Melatonin Synthesis and Signaling for Seasonal Reproductive Timing

By

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Abstract

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This dissertation critically investigates research in melatonin across species and disciplines. It offers a broader perspective within the field of reproductive neuroendocrinology by integrating research in pharmacology, genetics, and evolution. The first chapter of this dissertation reviews melatonin administration techniques across fields. The timing, dose, and mode of administration is compared across species. This juxtaposition of previous protocols using melatonin administration illustrates the importance of context to help inform future experimental designs. Furthermore, details of the experiment that affect the findings are emphasized to improve replicability of melatonin administration studies. The second chapter focuses on melatonin synthesizing enzymes in two different songbirds. The zebra finch (*Taeniopygia guttata*) is an opportunistic breeder in the wild and uses primarily food and water availability to time reproduction (Perfito et al., 2007). The European starling (*Sturnus vulgaris*) is a photoperiodic breeder whose reproductive state relies on changes in day length (Gwinner, 1977). Melatonin-synthesizing enzymes were expressed in photoreceptive sites of the brain. Key melatonin-synthesizing enzymes were differentially expressed in the hypothalamus of the photoperiodic breeder at certain times of the year, and this expression was not analogously observed in the opportunistic breeder. Melatonin may be a distinguishing physiological factor in reproductive timing between these two breeding types. The last chapter determines the physiological relevance of melatonin binding at the level of the gonad, specifically measuring how melatonin affects testosterone output from cultured testes from Syrian hamsters (*Mesocricetus auratus*), a photoperiodically-breeding mammal.

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To the people who have succeeded
To the people who have struggled
To the people who have changed the world
So I could have the privilege of writing this dissertation

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Introduction

"We begin in the dark, and birth is the death of us."
Antigonḗ, Translated by Anne Carson

The connection between darkness and birth is inextricable for photoperiodically and seasonally breeding animals. The tilt and rotation of the Earth creates geographical variation in temperature and day length. Daily and annual fluctuations of environmental conditions outside of the tropics, resulting from these astronomical circumstances, correspond to changes in relative resource abundance. Organisms that detect light with the physiological mechanisms to track and store photic information over time may use day length to anticipate environmental changes, subsequently altering behavior and physiology to optimize metabolism and seasonal reproductive timing. The synthesis of *N*-acetyl-5-methoxytryptamine, or melatonin, corresponds with darkness, thereby transducing photic information at the physiological level.

This introduction provides an overview of melatonin research in the context of seasonal reproduction in mammals, birds, and other vertebrates that integrate day length as a cue for reproduction, henceforth deemed *photoperiodic breeders*. Melatonin synthesis, binding, and signaling in the hypothalamo-pituitary gonadal (HPG) axis will be contextualized within existing models for molecular regulation of seasonal reproduction. I conclude by using the framework set forth by Tinbergen (1963) for ethology to pose questions that may guide future experiments studying the connection between melatonin and seasonal reproduction.

Melatonin Synthesis

Melatonin synthesis originated in mitochondria and chloroplasts (Tan et al., 2013). The antioxidant cascade of byproducts in the biosynthetic pathway are speculated to serve a key role in evolutionary history. Derived from the amino acid tryptophan, melatonin is synthesized with four key enzymes. Enzymes in the biosynthetic pathway optimized functionality by peaking expression during the time of day with lower temperatures and UV radiation: nighttime. The main source of circulating melatonin in vertebrates is the pineal, and photic information, transmitted via the phototransduction pathway, is the primary regulator of pineal melatonin synthesis and secretion (for review, see Reiter, 1991).

Phototransduction Pathways in Mammals and Birds. Melatonin is an established chemical transducer of photic information because its synthesis in photoreceptive organisms reaches its zenith in darkness. The inverse relationship between day length and the length of the night, which varies depending on the season outside of the tropics, is translated through the duration of melatonin synthesis at night. The duration of melatonin synthesis drives the reproductive state in a number of seasonal, photoperiodically-breeding mammals. Among these mammals, there are short-day breeders that breed in winter (e.g. sheep) and long-day breeders that breed in summer (e.g. hamsters). The gestation lengths vary to enable parturition at the predicted time of year with highest resource abundance, springtime. Long winter nights, corresponding to an extended duration of melatonin synthesis, stimulate the reproductive axis of short-day breeders and inhibit long-day breeders. In hamsters, a long-day breeder, induced testicular regression observed in extended darkness can be prevented by removing the pineal gland or through bilateral enucleation, illustrating the role light plays in reproductive state (Hoffmann & Reiter, 1965). Pinealectomies remove a significant portion of circulating melatonin

across vertebrates (for review, see Cassone, 1990), and the pineal gland continues to be researched as a key component of the pathway for photic and endogenous regulation of mammalian melatonin synthesis (for the phototransduction pathway see Klein & Moore, 1979; for review on pineal melatonin and reproductive state see Malpaux et al., 2001).

Even in the wild, artificial light at night corresponds to lower melatonin concentrations and compromises reproductive physiology in the tammar wallaby (Robert et al., 2015) and European starling (Dominoni et al., 2013), indicating there are ecological implications of the effects of light and melatonin on mammalian and avian breeding cycles. However, phototransduction in birds includes pineal and deep-brain photoreceptors situated beneath a translucent skull, whereas in mammals, photic information is transduced via the retino-hypothalamic pathway. Benoît conducted several experiments in ducks (*Anas platyrhynchos*) demonstrating that deep brain photoreception was sufficient to induce testicular development if the light administered includes blue wavelengths (Benoît & Ott, 1944; reviewed in Benoît, 1964). Bilateral enucleation and pinealectomy in tree sparrows (*Spizella arborea*) did not prevent testicular growth in long days (Wilson, 1991). Although this study removed the main sources of circulating melatonin, detectable concentrations of melatonin were measured in plasma after removal of the eyes and pineal in quail (*Coturnix japonica*). One-third of quail without these photoreceptive organs could still entrain to a light-dark cycle (Underwood & Siopes, 1984). These findings imply that melatonin synthesis may occur in yet another photoreceptive site in birds. Given that the other photoreceptive sites of birds, the eyes and pineal, use melatonin to transduce photic information, deep brain photoreceptors of the hypothalamus may use the same chemical signal. In fact, turkeys (*Meleagris gallopavo*) have melanopsin photoreceptors in the premammillary nucleus of the hypothalamus, along with key melatonin-synthesizing enzymes (Kang et al., 2007; El Halawani et al., 2009). Deep-brain photoreceptors in chickens (*Gallus gallus*) are capable of driving gonadal response (Kang & Kuenzel, 2015), in line with the findings Benoît observed nearly fifty years ago in ducks. The molecular mechanism of how light regulates melatonin synthesis may be understood through regulation of melatonin-synthesizing enzymes.

Melatonin-Synthesizing Enzymes. There are four enzymes involved in melatonin synthesis from its amino acid precursor, tryptophan: tryptophan hydroxylase, dopa-decarboxylase, arylalkylamine-N-acetyltransferase, and hydroxyindole-O-methyltransferase. The nomenclature of these enzymes and their variants, isozymes with the same functional roles but differentially regulated, continue to be modified as we learn more about when and how they are transcribed and translated. This overview accounts for how melatonin-synthesizing enzymes are regulated at the transcriptional level (Fig. 1a) and activation (Fig. 1b). A more detailed account of post-transcriptional modifications of these genes may be found in a book compiled by Pandi-Perumal and Cardinali (2007).

Tryptophan Hydroxylase. Tryptophan hydroxylase (TPH) initiates the melatonin biosynthetic pathway, and it is regarded as the rate-limiting enzyme in serotonin (5-HT) synthesis (Lovenburg et al., 1967). Nuclear factor-Y (NF-Y) transcription factor binds to enable the transcription of *tph* in mice (Reed et al., 1995). The CCAAT box binding factor (CBF)/NF-Y complex is involved in transcriptional activation of *tph* in human DNA derived from P815-HTR and HeLa nuclear protein extracts (Teerawatanasuk & Carr, 1998). Because NF-Y contributes to disease-response and photoreceptor cell differentiation in *Drosophila* (Ly et al., 2013), the possibility of TPH regulation in congruence with these processes is plausible. Specialty protein 1 (Sp1) is another known transcription factor for the *tph* gene (Simonneaux & Ribelayga, 2003).

In *Xenopus laevis*, *tph* expression in the retina is considered to be under circadian control because its expression fluctuates in a circadian fashion even in constant darkness (Green & Besharse, 1994). Additionally, transcriptional suppression of *tph* and the clock gene *bmal1* is mediated through circadian nuclear receptor REV-ERBα, shown through significant differences of *tph* expression observed in wild type and *Rev-erbα* KO mice (Chung et al., 2014). Given the functional similarities of TPH variants, TPH1 (NCBI Gene ID: 7166) and TPH2 (NCBI Gene ID: 121278), little has been done to distinguish transcriptional regulation of these two genes, found on different chromosomes in humans. Given that TPH plays a role in both serotonin and melatonin synthesis from the precursor, tryptophan, the end product to which transcriptional regulation of *tph* is directed cannot be determined. Therefore, transcriptional regulation of subsequent enzymes in the melatonin biosynthetic pathway must be considered alongside TPH.

Aromatic L-Amino Acid Decarboxylate. The second enzyme in the melatonin biosynthetic pathway, DOPA decarboxylase (DDC; NCBI Gene ID: 1644), or aromatic L-amino acid decarboxylase (AADC), decarboxylates the product of TPH (5-hydroxytryptophan) to synthesize 5-hydroxytryptamine (5-HT), serotonin. *Aadc* gene regulation varies depending on the tissue considered (for review, see Berry et al., 1996). The promoter regions of *ddc* has putative binding sites for octamer transcription factors (TF) and AP-2, suggesting alternative regulatory pathways (Hahn et al., 1993); however, neither of these transcription factors are regulated in a circadian manner. AADC depends on pyridoxal phosphate for functionality (Rahman et al., 1982). Because AADC is the rate-limiting step in neither serotonin nor melatonin synthesis, research on its regulatory mechanisms is sparse compared to the amount of research on the transcriptional regulation of the penultimate enzyme in the melatonin biosynthetic pathway, AANAT.

Arylalkylamine-N-Acetyltransferase. Arylalkylamine-N-Acetyltransferase (AANAT) or 5-HT-N-acetyltransferase (NCBI Gene ID: 15), is highly localized in the pineal gland (Axelrod & Weissbach, 1960) and converts serotonin into N-Acetylserotonin (NAS). NAS is an antioxidant (Oxenkrug, 2005) with its own circadian rhythm that binds and activates the TrkB receptor (Jang et al., 2010). Rhythmic transcriptional regulation of *AANAT* includes the cAMP response element modulator and its product, inducible cAMP early repressor (ICER) (Foulkes et al., 1997). In rat pinealocytes, adrenergic-cAMP regulation upregulated pineal *Aanat* synthesis in darkness and inhibit its own synthesis during the light period (Rosebloom et al., 1996). There are detectable levels of *AANAT* expression in the retina (Iuvone et al., 2005) and the rat brain (Rosebloom et al., 1996); however, the exact neural regions were not isolated in these particular studies. If comparable transcriptional regulatory mechanisms apply to other sites of Aanat synthesis, then *AANAT* expression may be regulated by light along the phototransduction pathway. This pathway includes specific nuclei of the hypothalamus in some mammals (e.g. the suprachiasmatic nucleus, or SCN, and paraventricular nucleus, or PVN) or deep-brain photoreceptors in birds (e.g. the premammillary nucleus). Interestingly, rhythmic melatonin synthesis in the sheep pineal did not correlate to *AANAT* gene expression, suggesting that sheep regulate pineal melatonin synthesis in a manner that differs from rodents or long-day breeders in general (Johnston et al., 2004). The AANAT protein has binding sites for casein kinase type II (CK-II), PKA, and PKC (Simonneaux & Ribelayga, 2003). Because CK-II phosphorylates PER2 in a circadian fashion (Tsuchiya et al., 2009), CK-II may refine circadian regulation of AANAT phosphorylation. An investigation into the evolution of AANAT revealed different subtypes across vertebrates (Cazaméa-Catalan et al., 2014; Li et al., 2015).

Hydroxyindole-O-Methyltransferase. The final enzyme of the melatonin biosynthetic pathway, hydroxyindole-O-methyltransferase (HIOMT, NCBI Gene ID: 438), synthesizes melatonin from NAS. Both light and time of day may influence *HIOMT* expression in the chicken pineal gland (Bernard et al., 1993). The avian pineal is directly photoreceptive, but the mammalian pineal gland receives photic input via the phototransduction pathway. A radioenzymatic assay detected direct β_1-adrenergic regulation of the *HIOMT* gene (Ribelayga et al., 1999). Additionally, AANAT may *not* be the rate-limiting enzyme in melatonin synthesis in rats, implying that HIOMT may play this role instead (Liu & Borjigin, 2005). Although AANAT is still regarded as the enzyme that drives the circadian rhythm of melatonin synthesis in some contexts, the amplitude of nocturnal melatonin synthesis that fluctuates with annual photoperiod may be regulated by HIOMT. In Siberian hamsters housed in short photoperiods, HIOMT activity is significantly higher than hamsters housed in long photoperiods, so there may be a seasonal relationship between HIOMT activity and melatonin synthesis (Ribelayga et al., 2000). The HIOMT protein, like AANAT, has binding sites for CK-II, PKA, and PKC (Simonneaux & Ribelayga, 2003). Because both enzymes are post-translationally activated by similar factors, it is possible that AANAT and HIOMT are both differentially regulated rate-limiting enzymes in the melatonin biosynthetic pathway.

Summary. The regulatory process of melatonin synthesis includes photoinhibition of photoreceptive sites as well as transcription, translation, and activation of melatonin-synthesizing enzymes. Instantaneous measurements of any one of these factors may differ based on the time of day and season of the year. The modes of transcription, translation, and activation reviewed here may not be comparable across tissues nor conserved across species. To date, the melatonin biosynthetic pathway and the enzymes it comprises have not been studied enough to claim there is circadian regulation of enzyme expression across vertebrates.

The main trend of research on the molecular underpinnings of melatonin synthesis focuses on mammalian pineal melatonin synthesis. In the following chapters of this dissertation, extra-pineal sites that may be capable of local synthesis and secretion of melatonin at undetectable levels in plasma were studied: the hypothalamus in birds and testes in mammals. By considering the expression of melatonin-synthesizing enzymes in the avian hypothalamus and within the mammalian testis, my research elucidates novel supplements to pineal melatonin synthesis in these seasonally breeding vertebrates. However, the endocrinological relevance of localized melatonin synthesis in extra-pineal tissue requires binding of melatonin and activation of its receptor.

Melatonin Binding

Once melatonin is synthesized, and its amphiphilic properties allow it to diffuse across the cellular membrane, it may either work as an antioxidant with byproducts that are a part of a cascade of free-radical scavengers (for review see Tan et al., 2003) or as a hormone and bind specific receptors. Two approaches are commonly used to research the endocrine role of melatonin: (1) receptor autoradiography in which radiolabeled ligands localize melatonin binding-sites or provide pharmacological evidence of melatonin receptor binding properties and (2) RNA extraction and/or *in situ* hybridization to label mRNA coding for specific melatonin receptor subtypes. The current section reviews recent findings from studies using these approaches to better understand melatonin binding. Additionally, the most extensive multiple

sequence alignment of melatonin receptor subtypes to date was conducted to fill gaps in our comparative understanding of melatonin receptor subtypes across vertebrates.

Melatonin and Receptor Autoradiography. Radioligand studies elucidate the localization of melatonin binding sites in peripheral tissues in rodents (Pang et al., 1994; Pandi-Perumal et al., 2008) and in the brain across vertebrates (Stankov & Reiter, 1990; Zawilska & Nowak, 1996; Vernadakis et al., 1998). The pars tuberalis (PT) of the anterior pituitary is a conserved melatonin binding site in mammals (Morgan et al., 1994). However, the localization of membrane receptors for melatonin in the hypothalamus varies across species, reproductive states, and lighting conditions. In ferrets (*Mustela putorius furo*), melatonin binds only in the pituitary and not in the brain (Weaver & Reppert, 1990). In other mammals, melatonin also binds in the hypothalamus. In Sprague-Dawley rats, the suprachiasmatic nucleus (SCN) of the hypothalamus and the median eminence (ME) have relatively higher melatonin radioligand binding (Vaněček et al., 1987). Furthermore, in male Wistar rats, there were no differences in the density and affinity of melatonin binding in the PT and the SCN, and daily fluctuations in circulating melatonin levels may regulate melatonin receptors in these sites (Gauer et al., 1993). Melatonin binds the SCN and ME in addition to the preoptic area (POA) and dorsomedial region of ventromedial nuclei (VMN) of Syrian hamsters (Williams et al., 1989). In C3H/HeN mice, 2-[^{125}I] iodomelatonin binding in the SCN is significantly higher two hours after lights on during the subjective day (Masana et al., 2000). Thus, the lighting condition, not independent of endogenously regulated circadian rhythms, affects melatonin binding in a given neural site. Although circulating concentrations of melatonin are undetectable in most species during the day (i.e. lights on in the laboratory), melatonin is still detectable at low concentrations in some tissues during the day (for review, see Huether et al., 1994), so there may be some biological relevance to this light-dependent change in melatonin binding. Syrian and Siberian hamsters also show 2-[^{125}I] iodomelatonin binding in neural regions not directly associated with the phototransduction pathway (Maywood et al., 1995). However, these neural sites have yet to be studied for potential circadian and/or developmental regulation of melatonin binding.

Non-rodent mammals have also been examined in trying to understand melatonin's functional role in seasonal reproductive timing. For example, sheep (*Ovis aries*) are commonly used to study the role of melatonin in seasonal reproductive timing is sheep. The premammillary nucleus (PMM) exhibits high melatonin binding in sheep (Malpaux et al., 1998). Ablation and replacement, or lesions, of sites with high density melatonin binding can provide information about the functional role of melatonin binding in a given site. However, this approach may disrupt other aspects of the network unrelated to melatonin binding. In non-mammalian vertebrates, day length and reproductive state affect melatonin binding as well. Melatonin binding in the forebrain of European starlings is observed in nuclei associated with the song control system, such as Area X. Total 2-[^{125}I] iodomelatonin binding was associated with reproductive state in starlings housed in the laboratory, not the lighting condition *per se* (Bentley & Ball, 2000). Annual fluctuations in the volume of these nuclei are affected by exogenous melatonin administration, even with removal of gonadal steroids via castration (Bentley et al., 1999). However, the changes in melatonin binding observed in the song-control nuclei over the course of a year are not directly correlated to changes in nuclei volume (Bentley et al., 2013). Also, reproductive state does not determine melatonin binding in these sites, for photostimulated male European starlings housed in semi-natural environments do not downregulate 2-[^{125}I] iodomelatonin binding in Area X, as expected in starlings housed in the laboratory (Bentley et al., 2013). These differences in 2-[^{125}I] iodomelatonin binding may result from synergistic

variables offered by semi-natural environments that are absent from laboratory settings (for review, see Calisi & Bentley, 2009). There are also ontological differences (in zebra finch, *Taeniopygia guttata*, see Gahr & Kosar, 1996) and sex differences for melatonin binding in the avian brain (in house sparrow, *Parus major*, see Whitfield-Rucker et al., 1996; in quail, *Coturnix japonica*, see Aste et al., 2001; in starling, *Sturnus vulgaris*, see Bentley et al., 2013). It is important to consider this intraspecies variation for comparative research in melatonin binding.

Radiolabeled ligand studies provide integral information to localize melatonin-binding sites in the brain and ascertain the density of melatonin receptors. The extent to which a given neural site binds melatonin depends on the time of day, season, and housing condition at the time of tissue collection. Sequencing and cloning techniques optimally parse out the different types of melatonin receptor subtypes.

Melatonin Receptor Subtypes. Melatonin's direct and indirect effects on reproduction are contingent upon its action as a hormone that binds particular receptor subtypes (Tan et al., 2010). Differences in regulatory functions of melatonin-binding proteins have procured categories of melatonin receptor subtypes. Melatonin receptor subtypes include membrane-bound G-protein coupled receptors and nuclear orphan receptors. Research on the functional role of melatonin receptors tends to focus on the membrane receptors, likely due to their higher affinity and specificity for melatonin. However, there is evidence that nuclear orphan receptors that directly bind melatonin may be primary targets downstream in the membrane receptor signaling pathway (Carlberg, 2000). Based on localization and administration studies, functions for melatonin membrane receptors likely regulate metabolic, cardiovascular, immune, and reproductive systems (for review see Dubocovich et al., 2010).

