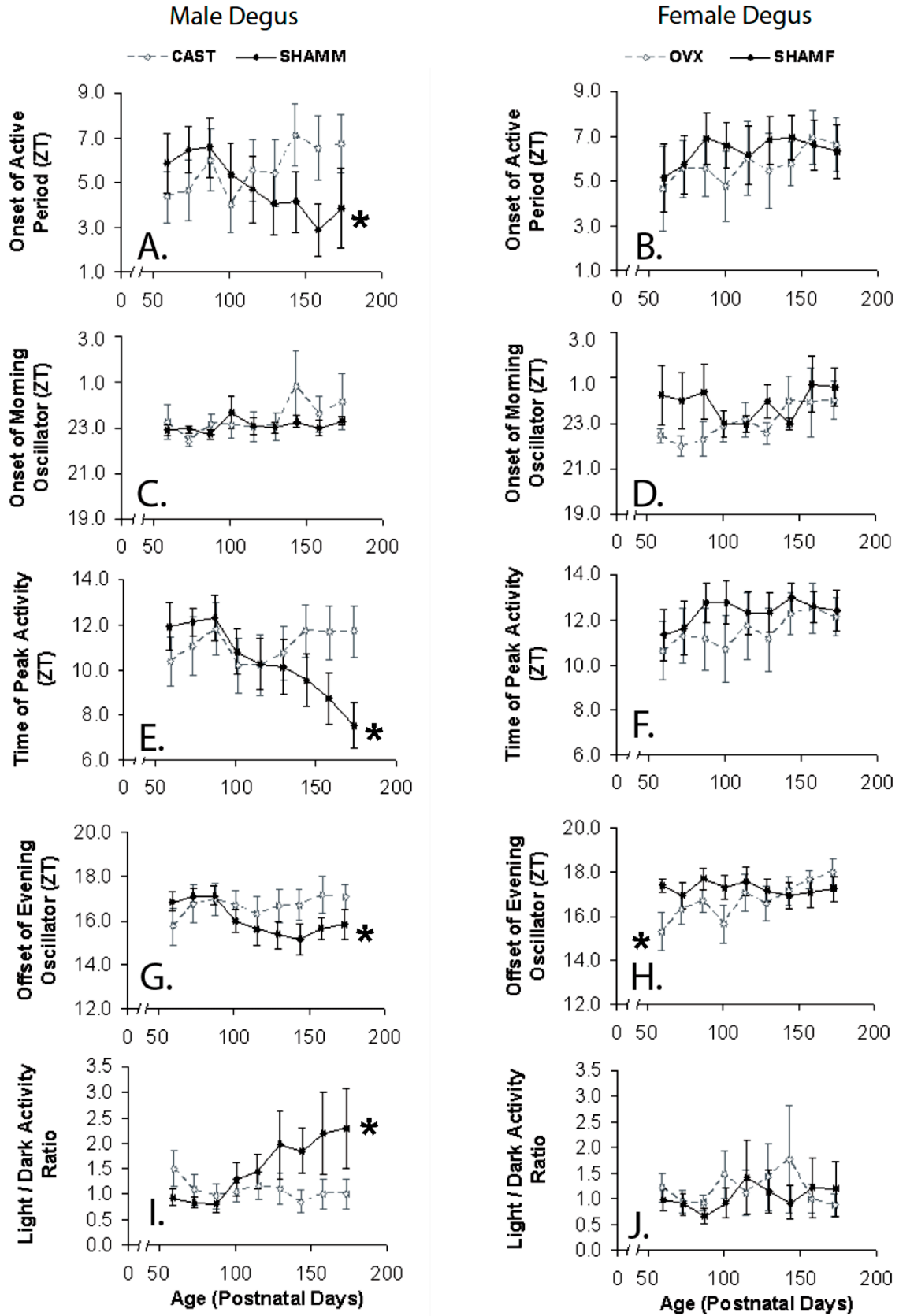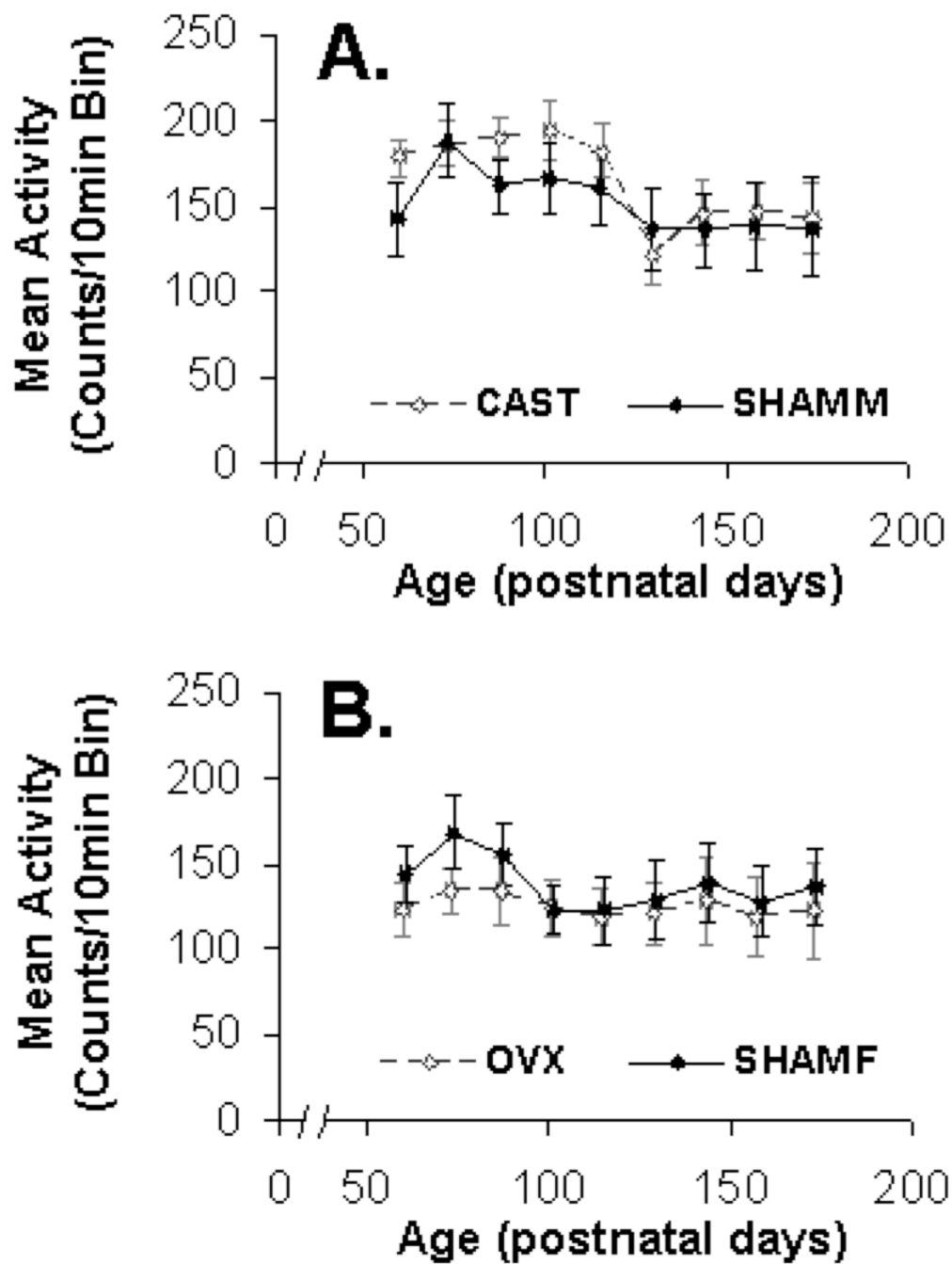
Figure 3.4

Figure 3.5. Mean activity levels decrease across development (*p*=0.040) in a manner that depends on sex (*p*=0.004). The daily mean activity (wheel turns/10 min bin) is illustrated for SHAM and castrated males (left) and SHAM and ovariectomized females (right). X- and Y- error bars represent +/-SE.

Supplementary Material

Supplemental Experiment 1: Pilot Methods

This experiment tracked activity rhythms in degus across the time period that had previously been identified as puberty (Hummer et al., 2007). Earlier work indicated that there were no sex differences in circadian phase changes during this period (Hummer et al., 2007), so both sexes were used indiscriminately in these preliminary experiments.

Subjects and Housing: Eight *Octodon degus* (P55-66) were selected from four litters within a week following weaning. The degus were placed into an environmental chamber that was maintained at a light intensity of 250 lux during the lighted phase of the 12:12 LD cycle. After several weeks of recording, it became clear that the majority of these animals (7/8) fit a nocturnal/crepuscular chronotype, which is unusual in our colony. We suspected that this unusual chronotype might have been produced by the bright, exposed housing conditions (the degus were kept directly under the lights (within 45 cm) in the boxes in order to maintain relatively equal light exposure). Therefore, we continued to record from the first set of animals but began a second set of eight degus (P60-72, selected from three litters within two weeks of weaning) under dimmer lighting conditions (around 100 lux, in dark grey opaque cages). Besides lighting intensity, both sets of degus were treated equivalently.

Monitoring activity rhythms: Pubertal changes in activity distribution and circadian parameters were quantified in an identical manner to Experiment 1. In the case of the brightly-lit animals, only mean activity, the time of peak activity, and LD ratio were scored because almost all animals (7/8) were found to be nocturnal and negatively masked by light. Therefore, even quick review demonstrated that their activity onset and offset were stably set at ZT12 and ZT0, respectively.

Statistical Analyses: To determine if there was a relationship between pubertal development and circadian parameters, we ran a linear and marginal model analyses. Within the marginal model, due to limited sample size, data from the earliest and latest age bins (before P71 and after P189 for the brightly housed animals, and before P71 and after P148 for the dimly lit animals) was removed from the analysis. The linear equation used to fit these data was:

$$Y_{it} = \beta_0 + \beta_1 Age + b_{0i} + b_{1i} Age + \varepsilon_{it}$$

In this linear equation, the variables are defined in an identical manner as in Experiment 1. The intercept terms reflected differences at the age of maturity by having the variable of "Age" centered around P140, which means that 140 was universally subtracted from all age values so that the linear fit would cross the y-axis at a meaningful location.

Supplemental Experiment 1: Pilot Results

The brightly-lit degus showed a nocturnal chronotype with strong negative masking, whereas the dimly-lit degus showed the diurnal chronotype typical of our degu colony. These differences in chronotype due to lighting are thoroughly discussed in another paper (Lee and Hagenauer, *in preparation*). However, it should be noted that the overall light intensity, as measured at cage-bottom, for the exposed, dimly-lit degus was more similar to the light intensity at the bottom of a cage on a shelf in the colony room than the light intensity for the exposed, brightly-lit degus.

Therefore, with the exception of the first week in the testing chambers, 7/8 of the brightly-lit degus were thoroughly masked and showed little change in phase over the recording period. With the first week included in the analysis, there was a trend towards the timing of peak activity changing with age ($F_{(1, 7.003)}=4.776$, $p=0.065$), with daytime activity disappearing during the first week and nighttime activity increasing. LD Ratio was not found to change with age ($F_{(1,7.343)}=1.303$, $p=0.289$), nor was mean activity ($F_{(1, 100)}=1.590$, $p=0.210$). With the first week of adjustment to the new lighting conditions removed from the analysis, the time of peak activity lost its significant relationship with age ($F_{(1, 7.022)}=1.759$, $p=0.226$, **Supplemental Figure 3.6**). None of the circadian variables showed a significant relationship with age when analyzed using the marginal model, regardless of whether data from the first week of recording was included or not ($p>0.1$).

The dimly-lit degus showed changes in activity rhythm chronotype during puberty. The degus became more diurnal, as indicated by an increase in the proportion of activity occurring during the lighted period of the LD cycle ($F_{(1, 7.989)}=5.733$, $p=0.044$), Simultaneously, there was a trend towards the timing of daily peak activity advancing ($F_{(1, 6.856)}=4.547$, $p=0.071$), as well as the time of activity offset ($F_{(1, 6.612)}=4.263$,

p=0.080). **Supplemental Figure 3.7**). Activity onset and mean activity did not change during this time period (*activity onset* $F(1,12.102)=0.481$, *p=0.501, mean activity:* $F(1, 11.219)=1.817$, *p*=0.204). These conclusions continued to hold true when the first week of recording was removed from the data set (α=0.05), although the trend towards age-related change in the timing of peak activity dissolved. None of the circadian variables showed a significant relationship with age when analyzed using the marginal model, regardless of whether data from the first week of recording was included or not (*p*>0.1).

Supplemental Experiment 2: Methods

These data were drawn from the baseline wheel-running data under a 12:12 LD cycle from two pilot experiments examining the entrainment mechanisms of pubertal and post-pubertal degus. Similar to both Experiment 1 and the Pilot Experiment, these animals were weaned around P60.

Subjects and Housing: Eleven of the animals (3 males, 9 females) came from an experiment that examined the phase response of degus to light at several points during development using a brief, Aschoff II style protocol (8-9 days in a baseline LD cycle, 10 days in constant darkness (DD) with a 15min-1 hr light pulse on the third day). Only LD recordings made between P66-104 (pubertal) and P135-220 (late/post-pubertal) were used for this comparison. During the protocol, these animals were housed in the brightly-lit chambers used in the Pilot Experiment. Between sampling periods, the degus were housed in cages on shelves in the colony room with siblings where they were exposed to dimmer lighting (an estimated 40-150 lux at cage bottom). Degus were always given at least 2 weeks recovery in the colony room between recording periods.

Fifteen of the animals (6 males, 9 females) came from an experiment that examined the free-running period of pubertal and post-pubertal degus under conditions of constant light. During this experiment, the animals were exposed to 3 weeks of constant light during the pubertal ages of P80-P110 and the post-pubertal ages of P172-P199. These sessions were preceded by 7-8 days in a baseline LD cycle. During the protocol, the animals were housed in cages on exposed tables directly below the light source (~250 lux). Puberty was carefully monitored using methodology identical to Experiment 2.

Activity Rhythm Monitoring and Analysis: The last four days of the LD baseline recording period was analyzed for both experiments. Pubertal changes in activity distribution and circadian parameters were quantified in an identical manner to Experiment 2. Statistical analysis was performed using a 2x2 Repeated Measures ANOVA (sex x age).

To address the question of whether masking could account for the lack of pubertal changes in circadian rhythms in the females, activity distribution and phase parameters from the entrained conditions were compared to those from the first two days after placement in DD for nine females. This analysis was possible because degus of this age typically do not free-run immediately after placement in constant darkness (Hummer et al. 2007). A similar analysis was done with males but they were not included in the final statistical comparisons because their sample size was small (n=3). Degus placed in constant light were also not included in this analysis because they frequently free-ran immediately. Several of the females were found to reverse their chronotype in response to placement in DD. Under these circumstances, it was ambiguous whether the phase shift for some variables should be scored as an advance or delay. Therefore, for consistency, a shift to a more diurnal pattern was scored as an advance, and a shift to a more nocturnal pattern was scored as a delay. The effects of masking (LD vs. DD) and age (pubertal vs. late/post-pubertal) were evaluated using a 2x2 Repeated Measures ANOVA.

Supplemental Experiment 2: Results

As males exited puberty they showed a decrease in evening activity and an increase in daytime and morning activity (**Supplemental Figure 3.8A**). Females remained strongly crepuscular across the developmental period (**Supplemental Figure 3.8B**). Therefore, age-related changes were found to show significant sex differences for the time of peak activity ($F(1, 24)=4.661$, $p=0.041$), and there was a trend towards sex differences in the change in the onset of the active period ($F(1, 24)=3.575$, $p=0.071$) and LD ratio ($F(1, 24)=3.011$, $p=0.096$). Overall, there was a main effect of development on the LD ratio ($F(1, 24)=6.734$, $p=0.016$) and offset of the evening oscillator ($F(1, 24)=9.364$, $p=0.005$), as well as a trend towards age-related changes in the onset of the active period ($F(1, 24)=3.676$, $p=0.067$) and the time of peak activity ($F(1, 24)=3.021$,

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p=0.095). Four variables were found to show overall sex differences: the onset of the active period ($F_{(1, 24)}$=5.137, *p*=0.033), the time of peak activity ($F_{(1, 24)}$=7.322, *p*=0.012), the offset of the evening oscillator ($F_{(1, 24)}$=6.533, *p*=0.017), and the LD ratio ($F_{(1, 24)}$=6.385, *p*=0.019; **Supplemental Figure 3.8C**). The onset of the morning oscillator and mean activity level were not affected significantly by either sex or development (*p*>0.10).

Both pubertal and late/post-pubertal females were found to have masked activity rhythms. During the first two days in DD, females shifted to a more diurnal activity pattern (**Supplemental Figure 3.9A&B**). This shift was reflected in an overall increase in the amount of activity occurring during the subjective day (as extended from the former LD cycle, $F_{(1, 8)}$=10.293, *p*=0.012), as well as a phase-advance in the offset of evening activity ($F_{(1, 8)}$=7.878, *p*=0.023) and the onset of the active period ($F_{(1, 8)}$=5.084, *p*=0.054; **Supplemental Figure 3.9C**). Significant changes were not observed in the timing of peak activity ($F_{(1, 8)}$=3.551, *p*=0.096), and morning onset ($F_{(1,8)}$=3.011, *p*=0.121). When the "unmasked" activity distributions from the first two days in DD were compared between pubertal and late/post-pubertal females there was still no indication of age-related changes (**Supplemental Figure 3.9D**). Therefore, age did not have an effect on any of the circadian phase variables (*p*>0.54), nor did it have an interacting effect with lighting condition (*p*>0.55; **Supplemental Figure 3.9E**). Although suggestive, these results demonstrating a lack of phase-change under "unmasked" conditions should be taken tentatively, as degus demonstrate a great deal of individual variability in how they adapt to DD. Similarly, it is not yet clear that the pubertal change that occurs in male rhythms could be detected using this technique, although there seemed to be indications of it in the three males that we monitored.

Cohort	1st day in box	1st day in wheel	1st day recorded	Last day recorded	# of Sham M	# of Cast M	# of Sham F	# of OVX F
A	58	58	59	178	2	2	1	1
B	55	55	63	169			1	1
C	51	51	59	132(1), 165	1	1		1
D	54	54	58	122	1			
E	54	54	55	156			1	1
F	38-40	43-45	46-48	194-196	2	2	1	1
G	40, 42	45, 47	55, 57	188, 190	2	2	2	2
H	35	40	43	188			1	
I	25	30	39	184	1	1		1
J	19	29	40	173	2	2	2	2
K	19	29	47	165, 178		1	3	
TOTAL					11	11	12	10

Table 3.1. The developmental cohorts of degus included in the final analysis of *Experiment 2.* Ages are presented in postnatal days. For cohorts #I-K, wheel-access was allowed before weaning. Grey boxes indicate that the first day of recording occurred less than 1 week after initial wheel access.

Figure 3.6. During the pilot experiment in bright, exposed housing conditions, degus maintained a nocturnal/crepuscular activity distribution across puberty. A. A representative single-plotted actogram tracking wheel-running activity rhythms across pubertal development in one degu. Each horizontal line on the graph sequentially represents 2 days of activity (# of wheel turns/ 10 min bin) and the LD cycle is indicated by bars at the top of the figure. Grey bars indicate multi-day gaps in the recording when the degu was returned to a cage with a sibling. The average postnatal age of the animal during each recording session is listed on the left. Note that as the degu grew older its activity remained firmly nocturnal/crepuscular. **B.** The average percent of daily activity occurring during each 10 min bin across the day is presented for several ages during pubertal development (n=8). Cool colors represent data from the youngest recording sessions (early pubertal), and warm colors represent the oldest recording sessions (late-pubertal). Two sessions are averaged for each line. The white and black bar at the top of the figure illustrates the time of lights on and lights off. **C.** Circadian parameters indicate little change in phase during pubertal development. The y-axis depicts the average within-subject change in each parameter. Positive values indicate that the time of peak activity is more advanced than the individual's mean for P74-P179 (earlier and later ages have a small *n*) and that the LD ratio is relatively more diurnal. X- and Y-error bars represent +/-SE.
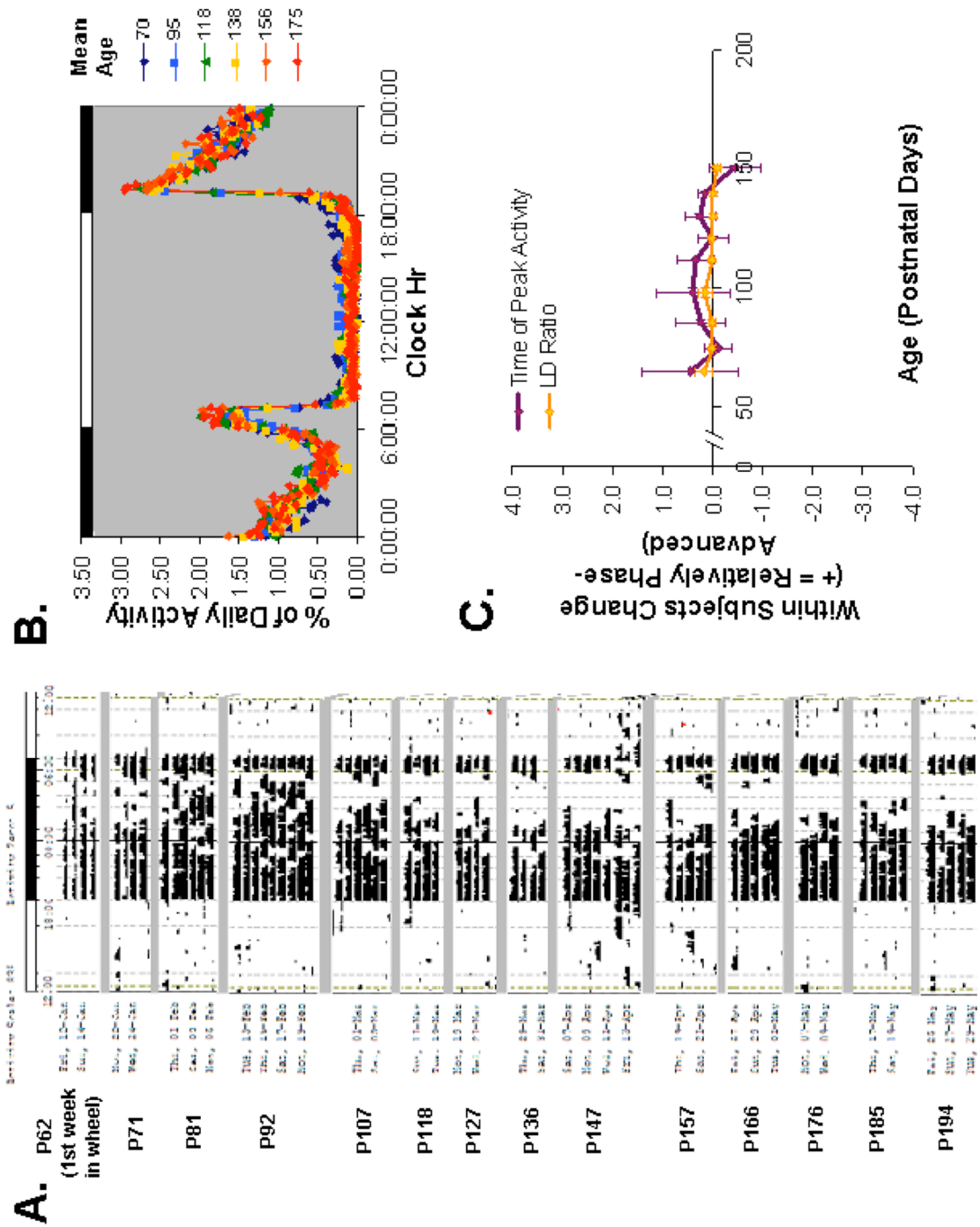
Figure 3.6

Figure 3.7. During the pilot experiment under dimmer conditions, degus showed circadian reorganization during puberty. A. A representative double-plotted actogram tracking wheel-running activity rhythms across pubertal development in one degu. Each horizontal line on the graph sequentially represents 2 days of activity (# of wheel turns/ 10 min bin) and the LD cycles for both days are indicated by bars at the top of the figure. Grey bars indicate multi-day gaps in the recording when the degu was returned to a cage with a sibling. The average postnatal age of the animal during each recording session is listed on the left. Note that as the degu grew older its activity during the hours immediately following lights-off (evening) diminished and daytime activity increased. **B.** The average percent of daily activity occurring during each 10 min bin across the day is presented for several ages during pubertal development (n=8). Cool colors represent data from the youngest recording sessions (early pubertal), and warm colors represent the oldest recording sessions (late-pubertal). Two sessions are averaged for each line. The white and black bar at the top of the figure illustrates the time of lights on and lights off. **C.** All circadian phase parameters suggest that degus phase-advanced during pubertal development. The y-axis depicts the average within-subject change in each parameter, with positive values indicating that the parameter is more advanced than the individual's mean for P71-P137 (earlier and later ages have a small *n*). In the case of the LD ratio, positive values indicate a relatively more diurnal activity distribution. X- and Y-error bars represent+/-SE.

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Figure 3.7

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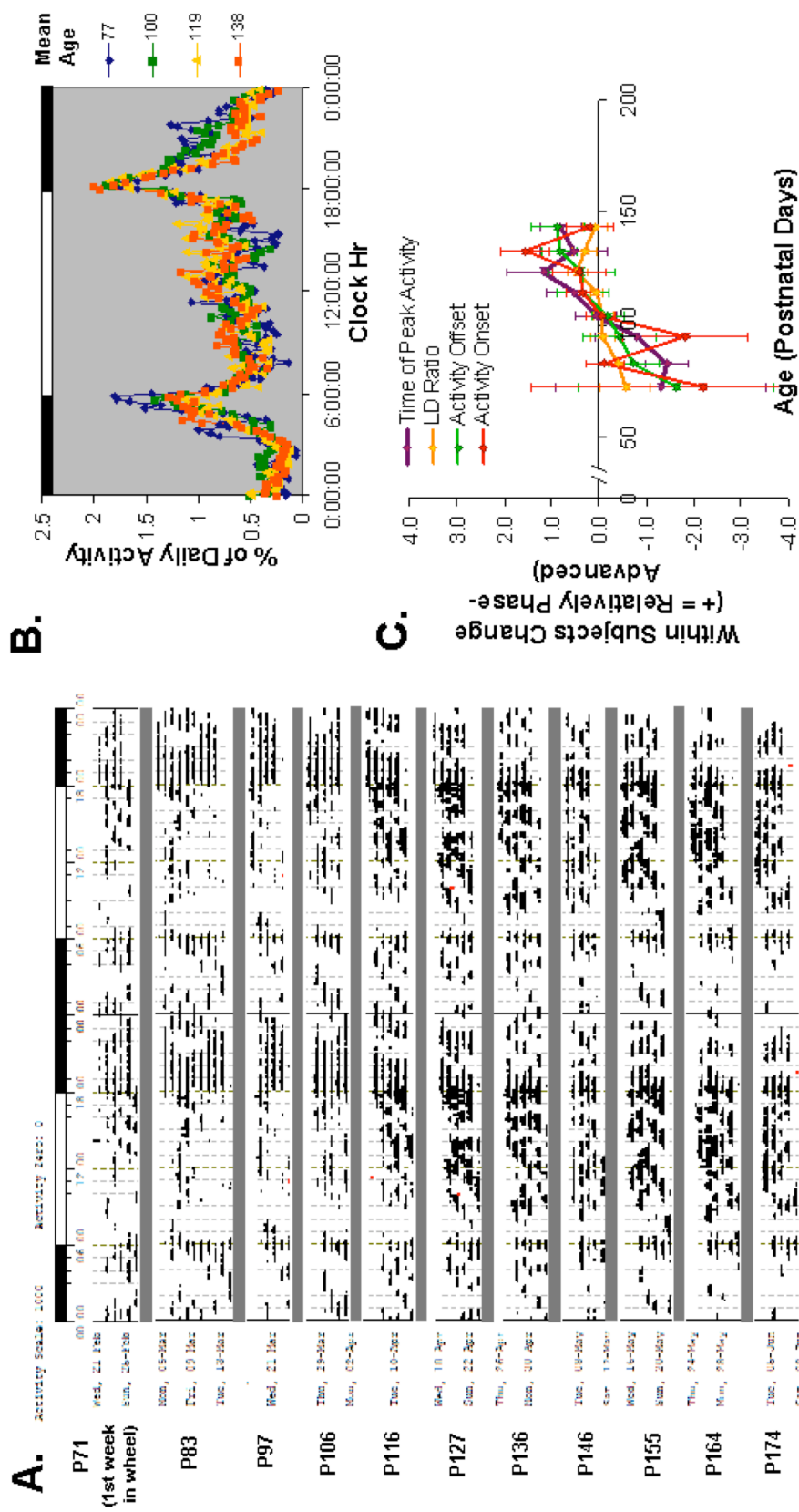
Figure 3.8. Male and female degus that had not received surgery still showed sex differences in circadian reorganization during puberty under bright, exposed housing conditions. A. The average percent of daily activity occurring during each 10 min bin across the day is presented for male (top, n=9) and female (bottom, n=17) degus at an age of mid-puberty (black line, age P66-104) and late-puberty (grey line, age P135-220). The white and black bar at the top of the figure illustrates the time of lights on and lights off. Males showed a circadian phase-advance during puberty, whereas females maintained a nocturnal/ crepuscular activity distribution. **B.** The average within-subject change is illustrated for each of five circadian phase parameters. Positive values indicate that the parameter phase-advances with development, except in the case of the LD ratio, where positive values indicate the development of a more diurnal activity distribution. Error bars represent +/-SE. **C.** A comparison of four circadian phase parameters at mid-puberty and late-puberty in male and female degus. Circadian phase is presented in terms of zeitgeber time (ZT), with ZT12 equal to the time of lights-off and ZT0 or ZT24 equal to the time of lights on. X- and Y- error bars represent +/-SE.