Research on melatonin receptor subtypes has its challenges. Autoradiography shows 2-[^{125}I] iodomelatonin binding density but fails to distinguish melatonin receptor subtypes. Furthermore, nomenclature for melatonin receptor subtypes varies across species and publications. Overall, research in mice and rats uses MT1 and MT2, but there are exceptions for some rodents, and Mel1a and Mel1b are used instead. MT3 is considered a mammalian melatonin receptor subtype as well, characterized as the enzyme quinone reductase 2 (QR2 or NQO2) (Nosjean et al., 2001). The discovery of a binding site for melatonin on QR2 justified its appellation of "MT3" as a putative melatonin membrane receptor (Dubocovich et al., 2005). The functional roles of MT1 and MT2 may not be analogous to Mel1a and Mel1b, respectively, in other non-mammalian vertebrates. Ideally, the nomenclature is not conflated without comparative evidence. Using the Percent Identity Matrix by Clustal Omega Multiple Sequence Alignment (Sievers et al. 2011), Fig. 2 compares mRNA sequences for Mel1a/MT1 and Mel1b/MT2. Furthermore, it shows MT3 does not share significant percent identity with mRNA sequences of other melatonin membrane receptors, supporting pharmacological evidence that it is not a melatonin receptor. This finding will be discussed later in this section on melatonin receptor subtypes.

Non-mammalian vertebrate research consistently uses Mel1a, Mel1b, and Mel1c for nomenclature. For the purpose of this review, the names are kept consistent with their use in the cited primary literature, but this is not to imply the Mel1a/MT1, Mel1b/MT2, or Mel1c/putative MT3 are interchangeable. Although there is evidence of some shared identity of melatonin receptor subtypes across vertebrates (Fig. 2), the names are assigned based on pharmacological binding properties, such as affinity and specificity. These binding properties have interspecies variation in subcellular regulation and differ across tissue types (Witt-Enderby et al., 2003; Morgan & Hazlerigg, 2008). In some vertebrates, gene polymorphisms and phylogenetic

analyses of melatonin subtype receptor amino acid sequences were studied (Li, D.Y. et al., 2003). Unlike Li, D.Y. et al. (2003), the percent identity matrix presented here (Fig. 2) is an alignment of mRNA sequences and includes QR2/NQO2, the putative MT3 receptor.

Besides binding melatonin, receptor subtypes share few conserved traits across vertebrates. The localization, activation, and regulation of receptor subtypes vary widely. Future studies should isolate and determine the affinity, specificity, and stability of melatonin and other agonist/antagonist binding for receptor subtypes in species which diverged further back in evolutionary history. Li, D.Y. *et al.* (2003) presented a phylogenetic tree of melatonin subtype receptor amino acid sequences, and Fig. 3 shows a cladogram by *Phylogeny.fr* (Dereeper et al., 2008 & 2010) of melatonin subtype receptor mRNA sequences from NCBI GenBank (Table 1 shows the list of NCBI Accession Numbers). Here, I focus on melatonin membrane receptors in mammals, birds, and other non-mammalian vertebrates, specifically elucidating the functional role of melatonin membrane receptor subtypes in the brain and gonads (for studies elucidating melatonin receptor subtypes in peripheral tissues in mammals, see Pang et al., 1994; Naji et al., 2004; Dubocovich & Markowska, 2005; Pandi-Perumal et al., 2008; and in birds see Lee & Pang, 1994; Natesan & Cassone, 2002; Jones et al., 2012; Guo et al., 2015).

In mammals. What regulates melatonin membrane receptor subtypes, and what does activation of different subtypes subsequently regulate in mammals? Each subtype receptor appears to have unique roles in different tissues and species. Methods used to isolate MT1, MT2, and the putative MT3 receptor are reviewed here.

Mel1a and MT1: Interchangeable names? Most mammalian Mel1a and MT1 research has focused on melatonin-binding in the suprachiasmatic nucleus (SCN) (for review of applications, see Pévet, 2016). As in most biomedical research, transgenic mice are used for their slight photoperiodicity and genetic homogeneity. In C3H/HeN mice, *mt1* mRNA and MT1 protein can be detected in the SCN, and there is evidence to suggest that this melatonin subtype receptor is regulated by diurnal and circadian mechanisms (Masana et al., 2000). In C3H/HeN mice housed in constant darkness for 6 weeks, a peak in the density of 2-[^{125}I] iodomelatonin binding in the SCN was observed at the beginning of the subjective day determined by free-running activity patterns (Masana et al., 2000). For C3H/HeN mice housed in light-dark cycles, low *mt1* mRNA levels were measured from SCN tissue collected during the day, and *mt1* mRNA expression peaked at the beginning of the dark period, coincident with increases in circulating melatonin (Masana et al., 2000). Because melatonin binding peaked in the SCN approximately 8 hours after the peak in *mt1* mRNA expression, it appears either that the melatonin receptor mRNA and protein are differentially regulated in C3H/HeN mice, or there is a time-lag in protein synthesis (Masana et al., 2000). Ultimately, studies in different strains of mice revealed that melatonin receptor MT1 is necessary and sufficient for transmitting the photoperiodic signal (Yasuo et al., 2009). There is the option of using "nature's knockout" to study Mel1a, for the Siberian hamster (*Phodopus sungorus*) Mel1b gene has nonsense mutations in the coding region (Weaver et al., 1996); however, there is still a Mel1b sequence stored in NCBI GenBank for *P. sungorus* (Accession Number U57555.1). Using this model organism and administering MT1/MT2 receptor agonist, Prendergast determined MT1 was necessary and sufficient to transduce photoperiodic information and alter reproductive and metabolic physiology (2010). The SCN in Syrian hamsters (*Mesocricetus auratus*), meanwhile, did not show 2-[^{125}I] iodomelatonin binding and was assumed to not have melatonin receptors. However, a study on post-natal (PN) Syrian hamsters revealed that Mel1a binding and expression was present in the SCN and highest before PN 8. While SCN binding of melatonin decreased after PN 8, the

expression levels of *Mel1a* mRNA decreased but not as significantly as autoradiographical binding, implying the developmental regulation of melatonin receptors in the Syrian hamster SCN is post-transcriptional (Gauer et al., 1998). Thus, we cannot disregard the effects of post-natal development on melatonin receptor regulation in general.

MT1 and MT2: Distinguishable receptor subtypes. Research on melatonin receptor subtypes becomes more complex when considering MT1 and MT2 together. In 1995, the early stages of research on melatonin receptor subtypes (at the time, named ML1 and ML2) were outlined by Dubocovich. Similarities and differences in the peripheral functions of MT1 and MT2 in mammals have been more recently revisited by Dubocovich and Markowska in 2005. Several steps were taken to distinguish these membrane melatonin-binding receptors. Firstly, pharmacological and functional characteristics of these two receptor subtypes are distinct (Ebisawa et al., 1994; Reppert et al., 1994). If the binding is stable, saturable, reversible, and specific, the ligand affinity of the receptors can be tested to determine if they are, in fact, distinguishable subtypes. Secondly, a specific radioligand was discovered to selectively target melatonin ML2 receptor across tissues in rodents (Molinari et al., 1996). Antagonists with a higher specificity for MT2 were used to isolate and distinguish the functional role of MT2 from MT1 receptor subtypes. Blocking specific receptor subtypes corresponded to downstream effects on behavior (e.g. activity rhythms and anxiety tests). For example, in C3H/HeN mice, 4-phenyl-2-propionamidotetraline (4P-PDOT), a specific antagonist for MT2, blocked melatonin-mediated phase advances in circadian rhythms (Dubocovich et al., 1998). In rats, luzindole blocked melatonin-induced antinociception (Yu et al., 2000). However, luzindole also may function as an antagonist for MT1 receptors, so the receptor-mediated effects observed in Yu et al., 2000 may include MT1. Another experiment, describing luzindole as a nonselective antagonist to MT1/MT2, observed luzindole and 4P-PDOT could block melatonin-induced phase advances in the SCN of Long-Evans rats when administered independently (Hunt et al., 2001). Because 4P-PDOT may have a higher specificity for MT2 (Dubocovich et al., 1998), the use of 4P-DOT blocked activation of MT2 in the SCN and prevented phase advances in circadian activity rhythms of mice and rats. Luzindole also had antidepressant effects on C3H/HeN mice subjected to the forced swim test (Dubocovich et al., 1990). These changes in behavior resulted from selectively targeting melatonin receptor subtypes with specific antagonists.

Other methods used to distinguish specific functional roles of melatonin receptor subtypes include western blots and southern hybridization, which localized MT1/MT2 in peripheral tissues (Naji et al., 2004). The limited distribution of MT2 protein in mice (restricted to the brain and lung) compared to the peripheral distribution observed of MT1 protein (including the brain, lung, heart, liver, and kidney) suggests a distinct functional role for MT2 in these tissues (Naji et al., 2004). It should be noted, however, that MT1/MT2 mRNA expression using RT-PCR method showed low expression of MT2 in the rat liver and heart (Sallinen et al., 2005). Either differences in species and methods affected results, or there is differential transcriptional/translational regulation of MT1 and MT2 in rodents.

Lastly, selectively bred rodents are ideal for parsing out the distinctions between MT1 and MT2 subtype expression and regulation. In C57BL/6 mice, there is a point mutation in the gene for AANAT (Rosebloom et al., 1998), and although the nocturnal peak in pineal melatonin concentration is not as high in C57BL mice relative to other strains (e.g. CBA mice), there are still significant circadian differences in pineal melatonin concentrations (Kennaway et al., 2002), suggesting there is still biological relevance for melatonin receptor subtypes. *In situ* hybridization and RT-PCR show that targeted disruption of *Mel1a* in selectively bred C57BL/6

mice disrupted 2-[^{125}I] iodomelatonin binding in the brain, suggesting Mel1a represents 99% of binding observed under this particular protocol (Liu et al., 1997). However, C57BL/6 mice with disrupted *Mel1a* are still capable of phase-shifting, so it was conjectured that relatively low levels of Mel1b may compensate (Liu et al., 1997). By using MT1-KO and MT2-KO mice, another study deduced the antidepressant effects of luzindole were mediated through the MT2 receptor (Sumaya et al., 2005). MT1-KO C57BL/6 mice have revealed the connection between the MT1 subtype and depressive or anxiety-like behaviors (Adamah-Biassi et al., 2014). In summary, transgenic mice provide a useful model alongside melatonin receptor antagonists to distinguish the functional roles of MT1 and MT2 in rodents (for review of targeted deletion of melatonin receptor subtypes, see von Gall et al., 2002). Mice models have been developed for studying therapeutic applications of selective blocking and activation of melatonin receptor subtypes (Tosini et al., 2014). However, transgenic mice are not representative of photoperiodically-breeding mammals. Research on the effects of melatonin receptor subtype knock-outs on seasonal reproductive timing should be conducted in other species.

Melatonergic Regulation of MT1/MT2 Receptor Subtypes. Seasonal changes in melatonin responsiveness are accompanied by daily changes of pineal synthesis and secretion of melatonin, and these fluctuations may be regulated by melatonergic negative feedback. Circulating concentrations of melatonin increase with the onset of darkness. However, this elevation in melatonin concentration is temporally constrained and not directly related to the absence of light. There is a detectable decrease in circulating melatonin concentrations before the onset of dawn, or before light directly inhibits melatonin synthesis. This finding implies that melatonin is regulated by something other than light during this pre-dawn trough. Specific melatonin receptor antagonists (luzindole and 4-P-PDOT) were administered to white-footed mice (Bedrosian et al., 2013). The MT1/MT2 antagonist, luzindole, prevented the light-independent drop in plasma melatonin typically observed late night/early morning while it was still dark (Bedrosian et al., 2013). This was not the first evidence to suggest lagging, homeostatic regulation of melatonin on itself via its own receptors. Melatonin affects MT2 functionality to regulate tissue sensitivity to the melatonin signal in rats as well. When administered at physiologically relevant concentrations and durations, melatonin desensitized MT2 in the rat SCN by preventing stimulation of PKC (Gerdin et al., 2004), providing yet another mechanism by which melatonin may regulate the circadian clock through melatonin receptor subtypes. The potential for negative feedback regulation of pineal melatonin via binding and activation of melatonin receptor subtypes MT1 and/or MT2 is an exciting possibility worth further investigation in other vertebrates. However, it is important to note that 2-[^{125}I] iodomelatonin binding was not observed in the pineal complex of fifteen avian and three testudine species previously studied (Cassone et al., 1995), and as described in mammals, binding properties of receptors may vary based on age, reproductive state, and the time of day the animal was used in the experiment.

Putative "MT3" receptor. There is relatively less research on the functional role of the putative mammalian MT3 receptor, part of the quinone reductase enzyme family and known as quinone reductase 2 (QR2) (Witt-Enderby et al., 2003; Tan et al., 2007). QR2 is an enzyme and not a classical seven transmembrane domains receptor (Boussard et al., 2006). MT3 has been described as the Syrian hamster homologue of human QR2 (95% identity) based on amino acid sequencing (Nosjean et al., 2000). Prazosin was used as an MT3 antagonist (Yu et al., 2000), and 5-MCA-NAT was used as an MT3 agonist in rabbits (Pintor et al., 2003) and monkeys (Serle et al., 2004). However, nuclear magnetic resonance studies using a biological source of QR2 [pcDNA3.1(+)/hQR2 plasmid, as in Nosjean et al., 2000] produced no evidence to suggest

melatonin is a substrate at all for MT3 (Boutin et al., 2008). This finding suggests that melatonin functions in the capacity of an antioxidant in conjunction with QR2. Despite this experiment, there are still countless publications referring to QR2 as the "putative MT3 receptor". 5-Methoxycarbonyl- amino-*N*-acetyltryptamine (MCA-NAT), a partial agonist of MT1 and MT2 at sub-micromolar ranges, does not elicit any detectable receptor-like responses from Chinese Hamster Ovary (CHO) cells overexpressing quinone reductase 2 (Vincent et al., 2010), even though MCA-NAT was used to study molecular responses to melatonin in chick retinal development (Sampaio, 2009) and bovine blastocysts (Sampaio et al., 2012). These studies assume that MCA-NAT specifically targets MT3, and luzindole non-selectively blocks MT1 and MT2. These assumptions, based on the premise the MCA-NAT targets MT3 and luzindole blocks MT1/MT2, ascribe the observed physiological effects to the functional role of MT3. Given insufficient evidence of MCA-NAT binding QR2, and contrasting evidence that QR2 even binds melatonin, studies that assume QR2 is functional melatonin subtype receptor should be reviewed with skepticism.

The inconsistent nomenclature QR2 in NCBI GenBank includes and is not limited to NAD(P)H quinone dehydrogenase 2, ribosyldihydronicotinamide dehydrogenase [quinone], and NRH:quinone oxidoreductase 2. The most frequently used acronym for QR2 identified for genes included in the phylogenetic analysis conducted here is NQO2 (see Fig. 2 and 3). Despite persistent claims of ambiguity on the matter (Dubocovich et al., 2005), the percent identity matrix shown here has no significant overlap with the mRNA sequences for NQO2 and mRNA sequences for membrane melatonin receptor subtypes (Fig. 2). This mRNA phylogeny supports pharmacological evidence (Boutin et al., 2008) that QR2/NQO2 is not a membrane melatonin subtype receptor.

Ecological relevance of melatonin binding sensitivity in mammals. Rodents that are considered more heterogeneous than transgenic strains of mice can be used to effectively study melatonin for more ecologically relevant questions. White-footed mice (*Peromyscus leucopus*) from Connecticut and Georgia are sensitive and insensitive to melatonin, respectively (Weaver et al., 1990). A longer duration of melatonin is observed in *P. leucopus* housed in short photoperiods (Petterborg et al., 1981). When maintaining short photoperiods (8L:16D) for long-term housing of *P. leucopus* mice from Connecticut, Georgia, and Maine, only mice from Georgia remained reproductively competent (Lynch et al., 1981). Meanwhile, mice from Connecticut and Maine underwent testicular regression and spontaneous recrudescence within this extended exposure to short photoperiods (Lynch et al., 1981). Daily injections of 50µg of melatonin in wild-caught mice from Connecticut and Georgia led to six out of fourteen mice from Connecticut molting into winter pelage with no observable effects on mice collected from Georgia (Heath & Lynch, 1982). In *P. leucopus* mice wild-caught and selectively bred from Virginia, strains that were responsive and nonresponsive to changes in photoperiod were studied for differences in melatonin binding (Heideman et al., 1999). Selectively bred, nonresponsive white-footed mice showed higher 2-[^{125}I] iodomelatonin binding in the medial preoptic area (mPOA) and nucleus stria terminalis, which may be due to differences in density or affinity of receptors in these areas (Heideman et al., 1999). These findings suggest that intraspecies geographical variation in melatonin sensitivity is fixed in the wild.

Melatonin receptor subtypes in mammalian gonads. Most melatonin receptor research in mammals focuses on the brain or eyes. Because melatonin receptor expression in hamster testes is studied in the third chapter, here an overview of MT1/MT2 in the gonads of rodents shall be discussed. The antioxidant role of melatonin in ovaries was reviewed previously (Tamura et al.,

2009), but the presence of melatonin receptors in ovarian tissue suggests melatonin plays an endocrine role as well. In rat ovaries, PCR and *in situ* hybridization showed *mt1*/*mt2* expression at various stages of the estrous cycles (Soares et al., 2003). The functional relationship between melatonin receptor subtypes and estrous cycles is unexplored to date. In future studies that assess the physiological effects of exogenous melatonin in the ovaries, concentrations of melatonin administered must be considered. Supraphysiological levels of melatonin administered to the Chinese hamster ovary (CHO) cell line increased MT1 detected and decreased affinity observed through competitive binding with 2-[^{125}I] iodomelatonin (Gerdin et al., 2004). These melatonergic effects on MT1 in the CHO cell line may be mediated through specific modifications of the subcellular signaling cascade (Bondi et al., 2008). However, melatonin administered at physiologically relevant levels had no effects on CHO cell line MT1 (Gerdin et al., 2004). The role of melatonin in testes on sperm production was studied across several groups of mammals. Although it was previously observed that 2-[^{125}I] iodomelatonin did not bind in the gonads of mammals other than shrews (family Soricidae) (Ayre & Pang, 1994), regulatory mechanisms of MT1/MT2 expression were later found in spermatozoa and ejaculate of five different breeding types of mammals (González-Arto et al., 2016). Melatonin's endocrine role in mammalian testes appears to, in part, regulate sperm maturation. In rat testes, *mt1* and *mt2* are expressed throughout development (Izzo et al., 2010), and melatonergic effects on rat spermatogenesis and steroidogenesis were studied (Ellis, 1972; Tijmes et al., 1996; Stefulj et al., 2001; and see Acuña-Castroviejo et al., 2014 for partial review). Because rats are not as photoperiodically responsive as hamsters, the potential for melatonin binding and receptor-mediated changes in testes of this relatively more seasonal species is investigated later in this dissertation.

In birds. The majority of reviews on melatonin receptor subtypes focus on mammals. This section provides a comprehensive review of melatonin receptor subtypes in birds. Early work focused on the general distribution and characterization of 2-[^{125}I] iodomelatonin binding in the brain of chicks (Rivkees et al., 1989) and quail (Cozzi et al., 1993). The affinities and densities of 2-[^{125}I] iodomelatonin binding observed in ducks, geese, pigeons, and turkeys (Zawilska & Nowak, 1996) were an order of magnitude lower than what was previously described in quail (Cozzi et al., 1993), suggesting that the pharmacological properties of melatonin receptors may not be conserved across different species of birds. 2-[^{125}I] iodomelatonin binding also was compared in brains collected from five orders of birds (Psittaciformes, Passeriformes, Columbiformes, Galliformes and Anseriformes) and turtles, and melatonin binding was not observed in the pineal, adenohypophysis, or tuberal hypothalamus (analogous to SCN) in any of the avian or testudine species studied (Cassone et al., 1995). Additionally, our understanding of melatonin binding in the avian brain was challenged by the discovery of how photoperiodic history affects 2-[^{125}I] iodomelatonin binding in quail (Panzica et al., 1994) and songbirds (Bentley & Ball, 2000; Bentley et al., 2013). 2-[^{125}I] iodomelatonin binding densities in the brain varied based on photoperiodic history, reproductive state, and sex of birds (Aste et al., 2001).