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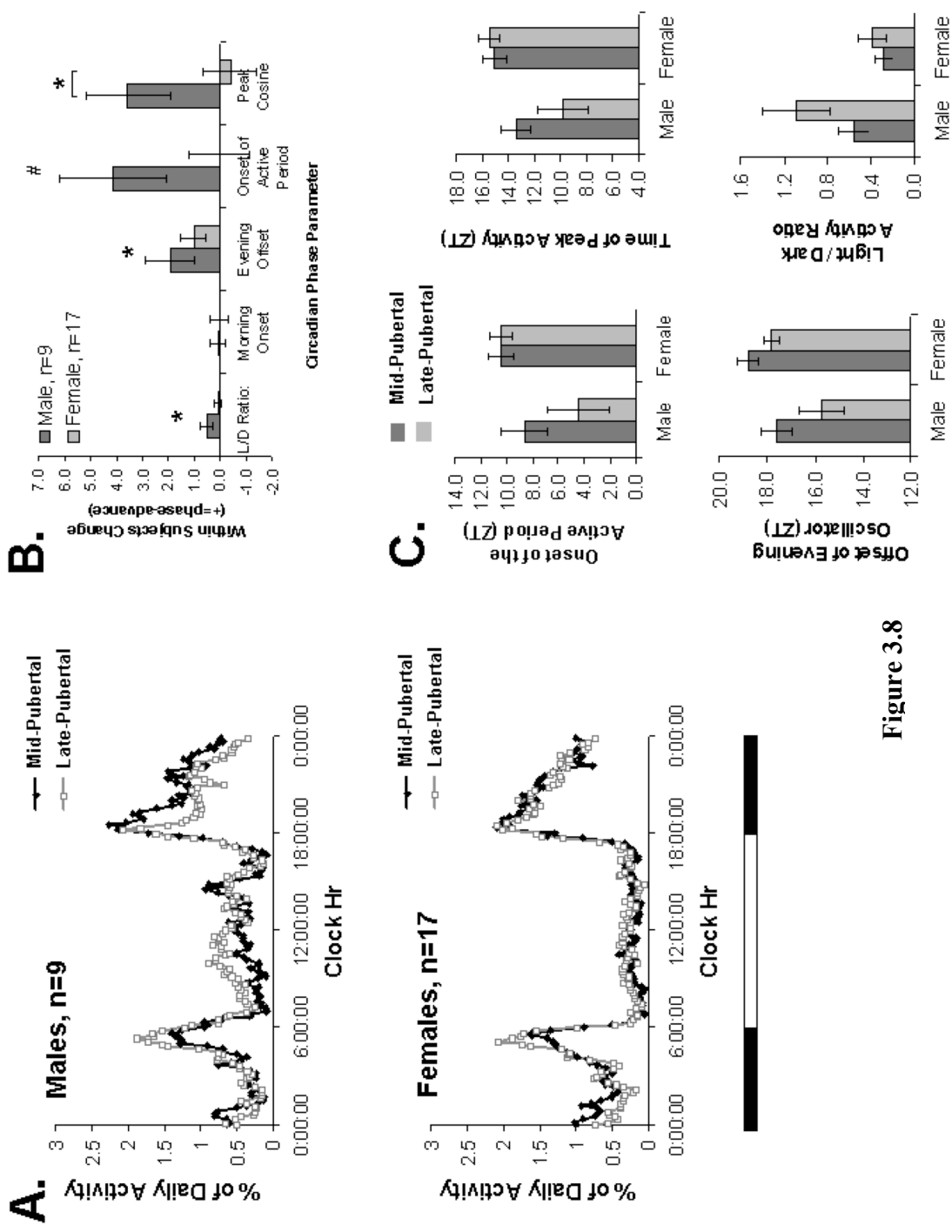
Figure 3.8

Figure 3.9. Female degus may have masked activity rhythms under a bright LD cycle in exposed housing conditions. This masking does not appear to account for their lack of pubertal change in activity distribution. **A&B.** The average percent of daily activity occurring during each 10 min bin across the day is presented for female degus (n=9) under a light dark cycle (LD, black line) and during the first two days after placement in constant darkness (DD, grey line). The grey and black bar at the top of the figure illustrates the time of lights on and lights off under the LD conditions. Note that the females demonstrate a nocturnal/ crepuscular activity distribution under LD conditions, but after placement in DD their activity increases during the subjective day and decreases in the evening. This same response to placement in DD occurs at **A.** a mid-pubertal age (black line, age 66-104) or **B.** a late- or post-pubertal age (grey line, age 135-165). **C.** The average within-subject change during the "unmasking" of rhythms in response to placement in DD is illustrated for each of five circadian phase parameters. Positive values indicate that the parameter phase-advances immediately after placement in DD, except in the case of the LD ratio, where positive values indicate an increase in the amount of activity during the subjective day. Asterisks indicate significant within-subject change (LD vs. DD, $p<0.05$). Error bars represent +/-SE. **D.** When the activity distributions for pubertal and late-/post-pubertal females during the first two days in DD, there is little indication for age-related change despite the fact that the rhythms are now free of the masking influences of the LD cycle. **E.** None of the circadian phase parameters show significant within-subject change when comparing mid-pubertal and post-pubertal females under a LD cycle or during the first two days in DD. Error bars represent +/-SE.
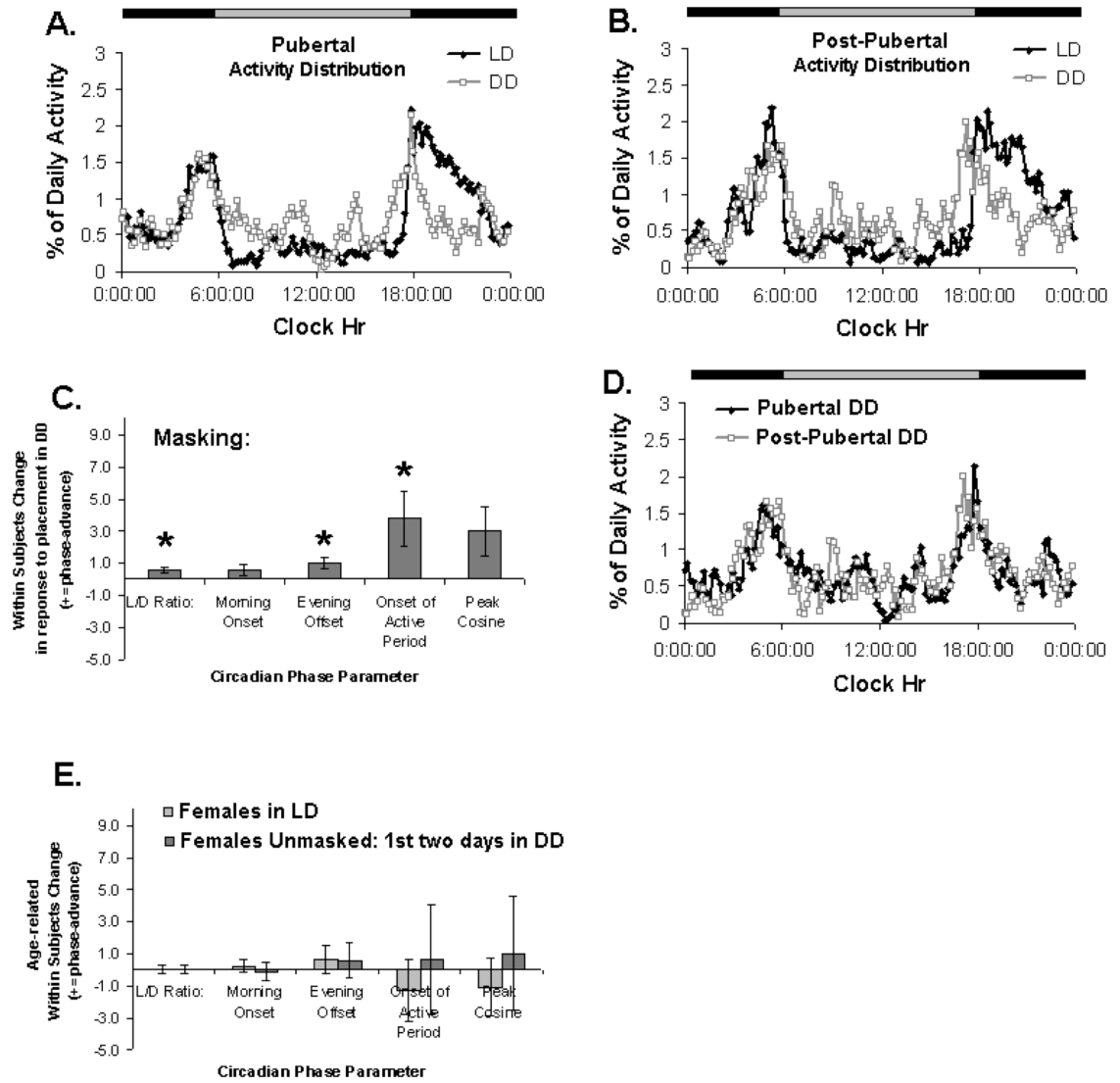
Figure 3.9

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Chapter 4

Period1 Rhythms in the Suprachiasmatic Nucleus Are
Delayed during Puberty in the Degu (*Octodon degus)*

Abstract

Human adolescents exhibit a dramatically different timing of sleep than children or adults. We recently demonstrated that two laboratory rodents, the degu and rat, similarly show significant changes in the distribution and phasing of activity rhythms during puberty. These changes were partially due to pubertal increases in gonadal hormones, and the circadian timekeeping system in the suprachiasmatic nucleus (SCN) of the hypothalamus is known to be sensitive to gonadal hormones. Thus, using *in situ* hybridization we examined the phasing of daily rhythms of *Per1*, a photosensitive element in the self-sustaining molecular feedback loop in the SCN that is essential for the production of circadian rhythmicity. We collected tissue at 3 hr intervals across the day in mid-pubertal (P83-98) and post-pubertal (P145-204) degus maintained in a 12: 12 light-dark (LD) cycle. Preliminary results indicate that the *Per1* rhythms in the SCN of pubertal degus are phase-delayed relative to those of post-pubertal degus or adults in a manner that parallels developmental changes in the phasing of activity rhythms.

Introduction

Human adolescents exhibit a well-characterized change in the timing of sleep during puberty. The timing of sleep grows later as puberty progresses, such that the latest sleep patterns occur around the time of achieving sexual maturity (between the ages of 15-21; Crowley et al. 2007; Roenneberg et al. 2004; Thorleifsdottir et al. 2002; Yang et al. 2005). These changes in the timing of sleep correlate with secondary-sex development (Carskadon et al. 1993; Sadeh et al. 2009). Thus, several authors have proposed that these changes may partially reflect pubertal influences on the circadian timekeeping system (*e.g.,* Carskadon, Acebo, and Jenni 2004).

Circadian rhythms are generated by an endogenous pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph et al. 1990). Endogenously generated circadian rhythms have a period (or day length, τ) that only approximates 24 hours. Consequently, under normal conditions, the endogenous rhythm must be entrained by external time cues (or "zeitgebers") to maintain a stable phase relationship with the outside world (Moore-Ede et al. 1982). Light is the dominant environmental zeitgeber, and under laboratory conditions the phase of behavioral and physiological rhythms is characterized by the relationship of phase markers – such as the onset, peak, or offset of a rhythm – to the laboratory light-dark (LD) cycle. An advance in circadian phase means that phase markers occur earlier during the LD cycle, whereas a delay means that phase markers occur later. Therefore, the sleep rhythms of human adolescents would be described as being delayed in phase relative to those of pre-pubertal children or adults.