Melatonin receptor subtypes in the avian brain. Parsing out different receptor subtypes in the avian brain is useful for understanding context-dependent differences. Melatonin receptor antagonists such as prazosin and luzindole selectively block specific subtypes in broiler chickens (Li, J. et al., 2013). However, the affinity and specificity of these antagonists cannot be guaranteed, especially considering differences in avian melatonin receptor pharmacology in different species (Zawilska & Nowak, 1996). Furthermore, expression analysis of subtype

receptor sequences identifies the presence of mRNA in different neural sites. RT-PCR was used to identify *Mel1a*, *Mel1b*, and *Mel1c* in the chick brain (Reppert et al., 1995). The same technique was used in zebra finch brain and peripheral tissues and found significant rhythms of both *Mel1a* and *Mel1b* expression in cerebellum, diencephalon, retina, and tectum opticum (Jones et al., 2012). *Mel1a* expression patterns showed significant rhythms in the telencephalon, and *Mel1b* showed significant rhythms in the pineal gland (Jones et al., 2012). However, the expression patterns of *Mel1c* in the zebra finch brain did not appear to be significantly rhythmic (Jones et al., 2012). *In situ* hybridization assesses the distribution of specific melatonin receptor subtypes in the avian brain (in quail, Ubuka et al., 2005; in blackcap and zebra finch, Fusani & Gahr, 2014). These findings provide some insight into the specific functional roles of melatonin receptor subtypes in birds. For instance, *Mel1c* expression was co-localized with gonadotropin inhibitory neurons (GnIH) (Ubuka et al., 2005). Several neural sites involved in sensory motor integration also co-express *Mel1a* & *Mel1b* or *Mel1a* & *Mel1c*, with few sites expressing all three subtypes or *Mel1b* & *Mel1c* (Fusani & Gahr, 2014). In male zebra finches (*Taeniopygia guttata*), the song motor control pathway expressed melatonin receptor subtypes, and administration of Mel1b antagonist (S20928) transiently shortened the length of the song (Jansen et al., 2005). Considering inter-species differences in melatonin subtype receptor characteristics previously described, the affinity and specificity of S20928 for the Mel1b receptor still needs to be determined in this species.

Melatonin receptor subtypes in the periphery of birds. Melatonin receptor subtypes have also been studied peripherally in birds. In chickens, melatonin subtype receptor mRNA temporal patterns and spatial distribution were found in the retina (Natesan & Cassone, 2002) and in the spleen (Gui et al., 2015). In the latter study, age-related changes in subtype receptor expression were discovered (Gui et al., 2015), indicating age in birds, as previously described in mammals, is an important variable to account for in future studies.

The presence of melatonin receptor subtypes in avian gonads has exciting implications for the endocrine role of melatonin in the avian HPG axis. 2-[^{125}I] iodomelatonin binding was observed in the testes and ovaries of chicken, duck, and quail (Ayre & Pang, 1994). Partial sequences of melatonin receptor subtypes from the chicken ovary were identical to subtype receptor sequences from the brain (Sundaresan et al., 2009). Follicles at varied stages of development expressed different levels of melatonin receptor subtypes. Small white follicles only expressed *Mel1b*, and small yellow follicles expressed all three subtypes (Sundaresan et al., 2009). *Mel1a* was restricted to the chicken thecal layer and *Mel1b* and *Mel1c* were expressed in both chicken granulosa and thecal layer (Sundaresan et al., 2009). In European starlings, we previously found *Mel1b* and *Mel1c* expression correlated with expression of gonadotropin inhibitory hormone (GnIH) and its receptor (GnIHR), respectively (McGuire et al., 2011). Furthermore, we found that *Mel1b* expression in starling testes appeared to correspond with day length and *Mel1c* with reproductive state, suggesting these receptor subtypes are differentially regulated and might serve different functions in songbird testes (McGuire et al., 2011). There is also differential photoperiodic regulation of melatonin subtype receptor expression in tropical bird testes (*Perdicula asiatica*, see Yadav & Haldar, 2013). The effects of monochromatic light on ovarian melatonin subtype receptor expression were studied in chickens (Li et al., 2015). Hens that were housed in red (660 nm) light expressed *Mel1a* and *Mel1c* at significantly higher levels than all other groups (Li et al., 2015). Furthermore, hens housed in blue (480 nm) light laid significantly more eggs than all other groups (Li et al., 2015). Blue light suppresses pineal melatonin synthesis in chickens (Csernus et al., 1998; Torii et al., 2007). Li et al. (2015) show an

inverse relationship between fecundity and melatonin subtype receptor expression in the chicken ovary. The implications of these findings are discussed in greater detail in the section of this introduction on melatonin signaling.

Methods used in mammals to study melatonin subtype receptor functionality, such as transgenics (von Gall et al., 2002; Tosini et al., 2014) and natural variation in intraspecies melatonin sensitivity (Weaver et al., 1990), have no obvious equivalents in birds. Mammalian melatonin receptor agonists or antagonists show variance in how they bind (i.e., specificity and affinity) based on the age and sex of the animal as well as the time of day of administration. To account for these variables across avian species would be an exhaustive undertaking. There are no established practices for taking advantage of natural variation in melatonin sensitivity within a species of bird found in the wild, as previously described in white-footed mice. Chapter 2 lays the foundation for considering different breeding types of birds, opportunistic and photoperiodic. Future work may explore binding properties of melatonin receptor subtypes in different breeding types with confounding variables held constant. Additionally, new gene editing technologies, such as CRISPR-Cas9 (Jenik et al., 2002; Burstein et al., 2016), provide a novel approach for melatonin subtype receptor research. Comparative research on melatonin receptor subtypes in other non-mammalian vertebrates may inform future experiments in birds.

In other vertebrates. The effect on pineal extractions on skin pigmentation of *Xenopus* tadpoles was discovered a century ago this year (McCord & Allen, 1917). The pineal complex and melatonin were causally connected to the diurnal rhythms of color change in lamprey much later (*Lampetra*) (Joss, 1973). Forty years after McCord and Allen (1917) published their findings in *Xenopus*, melatonin was isolated (Lerner et al., 1958), and nearly thirty years later, the relationship between melatonin and photoperiod and their effect on anuran larval development was empirically confirmed (Delgado et al., 1987). Despite this long history investigating the effects of melatonin on amphibia, research on melatonin receptor binding and subtypes in non-mammalian and non-avian species is relatively recent. 2-[^{125}I] iodomelatonin binding was studied in amphioxus (*Branchiostoma lanceolatum*), Atlantic hagfish (*Myxine glutinosa*), larval and adult lamprey (*Petromyzon marinus*), little skate (*Raja erinacea*), and rainbow trout (*Oncorhynchus mykiss*), and all but hagfish showed specific binding in the brain of these species (Vernadakis et al., 1998). *In vitro* culture of the pineal complex from lamprey (*Petromyzon marinus*) revealed fluctuations in melatonin secretion when kept in light:dark (12L:12D) cycles, and this rhythm did not persist in constant darkness (Bolliet et al., 1993). Melatonin secretion from the lamprey pineal complex is likely temperature dependent because the rhythm of melatonin secretion from the cultured lamprey pineal complex was maintained in 20°C but abolished in 10°C in constant darkness (Samejima et al., 1997), showing that melatonin secretion is regulated by more than light in other vertebrates. In turtles (*Chrysemys picta*), 2-[^{125}I] iodomelatonin binding was observed primarily in the visual system (Larson-Prior et al., 1996), potentially affecting photosensitivity and the capacity for light to affect melatonin synthesis.

The existence of melatonin receptor subtypes could not be confirmed until sequencing technologies became more accessible. *Mel1c* in *Xenopus* was cloned just over two decades ago (Ebisawa et al., 1994), around the same time mammalian *Mel1a* and *Mel1b* were cloned (Reppert et al., 1994). In the past 10 years, melatonin receptors were cloned and sequenced from sea bass (*Dicentrarchus labrax*) (Sauzet et al., 2008), sole (*Solea senegalensis*) (Confente et al., 2010), and the mudskipper (*Boleophthalmus pectinirostris*) (Hong et al., 2014). The study in sole showed seasonal and daily fluctuation in expression levels of melatonin receptor subtypes

(Confente et al., 2010), and melatonin receptor subtypes receptor subtypes in the mudskipper seemed to synchronize with the semilunar spawning rhythm (Hong et al., 2014). In the velvet belly lantern shark (*Etmopterus spinax*), melatonin stimulated light production by isolated photophore-filled skin patches (Claes & Mallefet, 2009). Luzindole and 4P-PDOT blocked this response, suggesting shark luminescence is mediated by the MT2 receptor (Claes & Mallefet, 2009). Research on melatonin and its receptor subtypes in aquatic craniates and poikilotherms extends beyond pure classification and is answering questions related to the functional roles of melatonin receptor subtypes (for review, see Filadelfi & Castrucci, 1996).

Recent research on the functional role of membrane melatonin receptors in Actinopterygii focused on reproductive and lunar cycles. 17α,20β-dihydroxy-4-pregnen-3-one (DHP) was used as a biomarker for the onset of spawning in mudskippers, and melatonin injections increased DHP *in vivo* and in cultured ovaries (Hong et al., 2014). Melatonin subtype receptor expression corresponded with the lunar cycles and with the spawning season in mudskippers (Hong et al., 2014). However, it is important to note only one reference gene (*ß-actin*) was used in the qRT-PCR analysis (Hong et al., 2014), and accurate normalization ideally includes multiple control genes (Vandesompele et al., 2002). In the orange-spotted grouper (*Epinephelus coioides*), *mt1* and *mt2* expression in the brain varied based on reproductive state (Chai et al., 2013). Again, only one reference gene (*18S*) was used in the qRT-PCR analysis (Chai et al., 2013). In the gold-lined spinefoot (*Siganus guttatus*) fluctuations in *MT1* and *Mel1c* expression corresponded to lunar brightness (Park et al., 2014). The overall relationship between lunar brightness, melatonin, and fish reproduction was previously reviewed (Ikegami et al., 2014). Cultured pineal of golden rabbitfish (*Siganus guttatus*) varied in melatonin content based on exposure to moonlight intensity (Takemura et al., 2006). In the grass puffer (*Takifugu niphobles*), *mel1a* (1.4 & 1.7), *mel1b*, and *mel1c* appear to be expressed in constant darkness with ultradian regulation in the pineal gland, so there may be light-independent lunar oscillations (Ikegami et al., 2015). Given that these results show lunar patterns in tissue cultured in darkness, there is more to lunar cycles than moonlight affecting melatonin receptor subtypes in fish, possibly relating to the type of environment in which the fish are found in the wild (fresh water, salt water, tidal patterns, still water, etc.).

Comparative research on melatonin receptor subtypes has broader implications across vertebrates. Findings connecting moonlight to melatonin synthesis in mammals are comparable to lunar patterns observed in fish. Moonlight suppressed pineal melatonin production in Syrian hamsters (Brainard et al., 1984). However, moonlight appears to have no effect on pineal AANAT activity and melatonin content in the cotton rat (*Sigmodon hispidus*) (Nürnberger et al., 1985), reminding us that moonlight, like all other variables to be considered, may not show conserved effects across species or individuals. The effects of moonlight in fish may be more conserved because the pineal itself is photoreceptive in this order. Whether the animals were caught in the wild or reared in the laboratory is also cause for such variance (for review, see Calisi & Bentley, 2009). The effects of moonlight on avian melatonin synthesis has yet to be investigated. Given that (1) the pineal in birds is photoreceptive, (2) the mRNA sequences of melatonin receptor subtypes in birds and other non-mammalian vertebrates are relatively conserved (see Fig. 2 and 3), and (3) urban light pollution at night affects melatonin content in birds, even at low levels (in European blackbirds, *Turdus merula*, Dominoni et al., 2013; and in western scrub-jays, *Aphelocoma californica*, Schoech et al., 2013), we should consider the potential for moonlight to affect melatonin and its receptor subtypes in birds as previously observed in fish. This possibility is considered in Chapter 2 because the European starlings and

zebra finches were exposed to moonlight at the field station where they were housed for the duration of the study.

Summary. The determined location of melatonin-binding sites and quantified expression levels of melatonin receptor subtypes set the conditions of possibility for melatonergic functionality in reproductive physiology. Given that binding densities revealed by autoradiography do not isolate specific melatonin receptor subtypes, we must use protein assays and RNA sequencing to determine the presence of melatonin receptor subtypes in a given tissue. The multiple sequence alignment of amino acid sequences previously determined shared identities of melatonin receptor subtypes across a subset of vertebrates (Reppert et al., 1994; Li D.Y. et al., 2003; Sauzet et al., 2008; Confente et al., 2010; Hong et al., 2014). Because NCBI GenBank offers an expansive list of melatonin subtype receptor mRNA sequences from more species now than ever before (see names of sequences and Accession No. organized in Table 1), the need for a multiple sequence alignment and phylogenetic analysis of mRNA sequences from known and putative melatonin receptor subtypes in vertebrates was fulfilled here (see Fig. 2 and 3). Amino acid sequences of melatonin membrane receptors share distinct clades, but this analysis was limited to alignment of amino acid sequences from four mammals, one fish, one bird, and one frog (Reppert et al., 1995). The mRNA multiple sequence alignment conducted here includes 11 mammals, seven birds, and 10 other vertebrates in Mel1A and a subset of this list for available sequences for other subtypes (for Accession No. see Table 1), rendering the most extensive multiple sequence alignment of melatonin subtype receptor mRNA sequences to date. Within the percent identity matrix (Fig. 2, analyzed with Clustal Omega), mRNA sequences of melatonin receptor subtypes in Aves share a higher identity with sequences from Osteichthyes, Testudines, Crocodilia, Anura, and Latimeria (~70.00%-99.99%) than with Mammalia (~60.00%-79.99%). The cladogram (Fig. 3, analyzed in *Phylogeny.fr*) shows higher parsimony with non-mammalian melatonin receptor subtypes (***nmvMel1A***, ***nmvMel1B***, ***nmvMel1C***) and mammalian Mel1B (***mMel1B***) than with mammalian Mel1A (***mMel1A***). This observation suggests that mammalian Mel1A (***mMel1A***) and ***nmvMel1A*** are not homologous. Furthermore, the clade of QR2/NQO2, the putative MT3 receptor, diverged prior to the clade of melatonin receptor subtypes than the outgroup (*Homo sapiens* opioid receptor, Accession No. L29301.1, selected based on rat μ Opioid R outgroup used in Reppert et al., 1995). Because the identity of QR2/NQO2 is less than 59.99% with all membrane melatonin receptor subtypes across vertebrates (Fig. 2), the findings of this matrix support pharmacological evidence (Boutin et al., 2008) that QR2/NQO2 is not a melatonin membrane subtype receptor.

In addition to quantifying mRNA sequences and localizing melatonin receptor subtypes, future studies must rigorously test the binding properties of agonists and antagonists to determine affinity and specificity with melatonin receptor subtypes. Binding properties of melatonin receptor subtypes not only vary across species but also can vary with time of day, age, and sex. Ligands that target melatonin receptor subtypes in one species may not have the same affinity and specificity in another species. If investigators do not test the binding affinities of melatonin receptor agonists/antagonists in the model organism being used, then we cannot be certain that the observed physiological and behavioral changes are the result of targeted activation or blocking, convoluting melatonin subtype receptor applications and therapies. Following melatonin binding to specific receptor subtypes, physiological changes that occur in the context of reproduction will be addressed in the next section on melatonin signaling.

Melatonin Signaling in Reproduction

> *To think that we understand survival value completely...is to think that, once it is obvious that sex hormones control mating behaviour, we need not inquire into the way they do this, nor into the interaction between various endocrine processes that are involved.* (Tinbergen, 1963, pp. 419)

The classical definition of a hormone is a chemical that is synthesized and secreted from a particular gland into circulation, binds to a specific receptor, and induces a physiological change. Distinguishing the endocrine function of melatonin from its role as an antioxidant is more complex than it first may seem. Although the main specialized gland for melatonin synthesis is considered to be the pineal, removing this gland does not remove all circulating melatonin (discussed further in Chapter 1 in a review of melatonin administration techniques). The four enzymes that synthesize melatonin from the precursor tryptophan are expressed in several other tissues (discussed further in Chapter 2 on the avian hypothalamus and Chapter 3 on the mammalian gonad), which strongly suggests that melatonin synthesis is distributed peripherally. Furthermore, melatonin endocrine action can be mediated by melatonin receptor subtypes, but melatonin and its precursor N-acetylserotonin also can function as free-radical scavengers as part of an antioxidant cascade, and the byproducts resulting from degradation are also antioxidants. To differentiate the classical endocrine role of melatonin from its antioxidant effects requires us to focus on receptor-mediated functions, and yet unless specific melatonin receptor agonists/antagonists are administered, we cannot isolate these receptor-mediated functions.

This section addresses physiological responses induced by melatonin in the context of seasonal reproduction. It is important to note that many of the studies reviewed here do not use specific melatonin receptor agonists/antagonists, so whether melatonin corresponds to specific reproductive physiological changes in its capacity as an antioxidant or hormone is inextricable unless the study determines if specific receptor subtypes are expressed or immunolabeled. Approaches that are commonly employed to study melatonin signaling in seasonal reproduction include (1) ablation, lesion, or culture of melatonin-synthesizing and/or binding sites, (2) co-localization of melatonin-synthesizing and/or binding sites within neural and/or peripheral reproductive pathways, and (3) exogenous administration of melatonin. This section will focus on studies that employ these approaches to study the physiological relevance of melatonin in vertebrate reproductive pathways with the hypothalamo-pituitary gonadal (HPG) axis as the point of convergence.

HPG axis. Among the various endocrine feedback loops fluctuating within age- and sex-dependent homeostatic ranges is the hypothalamo-pituitary gonadal (HPG) axis. A subset of hypothalamic neurons synthesizes and transports gonadotropin releasing hormone (GnRH, or luteinizing-hormone-releasing-hormone, LHRH, see Sandow, 1983). There are several different isoforms of GnRH across vertebrates (for reviews, see Millar et al., 2001; Pawson et al., 2003; and Roch et al., 2014), and these GnRH subtypes have yet to be studied in conjunction with melatonergic effects on seasonal reproductive timing in vertebrates. In mammals, kisspeptin (*Kiss*) and RFRP (or gonadotropin inhibitory hormone, GnIH) are hypothalamic neuropeptides that, respectively, stimulate and inhibit the synthesis and release of GnRH (for reviews on kisspeptin, see Revel et al., 2007 and Greives et al., 2007; for reviews on GnIH/RFRP, see Bentley et al., 2006; Tsutsui et al., 2010; and Kriegsfeld et al., 2015).

GnRH is secreted in a pulsatile fashion and binds the anterior pituitary to stimulate release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (for review, see Padmanabhan et al., 2001) in a frequency-dependent fashion (Thompson & Kaiser, 2014). It is important to note that gonadotropins may also be regulated by other factors, such as inhibins (for review, see Ying, 1988) and steroids (Karsch, 1987; for reviews see Burger et al., 2004; Dungan et al., 2006; and Smith, 2008). GnIH also binds in the pituitary and decreases gonadotropin synthesis and release (Ubuka et al., 2006). LH and human chorionic gonadotropin (hCG) both bind the same receptor, but they are differentially regulated and support different aspects of the reproductive cycle (for review, see Choi & Smitz, 2014). Upon synthesis and release from the pituitary, LH and FSH bind receptors in the gonads (testes/ovaries), stimulating steroidogenesis (for review, see Walker & Cheng, 2005) and regulating the ovulatory process (for review, see Chappel & Howles. 1991). Androgens and estrogens negatively feedback to the hypothalamus and pituitary to downregulate GnRH and gonadotropin secretion (for review, see Karsch, 1987; Gill et al., 2002). The literature on gonadal steroid negative feedback comprises a number of papers detailing the mechanisms at the level of the hypothalamus and pituitary, but there is not a comprehensive review found of this to date.

Interestingly, the classical HPG axis is expanded by recent research calling into question the precision of its nomenclature. For example, there are also GnRH and GnIH receptors in the gonads, which raises questions about how these "neuropeptides" function at the level of the gonads (for review, see McGuire & Bentley, 2010). Additionally, estrogens are necessary for proper spermatogenesis and testicular function (Carreau et al., 1999; Hess, 2003; Hess & Carnes, 2004). Referring to estrogen as the "female hormone" in scientific literature potentially postponed research on this important role for estrogens in testes. How we limit the questions we ask as researchers by using socially-constructed language in scientific nomenclature is evidenced by these few examples in the HPG axis.

The HPG axis is in constant interface with and influenced by factors such as social cues (Maruska & Fernald, 2011) and aging (Morley et al., 1997; Gill et al., 2002; Peper et al., 2010). This introduction focuses on the interaction between melatonin and seasonality of the vertebrate reproductive axis. Specifically, melatonin and the HPG axis are reviewed here in post-pubertal mammals, birds, and other non-mammalian vertebrates (for review of the HPG axis during puberty, see Peper et al., 2010).

In mammals. The mammalian brain and the gonads contain melatonin-synthesizing sites (for review, see Acuña-Castroviejo et al., 2014). Given that pinealectomies prevent light-induced changes in the reproductive state of photoperiodic mammals, we may deduce that pineal-derived melatonin is necessary for maintaining reproductive physiology in seasonal, photoperiodically-breeding mammals. Furthermore, there appears to be no compensatory mechanism for melatonin synthesized in other regions. This section focuses on the effects of melatonin on the mammalian hypothalamus, pituitary, and gonads in seasonal reproduction.

Melatonin in Mammalian Hypothalamus. The hypothalamus integrates environmental and physiological information to inform everything from satiety to osmoregulation to reproductive state. In previous sections of this introduction, the mammalian hypothalamus has been described in terms of its role as a conduit in the mammalian phototransduction pathway and as a melatonin binding site. Here, we focus on how the mammalian hypothalamus integrates photoperiodic and/or melatonergic signals to affect the reproductive condition of different breeding types of mammals (for an additional review on this topic, see Revel et al., 2009).

Although evidence for immediate and direct effects of melatonin on deiodinase regulation of reproductive state is accumulating (see next section on Mammalian Pituitary), there is also research demonstrating direct effects of melatonin on mammalian reproductive neuropeptides in the hypothalamus. In mammals, reproductive neuropeptides such as kisspeptin and RFRP (or GnIH) are involved in the transduction of photoperiodic signals into gonadal responses (for review, see Revel et al., 2009). Specific hypothalamic nuclei in the phototransduction pathway also regulate pineal melatonin synthesis. The suprachiasmatic nucleus (SCN) and paraventricular nucleus (PVN) are hypothalamic nuclei that must be intact to regulate melatonin synthesis in mammals (Klein et al., 1983). Lesions to the SCN or PVN remove the pineal melatonin signal (Bittman et al., 1989). However, appropriately-timed melatonin injections still induce testicular regression in hamsters (Bittman et al., 1989), so although intact hypothalamic sites of the phototransduction pathway are necessary for photic regulation of melatonin synthesis and secretion, there are neural sites outside of this pathway that respond to the melatonin signal itself. Lesions that extended to neighboring hypothalamic nuclei (i.e. the anterior hypothalamus, AH) were associated with the variability observed in testicular response (Bittman et al., 1989). By using neurotoxic lesions that destroy neuronal cell bodies of the AH without affecting fibers passage or glia, leaving the non-sensitive neurons of the SCN and PVN intact, Hastings et al. (1985) determined cell bodies of the AH are necessary for photoperiodic regression of Syrian hamster testes during short-days. Damage to pericellular areas of the AH, such as the POA, was not correlated with changes in testicular response associated with photoperiodic time measurement (Hastings et al., 1985). Intracranial application of melatonin suggested that melatonin acted on a site near the SCN but not the SCN directly (Hastings et al., 1988). Therefore, hypothalamic nuclei outside the phototransduction pathway were considered critical sites of melatonin action influencing seasonal changes in mammalian reproductive state.

Other methodological approaches isolated the impact of melatonin on the mammalian hypothalamic network overall. There is some evidence that melatonin affects gonadotropin-releasing hormone (GnRH) neuronal control via the stimulatory signal of Kisspeptin (*Kiss*) and inhibitory signal of RFRP (the mammalian homolog of gonadotropin inhibitory hormone, GnIH). For instance, in hypothalamic cell lines in rats, administration of melatonin downregulates *Kiss* and upregulates RFRP (Gingerich et al., 2009). Beyond cell lines, specific mammalian hypothalamic nuclei that are not a part of the phototransduction pathway also influence reproductive physiology. In hamsters housed in photo-inhibitory short days, *Kiss* expression was down-regulated in the anteroventral periventricular nucleus (AVPV) of both Syrian and Siberian hamsters and the arcuate (Arc) nucleus of just the Syrian hamster. Peripheral administration of kisspeptin to short-day housed Syrian hamsters with regressed testes significantly increased testicular volume and testosterone secretion, and this response was blocked by GnRH receptor antagonist (Ansel et al., 2011). Melatonin injections downregulated *Kiss1* expression, and removal of the pineal prevented short-day inhibition of *Kiss1* in the Arc of the Syrian hamster hypothalamus (for review see Revel et al., 2007 & Simonneaux et al., 2009). From these studies, we find that melatonin affected *Kiss1* expression and kisspeptin mediated a reproductive signal via a pathway responsive to GnRH (Ansel et al., 2011). This study corroborates previous findings that GT1–7 cells contain GnRH-secreting neurons expressed melatonin receptor subtypes (Roy et al., 2001) and that melatonin receptor activation regulated GnRH gene expression in these GT1-7 cells (Roy & Belsham, 2002). The results of Roy et al. (2001) and Roy & Belsham (2002) have yet to be determined *in vivo*. It is difficult to ascertain the

transferability of these findings from isolated cells to the whole animal. The underlying hypothalamic regulatory mechanisms vary for hamsters experiencing testicular regression, photostimulation of testicular growth, and spontaneous recrudescence of testes in extended photo-inhibitory conditions. The role for melatonin reflected by these different reproductive states will be contingent on photic conditions. Neither the subtleties of reproductive state nor the responsiveness to light (contingent on photoperiodic history) may be fully re-enacted *in vitro.*

The role for melatonin on the mammalian hypothalamus was studied *in vivo* by administering melatonin or changing in the lighting schedule. ICV administration of melatonin into the medial hypothalamus of pinealectomized male Syrian hamsters maintained photostimulated testicular volume, but this was not observed for melatonin administered to the lateral hypothalamus, midbrain, or amygdala (Hastings et al., 1988). Furthermore, androgen receptors (AR) and melatonin receptors are co-localized in the dorsomedial hypothalamus (DMH) (Maywood et al., 1996). Testicular regression in Syrian hamsters, typically induced by extended melatonin infusions, were prevented with DMH lesions (Maywood et al., 1996). DMH ablation prevented photoperiod-induced testicular regression without affecting negative feedback on FSH in the pituitary in Syrian hamsters (Jarjisian et al., 2013). Furthermore, RFRP-ir and mRNA expression was significantly reduced in Syrian hamsters housed in short-days relative to long-days (Mason et al., 2010). Testosterone administration does not appear to affect RFRP, suggesting a steroid-independent mechanism of RFRP regulation in the DMH (Mason et al., 2010). *Rfrp* neuronal expression, typically associated with inhibition of the reproductive axis, was highly down-regulated in the DMH of male Syrian hamsters housed in short days (Ancel et al., 2012). Chronic administration of RFRP-3 led to testicular recrudescence and *Kiss* upregulation in the Arc nucleus in the male Syrian hamster, despite being housed in photo-inhibitory conditions (Ancel et al., 2012). RFRP-3 expression in the DMH is strongly inhibited by chronic infusion of melatonin in male Syrian hamsters (Simonneaux et al., 2013), counter to observations in the rat hypothalamic cell line (Gingerich et al., 2009). These findings show the importance of considering individual hypothalamic nuclei in addition to considering the hypothalamus as a whole. Both Syrian and Siberian hamsters show variation in *RFRP* mRNA resulting from changes in melatonin levels due to ablation of the pineal and exogenous replacement of melatonin, and this relationship is not observed in the non-photoperiodic Wistar rat (Revel et al., 2007).

Thus far, the focus of research on melatonin's effect on reproductive timing in mammals has emphasized studies in males. However, there are sex differences in the regulation of reproductive neuropeptides in hamsters. More RFRP neurons were counted in female Syrian hamsters relative to males (Henningsen et al., 2013). Female Syrian hamsters housed in short-days had downregulated RFRP in the AVPV, so there are sex differences in how RFRP is regulated in Syrian hamsters (Henningsen et al., 2013). Although seasonal gene regulation was determined in male Siberian hamsters (Petri et al., 2016), seasonal gene regulation is likely to be different in females.

As for short-day breeding mammals, melatonin's effect on reproductive state has been studied mostly in sheep (for review, see Malpaux et al., 1996). The mechanism linking melatonin and kisspeptin in sheep is still not clear. Although the ovine premammillary nucleus binds melatonin (Malpaux et al., 1998), melatonin receptor subtype MTNR1A was not found to be expressed in kisspeptin neurons but was found in the ovine pars tuberalis to regulate prolactin, which may have indirect effects on kisspeptin (Li, Q. et al., 2011). Steroid-dependent effects on kisspeptin/RFRP in sheep show these neuropeptides work in opposition, alternating in peak

levels between reproductively active and quiescent cycles (Smith et al., 2008). While long-days are associated with upregulation of RFRP in the ovine hypothalamus (Dardente et al., 2008), the isolation of melatonergic, steroid-independent effects of seasonal changes in *RFRP* expression has yet to be determined in sheep.

Although the mode of signal transduction between melatonin and the mammalian kisspeptin/RFRP system is neither fully elucidated nor conserved, there is clearly an upstream role for melatonin on neuropeptides in the mammalian hypothalamus that influence GnRH via RFRP and kisspeptin. Future studies in mammals may also consider how melatonin interacts with the circadian pulsatility of the GnRH system (for review, see Knobil, 1999). Furthermore, the positive and negative feedback of estradiol on hypothalamic neuropeptides would need to be integrated into future studies on the relationship between melatonin and female reproductive state (for review, see Christian et al., 2010). The pars tuberalis of the pituitary, as a conserved binding site for melatonin across different breeding types of mammals, shall be discussed in the subsequent section.

Melatonin in Mammalian Pituitary. Melatonin binds the pituitary of rat (Williams et al., 1995), Syrian hamster (Williams et al., 1989), and in rhesus monkeys (*Macaca mulatta*) (Weaver et al., 2016). Melatonin binding was observed only in one out of eight human pituitaries (Weaver et al., 2016). As discussed in the section on Melatonin Binding, the pars tuberalis is a conserved melatonin binding site in mammals (for review, see Morgan et al., 1994). There is no single cohesive narrative to how melatonin acts in the mammalian pituitary to affect reproduction, so the literature reviewed here provides a heterogenous perspective of the physiological effects of melatonin binding in the mammalian pituitary.

Most research on melatonin action in the pituitary was conducted in rats. Nanomolar concentrations of melatonin, but not micromolar, administered *in vitro* to anterior pituitary harvested from neonatal rats suppressed LH/FSH release induced by GnRH (previously named luteinizing-hormone- releasing-hormone, LHRH) (Martin & Sattler, 1982). In other studies, melatonin prevented LHRH-induced cAMP and cGMP accumulation (Vaněček & Vollrath, 1989) and GnRH-induced intracellular free Ca2+ and depolarization of the plasma membrane (Vaněček & Klein, 1993) from cultured neonatal rat anterior pituitary. However, the pars distalis of the fetal rat has a higher responsiveness to melatonin that declines with development into adulthood (Johnston et al., 2003), so these subcellular changes resulting from melatonin signaling in cultured fetal rat anterior pituitary may not be extrapolated to describe melatonin action in the adult pituitary.

Melatonin action at the level of the pituitary of small mammals that are more photoperiodic than rats is also evident. In Syrian hamsters, a photoperiodic rodent, melatonin binds the pars tuberalis (Williams et al., 1989). Melatonin binding in the Syrian hamster changes based on photoperiod in the median eminence and in the anterior pituitary (Vaněček & Janský, 1988). The signal transduced by melatonin binding in the pars tuberalis (PT) of the pituitary includes regulation of thyroid-stimulating hormone (TSH), which in turn affects gene regulation in tanycytes, cells lining the third ventricle of the hypothalamus (for review, see Barrett & Bolborea, 2013). The enzymes that activate or deactivate thyroid hormones, deiodinases *Dio2* and *Dio3*, have been a focus of how melatonin mediates reproductive responsiveness in hamsters. Dio3 inactivates T3 and its prohormone, thyroxine (T4), while Dio2 converts T4 into its active form T3. Triiodothyronine (T3) injections stimulate testicular growth and modulate neuropeptide synthesis to activate reproductive physiology in photorefractory Siberian hamsters (Henson et al., 2013). While exogenous administration of thyroid hormones appears to override

the impact of photoperiod on reproductive state, exogenous melatonin also can affect the Dio2/Dio3 system. Melatonin injections increase *Dio3* expressions levels in juvenile Siberian hamsters, implicating a melatonergic effect in peripubertal maturation (Prendergast et al., 2013). Photostimulated adult Syrian hamsters that were injected with melatonin for one week showed levels of *Dio2* mRNA comparable to what is observed in hamsters kept in short days (Revel et al., 2006). However, the effect of melatonin injections on *Dio2*/*Dio3* expression may be inconsistent based on the time of day of the injection and the strain of mice used (Goto et al., 2013). A single melatonin injection administered in the late afternoon alters the temporal dynamics of *Dio2* expression the subsequent day in Syrian hamsters (Yasuo et al., 2007). Furthermore, melatonin injections used to simulate short-days and terminate breeding differentially affects Dio2/TSHβ relative to long-day induction, or photostimulation, of this pathway (Yasuo et al., 2010). Melatonin injections and light pulses have differing effects on *Dio2* in Siberian hamsters. *Dio2* expression decreased with melatonin injections but did not change with light pulses (Watanabe et al., 2004), suggesting that melatonin may serve as an intermediate between changes in lighting condition and *Dio2* regulation. In male Syrian hamsters, the rapid induction of TSHβ expression in the pars tuberalis, determined by in situ hybridization, following photostimulation is a strong indicator of the role of the pituitary in seasonality of the HPG axis (Yasuo et al., 2010). However, melatonin injections simulating short-days in this long-day breeder did not synchronously alter Dio2/TSHβ mRNA expression, which suggests that another mechanism is involved in the termination of breeding (Yasuo et al., 2010). Additionally, in male viscacha (*Lagostomus maximus maximus*), chronic melatonin administration (twice daily s.c. injections for 9 weeks) decreased the size of LH and FSHβ cells (Filippa et al., 2005). Chronic exposure (16hr) to melatonin in mediobasal hypothalamic explants including the pars tuberalis lowered melatonin binding in the pars tuberalis in mink (*Mustela vison*) at all times of the year (Messager et al., 1999). It is important to note that the use of the concept of chronic exposure is quite different in the methodologies of several studies applying melatonin administration techniques, discussed further in Chapter 1. In the context of melatonergic effects on pituitary function, it is difficult to directly compare the findings of these administration studies.

Larger seasonally breeding mammals provide certain advantages over small mammals in understanding the temporal dynamics and physiological nuances of melatonergic effects on pituitary function. Blood can be sampled from larger mammals more frequently. Also the anatomy is more visibly accessible for intricate surgical procedures. One example of the value of regular blood sampling in ascertaining temporal dynamics is a study conducted in reproductively quiescent dairy goat (*Capra*). The number of LH pulses was unaffected by lighting condition and melatonin treatment, but basal levels of LH increased with melatonin treatment (Chemineua et al., 1986). The temporal scale of blood sampling needed to distinguish LH pulsatility from estrous rhythms was more easily obtained by using a larger mammal. Additionally, it may seem impossible to distinguish the indirect effects of melatonin on the pituitary via hypothalamic neuropeptides from the direct effects of melatonin binding the pituitary without damaging other necessary physiological systems. pituitary disconnected rams (PDR) can be used to distinguish these effects, enabled by the size of the animal. The hypophyseal portal system is compromised in PDR. Melatonin implants in PDR affect prolactin secretion that is comparable to control groups, which suggest that elatonin has a direct effect on the pituitary that functions independently from hypothalamic input in this model (Lincoln & Clarke, 1994). As mentioned before, the mode of melatonin administration varies across mammalian pituitary studies (in these

examples, implant versus injection), and the results of these experiments may not be directly comparable in determining a conserved mode of action for melatonin on reproductive pituitary function across mammals (see Chapter 1). Lastly, the ovine pituitary itself is large enough to separate tissue fragments to study the effects of melatonin on the pars distalis and the pars tuberalis *in vitro* (Skinner & Robinson, 1997). Administering melatonin to cultured ovine pars distalis, but not the pars tuberalis, attenuated the GnRH-induced secretion of LH (Skinner & Robinson, 1997), revealing a functional role for the high density of melatonin receptors previously found in the pars tuberalis of sheep.

The role for melatonin binding specifically in the pars tuberalis and its effect on local gene regulation was studied extensively. It is impossible to isolate metabolic effects from reproductive state, especially since body weight and reproductive success are seasonally regulated by melatonergic pathways in photoperiodically-breeding mammals (for review, see Barrett & Bolborea, 2012). The question remains about the role of melatonin in the human pituitary. In healthy adult males, melatonin administration did not correspond to changes in LH, FSH, or testosterone (n=5 males over one night in Weinberg et al., 1980; n=6 males over 3 nights in Luboshitzky et al., 2000). However, results from pituitary gonadotropin research with healthy adult females show a stronger relationship between melatonin and reproductive state. A significant effect of season on melatonin and LH concentrations was observed, regardless of menstrual state (n=11 females providing serum and urine samples and n=21 providing serum samples for anterior pituitary and ovarian hormones in Kauppila et al., 1987; n=12 females, samples collected various days of menstrual cycle in summer and winter in Kivelä et al., 1988). Sex differences were previously observed in reproductive responses to stress (for review, see Handa et al., 1994). As extensively reviewed by Beery and Zucker (2011), the sex bias in animal research can learn from these differences in humans. The effects of melatonin on pituitary reproductive function may not be extrapolated across sexes, and consideration of sex differences extends beyond the gonads.

Melatonin in Mammalian Gonads. Research related to sexual dimorphism of melatonergic effects on reproductive physiology tends to focus on the gonads, despite research that shows that there is more than what meets the gonads (testes/ovaries) when it comes to sex differences in animal research (see Beery & Zucker, 2011).

Extensive reviews have been conducted on melatonin in seminal plasma (Cebrián-Pérez et al., 2014), on oocyte competence and blastocyst development (Cruz et al., 2014), on *in vitro* fertilization and embryo transfer success (Reiter et al., 2013), on the ovary (Tamura et al., 2009), and on male reproductive health overall (Reiter et al., 2013; Rocha et al., 2015). However, parsing out the effects of melatonin acting as a hormone from other confounding factors (e.g. other products of melatonin biosynthetic pathway, melatonin synthesizing-enzymes, melatonin as an antioxidant/free-radical scavenger, etc.) is a challenging undertaking. In several studies, the direct endocrinological relevance of melatonin in the gonads is left to speculation. Since AANAT and ASMT show highest activity in the interstitial cells of rat testes (Tijmes et al., 1996), and early research shows melatonin and serotonin can inhibit key enzymes in androgen steroidogenesis *in vitro* (Ellis, 1972), melatonin and the products of its biosynthetic pathway (see Fig. 1) may also affect steroidogenesis in a manner that cannot be isolated through the methodologies implemented in these studies. The foundation of research on melatonin in reproductive organs provides key insights into the function of melatonin on reproductive state in seasonal reproductive breeding mammals.

Melatonergic effects on testicular physiology vary: sperm motility may be unaffected by melatonin (Bornman et al., 1989) in humans, inhibited by high concentrations of melatonin in Wistar rats (Gwayi & Bernard, 2002) and hyperactivated in Syrian hamster (Fujinoki, 2008). These varying effects of melatonin on sperm motility may be due to time of day the tissue was harvested, photoperiodicity of these particular mammals, or even melatonin-receptor expression in spermatozoa. Melatonin implants during the nonreproductive season (spring) in Rasa Aragonesa rams (short-day breeders) increase seminal plasma testosterone concentrations after four weeks and 17β-estradiol after eight weeks (Casao et al., 2013). From this observation, we can predict that melatonin administration can override other endogenous circannual rhythms to activate the mammalian reproductive axis in seasonally-breeding males that respond to melatonin to time reproduction. In fact, fifteen weeks of melatonin administration in the breeding season (winter) compared to the non-breeding season (spring) significantly increased plasma testosterone concentrations in both winter and spring in Chios rams, and this increase was higher in winter (Kokolis et al., 2002). In Syrian hamster (long day breeders), testes collected at different reproductive states (photostimulated and photorefractory) showed melatonin concentration is significantly higher in the testes of hamsters kept on short days (photorefractory) relative to their long days (photostimulated) counterparts (Mukherjee & Haldar, 2014). The concentration of melatonin in the testes correlated with the nanomolarity of N-acetyltryptamine formed, which was used as an indicator of AANAT activity (Mukherjee & Haldar, 2014). In addition to the implication of seasonal changes in testicular melatonin synthesis, Mukherjee and Haldar also found MT1 receptor was significantly higher in testes collected from the short-day group (2014). It is worth noting that the time of day when the testes were collected was not specified in this study (Mukherjee & Haldar, 2014), so we are not able to assume the testes in different groups were collected at the same time. Across different breeding types, *MT1* and *MT2* are expressed in the spermatozoa of long-day breeders, short-day breeders, and non-seasonal breeders (González-Arto et al., 2016), so melatonin may affect sperm in a receptor-mediated fashion. This is supported by the finding that the melatonin receptor antagonist, luzindole, inhibits melatonin-induced hyperactivation of sperm motility in Syrian hamsters (Fujinoki, 2008).