We have found that pubertal changes in the timing of physiological rhythms do not occur exclusively in humans, but are instead common among other mammalian species as well (for review see Hagenauer et al. 2009). Our recent detailed reports on two laboratory rodent species, the slow-developing, diurnal degu and fast-developing, nocturnal rat, demonstrate that the phasing and distribution of daily activity rhythms is altered by pubertal development (Chapter 2; Chapter 3; Hummer et al. 2007). In both species, we observed that juvenile and pubertal animals had a phase of activity rhythms that was 3-5 hrs delayed compared to that of post-pubertal animals or adults. This delayed phase was due to the younger animals having a crepuscular (or bimodal) distribution of activity, with the majority of activity clumping near the end of the active phase (late

afternoon/early evening for degus; late night/early morning for rats). As the males progressed through puberty, this secondary bout of activity phase-advanced until it consolidated into a strong, unimodal rhythm that peaked near the beginning of the animal's active phase. These developmental changes in activity rhythms showed sex differences, and were smaller in females as well as animals that had been gonadectomized in pre-puberty. Thus, these developmental changes in activity rhythms were likely to be caused by pubertal increases in gonadal hormones (Chapter 2; Chapter 3; Hummer et al. 2007).

By using animal models, we can explore which aspects of the circadian timekeeping system might be altered by pubertal hormones. It is already well-known that gonadal hormones can alter key aspects of SCN physiology (for review see Karatsoreos and Silver 2007). Circadian rhythms in mammals are generated in the SCN by a transcriptional-translational feedback loop involving a group of genes commonly referred to as "Clock Genes" (Hastings and Herzog 2004). The core feedback loop consists of a positive arm, which contains proteins (BMAL1 and CLOCK) that drive transcription, and a negative arm, which contains proteins (PER, CRY, REVERBα) that inhibit transcription. To initiate the loop, a heterodimer of BMAL1 and CLOCK protein drives the transcription of *Per, Cry, Reverbα*. The protein REVERBα then feeds back to inhibit the transcription of *Bmal1*. The PER and CRY proteins (PER1, PER2, PER3, CRY1, CRY2) form heterodimers that inhibit their own transcription and the transcription of *Reverbα* (Bae et al. 2001; Zheng et al. 2001; for review see Hastings and Herzog 2004). The phasing and amplitude of rhythmic components in this feedback loop (*Per2, Cry2*) are known to be sensitive to estrogen in females (Nakamura et al. 2001; Nakamura et al. 2005).

Gonadal hormones can also alter aspects of the photic entrainment pathway. Light influences the SCN via a direct pathway between the retina and ventrolateral SCN (vlSCN, Morin and Allen 2006). Light exposure on the retina causes a rapid release of glutamate in the vlSCN, which binds to NMDA receptors, producing a cascade of second messenger signaling. These signaling pathways then quickly induce the transcription of immediate early genes, such as *cFos* (Meijer and Schwartz 2003), as well as two components of the clock gene feedback loop, *Per1* and *Per2* (Miyake et al. 2000;

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Shigeyoshi et al. 1997). Induction of *Per1* and *Per2* causes an overall phase-shift of the molecular feedback loop (Shigeyoshi et al. 1997) as well as rhythms in membrane potential (Kuhlman 2010). This phase-shift propagates to the dorsomedial (dm) SCN (Antle and Silver 2005; Nakamura et al. 2005; Yan and Silver 2002) and eventually to circadian output pathways. Consequently, phase-shifts in the molecular feedback loop in the SCN are thought to underlie the photic phase-shift of behavioral rhythms (Akiyama et al. 1999; Albrecht et al. 2001; Shigeyoshi et al. 1997; Tischkau et al. 2003). Thus, it has become common to examine the phasing of *Per1* and *Per2* rhythms within the SCN as a manner of characterizing the entrainment of the central circadian pacemaker (e.*g*. Abe, Honma, and Honma 2007; Yamanaka, Honma, and Honma 2008).

Both androgens and estrogens can reversibly increase photic sensitivity within the entrainment pathway, as measured by the induction of immediate early gene expression (Abizaid, Mezei, and Horvath 2004; Karatsoreos et al. 2007). Estrogen can also alter the phasing of neurotransmitter rhythms in the SCN (Cohen and Wise 1988; Krajnak et al. 1998; Mahoney et al. 2009) as well as increase electrical intercellular coupling (Shinohara et al. 2000; Shinohara et al. 2001) in a manner that is opposed by progesterone (Shinohara et al. 2001). Gonadal hormones can modulate SCN neuron excitability in both sexes as well (Fatehi and Fatehi-Hassanabad 2008; Kow and Pfaff 1984). Little work has examined pubertal hormone effects on the SCN, but there are indications of anatomical changes (growth in nuclear size and nucleoli size) around mid-puberty in rats (Anderson 1981; Morishita et al. 1978; Morishita et al. 1974), as well as an increased number of cells expressing a neuropeptide that is important for photic entrainment (VIP) during late adolescence in humans (Swaab et al. 1994). Behavioral data also suggests that pubertal rodents have an altered sensitivity to light (Hummer et al. 2007; Weinert and Kompauerova 1998),

We were interested in determining whether the large hormone-driven phase shifts in behavioral rhythms during puberty were reflected in the phase of components of the central circadian pacemaker in the SCN. Previous developmental studies in rodents suggest that clock gene rhythms in the SCN may change during the pubertal period. In traditional laboratory rodents, components of the central oscillator (*Per1, Per2, Bmal1, Cry1*) gradually develop pronounced rhythmic gene expression between the late prenatal

period and the second week of postnatal life (Isobe, Tauchi, and Kawaguchi 2005; Shimomura et al. 2001; Sladek et al. 2004). After this age, the phasing of *Per1* and *Per2* rhythms appear to shift by several hours before reaching their adult pattern of expression (Shimomura et al. 2001; Yamazaki et al. 2009). The amplitude of *Per1* rhythms also significantly increases (Shimomura et al. 2001). However, to our knowledge no studies to date have explicitly examined the development of central molecular oscillator during postnatal development following weaning.

As previous evidence suggests that elements of the photic entrainment pathway are altered during puberty (Hummer et al. 2007; Weinert and Kompauerova 1998), we hypothesized that the phase-advance that occurs during puberty in male rodents would be reflected in a phase-advance of transcript rhythms for the photosensitive clock gene *Per1*. We chose to use the degu as an animal model for two primary reasons. First, large behavioral phase-shifts had already been well-characterized during puberty in male degus, and these phase-shifts were clearly related to pubertal hormones (Hummer et al. 2007; Chapter 3). Second, degus are a slow-developing, precocial species, and therefore we were able to collect tissue from mid-pubertal degus that had already been weaned for over a month. This meant that any developmental changes we observed in *Per1* rhythms were unlikely to be due to lingering influences of the dam or sire on the entrainment of the youngest animals (Weinert 2005). Finally, we had already carefully characterized rhythms of *Per1* transcript in the SCN of adults of this species (>1 year of age) under entrained conditions (Vosko et al. 2009), as well as its photic sensitivity (Koch, Hagenauer and Lee 2009).

Methods

As a preliminary analysis of whether the central circadian pacemaker is phase-delayed in during puberty, we examined the expression of *Per1* RNA in the SCN across the day in mid-pubertal and post-pubertal degus. Extensive details regarding animal husbandry can be found in Hagenauer et al. (Chapter 3), and the methodology used for tissue preparation and *in situ* hybridization is similar to that used by Vosko et al. (2009).

Animals and Housing

Male degus (n=72) were obtained from the University of Michigan breeding colony. In the colony, breeding degus were kept in large (42.5 x 46 x 19.5 cm), transparent acrylic cages containing one sire, two dams, and their respective young. One side of each cage was kept on a heating pad at low heat in a room maintained at 20 +/- 1°C with a 12:12-h light-dark (LD) cycle (light intensity 250 lx). During breeding, pregnancy, and early development (postnatal days P0-90) the animals were provided *ad libitum* with Prolab Laboratory Animal Diet Product 5P06 and acidified water (2.5 x 10^{-5}% HCl) to prevent infections. At the age of 3 months (P90), they began receiving adult chow (5001 Rodent Diet, PMI Nutrition) and tap water. Before weaning, they were also given generous handfuls of dried alfalfa 2 times weekly. Weaning took place between ages P35-P50. The animals were then housed in 48 x 26.8 x 20.3 cm opaque plastic cages under a 12:12 Light-dark (LD) cycle (40-150 lux) with food and water available *ad libitum* and 1-2 same sex companions.

Degus were divided into two experimental populations based on age: mid-pubertal (P83-98) and post-pubertal (P145-204). Brain samples were collected every three hours across the day at ZT times: 0, 3, 6, 9, 12, 15, 18, and 21. Brain collection occurred within twenty minutes before or after the given time point. Degus were anesthetized and decapitated under isoflurane anesthesia. Care was taken to extract and flash-freeze the tissue within four minutes of decapitation so as to prevent mRNA degradation. Tissue was stored at -80°C until the time of sectioning. During the dark phase of the LD cycle, brains and blood were collected under dim red lights. All procedures were approved by the University Committee for the Care and Use of Animals at the University of Michigan.

Tissue Preparation

Tissue frozen at -80°C was brought to -20°C for sectioning with a cryostat. Four series of serial coronal sections of 16 μm were taken through the SCN. Sectioning began from the anterior brain where the optic chiasm appeared and the anterior commissure met medially. This region corresponds to 0.12 mm posterior to bregma in the rat brain. While the SCN has been anatomically verified in the degu (Goel, Lee, and Smale 1999), regional neuroanatomy has not been anatomically verified in this species and visualization was the primary means of orientation. From these landmarks, 96 sections

were taken in the posterior direction to ensure the capture of the complete SCN and placed on Fisherbrand Superfrost/Plus microscope slides. Sections were stored at -80°C until they were processed for *in situ* hybridization.

Radioactive Probe Production

Radioactive cRNA probes were synthesized from bacterial plasmids containing the degu *Per1* fragment (pGEMT) (courtesy of Drs. Jeremiah Shepard and Steven McKnight, Southwest Medical School, Texas, GenBank EU 715821). These plasmids were purified with a QIAprep Spin Miniprep Kit (Qiagen, Valencia CA). The plasmids were linearized using restriction enzymes, and fragments were characterized by gel electrophoresis and purified using a PCR purification kit (Qiagen, Valencia CA). Once pure, linear DNA was produced, radioactive double-labeled antisense mRNA probes were synthesized by incubating the DNA and RNA polymerase in a buffer containing ^{35}S-UTP and ^{35}S-CTP (Perkin-Elmer, Waltham, MA) and unlabeled adenine and guanine for 2 hours in 37°C water bath. After polymerization, plasmid DNA was digested with RNase-free DNase I. The labeled probe was separated from free nucleotides using Micro Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Hercules, and CA). The radioactivity of the cRNA probes was then quantified with a liquid scintillation counter (Perkin-Elmer, Tri-Carb 2800).

In situ Hybridization

Slides containing one series of sliced tissue (24 sections) per animal were fixed in 4% paraformaldehyde for 1 hr, washed 3 times in 2X SSC solution, and then incubated for 10 min in 0.1M TEA with 0.25% acetic anhydride. Slides were then washed in dH$_2$O and dehydrated in graded alcohols before being left to air dry. The ^{35}S-labeled probe was diluted in hybridization buffer until the concentration of radioactivity was 4 million cpm per 100 uL of solution. The hybridization solution was then applied to each slide and cover-slipped. Slides were then placed in a hybridization box with 50% formamide-soaked filter paper in the bottom and incubated at 55°C overnight. The following day, cover slips were removed in a 2X SSC solution. Slides were then washed three times in 2X SSC solution and incubated at 37°C in RNase solution (200 ng/mL) for 1 hour to remove unhybridized probe. Slides were then washed in increasingly stringent SSC solutions and incubated at 65°C in 0.1X SSC for 1 hour to decrease non-specific labeling.

After incubation, the slides were rinsed with distilled water before being dehydrated through graded alcohols and left to air dry. To visualize the radioactive labeling, Biomax film (Kodak, Rochester, NY) was exposed to the slides for one week. The film was then digitized with a Microtek ScanMaker 1000XL (Microtek, Cerritos, CA) and SilverFast Ai software (Lasersoft Imaging Inc., Sarasota, FL).

Analysis

The location of the SCN was identified using ventral landmarks (*e.g.,* optic chiasm, 3rd ventricle) in reference to previous anatomical characterization performed in our lab (Goel, Lee, and Smale 1999). *Per1* signal was then measured from the digitized images of two mid-SCN slices representative of each degu using ImageJ software (NIH, Bethesda, MA). Greyscale units were converted to standard optical density units using a 21-step sensitivity guide (Stouffer, Mishawaka, IN) and the Rodbard calibration function. For each image, the optical density of the corpus callosum was measured, and any labeling that did not surpass 2.0 standard deviations above this density was considered background. The number and density of pixels within the SCN that surpassed background was then quantified (referred to as labeled area and optical density, respectively). Background values were subtracted from optical density measurements to remove the influences of background variation from the final results. Optical density was then multiplied by labeled area to produce a measurement of integrated optical density. Slides deemed low quality (unsalvageable SCN, excessive background signaling) were excluded from the data analysis. All image analysis was performed by an experimenter blind to the identity of the tissue. A 2 X 7 ANOVA was used to analyze the effects of age and time (ZT) on SCN *Per1* optical density, labeled area and integrated optical density.

Preliminary Results

During analysis, fifteen of the original brains were discarded due to poor tissue quality from improper freezing. Tissue from the remaining 57 subjects was analyzed for *Per1* expression. Within these 57 subjects, there were 6 animals that had particularly high background staining (optical density >0.40): two degus from ZT3 (one pubertal and one post-pubertal), two animals from ZT18 (one pubertal and one post-pubertal), and two

pubertal animals from ZT15. Results are presented below excluding these animals (n=51, with 2-4 degus per group/time point; **Table 4.1**).