Melatonin also affects ovarian physiology. Melatonin administered with human chorionic gonadotropin (hCG) decreased progesterone and estradiol production in preovulatory follicles in adult cyclic female hamsters (Nakamura et al., 1998). Melatonin concentrations peaked in the hamster ovary in the middle of the scotophase, as observed in serum and in the pineal gland, suggesting melatonin in the ovary may inform this cyclicity (Nakamura et al., 1998). In human patients, melatonin and progesterone concentrations correlated, but *in vitro* administration of melatonin did not affect progesterone levels significantly, implying an indirect relationship between melatonin and progesterone in humans (Nakamura et al., 2003). In early preantral follicles harvested from C57BL x CBAca mice cultured with varying concentrations of melatonin, androstenedione and progesterone concentrations increased with 100μM melatonin, but any concentration higher was toxic or negatively influenced oocyte maturation while lower concentrations had no effect (Adriaens et al., 2006). In CD-1 mice, with stimulated follicle growth by pregnant mare serum gonadotropin (PMSG) and triggered ovulation by hCG, ovarian fluid showed increased concentrations of melatonin as well as *MT1* and *SNAT* (AANAT) expression in cumulus cells (He et al., 2016). Additionally, melatonin administration increased progesterone and rates of successful implantation (He et al., 2016). Similar findings of melatonin receptor and enzyme expression are observed in bovine cumulus-oocyte complexes (El-Raey et

al., 2011). Although these studies show melatonin-receptor expression alongside ovarian physiological changes, the lack of a melatonin-receptor antagonist means we cannot distinguish with certainty the antioxidant effects of melatonin from receptor-mediated signal transduction.

The seasonal patterns of intra-gonadal melatonin synthesis and binding have yet to be studied. Measurements of melatonin from seminal fluid does not exclude extra-gonadal sources of melatonin (e.g. exogenously administered melatonin, see Casao et al., 2013), and the amphiphilic properties of melatonin may allow diffusion across the blood-testis barrier. Furthermore, several studies do not use receptor antagonists to distinguish melatonin receptor-mediated changes from actions of melatonin as an antioxidant. There are also seasonal variations of melatonin synthesis and secretion worth considering. The relationship between melatonin and the mammalian reproductive axis is reviewed in Chapter 3 as well, which investigates the effects of melatonin on testosterone production of Syrian hamster testes *in vitro*.

In birds. The effects of melatonin in timing reproduction in birds are not consistent across species or seasons. Synchronizing egg laying and care for fledglings with adequate environmental resources is necessary in a vertebrate class for which storing fat and lactating could compromise mobility. Seasonal changes in the avian HPG axis were reviewed previously (Leska & Dusza, 2007). However, the review of Leska and Dusza (2007) focuses on the role of thyroid hormone. Another review investigated the role of melatonin in non-mammalian vertebrate reproduction by closely considering the methodologies of the research (Mayer et al., 1997) which included studies that administered of melatonin (Homma et al., 1967; Sayler & Wolfson, 1968); Storey & Nicholls, 1978; Chaturvedi, 1984; Haldar & Ghosh, 1990; Juss et al., 1993; Ramachandran et al., 1996; Kumar Maitra & Dey, 1996) or its antisera (Ohta et al., 1989) in birds. Chapter 1 critically investigates how experimental factors, such as concentration of dose and time of day of administration, vary widely across melatonin administration studies, and we may attribute the inconsistent effects of melatonin on avian reproduction to methodological variation of these administration studies.

Additionally, the methodology of most of the studies investigating the effects of melatonin on avian reproduction conducted bilateral enucleation and/or pinealectomies (Mayer et al., 1997). However, melatonin rhythms are still detectable in pinealectomized and bilaterally enucleated male Japanese quail (*Coturnix japonica*) at 13% of the concentration measured in intact controls (Underwood et al., 1984). This may be due to post-pinealectomy compensatory mechanisms that develop over time, as observed in other birds. Although plasma melatonin is undetectable in pinealectomized White leghorn cockerels (*Gallus gallus domesticus*) (Pelham, 1975), melatonin rhythms are maintained post-pinealectomy in the eye and Harderian gland, with local melatonin concentrations peaking in amplitude during the dark phase (Cogburn et al., 1987). In pigeons (*Columba livia*), extra-pineal melatonin was found in the hypothalamus, eyes, Harderian gland, and duodenum (Vakkuri et al., 1985a), and allowing two weeks recovery post-pinealectomy showed increases in detectable concentrations of circulating melatonin up to 64% of amount quantified in sham-operated controls (Vakkuri et al., 1985b). Unlike photoperiodic mammals, American tree sparrows (*Spizella arborea*), even after pinealectomy and bilateral enucleation, experienced testicular growth when photostimulated (Wilson, 1991). It is important to consider that these birds have extra-pineal, extra-ocular sources of rhythmic melatonin synthesis not found in mammals (a comparative discussion of melatonin in relation to thyroid hormone is presented in Watanabe et al., 2004).

Furthermore, some birds, unlike mammals, have photoreceptors in the pineal situated beneath a translucent skull and deep brain photoreceptors localized to the hypothalamus (see

Phototransduction Pathways in Mammals and Birds in this Introduction). Chapter 2 of this dissertation shows the hypothalamus of a photoperiodically-breeding bird, the European starling, may synthesize melatonin in a circadian fashion since *Aanat* expression is significantly higher in birds collected at nighttime relative to daytime. The role of melatonin in circadian rhythms, seasonal immune function, and neuroplasticity in the song control network was reviewed previously (Gwinner et al., 1997; Bentley, 2001). Here, the mechanisms underlying how melatonin may interact with the hypothalamus, pituitary, and gonads to affect avian reproduction are reviewed in different breeding types of birds (for more extensive reviews on the effects of photoperiod on avian reproductive state, which includes but is not limited to melatonergic effects, see Dawson et al., 2001 and Bentley, 2010).

Melatonin in Avian Hypothalamus. The relationship between reproductive neuropeptides and photoperiodic time measurement (PTM) has been extensively reviewed (see Ubuka et al., 2013 and Kriegsfeld et al., 2015). The role of hypothalamic melatonin may be partially understood through an exploration of PTM. The avian mediobasal hypothalamus (MBH) includes the infundibular nucleus, inferior hypothalamic nucleus, and median eminence, and numerous studies indicate the MBH is necessary for PTM to induce gonadal growth or regression to correspond with changes in photoperiod (MBH lesions conducted in Sharp & Follet, 1969; Davies & Follett, 1975; MBH electrical stimulation in Ohta et al., 1984; and photo-induction of immediate early gene c-Fos expression in Meddle & Follet, 1995; Meddle & Follet, 1997). Although i.v. injections of anti-melatonin serum before lights-out in Japanese quail enabled testicular development during short-days (Ohta et al., 1989), we cannot isolate which part(s) of the HPG axis the anti-melatonin serum bound. Whether anti-melatonin serum acted at the level of the hypothalamus, pituitary, the gonads, or some combination of these classical target tissues to enable photostimulation in quails housed in short-days is worth investigation in future studies. It is worth noting that these studies use only male Japanese quail, and other studies have indicated there are interspecies and sex differences (e.g. Sayler & Wolfson, 1967). To generalize from findings in male Japanese quail to birds of all sexes and breeding types may not depict the most accurate representation of the role of melatonin in the avian reproductive axis.

More species have been integrated in research using 2-[^{125}I] iodomelatonin binding to identify melatonin receptors in the avian brain, which labels sites where the affinity and density are high enough to cross a critical threshold (reviewed in the section on Melatonin Binding in this Introduction). Previous 2-[^{125}I]iodomelatonin studies in birds identified binding in the hypothalamus to be restricted to the visual pathway, specifically to the visual suprachiasmatic nucleus, or vSCN (in chicks, Rivkees et al., 1989; in house sparrow *Passer domesticus*, Cassone & Brooks, 1991; in *Melopsittacus undulatus*, *Nymphicus hollandicus*, *Cardinalis cardinalis*, *Melospiza melodia*, *Sturnus vulgaris*, *Gallus gallus*, *Phasianus colchicus*, *Numida meleagris*, *Colinus virginianus*, *Columba livia*, *Streptopelia risoria*, *Zenaida macroura*, and *Anas platyrhynchos*, Cassone et al., 1995). However, sequencing of specific receptor subtypes increased the resolution to detect melatonin receptors to individual neurons. When the relationship between melatonin and GnIH mRNA and peptide in the diencephalon of Japanese quail was found to be dose-dependent, the mode of melatonergic action on GnIH in the paraventricular nucleus (PVN) was investigated (Ubuka et al., 2005). In addition to *Mel1c* expression in the PVN, *in situ* hybridization co-localized this melatonin subtype receptor with immunolabeled GnIH cell bodies (Ubuka et al., 2005). Because the melatonin receptor autoradiogram showed relatively low binding in the PVN compared to neural regions with high

binding (Ubuka et al., 2005), it seems that multiple methods are necessary for identifying melatonin binding and subsequent action in the avian hypothalamus.

In addition to studies on circadian clock gene expression in the MBH and SCN (Yasuo et al., 2003), along with differential subtractive hybridization analysis in quail (Yoshimura et al., 2003), a photoperiodic model in quail appears to include thyroid hormone synthesizing-enzyme *Dio2* similarly to photoperiodically-breeding mammals (see section on Melatonin signaling, In Mammals). In European starlings, *Dio2* varied seasonally but did not correspond to testicular volume or GnRH regulation in males (Bentley et al., 2013), and in females, first evidence was presented for *Dio2* expression to be regulated by social cues (i.e. the presence of a mate) (Perfito et al., 2015). The direct relationship, if any, between melatonin and Dio2 in birds remains unclear to date. Melatonin in birds is mostly reviewed in relationship to its daily rhythm, dissociated from the seasonal photo-neuroendocrine reproductive axis (for example, see Nakao et al., 2008), because of results of melatonin administration studies in birds, which are more inconsistent than administration studies in photoperiodically-breeding mammals.

Because melatonin administration studies have inconsistent results in birds (investigated in Chapter 1) and the regulation of thyroid hormones is directly related to changes in photoperiod (Nakao et al., 2008), it seems as though melatonin itself is unnecessary for photoperiodic changes of the avian hypothalamic network in the HPG axis. However, recent studies uncover the potential for melatonergic action in the avian hypothalamus. In addition to the discovery of *Mel1c* expression in GnIH neurons of the quail PVN previously described (Ubuka et al., 2005), circadian patterns of melatonin and AANAT were characterized in the turkey (*Meleagris gallopavo*) premammillary nucleus (PMM) with an inverse relationship with the co-localized expression of dopamine and tyrosine hydroxylase (Kang et al., 2007; El Halawani et al., 2009). The photoperiodic condition and corresponding reproductive state of the turkey also affected these expression patterns and associated with changes in *GnRH-I* mRNA expression (El Halawani et al., 2009). Additionally, changes in mRNA expression of the first enzyme in melatonin synthesis (*TPH1*) corresponded to expression of a photoreceptor gene (melanopsin, or *OPN4*), both of which decreased in a time-dependent manner in the PMM of photosensitive female turkey hens exposed to light (Kang et al., 2010), indicating a relationship between deep-brain photoreception and melatonin synthesis.

In another study, hypothalamic explants (including the PVN and GnIH projections to the median eminence) from adult male Japanese quail were cultured to determine the effect of melatonin on GnIH release (Chowdhury et al., 2010). *GnIH* mRNA and GnIH peptide dose-dependently increased in tissue cultured with higher melatonin concentrations (Chowdhury et al., 2010). Tissue also was collected at different times, and GnIH peptide release peaked in the dark period in coincidence with melatonin concentration in the diencephalon and inversely related to LH concentration in plasma (Chowdhury et al., 2010). Furthermore, quail that were housed in short days for 3 weeks had significantly higher *GnIH* mRNA and peptide released in the culture media and significantly lower concentrations of LH in plasma and testicular volume (Chowdhury et al., 2010), correlating observations in cultured quail hypothalami with the HPG of quail in different reproductive states. The remaining detectable melatonin concentrations in the diencephalon were hypothesized to be derived from pineal and ocular sources, since their previous study showed that removal of these tissues significantly decreased melatonin concentrations in the diencephalon (Ubuka et al., 2005). However, there was still melatonin detectable in the diencephalon of pinealectomized and bilaterally enucleated quail (Ubuka et al., 2005). Chapter 2 sequenced all four melatonin-synthesizing enzymes from the hypothalamus of

two different breeding types of birds, supporting the hypothesis that melatonin may be synthesized *de novo* within the avian hypothalamus.

In summary, the direct action of melatonin on GnIH neurons may be key to understanding the physiological mechanism by which photoperiodic information is chemically transduced to affect GnRH-I and GnRH-II (in European starlings Ubuka et al., 2008; in Indian weaver bird: *Ploceus philippinus* Kumari et al., 2015; reviewed in Chowdhury et al., 2013). The hypothalamus hosts GnIH neurons, which express Mel1c receptors (Ubuka et al., 2005), and also deep-brain photoreceptors that may regulate local melatonin synthesis (Kang et al., 2010). Furthermore, given the distribution of GnIH and its receptors throughout the avian reproductive system (for review see Bentley et al., 2008), melatonin may also regulate gonadal development and regression via GnIH in these other sites. Melatonergic action in the avian pituitary and gonads discussed in subsequent sections.

Melatonin in Avian Pituitary. The avian pituitary hosts a wide variety of hormones broadly related to reproduction. Several studies have investigated gonadal or behavioral response to changes in prolactin (for review, see Goldsmith, 1983 and Ziegler, 2000), adrenocorticotropic hormone (ACTH) (Flickinger, 1966; Deviche et al., 1980; Chaturvedi & Suresh, 1990; Astheimer et al., 1994), arginine vasotocin (Rząsa & Ewy, 1970; Baeyens & Cornett, 2006), and mesotocin, the avian homologue of oxytocin (Rząsa & Ewy, 1970; Pedersen & Tomaszycki, 2012). The seasonal fluctuations of these pituitary hormones directly precede, coincide, or are inversely related to gonadal steroids. These hormones have been studied in connection to the onset of broodiness (Burke & Dennison, 1980; Sharp et al., 1998), yolking (Sockman et al., 2006), nesting & incubation (Dawson & Goldsmith, 1982; Sharp et al., 1998), and cessation of laying (Burke & Dennison, 1980). This section will focus on the relationship between melatonin and pituitary hormones that are most strongly connected to avian reproductive activation include gonadotropins, LH/FSH, and thyrotropin, TSH.

Melatonergic actions at the level of the avian pituitary may regulate reproductive gonadotropins indirectly via GnIH as described in the previous section. Although mammals share a conserved melatonin binding site in the pars tuberalis, no 2-[^{125}I] iodomelatonin binding has been observed in the median eminence/pars tuberalis of avian species (Aste et al. (2001). However, Japanese quail show binding in the hypophyseal pars tuberalis (Cozzi et al., 1993). Binding was not detectable in the adenohypophysis, neurohypophysis, nor the median eminence in house sparrows (Cassone & Brooks, 1991) nor was pituitary binding reported in an autoradiographic study in the chicken brain (Yuan et al., 1990). However, expression of Mel1c subtype receptor is found in the chick pars tuberalis (Kameda et al., 2001).

In adult male Lal munia (*Estrilda amandava*), melatonin was injected with four different dose regimes of melatonin over thirty days (either mid-day or mid-night, low or high dose, and increasing or decreasing dosage over time), and seasonal gonadal growth was inhibited regardless of the time of day melatonin was administered (Gupta et al., 1987). Interestingly, melatonin injections increased colloid in the follicular lumen of the thyroid, and more thyroidal follicular cells become squamous, or inactive. As a proxy for the effects of melatonin on LH, feather and beak coloration were assessed. Melatonin injected birds lacked LH-induced changes in plumage pigmentation observed in controls. Circulating concentrations of FSH were not quantified, but it was concluded that because melatonin did not affect LH-induced changes in beak coloration, that melatonin may inhibit FSH as explanation for inhibition of testicular growth observed in the melatonin treated group. This may be due to localized changes in LH-receptor. Neither circulating LH or its receptor, however, were quantified in this study (Gupta et al.,

1987), so we may not conclude with certainty how melatonin affected LH synthesis or secretion from the anterior pituitary in this species.

In castrated White Leghorn roosters, melatonin injections at different concentration and administered for different frequencies or over extended periods of time consistently supported the hypothesis that melatonin dose-dependently reduces circulating LH concentrations (Rozenboim et al., 2002). Neither the mechanism between the photoperiodic signal to melatonin nor from melatonin to the LH signal are fully understood.

One possible mechanism for photoperiodic regulation of the melatonin signal on the avian pars tuberalis is via clock gene regulation (for a review on avian clock genes, see Kumar et al., 2004; Bell-Pederson et al., 2005; and Helfer et al., 2006). There is a rhythmic expression of *Cry1* and *Per2* in the pars tuberalis of Japanese quail, and this rhythm delays under longer photoperiods (Yasuo et al., 2004). Furthermore, a light pulse administered during the dark period can induce *Cry1* expression (Yasuo et al., 2004). Lastly, the role of clock genes in regulating the effects of melatonin on the avian pituitary is shadowed by research on thyrotrophin of the pars tuberalis. A seminal study was conducted in male Japanese quail that were 8 weeks old investigating photoinduced changes in gene expression upon exposure to the first long day (Nakao et al., 2008). Thyrotrophin (*TSH*) β-subunit expression was significantly upregulated in the pars tuberalis 14 hours after lights-on of the first long day, and this was followed 4 hours later by increased *DIO2* expression (Nakao et al., 2008). Furthermore, administration of TSH to quail housed in short days stimulated testicular development, and *DIO2* expression appears to be regulated in part through a signaling pathway involving TSH receptor-cyclic AMP (Nakao et al., 2008). This study provides compelling evidence for thyrotrophic-mediated signaling between photostimulation and testicular development in quail. However, there are notable details of the study that limit the potential ecological relevance of these findings. Quail were housed in square-wave pattern (on-off) lighting and were switched directly from short days (6L:18D) to an extremely long-day (20L:4D). The missing transitory cues provided by natural simulation of dawn and dusk, including color (Pauers et al., 2012) and irradiance (Tang et al., 1999; for review see Boulos & Macchi, 2005), in addition to the dramatic shift in day length that might only be experienced by migratory birds in the wild, limits the ecological generalizability of this study. Additionally, quail were selectively bred and are considered to be weakly photoperiodic, so the implications for other breeding types remain unclear.

The first study to consider the ecological relevance of early gene expression in photoperiodic time measurement was conducted in wild-caught great tits (*Parus major*) (Perfito et al., 2012). Male great tits were collected in Sweden (57°42′N) and Germany (47°43′N). Upon exposure to one long day, the great tit population from Sweden significantly changed hypothalamic gene expression while the population from Germany did not (Perfito et al., 2012). Despite only one population showing changes in hypothalamic gene expression, both populations significantly increase gonadotropin secretion and synthesis (Perfito et al., 2012). It is plausible that the pituitary in this species is differentially responding to photoperiodic cues beyond changes in hypothalamic gene expression, and it may also be worthwhile to consider that hypothalamic early gene expression is not conserved mechanism underlying photoperiodic regulation of the avian HPG axis. Another aspect investigated here is the relationship between melatonin and the proxy for reproductive state in photoperiodically-breeding birds: the gonads.

Melatonin in Avian Gonads. In the Melatonin Binding section of this introduction, melatonin binds in the testes and ovaries of chicks, ducks, and quail, but not in mammals (Ayre & Pang, 1994; Ayre et al., 1994). Only recently could we begin to hypothesize the functional

role for melatonin within the avian gonad in its effect on reproductive state and timing. Avian gonads express reproductive neuropeptides (such as GnRH and GnIH) and their receptors (for review, see McGuire & Bentley, 2010). European starling testes express melatonin receptor subtypes Mel1B and Mel1C (McGuire et al., 2011) and also express melatonin synthesizing enzymes (unpublished data). The potential for autocrine or paracrine regulation of gonadal melatonin on its own synthesis or the synthesis of its receptors may be corroborated by evidence of melatonin regulating itself in this fashion in the mammalian brain (Bedrosian et al., 2013). Here, I will focus on how melatonin interacts with the avian HPG axis at the level of the gonads.

The reproductive state the testes were cultured in also affected the effect of melatonin on testosterone production. Before the breeding season, melatonin upregulated *GnIH* mRNA in the testes, and GnIH and melatonin combined led to a significant reduction in testosterone secretion of starling testes *in vitro* as well (McGuire et al., 2011). Although GnIHR expression increased with photoperiod, GnIH expression appeared to correspond with testicular volume, for GnIH significantly higher in photosensitive (February) and photorefractory (June) birds than in photostimulated (April) birds (McGuire et al., 2011). Additionally, there was a significant correlation between *Mel1B* and *GnIHR* as well as between *Mel1C* and *GnIH* (McGuire et al., 2011). Interestingly, since *Mel1C* expression significantly decreased in testes treated with LH/FSH, there appears to be a causal relationship between reproductive state and this melatonin subtype receptor expression in starling testes (McGuire et al., 2011).

In avian ovaries and follicles, melatonin receptors have been sequenced varied stages of follicular development (Sundaresan et al., 2009) and egg laying (Jia et al., 2016). Melatonin implantation (10mg) to chickens 300-470 days of age increased egg-laying rate (Jia et al., 2016). Chickens supplemented with melatonin had no significant difference relative to controls with LH-receptor, FSH-receptor, estradiol receptor alpha (ERα), nor MT2 receptor expression in the ovaries (Jia et al., 2016). However, chickens supplemented with melatonin implants did show significantly higher serum estradiol-17β concentrations and ovarian MT2 expression with significantly lower ovarian GnIH-receptor expression (Jia et al., 2016). It is unclear what ecologically relevant phenomenon a tonic-release melatonin implant is simulating. However, these findings illustrate the potential for melatonin to bind the ovary and correspond to significant changes in reproductive physiology and fitness in chickens.

The effects of melatonin on egg laying were tested in free-living great tits (*Parus major*) (Greives et al., 2011). Without affecting body mass, clutch size, or the daily onset of activity, silastic implants containing melatonin led to a significant delay in laying of the first clutch in female great tits in the wild (Greives et al., 2011). The rate of egg laying, however, was not determined in this study, so we cannot make broader comparisons yet of the effects of melatonin on avian egg laying in general. In Indian jungle bush quail (*Perdicula asiatica*) housed in open air aviaries, an inverse relationship in the mass of the pineal and the mass of the ovary over the course of a year corresponded to seasonal changes in circulating melatonin (Dubey & Haldar, 1997). Furthermore, peak adrenal activity corresponded to peak gonadal activity, which occurred in the season when plasma melatonin concentrations were lowest (Sudhakumari et al., 2001). Whether these systems are independently affected by photoperiodic cues or responding to indirect endogenous signals is yet to be determined.

The relationship between melatonin, reproduction, and other cues cannot be entirely extrapolated. Given that there is seasonal responsiveness to cues of stress affecting testosterone and estradiol production in cultured testes and ovaries from starlings (McGuire et al., 2013), then we may predict that melatonin also has a seasonal efficacy. This hypothesis is supported by

findings showing seasonal regulation of melatonin receptor subtypes in the testes of birds (McGuire et al., 2011; Yadav & Haldar, 2013). The seasonal pattern of peripheral melatonin coincides with photoperiod and is inversely related to circulating testosterone concentrations even in male Indian tropical bird (*Perdicula asiatica*) (Singh & Haldar, 2007). These findings would have extensive implications for research on melatonin and avian reproduction. If research showing melatonin has no significant effect on gonadal development were administering melatonin in a time or fashion when gonadal responsiveness/sensitivity was low (Storey & Nicholls, 1978; Turek & Wolfson, 1978; Juss et al., 1993; Kumar Maitra & Dey, 1996), then the lack of a response would not be attributed to the generalized role (or lack thereof) for melatonin in avian reproductive development but rather a consequence of improper timing. Chapter 1 overviews methodologies of melatonin administration techniques.

Summary. There is variable evidence regarding the extent to which melatonin influences reproductive state in different breeding types of birds. Therefore, multiple non-photoperiodic cues should be considered in conjunction with seasonal changes in melatonin for a comprehensive picture of the avian HPG axis. These cues may include steroid dependent changes in sexual behavior or hypothalamic activation in response to social cues. In the non-photoperiodically-breeding zebra finch (*Taeniopygia guttata*), castrated males were supplemented with androgenic and estrogenic compounds restored courting and mounting sexual behaviors (Harding et al., 1983). Interestingly, females solicited castrated males supplemented with estrogenic compounds at a significantly higher rate, even if the male was not exhibiting courtship behavior (Harding et al., 1983). A similar finding was observed in castrated male red-winged blackbirds administered exogenous sex steroids (*Agelaius phoeniceus*). Additionally, social cues such as the presentation of female zebra finches to males did not significantly affect hypothalamic expression of *Dio2*, *Dio3*, *GnRH*, nor *GnIH*, but an immediate early gene (early growth response protein-1, or EGR1) did significantly increase relative to isolated males (Ernst & Bentley, 2016). However, these studies focus on the male response to the presentation of a female and do not investigate the active role of the female. Our terminology may limit us to some extent because female songbirds "solicit" and male songbirds "court," depicting historically constructed, anthropomorphic projections that limit the questions we ask and how we answer them scientifically. We may want to reconsider how we frame sexual behavior in animal research, so we do not limit ourselves with our terminology. There are countless more questions to be addressed in the role of melatonin in sexual behavior and accompanying physiological changes in future studies.

Overall, there is more evidence supporting the role of melatonin in seasonal changes in song regulation (for review see Ball et al., 2002), but melatonin does not have a consistent role in affecting avian gonadal state (for review see Cassone et al., 2009). It may be that research on melatonin in avian gonads is limited since injections, oral administration, and subcutaneous implants containing a range of melatonin concentrations (reviewed in Chapter 1) have widely variable results that are incomparable to the effect of melatonin observed in photoperiodically-breeding mammals. This observation has led to the dismissal of melatonin as a necessary and sufficient contributor to seasonal changes in the avian HPG overall. However, from the studies listed above, we cannot dismiss that there is still a great deal to understand if and how the photoperiodic signal is relayed to the gonads in different breeding types of birds if it is not primarily by melatonin. This signal is likely mediated through indirect changes in the hypothalamus and pituitary as well as direct autocrine/paracrine regulation within the gonad.

In other vertebrates. There is an impressive range of ways in which melatonin integrates (or not) with reproductive signals in reptiles, amphibia, bony fishes, and cyclostomes (for review, see Mayer et al., 1997), and many of these studies were conducted in controlled lab environments. The relationship between melatonin and seasonal reproduction may vary through the anatomy and photoreceptivity of the pineal (Oksche, 1971; for review, see Ekström & Meissl, 2003). The structure of pinealocytes in snakes resembles that of mammals, lacking photosensory anatomical features (Petit, 1971; reviewed in Ekström& Meissl, 2003). Hagfish (*Eptatretus burgeri*) appear to lack a pineal complex (reviewed in Underwood, 1989; Ooka-Souda et al., 1993). A pineal complex has not been identified in crocodile, and circulating melatonin rhythms are absent in free-living crocodiles (*Crocodylus johnstoni*) but are present in captive populations exposed to natural light and temperature cycles (Firth et al., 2010), suggesting an extra-pineal source of circadian melatonin in this species. Additionally, the presence of a melatonin-synthesizing pineal gland in a given class of vertebrates does not imply that it has been studied in the context of seasonal reproduction. Lamprey pineals maintain diurnal patterns of melatonin synthesis *in vitro* (in *Petromyzon marinus*, Bolliet et al., 1993; in *Lampetra japonica*, Samejina et al., 1997), but melatonin was not mentioned in recent reviews on the lamprey HPG axis (Sower et al., 2009; Sower, 2015). This section focuses on research that has investigated the connection between melatonin and reproductive state in seasonal breeders across non-mammalian and non-avian vertebrate classes.

The daily cycle of melatonin and the effects of temperature on melatonin synthesis in poikilotherms were previously reviewed (see Mayer et al., 1997). The majority of studies in reptiles have been conducted in lizards. In the female Carolina anole lizard (*Anolis carolinensis*), pinealectomies at different times of the year have differential effects on ovarian status (Levey, 1973). The gonadosomatic index (GSI) and the number of follicles yolked were significantly higher in the pinealectomized (Px) group administered saline relative to the Px group administered melatonin (daily s.c. injections of 10µg) and all other control groups in January (Levey, 1973). In June, the number of yolked follicles was significantly higher in the Px group administered saline, and in November, the ovarian GSI was significantly larger in the Px group administered saline, suggesting that the way in which pineal-derived melatonin affects ovarian status varies seasonally in this species (Levey, 1973). It is important to note these animals were kept in constant 6L:18D lighting conditions (Levey, 1973), so the differential effects of melatonin vs. saline injections were not photoperiodically driven. Additionally, the effects of photoperiod on reproductive state in Px individuals were studied in male Carolina anole lizards (Underwood, 1985). Firstly, pinealectomy only affected reproductive state of the male lizard when the surgery was conducted in December, accelerating testicular response to photostimulation (14L:10D) relative to sham-operated individuals (Underwood, 1985). Secondly, also housed in the 14L:10D lighting condition in December, lizards of the Px group with blank silastic implants had significantly higher testicular volume compared to the Px group with implants continuously releasing melatonin (Underwood, 1985). The effects of pinealectomy and subsequent melatonin administration are comparable between male and female Carolina anole lizards (Levey, 1973; Underwood, 1985). In male Indian garden lizards (*Calotes versicolor*), if the pinealectomy surgery took place in summer, the pinealectomy inhibited the regression of gonads that accompanied exposure of the animals to a shorter day length (Thapliyal & nee Haldar, 1979). If the pinealectomy surgery took place in winter, the testes accelerated in growth at a significantly faster rate once the garden lizards were exposure to a longer day length (Thapliyal & nee Haldar, 1979). These findings support a photoperiod-dependent role for

seasonal, pineal-derived melatonin on the reproductive state of male lizards. However, the differences in lighting conditions for animals housed for these experiments should be noted. Interestingly, pinealectomies in parthenogenetic whiptails (*Cnemidophorus uniparens*) did not disrupt circannual reproductive cycles (vitellogenesis, ovulation, and oviposition) in eleven out of thirteen females, but the pinealectomy itself appeared to cause high mortality with only thirteen out of thirty-two animals surviving the surgery (Cuellar, 1978). However, it is possible that this high rate of mortality was due to systematic error.

Among amphibia, Anura and Urodela are the foci of pineal and melatonin research to date (for review, see Mayer et al., 1997). Melatonin is involved in the neuroendocrine control of spawning in several anuran species (for review, see Vu & Trudeau, 2016). In *Pelophylax perezi* (previously *Rana perezi*), circulating concentrations of melatonin are more closely linked to ocular production of melatonin than to pineal, but diurnal, rhythmic fluctuations of plasma melatonin concentrations are only observed at higher temperatures in summer and are abolished at lower temperature ranges (Delgado & Vivien-Roels, 1989). Additionally, tissue sensitivity to melatonin in *P. perezi* appears to fluctuate diurnally in a light-dependent manner but varies with neither season nor temperature (Isorna et al., 2004). In female *P. perezi*, pinealectomy and partial blinding corresponded to significantly higher circulating oestradiol concentrations relative to sham-operated controls (Alonso-Gómez et al., 1990). Furthermore, ovarian regression typically induced by high temperature ranges was prevented by pinealectomy and partial blinding (Alonso-Gómez et al., 1990). However, these physiological consequences may not be solely ascribed to possible changes in melatonin, for changes in melatonin concentrations following pinealectomy were not determined in Alonso-Gómez et al. (1990). Because Delgado and Vivien-Roels (1989) found male *P. perezi* ocular melatonin production correlated with plasma melatonin concentrations, but melatonin concentrations within the pineal complex did not, it is reasonable to predict that partial blinding and pinealectomy in female *P. perezi* would fail to eradicate diurnal fluctuations in the melatonin production of this anuran. In male bullfrogs (*Rana catesbeiana*) raised in the lab, photoperiod did not significantly influence spermiation following LH/FSH injection, and temperature was a driving factor: Recrudescence was prevented at 30° C and spermiation was reduced significantly at 15° C/20° C (Easley et al., 1979). This effect may be attributed to the unnatural photoperiodic history of this lab-reared population. Female bullfrogs (*R. catesbeiana*), both wild-caught and lab-reared, housed in 12L:12D, but not longer (20L:4D) nor shorter (4L:20D) photoperiods, had significantly reduced atresia and prevented ovarian regression (Horseman et al., 1978). However, this study used unnaturally long and short photoperiods and does not address the influence of more subtle fluctuations of seasonal photoperiod. Ecologically relevant evidence in support of photoperiod as the primary cue informing anuran reproductive state may be supported by acoustic identification of anuran activity at Espinas stream in Maldonado, Uruguay over the course of two years (Canavero & Arim, 2009). Neither rainfall nor temperature significantly affected the richness of the identified calls, but photoperiod positively correlated with species richness based on acoustic identification (Canavero & Arim, 2009). Although this effect may be due to only a subset of species calling during long days, hence the observed increase in richness during long days, this study elegantly captures a behavior associated with breeding activity (related to territoriality or mate solicitation) in a collective wild population. Given previous work reviewed here associating seasonal fluctuations in nocturnal melatonin with photoperiod, melatonin is a strong candidate for the chemical transducer of photic information and should be investigated in the subset of species that only called during long days.

In fishes, Teleostei is the largest group studied to date on melatonin and reproductive state, but studies in Chondrichthyes are limited to the role of melatonin in luminescent photophore patterns (in lantern shark, *Etmopterus spinax*, Claes & Mallefet, 2009; in pygmy shark, *Squaliolus aliae*, Claes et al., 2012) or in non-visual receptors (in the elephant shark, *Callorhinchus milii*, Davies et al., 2012), which are outside the realm of melatonin's involvement in the HPG axis reviewed here (for general review on HPG axis in elasmobranchs, see Pierantoni et al., 1993). However, there are extensive reviews on the role of melatonin in teleost reproduction, including melatonin receptor distribution in the hypothalamus (Falcón et al., 2007), reproduction (Zachmann et al., 1992; Falcón et al., 2010; Maitra et al., 2013), circannual rhythms and mate selection (Reiter et al., 2010). Some teleosts spawn in long days, but others use decreasing day length as a cue for spawning, and melatonin injections seems to have no significant effect on the reproductive state of the former and suppresses reproductive development in the latter (for review, see Mayer et al., 1997). Furthermore, continuous administration of melatonin in silastic capsules or in water of different species has inconsistent effects on variables related to reproductive development (for review, see Mayer et al., 1997). However, photoperiod plays an important role in seasonal reproduction in teleosts (for review, see Mayer et al., 1997). Given that salinity (Gern et al., 1984) and temperature (Zachmann et al., 1992) affect daily melatonin rhythms in some teleosts, we must consider how smolting and other fish-specific behaviors coincide with photoperiod. Although there is substantial evidence for hypothalamic binding of melatonin in teleosts (Falcón et al., 2007), the field has limited studies testing melatonin receptor antagonists to distinguish the action of melatonin via its receptor from its role as an antioxidant (for review of the antioxidant role of melatonin in fish reproduction, see Maitra & Hasan, 2016). Furthermore, we have only begun to scratch the water's surface to the effects of anthropogenic sources of light at night that, even with diffraction, are detectable by underwater species (for review, see Collin & Hart, 2015).

Conclusion

> *When we turn from description to causal analysis, and ask in what way the observed change in behaviour machinery has been brought about, the natural first step to take is to try and distinguish between environmental influences and those within the animal. It is about this very first, preliminary step that confusion has arisen.* (Tinbergen, 1963, pp. 424)

The classical HPG axis and melatonin do not operate in a vacuum. Interdependent variables are fundamentally changed when they are experimentally isolated to ascertain causality. Countless endogenous and plastic physiological factors, as well as the perception and transduction of predictable and unpredictable environmental cues, coordinate in a manner that has evolved over time to adapt to ever-changing environments. These endogenous or entrained physiological responses are not guaranteed to enhance survival and reproductive fitness across contexts. Reviews lab-field experiments (Calisi & Bentley, 2009), eco-endo-immunology (O'Neal, 2013), and the influences of stress response on breeding (Lattin et al., 2016) offer a few examples of how experimental and environmental context affects the HPG axis. Inspired by Tinbergen's four questions (Tinbergen, 1963) and application for integrating research in GnIH (Calisi, 2014), procedural questions for future studies in melatonin and reproduction include:

Proximate:

Mechanism

- Has a circadian rhythm for melatonin synthesis/secretion been determined in this species as is being used for the experiment (e.g. age, sex, photoperiodic history, lighting schedule, wild-caught in the same region vs. laboratory raised, etc.), enabling melatonin to serve as a chemical transducer of photoperiodic information in this species?
- Are there daily or annual fluctuations in concentrations of melatonin within specific tissues or circulating in plasma?
- How does melatonin receptor affinity and density vary within the brain and/or gonads of this species at different times of the day/year?

Ontogeny

- How does melatonin synthesis/secretion change with development?
- At what stage of development does the species become fertile? How does the onset of puberty compare to seasonal gonadal development or recrudescence? How does senescence compare to seasonal gonadal regression? (for review, see Perfito & Bentley, 2009)

Ultimate:

Adaptive Value

- Is there a selective advantage to using photoperiod as a cue for reproduction in this species, given their geographical place of origin or current distribution in the wild?
- Has this species been selectively bred in an environment that is comparable to where they adapted/evolved in the wild?
- How many seasons is this species fertile? Would using photoperiod over multiple seasons provide an advantage for survival or resource acquisition in the environment where they breed?

Phylogeny

- Are there species of other vertebrate classes that may be used for comparison? What examples for homology or convergence may be considered for understanding melatonin and reproductive timing in this species?
- May controlled selective pressure to become a photoperiodic breeder or lose photoperiodic responsiveness change how melatonin affects the reproductive axis over multiple generations?

Regarding the questions related to phylogeny, it is important to note Tinbergen's parameters on such studies in ethology:

> *With the growing trend towards experimentation it is important, however, to point out that even the most perfect experiment of this kind does not give us direct proof of what selection has done in the past. The interpretation of such experiments as contributions to evolution theory will always include an extrapolation: while they demonstrate what selection can do, the best they can tell us is that selection can have happened in the way demonstrated, and that the results obtained are not contradictory to what other indirect evidence has led us to suppose. They really deal merely with "possible future evolution", and only indirectly with past evolution.* (Tinbergen, 1963, pp. 424)

Studies previously addressed in this introduction procure conflicting conclusions about whether or not melatonin is a key player in the HPG axis of seasonally breeding animals. These inconsistent results likely stem from uncontrolled variables across experiments, be it photoperiodic history, lighting/temperature in the lab or wild, and the reproductive state of the animal during experimental procedures such as pinealectomy or melatonin administration. These variables are extensively reviewed in Chapter 1, which investigates an array of melatonin administration techniques in different vertebrates.

The variability in melatonin synthesizing and binding sites across species was reviewed here. In Chapter 2, we sequenced and quantified melatonin-synthesizing enzymes in the hypothalamus of two different breeding types of birds. Studies using pinealectomies that concluded melatonin has no effect on the reproductive state of a non-mammalian species may only mention potential compensatory mechanisms of extra-pineal sources of melatonin to transduce photoperiodic information. The expression of all four melatonin-synthesizing enzymes in the hypothalamus of European starlings and zebra finches casts a new light on the hypothesis of the role for compensatory melatonin derived from extra-pineal and extra-ocular photoreceptive sites in non-mammalian vertebrates. Future studies should consider using gene editing methodologies to locally downregulate or upregulate melatonin-synthesizing enzymes and/or melatonin receptors to ascertain the effects on reproductive nucleotide transcription, translation, and associated changes in reproductive physiology.

There is also evidence in support of the role of melatonin in driving reproductive state in photoperiodic, seasonally breeding mammals. The mechanism underlying melatonergic regulation of mammalian gonadal steroid production may be conserved because melatonin appears to have similar effects in non-mammalian vertebrates as well (in European starling testes, McGuire et al., 2011; in *Bufo arenarum* oocytes, de Atenor et al., 1994; in teleost testes, *Fundulus similis*, and in tree frog testes, *Hyla cinerea*, de Vlaming et al., 1974). In Chapter 3, I describe how melatonin cultured with Syrian hamster testes for an extended period of time (12

hours) suppressed testosterone production, whether the testes were cultured with gonadotrophins or not. This study is the longest reported time period that photostimulated hamster testes have been cultured with melatonin.

To conclude, physiological effects of melatonin are observed at all levels of the classical HPG axis in seasonally breeding species classified from different vertebrate classes. Melatonin may influence these effects through its role as an antioxidant or by binding and activating its specific receptor subtypes. The para- and autocrine signaling taking place in the brain has far more extensive research than what has been studied in the pituitary and the gonads. The presence of GnRH and GnIH receptors in the gonads, as well as local synthesis of these "neuropeptides" (McGuire & Bentley, 2010), opens a whole new field for research in the effects of melatonin on gonadal neuropeptides and how these effects compare to effects on neuropeptides in the brain. The potential for melatonergic mechanisms to alter ontologically, provide adaptive value, and share conserved characteristics that are comparative in nature stimulates innumerable inquiries for seasonal reproductive endocrinology.