Both pubertal and post-pubertal male degus exhibited rhythmic expression of *Per1* transcript in the SCN, where *Per1* peaked during the middle of the light period and then fell to minimal levels during the dark period **(Figure 4.1, Figure 4.2)**. A two-way ANOVA found a significant main effect of time (ZT) on *Per1* optical density in the SCN (F(7, 35)=18.663, *p*<0.001), SCN labeled area (F(7, 35)=4.690, *p*=0.001), and integrated optical density (F(7, 35)=9.867, *p*<0.001). The pattern of *Per1* expression differed between groups. In post-pubertal degus, *Per1* began to increase steadily near the end of the dark period, peaked at the beginning of the light period (ZT 3), and declined to minimal levels at the beginning of the dark period (ZT 12). In pubertal degus, *Per1* began to increase following lights-on (between ZT 0 and ZT3), peaked at ZT 9, and dropped to minimal levels at ZT 12. Thus, the peak in *Per1* transcript in the SCN of pubertal degus was delayed by 3-6 hours relative to the post-pubertal group. The duration of peak expression, as defined by the number of hours during which *Per1* was elevated above 50% of amplitude (peak-trough) was also longer in pubertal degus. Thus, there was a significant interaction between the effects of age group and time (ZT) on *Per1* optical density (F (7, 35) = 4.024, p = 0.002), although not on labeled area (F(7, 35)=0.776, *p*=0.612) or integrated optical density (F(7, 35)=1.748, *p*=0.130). There was no overall age group difference in the levels of *Per1* at the peak or trough, and therefore we found no significant main effect of age group on labeled area (F(1, 35)=0.008, *p*=0.929), mean-background (F(1, 35)=0.379, *p*=0.542), or optical density (F(1, 35)=0.000, *p*=1.000).

Discussion

The daily pattern of *Per1* expression in the SCN of post-pubertal degus (145-204 days of age, or approximately 5-7m) was almost identical to that observed in adult male degus (>12m of age) in our previous study (Vosko et al. 2009). In contrast, during puberty, when degus exhibit a delayed phasing of activity rhythms (Hagenauer et al. *in preparation*), *Per1* rhythms in the SCN appeared either delayed or distorted. This evidence supports our hypothesis that the delayed activity rhythms observed during puberty are due to changes in the central circadian pacemaker.

We originally chose to examine *Per1* specifically because of its photosensitivity. *Per1* rhythms are directly entrained by the LD cycle (Shigeyoshi et al. 1997), therefore we presumed that any changes in the *Per1* rhythm would reflect pubertal changes in the photic entrainment mechanism. However, our recent data in the developing rat suggests that pubertal reorganization of circadian rhythms still occurs under constant conditions, suggesting that changes in the photic entrainment cannot fully explain this phenomenon (Chapter 2). The developmental changes in activity rhythms appear similar in these two species. If the mechanism driving these changes is also the same, then we should consider whether *Per1* SCN transcript rhythms could be entrained to a delayed phase at the same time as other critical changes in the circadian system occur downstream. For example, gonadal hormones are already known to strengthen the coupling between oscillators in the SCN (Shinohara et al. 2000; Shinohara et al. 2001). Therefore, the younger animals could have weaker coupling, which might lead to a delayed rise in *Per1* in the morning, as well as a delayed phase relationship between the photic-entrained oscillators in the SCN and rhythmic output pathways.

Whether these effects are due to the direct binding of pubertal hormones in the SCN is debatable. Some researchers have found clear evidence of steroidal receptors in the SCN (Hummer et al. 2006; Iwahana et al. 2008; Kruijver et al. 2003; Li et al. 2002; Mitra et al. 2003; Wilson et al. 2002) whereas others have not (Lauber, Romano, Pfaff 1991; Li et al. 1993; Shughrue, Komm, Merchenthaler 1996; Shughrue, Lane, Merchenthaler 1997: Shughrue and Merchenthaler 2001; Warembourg and Leroy 2004). However, the degu exhibits both estrogen and androgen receptors in the SCN throughout development (Hummer et al. 2006), thus the mechanism is in place for direct steroid effects on the SCN in this species. Also, the functioning of many input structures to the SCN (*e.g.*, the intergeniculate leaflet, medial preoptic area, or raphe nuclei) is sensitive to gonadal hormones (Abizaid et al. 2005; De La Iglesia 1999; Karatsoreos and Silver 2007). Additionally, classical steroidal hormones as well as their metabolites can directly affect synaptic transmission, most notably at NMDA and $GABA_A$ receptors (Melcangi et al. 2001). Therefore, pubertal increases in gonadal hormones could modulate SCN function regardless of direct binding in the SCN.

However, these results should be treated as preliminary until replicated, as final sample sizes were small due to technical difficulties. Future directions for this study also include a characterization of another photosensitive element in SCN transcriptional-translational feedback loop, *Per2*, as well as investigation into positive elements in the loop, such as the expression of *Bmal1*. This would reinforce the anticipated conclusion that the entire circadian pacemaker system exhibits a delayed phase during puberty. Furthermore, as the vlSCN and dmSCN are known to play separate roles in circadian rhythm generation and entrainment (Antle and Silver 2005), ideally our experiments should analyze these two regions separately. This might be facilitated by using non-radioactive labeling. Finally, while the mRNA level of analysis is useful, protein expression profiles derived using immunocytochemistry or western blotting are ultimately needed for a complete functional picture.

Future investigations will also include an investigation of daily rhythms in clock gene expression located in "slave oscillators" in brain areas outside the SCN. Previous work indicates that the phase relationship between the central SCN oscillator and slave oscillators in other regions of the brain closely relates to the phase of behavioral activity rhythms (Abe et al. 2001; Masubuchi et al. 2000; Mrosovsky et al. 2001; Vosko et al. 2009; Wakamatsu et al. 2001). Therefore, pubertal influences on the phasing of molecular clocks in the brain outside of the SCN might also be important for pubertal changes in chronotype.

Interestingly, the molecular oscillators outside of the SCN in traditional laboratory rodents (rats, mice) are already known to develop rhythmicity later than those in the SCN and show dramatic changes in the amplitude and phasing of their component rhythms during the post-weaning period. For example, the striatum and cortex still lack rhythms in core components one week before weaning (*Bmal1, Clock, Per1, Per2, Cry1;* Cai et al. 2009; Shimomura et al. 2001). Those components that are rhythmic before weaning (*Reverbα* and *Npas2*) undergo almost a complete phase reversal by adulthood (P60; Cai et al. 2009). In the liver, significant rhythms in *Cry1* and *Clock* do not appear until after weaning, at an age barely preceding typical pubertal development (Ojeda and Urbanski 1994; Sladek et al. 2007). Other components are rhythmic around the age of weaning (*Per1, Per2,* and *Reverbα*) but still seem to phase-advance by at least 4 hrs by adulthood

(Sladek et al. 2007; Yamazaki et al. 2009). Similarly, there also appears to be a change in the amplitude of phasing of rhythms in the heart, pineal gland, and adrenal glands between weaning and adulthood (Sakamoto et al. 2002; Yamazaki et al. 2009). As these measurements were taken in fast-developing, altricial rodents it is not clear whether post-weaning changes in molecular oscillators outside the SCN are related to pubertal development or the transition from nursing predominantly during the day (the dam's rest period) to foraging independently for food during the night (Sumova et al. 2006; Yamazaki et al. 2009). Our future studies using the slow-developing, precocial degu may clarify this point.

Acknowledgements

Table 4.1. Final sample size per group at each time point.

ZT	Pubertal *n*	Post-pubertal *n*
3	3	4
6	2	4
9	2	3
12	4	3
15	3	3
18	3	2
21	4	3
24	4	3
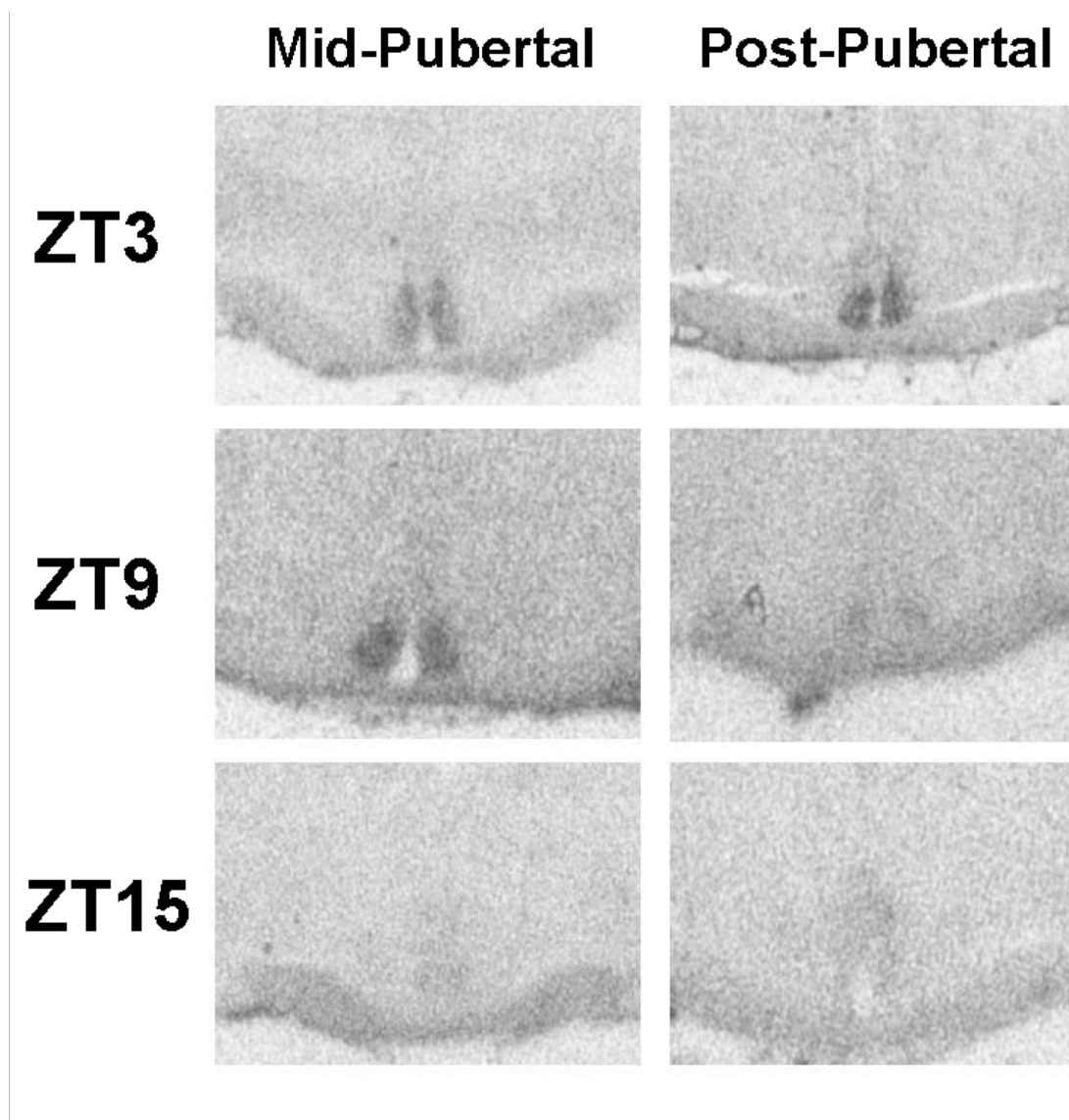
Figure 4.1. Representative autoradiographs showing *Per1* mRNA levels in the SCN of mid-pubertal (left) and post-pubertal (right) male degus. Brains were sampled every 3 h from animals housed in LD 12:12. *Per1* mRNA levels in the SCN were visualized using *in situ* hybridization. Time points are presented in zeitgeber time (ZT, ZT0= the time of lights on). Depicted are coronal slices from individuals at three representative time points: ZT3 and ZT9, which were time points containing peak *Per1* expression for post-pubertal and mid-pubertal degus, respectively, and ZT15, a time point containing trough *Per1* expression for both age-groups.

Figure 4.2. The daily expression of *Per1* mRNA in the SCN differs between mid-pubertal and post-pubertal male degus. *Per1* mRNA levels in the SCN at each timepoint were compared using *in situ* hybridization. Error bars represent standard error from the mean. Time points are presented in zeitgeber time (ZT, ZT0= the time of lights on). ZT/CT0 is double-plotted as CT/ZT24. **A.** Optical density refers to the intensity of the signal produced by radioactive *Per1* probes in the SCN, as quantified using autoradiography. Background was subtracted from all measurements, and was defined as any signal that didn't surpass 2.0 SD above the density of the corpus callosum. Mid-pubertal degus exhibited a daily rhythm in *Per1* optical density that appeared to be phase-delayed relative to that of post-pubertal degus (main effect of time: $p<0.001$; age-group x time: $p=0.002$). **B.**   Labeled area was defined as the number of pixels within the SCN image that had an optical density that surpassed background. *Per1* labeled area was significantly rhythmic for both age groups (main effect of time: $p=0.001$), but there were no significant differences between age groups (age-group x time: $p=0.612$). **C.** Integrated optical density is the product of optical density and labeled area. *Per1* integrated optical density was significantly rhythmic for both age groups (main effect of time: $p<0.001$), but there were no significant differences between age groups (age-group x time: $p=0.130$).

Figure 4.2

Legend: Post-Pubertal, Mid-Pubertal

A. Optical Density vs Zeitgeber Time (ZT)

B. Labeled Area vs Zeitgeber Time (ZT)

C. Integrated Optical Density vs Zeitgeber Time (ZT)
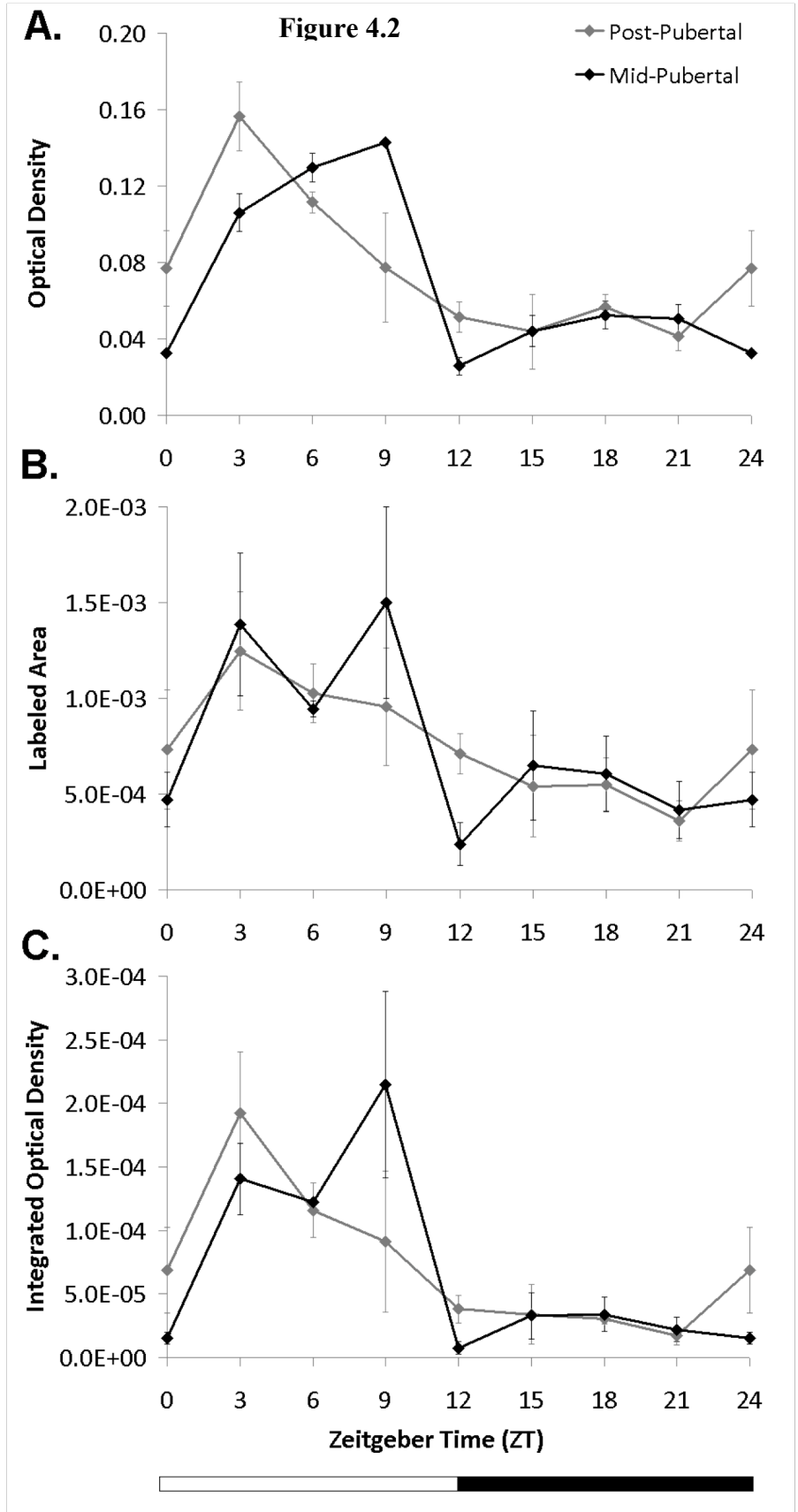
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Chapter 5

Conclusion

"Without due consideration of the neural and behavioral correlates of differences between higher taxa and between closely-related families, species, sexes, and stages, we cannot expect to understand our nervous systems or ourselves."
      - Bolluck (1984)

"We have evolved a nervous system that acts in the interest of our gonads, and one attuned to the demands of reproductive competition."
      - Ghiselin (1974), as cited in Crews (2002)

Biological Timekeeping, Gonadal Hormones, and Reproductive Diversity: Moving Beyond the Use of Pubertal Laboratory Rodents as Models for Human Adolescents

This dissertation began with the concept that the use of animal models could address fundamental questions regarding the delayed sleep patterns of human adolescents, while avoiding the technical and ethical issues associated with using human subjects. Two rodent species were chosen with very different developmental trajectories and natural histories, the laboratory rat (*Rattus norvegicus*) and degu (*Octodon degus*). Both species exhibited dramatic changes in the timing and organization of daily activity during puberty not unlike human adolescents. These changes exhibited sex differences and were, at least partially, dependent on gonadal hormones. Preliminary results in the degu indicated that developmental changes in the phasing of behavioral rhythms correlated with changes in the phasing of a photosensitive element of the circadian pacemaker. However, results in the rat indicated that changes in the photic entrainment mechanism were not required for the reorganization of activity rhythms during puberty. Thus, we conclude that multiple aspects of the circadian timekeeping system are altered by puberty.

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Taken in tandem with the results of previous studies suggesting that circadian phase changes occur during puberty in three other species (the rhesus macaque, fat sand rat, and mouse; Hagenauer et al. 2009; Chapter 1), these results indicate that pubertal changes in circadian timekeeping may be a relatively common phenomenon in mammals. This conclusion is broadly relevant for the circadian community beyond the scope of these studies, as immature animals are frequently used as the source of tissue for patch-clamp investigations of SCN electrophysiology. Our data indicate that the SCN tissues from young animals may be representative of only a short period of the life history of the species. Our data also have potential to inform unresolved questions regarding the prevalence of circadian, sleep and mood disorders in adolescents, as well as the national debate regarding high school start times.

However, before going into depth on these topics of relevance, it seems important to first answer a fundamental question: Are we observing the same phenomenon in these species? How generalizable are the conclusions from each experiment? In 1979, Beach laid out two cardinal rules regarding the construction of animal models for human behavior as well as for behavioral comparisons between species. One rule was that any "significant comparison of a particular type of behavior in two different species is impossible unless and until the behavior has been adequately analyzed in each species by itself." In this sense, the research presented in this dissertation has greatly furthered the field. This detailed analysis of circadian development has expanded upon several conclusions from our initial cross-species comparison (Hagenauer et al. 2009; Chapter 1).

First, these detailed analyses of activity rhythms across postnatal development supported the assertion that pubertal phase changes show sex differences in their timing and magnitude. In the rat, where females exhibit external signs of puberty 8-10 days earlier than males, we found that females also showed circadian phase changes at an earlier age, although, in the end, male rats showed larger phase changes over the pubertal period (Chapter 2). Circadian phase changes in the degu showed strong sex differences, with males exhibiting a large phase-advance during the pubertal period and females exhibiting little change (Chapter 3). Since the timing of adolescent phase changes in humans also displays sex differences and human males show larger phase changes than females (Roenneberg et al. 2004), these findings support the hypothesis that the

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phenomenon observed in each species shares some common origins. The magnitude of phase change in male degus and rats (3-5 hrs) also resembled that observed in studies of human males (Roenneberg et al. 2004).

Second, the pubertal phase changes occurring in these rodent species are directly related to a consolidation of ultradian components in the rhythms (Chapters 2 & 3). This conclusion is consistent with previous data indicating that both androgens and estrogens can consolidate rhythms at the beginning of the active period in rodents (Iwahana et al. 2008; Morin et al. 1981; Thomas and Armstrong 1989; Wollnik and Dohler 1986), but has not been discussed in previous studies of pubertal phase change (Golub and Takeuchi 2002; Hummer et al. 2007; Tate, Richardson, and Carskadon 2002). Earlier research on the post-weaning period in altricial rodents discussed this rhythm consolidation in terms of the switch from maternal to photic entrainment and in terms of the development of circadian output systems (*e.g.,*Weinert 2005). These data are the first to note that similar changes can occur in precocial species that presumably rely on photic entrainment soon after birth and that would have emerged from the burrow in the wild many weeks before the age of our initial measurements (Chapter 3). Thus, consolidation of rhythms at the beginning of the active period cannot be exclusively attributed to the pups developing independence from the dam (or dams and sire, in the case of communally-nesting degus; Jesseau 2004). These data are also the first to demonstrate that the developmental decrease in ultradian rhythms during post-weaning development is related to pubertal hormones (Chapters 2 &3).

The relationship between rhythm consolidation and phase change in rats and degus is one formal characteristic that differentiates the pubertal phase changes in these species from those occurring in human adolescents. A second characteristic that appears to differ between our rodent species and other species that have been studied in detail (human, macaque) was the developmental timing and the direction of the phase changes. These data did not provide evidence that circadian phase was relatively more advanced in the pre-pubertal animals than the pubertal animals (Chapters 2 &3) unlike previous studies in the degu and rat that briefly examined one or two pubertal time points (Alfoldi, Tobler, and Borbely 1990; Hummer et al. 2007; Tate, Richardson, and Carskadon 2002). However, we did replicate the observation that circadian phase is more delayed during

puberty than adulthood (Hummer et al. 2007; McGinnis et al. 2007) as it is in humans and macaques (Golub and Takeuchi 2002; Roenneberg et al. 2004). Thus, it is unclear whether the changes in rats and degus are more analogous to the circadian phase advance that occurs following sexual maturity in humans (Roenneberg et al. 2004) or to the phase delay that occurs between pre-puberty and sexual maturity in humans and macaques (**Figure 5.1;** Golub and Takeuchi 2002; Roenneberg et al. 2004). The relationship between phase changes and pubertal timing in each species, as well as the dependency on pubertal hormones, suggests that the latter comparison may be more appropriate, although the direction of phase change during puberty clearly differs between these species.

Consequently, Beach's second rule of comparative studies pertains, which is that meaningful comparisons should not be based "upon the formal characteristics of behavior, but upon its causal mechanisms and functional outcomes" (1979). The causal mechanisms of the phenomenon, as suggested by the relationship between circadian changes and pubertal timing, sex, and gonadal dependence, indicate that the phenomenon that we are observing is analogous between species, whereas the formal characteristics may differ in the ways discussed above. However, some possible differences in the causal mechanism driving pubertal phase changes in these species may also exist. First, the effect of pre-pubertal gonadectomy on activity rhythms in rats in our studies (Chapter 2) is very similar to the previously-published effects of adult gonadectomy (Thomas and Armstrong 1989; Wollnik and Dohler 1986). Thus, the hormonally-driven changes observed during puberty in the rat are likely to be activational in nature. On the other hand, adult male degus show a phase advance in activity rhythms when they are castrated (Jechura et al. 2000), which is the opposite of the response observed following pre-pubertal gonadectomy (Chapter 3; although see Hummer et al. 2007). Thus, the hormonally-driven changes observed during puberty in degus are potentially specific to the pubertal period (either organizational in nature or activational but subject to later organizational changes in hormone sensitivity). Second, both pubertal and post-pubertal female degus appeared to have activity rhythms that were masked by photic cues, and responded to constant darkness by decreasing their crepuscularity (Chapter 3), whereas juvenile male and female rats actually became more bimodal in response to placement in

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constant conditions (Chapter 2). Finally, in general the developmental changes in activity distribution in rats progressed similarly under entrained and constant conditions (Chapter 2), whereas developmental changes in degus seemed to correlate with the altered phasing of a photo-responsive element in the transcriptional-translational feedback loop in the SCN (*Per1*; Chapter 4). This evidence regarding the role of pubertal changes in the photic entrainment mechanism is not inherently contradictory (*e.g.,* both could be related to pubertal changes in SCN coupling), but the coexistence of both traits would be counterintuitive. Thus, further studies are needed to determine the similarity of the causal mechanisms driving phase changes during puberty in these species.

> *"There is…great diversity in the ways in which hormones can affect an animal's nervous system and behavior. To the student, this diversity might seem like an unwanted complexity, something else to complicate the story."*
>   *- Crews (2002)*

Indeed, in some ways it seems like it would be surprising if we actually did find an identical phenomenon occurring during puberty in each of these species because there are several striking differences in the progression of puberty between laboratory rodents, such as the degu and rat, and primates, such as macaques and humans. As discussed in the introduction, puberty in primates is characterized by several years of preceding gonadal quiescence (Plant 1994), whereas puberty in rodents follows low-level steroidogenesis throughout the infantile and juvenile periods (Ojeda and Urbanski 1994). Thus, it is possible that we do not observe a period of relatively advanced phase during pre-puberty in rats and degus because they have already been exposed to gonadal hormones during juvenile development (Hummer et al. 2007; Ojeda and Urbanski 1994). However, it seems unlikely that early-pubertal or juvenile hormone production in the degu and rat delayed circadian phase before our recording period in a manner analogous to the delay observed in human adolescents and pubertal rhesus macaques, as the activity rhythms of rodents gonadectomized prior to puberty appeared similar to those of pre-pubertal rodents (Chapters 2 & 3). Since long-term hormone withdrawal is known to alter hormone receptor levels (Mohamed and Abdel-Rahman 2000; Rose'Meyer et al. 2003), it seems more likely that following quiescence in primates, hormones transiently

have different effects on the circadian system than those observed in species with low-level steroidogenesis throughout the juvenile period.

Another fundamental difference between the progression of puberty in rodents and primates is the role of adrenal hormones (Belanger et al. 1989). During puberty in primates, there is not only a maturation of the hormone-secreting gonads (gonadarche), but also the maturation of the zona reticularis of the adrenals (andrenarche, Havelock et al. 2004). During andrenarche, the zona reticularis increases its production of steroid hormones with weak androgenic properties (androstenedione, dehydroepiandrosterone or DHEA, and DHEA- Sulfate, or DHEA-S; Campbell 2006; Havelock et al. 2004). Recently, these hormones were found to have neuroactive properties, modulating the GABA, NMDA, serotonergic, and nicotinic neurotransmitter systems (Rupprecht 2002). These data are potentialy interesting because GABA is the primary neurotransmitter of the SCN (Morin and Allen 2006), and DHEA-S exposure has already been shown to cause circadian phase shifts in a manner that depends on the time of administration, as well as to diminish phase-advances caused by early morning light exposure (de Tezanos Pinto and Golombek 1999). Similarly provocative is the correlation between the sex differences in the profile of DHEA-S secretion across the lifetime and the sex differences in lifetime changes in chronotype in humans (**Figure 5.2;** Campbell 2006; Havelock et al. 2004; Roenneberg et al. 2004). To the best of my knowledge, this is not true of other hormonal candidates (*testosterone*: Juul and Skakkebaek 2002; Kaufman and Vermeulen 2005; Mitchell et al. 2001; Moroz and Verkhratsky 1985; Stearns et al. 1974; Vermeulen, Rubens, and Verdonck 1971; *progesterone:* Genazzani et al. 1998; Kaufman and Vermeulen 2005; Moroz and Verkhratsky 1985; *inhibin A:* Burger et al. 2002; Sehested et al. 2000; *inhibin B:* Sehested et al. 2000; *growth hormone:* Zadik et al. 1985; *estrone:* Labrie et al. 1997; *estradiol:* Burger et al. 2002; Kaufman and Vermeulen 2005; Labrie et al. 1997; *pregnenolone:* Labrie et al. 1997; *pregnenolone sulfate:* Halikova et al. 2002; *epitestosterone:* Halikova et al. 2002; *allopregnanolone:* Genazzani et al. 1998; *androstenedione:* Kaufman and Vermeulen 2005; *FSH:* Burger et al. 2002; Moroz and Verkhratsky 1985; Sehested et al. 2000; Stearns et al. 1974; *LH:* Moroz and Verkhratsky 1985; Sehested et al. 2000: Stearns et al. 1974), with the possible exception of free testosterone (Kaufman and Vermeulen 2005; Mitchell et al. 2001; Vermeulen, Stoica, and

Verdonck 1971; although see Juul and Skakkebaek 2002; Stearns et al. 1974). Clearly, however, plasma hormone levels are not a full reflection of a hormone's efficacy, particularly since hormone receptors and plasma binding proteins also change across the lifetime (Karatsoreos and Silver 2007; Vermeulen, Rubens, and Verdonck 1971). Thus, the speculation above should be treated cautiously.