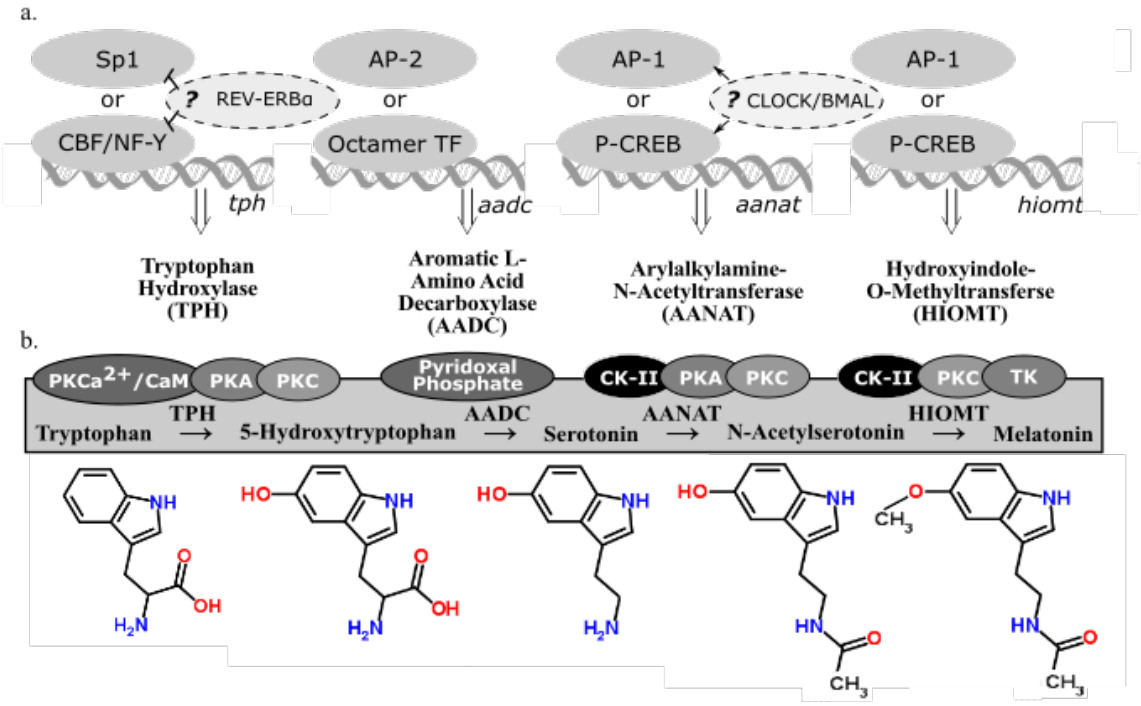

Figure 1. Transcription (a) and Activation (b) of Melatonin-Synthesizing Enzymes. Transcription factors of *tph* include Sp1 and CBF/NF-Y complex (Simonneaux & Ribelayga, 2003). REV-ERBα may inhibit *tph* transcription (Chung et al., 2014), implying some connection with circadian regulation, but mechanism is not known. TPH is phosphorylated/activated by calmodulin (CaM), phosphokinase A (PKA) and phosphokinase C (PKC) (Simonneaux & Ribelayga, 2003). Transcription factors of *aadc* may include AP-2 or octamer transcription factors (Hahn et al., 1993), neither of which are known for circadian regulation. AADC depends on pyridoxal phosphate for functionality (Rahman et al., 1982). Transcription factors of *aanat* and *hiomt* include AP-1 and phosphorylated CREB (P-CREB). The *aanat* gene is likely regulated in a circadian fashion via the CLOCK/BMAL heterodimer (Simonneaux & Ribelayga, 2003). AANAT and HIOMT have binding sites for casein kinase type II (CK-II) and PKC, and activation of HIOMT is enabled by tyrosine kinase (TK) (Simonneaux & Ribelayga, 2003).

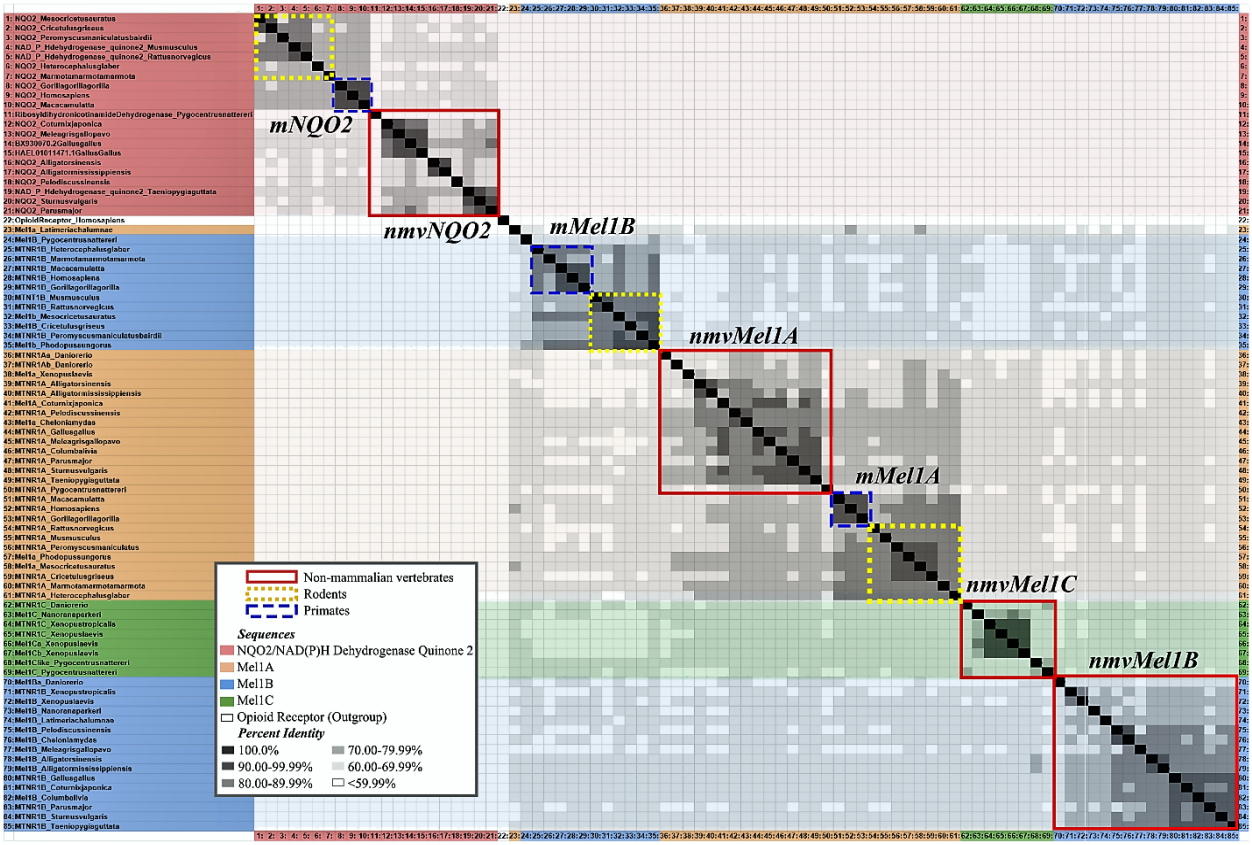

Fig. 2 Percent Identity Matrix of Melatonin Membrane Receptor Subtypes and Quinone Reductase 2. Analysis run by Clustal Omega 2.1 using NCBI GenBank. Red solid lines outline non-mammalian vertebrates (***nmv***), yellow dotted lines outline rodents, and blue dashed lines outline primates. Shades of grey indicate ranges of percent identity, black 100% and progressively lighter shades of grey down to 60%. Values <60% identity are white. Non-mammalian vertebrate (***nmv***) Mel1B sequences (lower right corner) do not have significant similarity with mammalian (***m***) Mel1B sequences (mid-upper left), suggesting that these are phylogenetically distinct melatonin membrane receptors. There is higher similarity within Mel1A receptor subtypes across vertebrates (mid-lower right). The quinone family in the upper-left [named NAD(P)H dehydrogenase quinone 2, ribosyldihydronicotinamide dehydrogenase, or NQO2] shows no significant similarity with other membrane melatonin receptor subtypes with percent identity across vertebrates and melatonin receptor subtypes <60%, supporting the position that QR2/NQO2 is not a putative MT3 membrane subtype receptor.

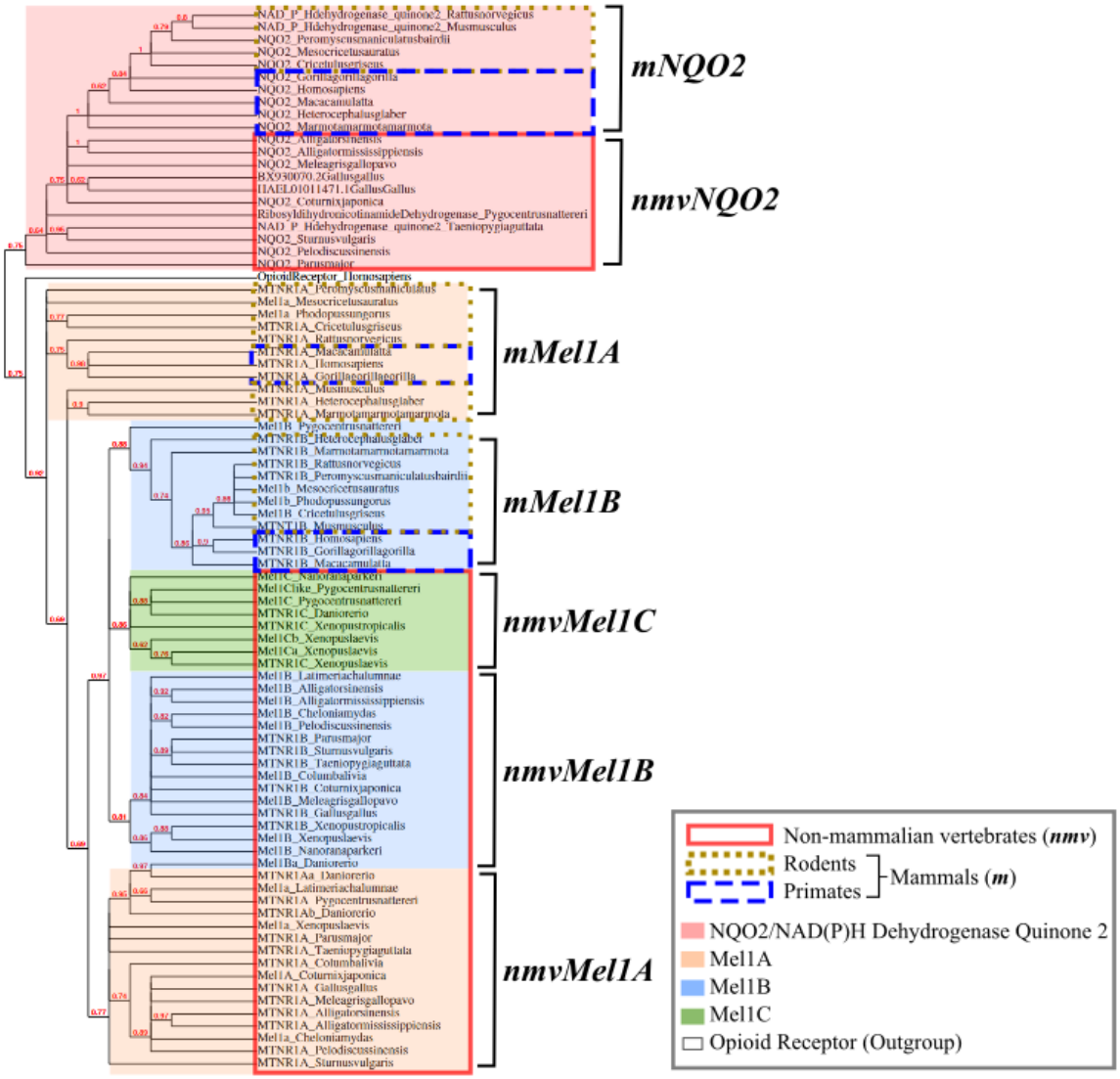

Fig. 3 Cladogram of Melatonin Membrane Receptor Subtypes and Quinone Reductase 2 Modified from neighbor-joining tree (without distance corrections), generated by Phylogeny.fr (Dereeper et al., 2008 & 2010). Red numbers represent branch support values. Branch support values smaller than 50% are collapsed. The quinone family is named NAD(P)H dehydrogenase quinone 2, ribosyldihydronicotinamide dehydrogenase, or NQO2, based on how it is named in the NCBI database. Opioid Receptor for *Homo sapiens* (NCBI Accession No. L29301.1) served as the outgroup. NCBI Accession Numbers and full names of sequences are in Fig. 4. The mRNA sequences of non-mammalian vertebrates (***nmv***) ***Mel1A*** diverged more recently than mammalian (***m***) ***Mel1B*** from ***mMel1A***. The pharmacological evidence that Mel1A/MT1 in birds has one order of magnitude lower affinity for 2-[^{125}I]iodomelatonin than MT1 in some mammals (rabbit, sheep and horse) and one order of magnitude higher than MT2 other mammals (Syrian and Siberian hamsters) supports the phylogenetic evidence presented here that avian Mel1A mRNA is not evolutionarily homologous with mammalian Mel1A/MT1 mRNA.

Mel1a/MTNR1A		Mel1b/MTNR1B		Mel1C/MTNR1C		NAD(P)H Dehydrogenase Quinone 2/NQO2 (Ribosyldihydronicotinamide Dehydrogenase)	
MTNR1A_Homo sapiens	NM_005958.4 Homo sapiens melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Homo sapiens*	NM_005959.3 Homo sapiens melatonin receptor 1B (MTNR1B), mRNA			*NQO2_Homo sapiens*	J02888.1 Human quinone oxidoreductase (NQO2) mRNA, complete cds
MTNR1A_Gorilla gorilla gorilla	XM_004040725.2 PREDICTED: Gorilla gorilla gorilla melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Gorilla gorilla gorilla*	XM_004051965.2 PREDICTED: Gorilla gorilla gorilla melatonin receptor 1B (MTNR1B), mRNA			*NQO2_Gorilla gorilla gorilla*	XM_019029756.1 PREDICTED: Gorilla gorilla gorilla NAD(P)H quinone dehydrogenase 2 (NQO2), transcript variant X8, mRNA
MTNR1A_Macaca mulatta	XM_001090972.3 PREDICTED: Macaca mulatta melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Macaca mulatta*	XM_001084265.3 PREDICTED: Macaca mulatta melatonin receptor 1B (MTNR1B), mRNA			*NQO2_Macaca mulatta*	XM_015135430.1 PREDICTED: Macaca mulatta NAD(P)H dehydrogenase, quinone 2 (NQO2), transcript variant X3, mRNA
MTNR1A_Marmota marmota marmota	XM_015486060.1 PREDICTED: Marmota marmota marmota melatonin receptor 1A (Mtnr1a), mRNA	*MTNR1B_Marmota marmota marmota*	XM_015489993.1 PREDICTED: Marmota marmota marmota melatonin receptor 1B (Mtnr1b), mRNA			*NQO2_Marmota marmota marmota*	XM_015507543.1 PREDICTED: Marmota marmota marmota NAD(P)H dehydrogenase, quinone 2 (Nqo2), transcript variant X2, mRNA
MTNR1A_Heterocephalus glaber	XM_004853058.2 PREDICTED: Heterocephalus glaber melatonin receptor 1A (Mtnr1a), mRNA	*MTNR1B_Heterocephalus glaber*	XM_004838123.1 PREDICTED: Heterocephalus glaber melatonin receptor 1B (Mtnr1b), mRNA			*NQO2_Heterocephalus glaber*	XM_013073256.1 PREDICTED: Heterocephalus glaber NAD(P)H dehydrogenase, quinone 2 (Nqo2), transcript variant X2, mRNA
MTNR1A_Peromyscus maniculatus	XM_006970866.1 PREDICTED: Peromyscus maniculatus bairdii melatonin receptor 1A (Mtnr1a), mRNA	*MTNR1B_Peromyscus maniculatus bairdii*	XM_006990545.1 PREDICTED: Peromyscus maniculatus bairdii melatonin receptor 1B (Mtnr1b), mRNA			*NQO2_Peromyscus maniculatus bairdii*	XM_006983508.2 PREDICTED: Peromyscus maniculatus bairdii NAD(P)H dehydrogenase, quinone 2 (Nqo2), mRNA
Mel1a_Phodopus sungorus	U14110.1 Phodopus sungorus melatonin receptor Mel-1a mRNA, complete cds	*Mel1b_Phodopus sungorus*	U57555.1 Phodopus sungorus Mel1b melatonin receptor mRNA, partial cds				
MTNR1A_Mus musculus	NM_008639.2 Mus musculus melatonin receptor 1A (Mtnr1a), mRNA	*MTNR1B_Mus musculus*	NM_145712.2 Mus musculus melatonin receptor 1B (Mtnr1b), mRNA			*NAD(P)Hdehydrogenase_quinone2_Mus musculus*	BC027629.1 Mus musculus NAD(P)H dehydrogenase, quinone 2, mRNA (cDNA clone MGC:41088 IMAGE:1225122), complete cds
MTNR1A_Cricetulus griseus	XM_007617174.2 PREDICTED: Cricetulus griseus melatonin receptor 1A (Mtnr1a), mRNA	*Mel1B_Cricetulus griseus*	XM_007636225.1 PREDICTED: Cricetulus griseus melatonin receptor type 1B-like (LOC103163272), mRNA			*NQO2_Cricetulus griseus*	XM_007638944.2 PREDICTED: Cricetulus griseus NAD(P)H quinone dehydrogenase 2 (Nqo2), transcript variant X2, mRNA
Mel1a_Mesocricetus auratus	AF061158.1 Mesocricetus auratus melatonin receptor Mel1a mRNA, partial cds	*Mel1b_Mesocricetus auratus*	AY145849 Mesocricetus auratus Mel1b melatonin receptor pseudogene, partial sequence			*NQO2_Mesocricetus auratus*	XM_013110593.1 PREDICTED: Mesocricetus auratus NAD(P)H dehydrogenase, quinone 2 (Nqo2), transcript variant X1, mRNA
MTNR1A_Rattus norvegicus	NM_053676.2 Rattus norvegicus melatonin receptor 1A (Mtnr1a), mRNA	*MTNR1B_Rattus norvegicus*	NM_001100641 Rattus norvegicus melatonin receptor 1B (Mtnr1b), mRNA			*NAD(P)Hdehydrogenase_quinone2_Rattus norvegicus*	BC079157.1 Rattus norvegicus NAD(P)H dehydrogenase, quinone 2, mRNA (cDNA clone MGC:94180 IMAGE:7128990), complete cds
MTNR1A_Gallus gallus	NM_205362.1 Gallus gallus melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Gallus gallus*	NM_001293103.1 Gallus gallus melatonin receptor 1B (MTNR1B), mRNA			*NQO2_Gallusgallus*	HAEL01011471.1 TSA: Gallus Gallus, breed ISA Brown, contig c17404/1/1559, transcribed RNA sequence
Mel1A_Coturnix japonica	XM_015862146.1 PREDICTED: Coturnix japonica melatonin receptor type 1A (LOC107313685), mRNA	*MTNR1B_Coturnix japonica*	XM_015852347.1 PREDICTED: Coturnix japonica melatonin receptor 1B (MTNR1B), transcript variant X1, mRNA			*NQO2_Coturnix japonica*	XM_015854496.1 PREDICTED: Coturnix japonica NAD(P)H dehydrogenase, quinone 2 (NQO2), mRNA
MTNR1A_Meleagris gallopavo	XM_003205776.3 PREDICTED: Meleagris gallopavo melatonin receptor 1A (MTNR1A), mRNA	*Mel1B_Meleagris gallopavo*	XM_010706129.2 PREDICTED: Meleagris gallopavo melatonin receptor type 1B (LOC104909381), mRNA			*NQO2_Meleagris gallopavo*	XM_010708413.2 PREDICTED: Meleagris gallopavo NAD(P)H quinone dehydrogenase 2 (NQO2), mRNA
MTNR1A_Sturnus vulgaris	XM_014873402.1 PREDICTED: Sturnus vulgaris melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Sturnus vulgaris*	XM_014877499.1 PREDICTED: Sturnus vulgaris melatonin receptor 1B (MTNR1B), mRNA			*NQO2_Sturnus vulgaris*	XM_014872375.1 PREDICTED: Sturnus vulgaris NAD(P)H dehydrogenase, quinone 2 (NQO2), mRNA
MTNR1A_Taeniopygia guttata	NM_001048257.1 Taeniopygia guttata melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Taeniopygia guttata*	NM_001048258.1 Taeniopygia guttata melatonin receptor 1B (MTNR1B), mRNA			*NAD(P)Hdehydrogenase_quinone2_Taeniopygia guttata*	DQ216354.1 Taeniopygia guttata clone 0065P0009D12 putative NAD(P)H dehydrogenase quinone 2 variant 2 mRNA, complete cds
MTNR1A_Columba livia	XM_005500227 PREDICTED: Columba livia melatonin receptor 1A (MTNR1A), mRNA	*Mel1B_Columba livia*	XM_005512954.2 PREDICTED: Columba livia melatonin receptor type 1B (LOC102095424), mRNA				
MTNR1A_Parus major	XM_019006224 PREDICTED: Parus major melatonin receptor 1A (MTNR1A), mRNA	*MTNR1B_Parus major*	XM_015647149 PREDICTED: Parus major melatonin receptor 1B (MTNR1B), mRNA			*NQO2_Parus major*	XM_015616869.1 PREDICTED: Parus major NAD(P)H quinone dehydrogenase 2 (NQO2), partial mRNA
MTNR1A_Alligator sinensis	XM_014521485 PREDICTED: Alligator sinensis melatonin receptor 1A (MTNR1A), mRNA	*Mel1B_Alligator sinensis*	XM_006021055.1 PREDICTED: Alligator sinensis melatonin receptor 1B (MTNR1B), partial mRNA			*NQO2_Alligator sinensis*	XM_014519268.1 PREDICTED: Alligator sinensis NAD(P)H dehydrogenase, quinone 2 (NQO2), transcript variant X1, mRNA
MTNR1A_Alligator mississippiensis	XM_006262998 PREDICTED: Alligator mississippiensis melatonin receptor 1A (MTNR1A), transcript variant X1, mRNA	*Mel1B_Alligator mississippiensis*	XM_006276749.3 PREDICTED: Alligator mississippiensis melatonin receptor type 1B (LOC102567490), partial mRNA			*NQO2_Alligator mississippiensis*	XM_019490389.1 PREDICTED: Alligator mississippiensis NAD(P)H quinone dehydrogenase 2 (NQO2), transcript variant X2, mRNA
MTNR1A_Chelonia mydas	XM_007062677 PREDICTED: Chelonia mydas melatonin receptor type 1A-like (LOC102946412), mRNA	*Mel1B_Chelonia mydas*	XM_007072335.1 PREDICTED: Chelonia mydas melatonin receptor type 1B-like (LOC102947942), mRNA				
MTNR1A_Pelodiscus sinensis	XM_006128505 PREDICTED: Pelodiscus sinensis melatonin receptor 1A (MTNR1A), mRNA	*Mel1B_Pelodiscus sinensis*	XM_014577564.1 PREDICTED: Pelodiscus sinensis melatonin receptor type 1B (LOC102444642), mRNA			*NQO2_Pelodiscus sinensis*	XM_014581445.1 PREDICTED: Pelodiscus sinensis NAD(P)H dehydrogenase, quinone 2 (NQO2), mRNA
Mel1A_Xenopus laevis	XM_018243177 PREDICTED: Xenopus laevis melatonin receptor type 1A (LOC108706613), mRNA	*Mel1B_Xenopus laevis*	XM_018250356 PREDICTED: Xenopus laevis melatonin receptor type 1B (LOC108710014), mRNA	*Mel1Ca_Xenopus laevis*	U67880 Xenopus laevis Mel-1c(a) melatonin receptor long mRNA, complete cds		
				Mel1Cb_Xenopus laevis	U67882 Xenopus laevis Mel-1c(b) melatonin receptor long mRNA, complete cds		
				MTNR1C_Xenopus laevis	KF486518 Xenopus laevis melatonin receptor type 1C (MTNR1C) mRNA, complete cds, alternatively spliced		
MTNR1A_Xenopus tropicalis	XM_002940864.3 PREDICTED: Xenopus (Silurana) tropicalis melatonin receptor 1A (mtnr1a), mRNA [removed from NCBI]	*MTNR1B_Xenopus tropicalis*	XM_004920744 PREDICTED: Xenopus tropicalis melatonin receptor 1B (mtnr1b), partial mRNA	*MTNR1C_Xenopus tropicalis*	XM_004916882 PREDICTED: Xenopus tropicalis melatonin receptor type 1C (mtnr1c), mRNA		
MTNR1A_Nanorana parkeri	XM_018563328 PREDICTED: Nanorana parkeri melatonin receptor 1A (MTNR1A), mRNA	*Mel1B_Nanorana parkeri*	XM_018558631 PREDICTED: Nanorana parkeri melatonin receptor type 1B (LOC108788772), mRNA	*Mel1C_Nanorana parkeri*	XM_018552598 PREDICTED: Nanorana parkeri melatonin receptor type 1C (LOC108783900), mRNA		
Mtnr1Aa_Danio rerio	NM_131393 Danio rerio melatonin receptor 1A a (mtnr1aa), mRNA	*Mel1Ba_Danio rerio*	BC163408 Danio rerio melatonin receptor type 1B a, mRNA (cDNA clone MGC:194859 IMAGE:9038309), complete cds	*MTNR1C_Danio rerio*	NM_001161484 Danio rerio melatonin receptor 1C (mtnr1c), mRNA		
Mtnr1Ab_Danio rerio	XM_688989 PREDICTED: Danio rerio melatonin receptor 1A b (mtnr1ab), mRNA						
MTNR1A_Pygocentrus nattereri	XM_017704098 PREDICTED: Pygocentrus nattereri melatonin receptor 1A (mtnr1a), transcript variant X1, mRNA	*Mel1B_Pygocentrus nattereri*	XM_017691296.1 PREDICTED: Pygocentrus nattereri melatonin receptor type 1B-B (LOC108423763), mRNA	*Mel1Clike_Pygocentrus nattereri*	XM_017713350 PREDICTED: Pygocentrus nattereri melatonin receptor type 1C-like (LOC108436701), mRNA	*Riboyldihydronicotinamide Dehydrogenase_Pygocentrus nattereri*	XM_017684980.1 PREDICTED: Pygocentrus nattereri riboyldihydronicotinamide dehydrogenase [quinone]-like (LOC108412776), mRNA
				Mel1C_Pygocentrus nattereri	XM_017721904 PREDICTED: Pygocentrus nattereri melatonin receptor type 1C-like (LOC108442058), mRNA		
Mel1Alike_Latimeria chalumnae	XM_014496132 PREDICTED: Latimeria chalumnae melatonin receptor type 1A-like (LOC102351371), partial mRNA	*Mel1B_Latimeria chalumnae*	XM_014492552 PREDICTED: Latimeria chalumnae melatonin receptor type 1B (LOC102361192), transcript variant X2, mRNA				