In general, in the realm of reproduction, diversity is certainly the rule and not the exception (Crews 2002). Therefore, alternatively, it could be that pubertal hormones have different effects on the circadian system of primates and rodents. This would not be an unprecedented conclusion, as gonadal hormones are already known to have species-specific effects during adulthood. For example, testosterone shortens τ in adult mice (Karatsoreos et al. 2007), but not in adult hamsters (Morin and Cummings 1980). Estrogen (17β-Estradiol) shortens τ in adult female hamsters (Morin, Fitzgerald and Zucker 1977; Zucker, Fitzgerald, and Morin 1977) and adult female rats (Albers 1981) but not adult female degus (Labyak and Lee 1995). Testosterone treatment consolidates rhythms in adult male mice (Daan et al. 1975, Iwahana et al. 2008), whereas it induces rhythm splitting in starlings (Gwinner, 1974). Indeed, since there are pronounced species differences in the degree of circadian regulation of the HPG Axis (Chappell 2005), it makes sense that gonadal hormone feedback should be similarly diverse.

Finally, the role of artificial lighting in prolonging or exaggerating delayed phase in human adolescents should not be underestimated. During the earliest human circadian experiments, unbeknown to researchers at the time, subjects lengthened their own free-running period by controlling their daily light exposure (Aschoff 1965). Now it is widely-acknowledged that behavioral habits in humans can lead to altered zeitgeber exposure and atypical circadian phase (*e.g.* Barion and Zee 2007; Kohyama 2008). If an individual is regularly awake later in the evening and exposes himself to evening light, he will delay his circadian phase even more. Likewise, if a subject is regularly up early, and exposes herself to morning light, she can shift her phase earlier.

In summary, it seems that the pubertal changes in circadian behavior observed in each of these species may be best understood in the context of the species' specific endocrinological and physiological context. How about the functional outcomes of phase changes during puberty? Beach's second rule argues that meaningful comparisons of

behavior between species can also be made based upon the "functional outcomes" of that behavior. However, the role for gonadal hormone effects on circadian rhythms has been a source of mystery for years. On this front, these new data have the ability to inform two pre-existing theories.

According to the gonadal hormone "feedback" theory, the circadian timekeeping system is sensitive to gonadal hormones because it plays an integral role in the HPG axis (as reviewed in the introduction; Karatsoreos and Silver 2007). In support of this theory, many physiological systems that are regulated by circadian rhythms are also known to influence circadian function. For example, the circadian system drives rhythms in pineal melatonin, and melatonin is an effective zeitgeber (Lewy et al. 1992). Rhythms in food consumption can also feed back on circadian function (Mistlberger et al. 2009), as well as rhythms in behavioral arousal (Maywood and Mrosovsky 2001). In each of these cases, it seems that the feedback effects of these systems serves a similar purpose across species to align output rhythms with appropriate cues from the internal and external environment. In this case, the diversity of effects of gonadal hormones on circadian rhythms sets them apart from other known feed back effects. Also, the feedback model seems to make the most sense when applied to traditional laboratory rodents (rats, mice, hamsters) that have strong circadian regulation of ovulation and mating (Chappell 2005). On the other hand, the reproduction of many primates, including humans, as well as the degu does not seem to be so strictly tied to a circadian cycle (Kennaway 2005; Mahoney et al., *submitted*; Mahoney, *personal communication;* Turek and Van Cauter 1994). Thus, it is unclear why pubertal hormones would have a similar magnitude of effect on the circadian system of these various species.

Another theory that is regularly discussed regarding the influence of gonadal hormones on circadian timekeeping is the "early bird gets the worm" theory. According to this theory, when females are most receptive during their cycle they become hyperactive and active earlier in the day to increase their chance of encountering a mate. In this case, it is in the best interest of reproductively-active males to be active earlier in the day, to increase their probability of gaining first access to females during their receptive period. Although this theory seems to have some explanatory power regarding the pubertal advances in circadian phase observed in males in our two rodent models, it

does not seem to explain the pubertal delays in phase observed in humans and macaques. There is a tendency for humans to be more sexually active in the evening, which could provide incentive for individuals to stay up later. However, there is also increased sexual activity on weekends, which suggests that the daily rhythm is an artifact of the modern work week (Palmer, Udry, and Morris 1982). On weekends, there is a secondary peak in sexual activity that develops in the morning (Palmer, Udry, and Morris 1982), which occurs around the time that testosterone peaks in men (Turek and Van Cauter 1994), again suggesting that the weekday evening peak may be an artifact of modern conditions. Rhesus macaques are known to be regularly sexually active during the morning hours following dawn, although this is unlikely to be their only time of sexual activity (Wilson and Gordon 1980).

Without an obvious reproductive function, is it possible that changes in circadian rhythms during puberty could serve a purpose that is not strictly reproductive? Outside of the mammalian kingdom, there are numerous examples of developmental shifts in preferred activity times around the time of maturity. For example, juvenile chubs (a variety of fish) are diurnal, whereas adult chubs are nocturnal. These chronotype differences are likely to be related to either risk of predation or optimal hunting time, since juvenile chubs have smaller mouths and consume different prey (Magnan and Fitzgerald 1984). Amongst toad species, it is extremely common for younger toads (metamorphlings) to be diurnal, whereas juveniles are crepuscular, and adults are active early in the night. The reason for this chronotype shift appears to be related to the risk of predation for the young toads as well as the need to avoid dehydration. The need to avoid dehydration restricts their habitat to wetter regions, and the high density of the metamorphlings, as well as their small size, increases the risk of predation. During the day the small toads can make use of their adaptive coloring to advertise toxicity and discourage predators (*Bufo bufo:* Freeland and Kerin 1991). Diurnal behavior can also allow young toads to avoid cannibalistic older toads (*Bufo marinus:* Pizzatto et al. 2008).

Although, clearly, most human teenagers are not at high risk for being consumed by their parents, there is an argument to be made that the risks, needs, and social interactions of mammalian species, including humans, change over post-weaning development. Similarly, there is growing evidence that within-species competition can

influence the timing of an individual's activity within the species' normal active period (called "Temporal Partitioning"; Kronfeld-Schor and Dayan 2003). This temporal partitioning regularly results in the dominant individual gaining primary access to the early part of the active period, whereas lower-ranking individuals are shunted to either a more unconsolidated or delayed activity pattern (*bellbirds:* Craig & Douglas 1984; *trout:* Alanara et al. 2001; *rats:* Calhoun 1962), in a manner that also resembles the division of time between younger and older individuals in the examples presented above (*toads:* Freeland and Kerin, 1991; Pizzatto et al. 2008). As a result, this temporal partitioning allows dominant individuals to forage during times when there are higher rewards (*bellbirds:* Craig & Douglas 1984; *trout:* Alanara et al. 2001), whereas lower-ranking individuals can gain access to resources during a time when there is less personal risk of intimidation and attack *(bellbirds:* Craig & Douglas 1984). Therefore, the pubertal shift that we observe in degus and rats from an activity pattern that is more crepuscular/delayed to one that is consolidated earlier during the active period resembles circadian changes associated with a shift in dominance status.

Gonadal hormones correlate with competitive status during adulthood in many species (Hirshenhauser and Oliveira 2006) as well as during puberty (Anestis 2006). For example, testosterone levels are lower in individuals that are frequent recipients of aggressive behavior, and higher in individuals that are frequent initiators (*chimpanzees:* Anestis 2006). During puberty in primates, individuals become increasingly more involved in the adult social hierarchy, engaging in more affiliative behaviors and associating with same sex adults. Pubertal animals also start to be treated competitively by adults, and aggressive interactions can peak during the pubertal period in some species (Spear 2000). Amongst rodent species, puberty overlaps with a play period (Spear 2000, Bolles and Woods 1964), but late puberty is also a time of intense competition (Bolles and Woods 1964), as many individuals are eventually evicted or emigrate from the colony (especially males; Ebensperger et al. 2009). Thus, potentially having a circadian system that is sensitive to signals regarding dominance status may be particularly important during the pubertal period. In that case, it is possible that when a temperamental adolescent avoids his parents while staying up late socializing with peers he might actually be responding to a hormonal drive to establish an independent life at a time of day that is not dominated by older individuals.

Future Experiments

If we theorize that pubertal hormones are influencing circadian rhythms in a manner that signals dominance status, then pubertal hormones may produce a consolidation and phase advance of activity rhythms in rodents under conditions of low competition (*e.g.* individual laboratory housing) but not under conditions of high competition (*e.g.* social housing with older, dominant individuals; Calhoun 1962). One might also predict that the degree of phase shift and rhythm consolidation would correlate with plasma testosterone levels. Recently the technology to run circadian experiments in socially-housed individuals became available (Paul and Schwartz 2007), so such experiments are now feasible.

Before testing larger theories, however, there are still several critical experiments that need to be performed to confirm and build-upon the conclusions presented in this dissertation. The first and foremost is a hormone replacement experiment. Traditionally, to demonstrate causality a researcher needs to not only examine the necessity of a physiological substrate for behavior, but also its sufficiency to produce that behavior. Our experiments demonstrated that pre-pubertal removal of the gonads is capable of reducing developmental changes in activity rhythm phase and consolidation but these experiments have not irrefutably demonstrated that hormones produce these changes.

The fact that the circadian changes during puberty in male rodents so closely-resembled those previously observed in response to castration and androgen replacement in adults (Daan et al. 1975; Iwanahana et al. 2008) suggests that an intuitive follow-up experiment would be to replace testosterone during puberty in males gonadectomized prior to puberty. Simultaneously, testosterone could be replaced in gonadectomized adults to determine whether the effects are specific to the pubertal period in these species (as would be expected for degus due to the results of Jechura, Walsh and Lee 2000). If testosterone replacement successfully produces circadian phase advance and activity rhythm consolidation, then we could subsequently investigate whether testosterone is acting via estrogen receptors following local conversion by the enzyme aromatase (as would be suggested by recent studies of τ in pubertal male degus; Hummer 2006), or acting via traditional androgen receptors (as suggested by studies of adult mice: Iwanahana et al. 2008). We could also determine whether estrogen is effective at phase-

advancing and consolidating activity rhythms in pubertal rats, as is already suggested by estrous-related variations in activity rhythms at this age (Chapter 2).

It is possible that the relationship between pubertal hormones and circadian rhythms may be more complicated. Hormonal effects during puberty could be due to the slow actions of steroid hormones at traditional nuclear receptors (which act as transcription factors), but they could also be due to the rapid actions of hormones at non-traditional membrane receptors or due to direct hormonal modulation of neurotransmitter receptors (McCarthy and Crews 2002). There is already evidence that steroid hormone receptors in the SCN of the degu are primarily non-traditionally located on cell membranes (Hummer 2006), and estradiol modulates SCN cell excitability in rat tissue within minutes of application (Fatehi and Fatehi-Hassanabad 2008). If pubertal hormones are acting rapidly on the SCN, then the timing of hormone exposure is likely to matter. The circadian system is already known to be phase-dependently sensitive to neuroactive steroid exposure (de Tezanos Pinto and Golombek 1999), and estrogen receptor mRNA is expressed rhythmically in the SCN, suggesting that rapid effects of estrogen could be similarly phase-dependent (Wilson et al. 2002). In this case, it is possible that the daily rhythm of hormone exposure (or plasma binding of hormone: Ankarberg and Norjavaara 1999) during an experiment could determine the effect of that hormone on circadian phase. This possibility is intriguing, since the daily rhythmicity of natural gonadal hormone secretion changes during puberty (Turek and Van Cauter 1994).

Other future experiments are much more straightforward. In the introduction, the model predicted that the circadian pacemaker is delayed in phase in pubertal animals relative to adult animals due to altered photic entrainment. The experiments presented in this dissertation are only a preliminary test of that hypothesis, and the results in the two species appeared contradictory. To address this, the first experiment would be a replication of the preliminary *Per1* in situ hybridization in the pubertal degus, as the sample size was not large enough at some time points due to technical difficulties. This experiment should be followed up by characterization of other elements in the transcriptional-translational feedback loop (*Per2*, *Bmal1*), as well as a careful analysis of rostral-caudal, dorsal-ventral distribution of these transcripts across the day to elucidate whether rhythms in a particular functional subsection of the SCN is altered during

puberty (Antle and Silver 2005). This same tissue could be simultaneously analyzed for the phasing and organization of oscillators outside the SCN (*e.g.* striatum, parietal cortex) that are known to correlate with behavioral rhythms (Masubuchi et al. 2000; Vosko et al. 2009). For a true comparison between species, a similar analysis would need to be performed in the rat.

Second, the phasing of melatonin rhythms could be examined as an indication of the phasing of the integrated output signal of the SCN, as has been previously done in human adolescents (Carskadon, Acebo, and Jenni 2004; Crowley et al. 2006). In phylogenetically older species (birds and reptiles), the pineal gland serves as the master circadian pacemaker. In mammals, pineal rhythms are regulated by the SCN, but the two regions are still closely linked (Gorman and Lee 2002). Therefore, under circumstances in which the phase of behavioral rhythms is determined downstream from the SCN, the phase of melatonin rhythms still remains coupled to the circadian pacemaker in the SCN (*e.g.* diurnality: Vivanco et al. 2007; or scheduled feeding: Ho et al. 1985). For these reasons, the daily rhythm in melatonin is treated as the most direct peripheral indicator of the phase of the circadian pacemaker (Arendt 2005; Lewy and Sack 1989). If the circadian pacemaker is relatively delayed in pubertal degus and rats compared to adults, then it would be expected that the melatonin rhythm would also be delayed. This hypothesis could be tested using either radioimmunoassay or pineal microdialysis.

Finally, as described in the model, another traditional manner of inferring the phase of the circadian pacemaker is by measuring the magnitude of circadian phase shift that is produced by a discrete light exposure at different times of day (a behavioral PRC) (Johnson 1992). This is because circadian light-sensitivity is dependent on the phase of the pacemaker. This method for measuring pacemaker phase has the additional benefit of indicating specific alterations in discrete entrainment mechanisms. Preliminary data from the mouse and human already suggest that the photic sensitivity is altered during puberty (Carskadon, Acebo, and Arnedt 2002; Weinert and Kompauerova 1998), so this experiment is likely to produce interesting results.

Societal Relevance

These data indicate that pubertal changes in the circadian timing of activity are common across mammalian species. What do these results mean for society?

For the research community, these results have two important implications. First, the circadian mechanisms in the SCN cannot be assumed to be static after prenatal/infantile development. These data, along with that reviewed in the introduction, suggests that a number of components of the circadian system change during puberty, including free-running period, continuous and discrete entrainment mechanisms, and recovery from photic phase-shift (jet lag). On a practical level, this means that the results of electrophysiological studies performed on young animals cannot be immediately generalized to adults. This point is particularly significant, because pre-pubertal and pubertal rodents are frequently used for voltage clamp studies (e.g., Klisch et al. 2009; Rash et al. 2007; Wong, Graham and Berson 2007) due to the greater ease of creating an electrode seal (or "patch") on younger cell membranes of (Vosko, *personal communication*). A reconsideration of the importance of the age of the animals used in these studies has the potential to inform current debates. For example, GABA is the primary neurotransmitter in the SCN, but it is still unclear whether its actions are exclusively inhibitory due to a contradictory series of results (Albus et al. 2005; Choi et al. 2008; Gribkoff, Pieschl and Dudek 2003; Gribkoff et al. 1999; Jeu and Pennartz 2002; Wagner 1998; Wagner 2001). If we examine these findings in relationship to the age of the animals, the studies that show the greatest GABA-related excitation (during the day) come partially from very young animals (P14-P28 rats in Wagner 2001, P21-P56 in Wagner 1998), when many circadian output rhythms are diurnal (pre-weaning) or strongly crepuscular (post-weaning) and the rats are still sensitive to maternal zeitgebers (Weinert 2005). There are two studies that show GABA-related excitation, but only during the night, in animals at the age of puberty (pubertal: 150-300 g = P35-P50 in Jeu and Pennartz 2002; P30-P40 in Choi et al. 2008) as well as in one multiunit study that doesn't give the age of the animals (Albus et al. 2005). In contrast, it appears that all of the studies that exclusively show inhibition from GABA are from older animals (>P40 Gribkoff et al. 1999) or from multiunit or single-unit recordings that are likely to come from adults (frequently there is no age given, but for these studies there would be no benefit to using young animals; *e.g.,* Gribkoff, Pieschl and Dudek 2003; Gribkoff et al.

1999).

For clinicians, these results stress the need to consider differential diagnostic considerations for sleep and circadian disorders in adolescents. This need appears of special importance for the diagnosis of circadian phase disorders, such as delayed (DSPD) or advanced (ASPD) sleep phase disorders, as well as for insomnia and narcolepsy. Indeed, an earlier study of human adolescents indicated that 10th grade students maintaining their typical sleep schedule could fall asleep within 5 minutes during a Multiple Sleep Latency Test (MSLT) administered during the morning (8:30 a.m.). Furthermore, 48% of these teens showed at least one episode of sleep onset REM sleep (Carskadon et al.1998). This combination of findings within a clinical screening are hallmarks of narcolepsy (Mitler et al. 1979). Such findings highlight the importance of revising clinical assumptions in the face of the prevalence of sleep deprivation amongst teens asked to be awake at the "wrong" circadian phase. In addition, interpretations of teenagers' complaints of malaise, fatigue, and sadness need to take into account these developmental sleep/circadian issues that are exacerbated by the exigencies of life in the 21st century (Carskadon 2004).

Finally, for policy makers, teachers and parents, these results provide a clear mandate. The effects of sleep deprivation on grades, car accident risk, and mood are indisputable (Carskadon 2002; Wolfson and Carskadon 1998, 2003). If there is truly an adolescent physiological drive for later bedtimes, it is not enough merely to tell teens that they need to go to bed earlier. A number of school districts have moved middle and high school start times later (Lofgren 1999; Rosenthal 2007; Wahlstrom 2002) with the goal of improving teens' sleep-wake patterns by easing the pressure for early mornings. This approach is supported both due to its inherent logic as well as due to evidence from pilot programs where later school start times were associated with decreased truancy and drop-out rate (Wahlstrom 2002). On the other hand, societal complications can arise if the alternative is sending pre-adolescent children to school earlier and requiring after-school care.
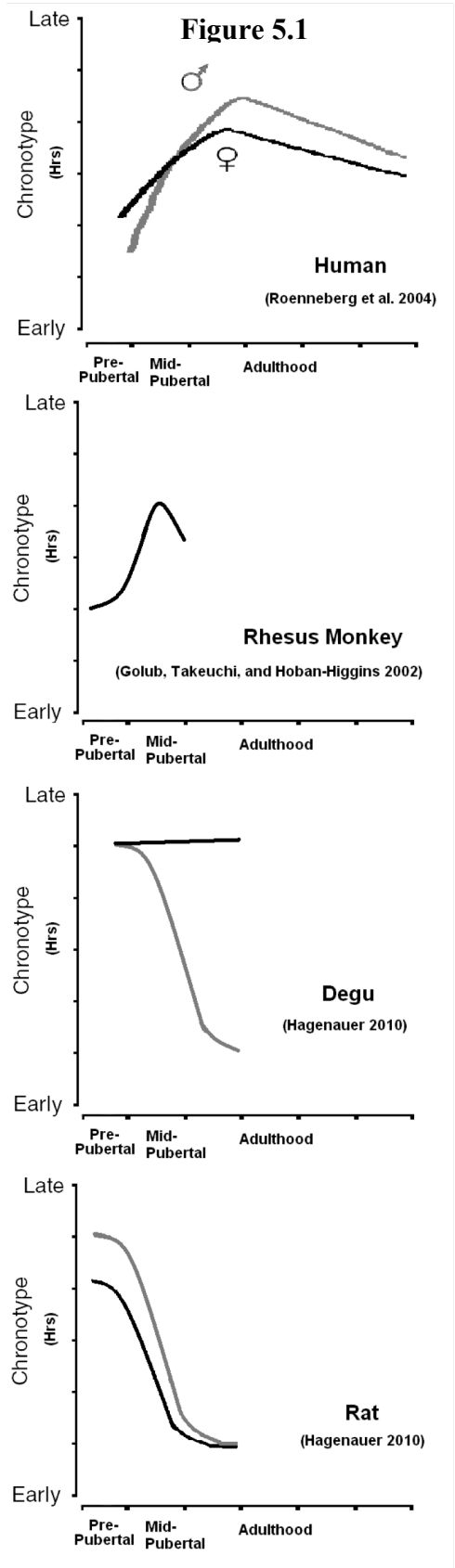
Schools can help teenagers gain control over their own sleep patterns by teaching sleep and circadian principles in middle and high school health education. Reducing light at night as well as TV or computer usage before bedtime can naturally advance circadian

phase. Similarly, incorporating outdoor morning activity into an adolescent schedule might also produce a phase advance. A Japanese study found that adolescents who were exposed to greater light exposure during the day were less likely to have trouble falling asleep at night (Harada, Morisane, and Takeuchi 2002). In high or low latitudes, daylight exposure can also produce the added benefit of reducing seasonal affective disorder (SAD), a mood disorder that affects more adolescents than any other age group (Imai et al. 2003; Tonetti et al. 2007). Whether these changes should be achieved through legislative initiatives on either the local or national level is unclear. What is clear, however, is that encouragement, pronouncements, or advocacy from the medical/scientific community is essential for driving such curricular enhancements (c.f., Committee on Sleep Medicine and Research 2006; National Commission on Sleep Disorders Research 1993).

"Of course, I shall never accomplish all the goals just listed, but that is unimportant. What counts is to have aims, to be able to work hard toward them and to experience the satisfaction of at least believing that progress is being made. I do not want to cross the finish line of this race – not ever – but I do hope I will be able to keep running at my own pace until I drop out still moving in full stride. It's been one hell of a good race."
  - Beach (1988) as cited in Glickman and Zucker (1994).

Figure 5.1. A comparison of chronotype changes during puberty in four well-studied species. Data from males and females are shown in grey and black, respectively. Chronotype was approximated from the phase variables measured in each experiment (citations given above), and is depicted in terms of relative change. Pubertal stage was assigned in the same manner as discussed in Chapter 1.
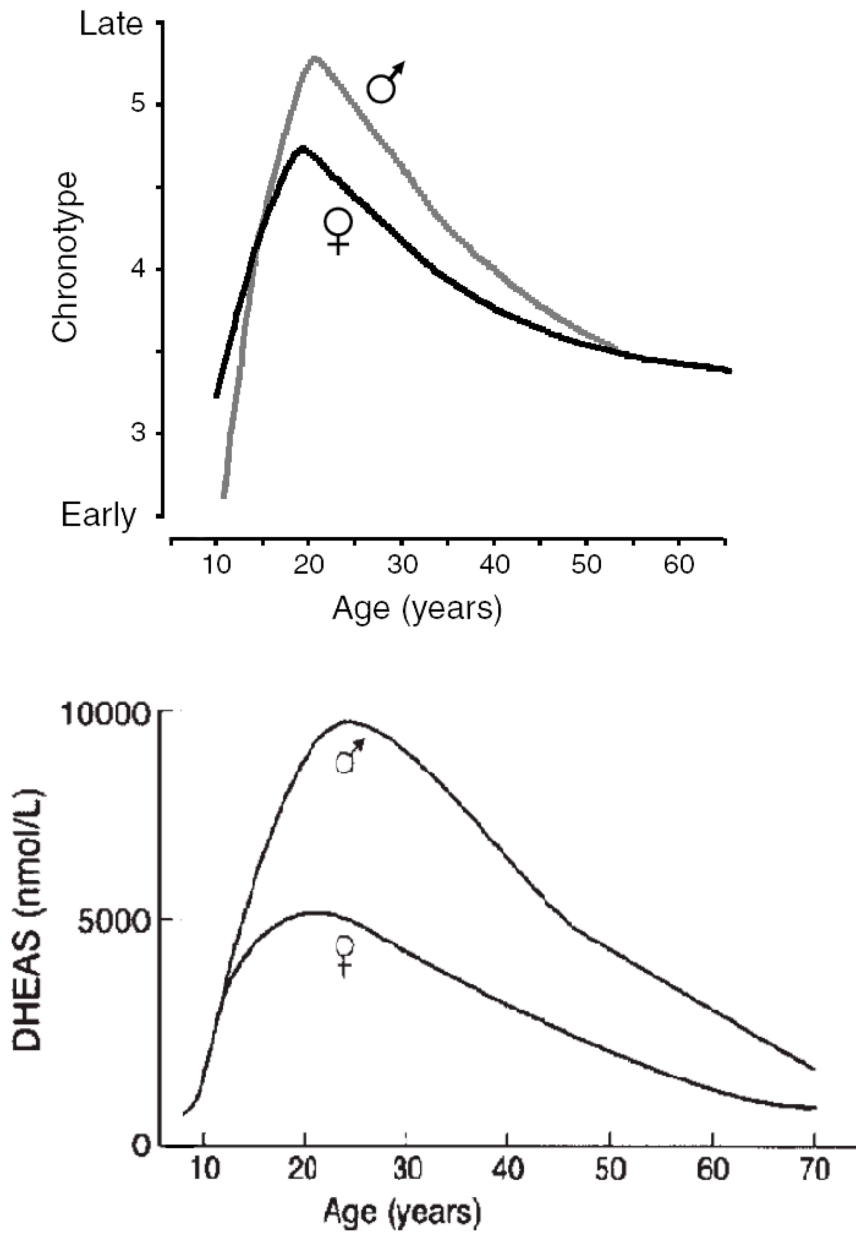
Figure 5.1

Human
(Roenneberg et al. 2004)

Rhesus Monkey
(Golub, Takeuchi, and Hoban-Higgins 2002)

Degu
(Hagenauer 2010)

Rat
(Hagenauer 2010)

Figure 5.2. Lifetime changes in serum DHEA-S concentration appear to correlate with changes in chronotype. The chronotype figure is identical to Figure 1.2 in the introduction and shows that individuals exhibited a transition into a more evening chronotype during adolescence (ages 10-20 yrs), as determined by self-reported mid-sleep phase. This trend showed strong sex differences, and then reversed for the rest of adult life (Adapted from Roenneberg et al. 2004). DHEA-S shows gradual increases in serum levels starting around the age of 6 years old, but these hormone increases accelerate at age 10-11 in a manner that exhibits sex differences. Serum concentration peaks at age 20-24 (concentration peaks earlier in females than males). Following this peak, DHEA-S levels decrease for the rest of adult life (adapted from Havelock, Auchus, and Rainey 2004).

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