Table 1. NCBI Accession Numbers and Full Names of Sequences used for Phylogenetic Analysis All sequences that were available for the selected primates, rodents, and non-mammalian species were used. Species line up horizontally on this table. Mel1C has not been reported in mammals. NQO2/QR2 has been reported to be the putative MT3 receptor. Sequences were curated using Clustal Omega Multiple Sequence Alignment.

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Chapter 1: Melatonin Administration Methods in Animal Research

Introduction

Melatonin has various biochemical properties that render exogenous administration a complex undertaking. The purpose of the current study is to elucidate the different methodological approaches used for administering melatonin. This critical investigation sheds light on which administration methods show consistent results, where variability emerges, and important considerations for future studies that adopt these techniques.

After the purification of melatonin from the bovine pineal over half a century ago, research on melatonin and the pineal glands across vertebrates has continued to advance (for review, see Chowdhury et al., 2008). The process of removing the pineal and administering exogenous melatonin has been applied as a basic method to study countless biological phenomena, from sleep regulation to seasonal reproductive timing in photoperiodic breeding animals. Given that melatonin is available over-the-counter in the United States and its prolific internet fame as a cure-all for aging and sleep issues, melatonin research in humans primarily relates to resetting the circadian clock for shift workers (Dawson et al., 1995), individuals with blindness (Lockley et al., 2000), or jet-lagged travellers (Suhner et al., 1998). However, the plethora of options on how melatonin is ingested or administered leads to variable administration techniques that are not easily comparable across research studies. The time of day of administration, slow- versus fast-release, concentration, solvent, duration of a single exposure, and frequency of administration are all independent variables in melatonin administration methodologies. Furthermore, these factors can impact the change in receptiveness over time of a given model organism to exogenous melatonin administration. Age, sex, physiological conditions at the start of an experiment (e.g. reproductive state, pinealectomy, bilateral enucleation), and body weight of selected species collectively influence the effects of melatonin treatments. The context of the surrounding environment (e.g. laboratory, field, semi-natural enclosure) also contributes to the extent to which melatonin has an observable effect (for review, see Calisi & Bentley, 2009).

External environmental factors and internal physiological variables may obscure which methodologies are the most relevant for particular research questions on melatonin. This review will chart methodologies across fields to inform research involving melatonin administration. We begin with an overview of approaches used to distinguish the effects of exogenous melatonin administration from endogenous production, focusing on the photochemical effects of light/dark cycles and the use of pinealectomies. Next, common administration methods in mammals and birds are investigated: microinfusion pumps, beeswax pellets, oral administration via food/water, injections, and SILASTIC® capsules. Finally, other melatonin administration and modeling options emerging on the market will be discussed with their potential costs and benefits for advancing research in melatonergic pathways.

Experimental Manipulations: Lighting and Pinealectomies

Administering exogenous melatonin may replace, extend, or amplify the endogenous signal. To parse out how exogenous melatonin relates to the endogenous signal, several experimental manipulations have been utilized. The main environmental/laboratory condition to consider prior to exogenous melatonin administration is lighting. Photic inhibition of circulating

melatonin may be used to downregulate endogenous melatonin signals without affecting sleep and other important physiological demands.

Lighting. Light has been controlled in a variety of ways in the laboratory, and temporal conditions of photic exposure come in several forms. Bright light, darkness, or dim light may be kept constant throughout the duration of the experiment. Continuous darkness (DD) or dim-light allows animals to free-run. Because endogenous circadian rhythms are not exactly 24 hours, a free-running animal kept in constant darkness or dim-light may exhibit sleep-wake cycles that add up to slightly more or less than 24 hours, depending on the phenotype. Free-running animals in constant photic conditions lack light cues as a *Zeitgeber*, "time giver" or external cue that resets the circadian clock (for reviews on light as a *Zeitgeber* in humans, see Arendt & Broadway, 1987). The inactive period of an animal is subjective night, and for diurnal animals housed in constant darkness, the melatonin rhythm corresponds to subjective night. Melatonin rhythms persist in DD (Ralph et al., 1971). The pineal melatonin rhythm is suppressed in constant light (LL) (Ralph et al., 1971), which demonstrates that different forms of constant photic conditions do not exhibit consistent results.

For alternating photic conditions, and in most indoor studies reviewed here, light has a square wave pattern. Light is either on or off to simulate the light and dark phase, respectively. In a square wave pattern, there is no gradient of intensity to simulate dawn and dusk. The timing and duration of light may be used to entrain animals. Entrainment aligns the internal clock with an external *Zeitgeber*. Light:Dark (L:D) ratios can simulate long days (e.g. 18L:6D), associated with summer outside of the tropics, or short-days (e.g. 6L:18D), associated with winter. However, not all L:D periods must sum to 24 hours. T cycles are lighting regimes in which the sum of hours for the light and dark period is more or less than 24 hours (for review see Merrow et al., 2005). Additionally, not all L:D periods must be limited to a single light and dark period. Skeleton photoperiods are protocols in which there are brief pulses of light during extended periods of darkness. Below a threshold, pulses of light may simulate temporal patterns that reflect long or short days, acting as a *Zeitgeber* to simulate dawn and dusk. These experiments reflect the typical photic exposure of a burrowing diurnal or crepuscular animals. Light pulses can be shorter (e.g. 15 min) or longer (e.g. 1 hour), generating a unique lighting regime to address a wide range of research questions (for review, see Pittendrigh & Minis, 1964).

Upon the discovery that melatonin synthesis in the pineal is regulated by light (Wurtman et al., 1963), and because light exposure of particular wavelength during the middle of a dark phase significantly decreases pineal melatonin (Brainard et al., 1984; Honma et al., 1992), experiments that regulate light may blunt or permit the melatonin signal. Light as a *Zeitgeber* is typically parsed out from the endogenous circadian rhythm of melatonin synthesis by changing an animal's photic exposure T cycles, skeleton photoperiods, constant light (LL), and constant dark (DD) housing conditions (for review see Kriegsfeld & Bittman, 2010). Darkness coincides with the subjective night and a peak in circulating melatonin (Reiter, 1991). During the subjective night, animals may be exposed to pulses of light at varying intensities and/or wavelengths, each affecting melatonin synthesis in different ways (Brainard et al., 1984). Because bilateral enucleation in hamsters prevents photic inhibition of pineal melatonin production, and there is no evidence for extra-ocular photoreception in hamsters (Yamazaki et al., 1999), there appears to be a clear role for ocular photoreception in regulating the melatonin signal in hamsters.

In photoreceptive animals, changes in lighting conditions can have detectable effects on endogenous melatonin production. Melatonin conveys photic information to internal clocks

(Axelrod, 1974). Two enzymes involved in pineal melatonin synthesis are regulated by light via the retinohypothalamic phototransduction pathway (Klein & Weller, 1972; Klein & Moore, 1979). Light activates intrinsically photosensitive retinal ganglion cells (ipRGCs) of the retina, which contain the photopigment melanopsin. This signal is transduced via the retinohypothalamic tract, monosynaptic and direct axonal projections, to the suprachiasmatic nucleus (SCN) of the hypothalamus. Axonal projections from the SCN synapse in the PVN, and preautonomic neurons from the PVN synapse sympathetic preganglionic neurons of the intermediolateral cell columns of the spinal cord; ending with noradrenergic projections of the superior cervical ganglion synapsing on pinealocytes. Light inhibits pineal synthesis of melatonin, and the absence of light releases inhibition of melatonin synthesis (Klein & Weller, 1972; Klein & Moore, 1979; Perreau-Lenz et al., 2003). The SCN, a necessary conduit in this pathway, is often a high-affinity binding site for melatonin (Dubocovich & Markowska, 2005). Melatonin binds the SCN in almost all mammals studied to date, with the exceptions being the ferret (*Mustela putorius furo*), spotted skunk (*Spilogale*), and mink (*Mustela*) (for review see Bittman, 1993). Melatonin-binding in the SCN has been shown to affect the electrical activity rhythm of the SCN (for review, see Kriegsfeld & Silver, 2006).

Although the seemingly binary presence/absence of melatonin is regulated via the absence/presence of light transmitted through the phototransduction pathway, varying qualities of light produce differing results on pineal melatonin synthesis. Qualities of light include wavelengths, irradiance, and illuminance. However, studies that use square-wave patterns of lighting in a laboratory may not always take into consideration how differences in these qualities affect the outcome of an experiment. Studies that control lighting as an independent variable demonstrate exactly how these qualities affect melatonin. For example, pulses of two different monochromatic wavelengths of light at the same irradiance were administered to Wistar and Long-Evans rats during the midpoint of the dark phase, and green light (520 nm) suppressed pineal and plasma melatonin to levels comparable to the light phase for an extended period, but red light (660 nm) only temporarily suppressed pineal melatonin with no quantifiable effect to plasma melatonin levels (Honma et al., 1992). Green or red pulses were also administered two hours before lights-on, and at this time point, both wavelengths suppressed pineal and plasma melatonin (Honma et al., 1992). The time of subjective day at which a light pulse is administered and the wavelength have differing effects on circulating melatonin, so these factors must be considered in experimental design.

Once it was recognized that the duration of melatonin synthesis corresponds to the length of a dark period, the potential for melatonin to convey information to a seasonal breeder about time of year (for review see Tamarkin et al., 1985) spurred research on photoperiodic reproductive timing. As nights grow longer in winter outside of the tropics, so does the duration of melatonin synthesis and secretion. The extended duration of melatonin secretion denoting winter results in observable physiological changes that fluctuate annually in seasonal photoperiodic breeders. The length of the subjective night could be artificially extended in the lab by administering exogenous melatonin before the transition between lights on/lights off, inducing winter phenotypes in mammalian reproductive state. Short-day breeders (e.g. sheep, goats, deer) are reproductively active in winter but long-day breeders (e.g. hamsters, horses) are not (for review see Bartness et al., 1993).

Reviews on lighting and human physiology include research on night shift workers (Horowitz & Tanigawa, 2002) and people with jet-lag (Revel & Eastman, 2005). Lighting regulation (e.g. bright light therapy) may not be an option for people who are blind (Sack &

Lewy, 2001). Melatonin's effect on phase shifting the circadian pacemaker is determined by measuring circulating melatonin, core body temperature, and/or phase response curves, which illustrate the relationship between a stimulus (e.g. light) and a response (e.g. internal circadian phase) (Gronfier et al., 2004). Lighting regulation and melatonin administration have compounding effects on phase response curves. The effects of melatonin signals and photic signals on circadian rhythm research are nearly inextricable. One approach used to parse out the effects of light from endogenous melatonin patterns involves removing the main source of melatonin, the pineal.

Pinealectomies. Removing the pineal gland decreases serum melatonin within the first day post-surgery across vertebrates as shown in goldfish (*Carassius auratus*) (Delahunty et al., 1978), Atlantic salmon (*Salmo salar*) (Porter et al., 1996), sea bass (*Dicentrarchus labrax*) (Bayarri et al., 2003), tiger salamanders (*Ambystoma tigrinum*) (Gern & Norris, 1979), ruin lizards (*Podarcis sicula*) (Foà et al., 1992), quail (*Coturnix japonica*) (Homma et al., 1967), chickens (*Gallus gallus*) (Pelham, 1975), pigeons (*Columba livia*) (Foà & Menaker, 1988); European starlings (*Sturnus vulgaris*) and house sparrows (*Passer domesticus*) (Janik et al., 1992), sheep (*Ovis aries*) (Bittman et al., 1983), Syrian hamsters (*Mesocricetus auratus*) (Vaughan & Reiter, 1986), and rats (species not listed, Lewy et al., 1980 and Bubenik & Brown, 1997). A subset of studies that involved pinealectomies administered exogenous melatonin at controlled levels and assumed that removal of the main source of melatonin correspondingly removes the endogenous rhythm of its production. However, an extended post-pinealectomy recovery time permits compensatory melatonin-synthesizing organs to reinstate a detectable nocturnal peak of circulating melatonin. As few as two weeks after pinealectomy in pigeons, a circadian rhythm of melatonin production is compensated through increases in melatonin production in the Harderian gland (Vakkuri et al., 1985). Similarly, following one month recovery post-pinealectomy in rats, a diurnal rhythm in retinal melatonin production contributes to circulating levels that are comparable to the levels when the pineal is sham-operated or left intact (Yu et al., 1981). Reinstatement of melatonin rhythms in the Harderian gland of pinealectomized rats has also been observed (Reiter et al., 1983). Across different organisms, experimental variables such as the duration of recovery after the pinealectomy and how melatonin is measured do not guarantee removal of the endogenous melatonin signal, and the synthesis of extra-pineal melatonin could compensate for the assumed consequences of a pinealectomy. Exogenous administration of melatonin and endogenous, extra-pineal melatonin production may compound in undetected ways. It is especially difficult to account for highly localized melatonin synthesis (e.g. specific hypothalamic nuclei), which are not discernible through measures of plasma melatonin. These considerations for extra-pineal melatonin synthesis are comparable to experiments determining "steroid-independent" effects of light on the reproductive neuropeptides of photoperiodic breeders following gonadectomy (for review see Kriegsfeld & Bittman, 2010). Removing the gonads, the primary sources of circulating sex steroids, does not account for the potential compensatory mechanisms of neurosteroidogenesis (for a comparative review, see do Rego & Vaudry, 2016).

The effects of pinealectomy depend on the context in which it was removed. Pinealectomy in mammals kept in constant darkness appears to have little effect on circadian regulation, but in constant light, there are differences between how pinealectomized and sham-operated rats respond to light intensity, changing the period at which the rats free-run (Warren & Cassone, 1995). Furthermore, the outcomes of this procedure are not directly comparable between mammals and birds. Although removal of the pineal in photoperiodic mammals inhibits

photic stimulation of testicular development (for review see Bartness & Goldman, 1989), pinealectomies in birds have variable effects. Even bilateral enucleation and pinealectomies combined do not inhibit photic stimulation of avian gonadal development (for review see Oliver & Baylé, 1982). This finding has been observed in ducks (*Anas platyrhynchos*) (Benoît, 1935), white-crowned sparrows (*Zonotrichia leucophrys*) (Gwinner et al., 1971), golden-crowned sparrows (*Zonotrichia atricapilla*) (Gwinner et al., 1971), quail (Homma et al., 1972), and American tree sparrows (*Spizelloides arborea*) (Wilson, 1991). However, experiments removing the pineal at different reproductive states and/or photoperiods revealed that context matters. In long-day photostimulated quail, pinealectomies decreased ovarian weight and delayed reproductive development, but in short-day quail, pinealectomies have no observable effects on gonadal development (Saylor & Wolfson, 1968). The seasonal timing of pinealectomies in Indian weaver birds (*Ploceus philippinus*) also differentially affected reproductive state (Balasubramanian & Saxena, 1973). Precocious gonadal recrudescence was observed following pinealectomies in short-days, and gonadal development was accelerated when pinealectomies coincided with long-days Indian weaver birds (Balasubramanian & Saxena, 1973). Because previous work has alluded to the influence of photoperiod and reproductive state on the observable effects of pinealectomies, it is surprising that most studies reviewed here do not provide this background information explicitly. The photoperiod and reproductive state can sometimes be inferred, however, with enough background information on the lighting schedule and the animals used in the experiment.

The removal of an endocrine gland does not equate to the absence of its principal hormonal product from the body. Compensatory physiological systems may confound interpretations of results from experiments removing the pineal and administering exogenous melatonin. Furthermore, the timing of pinealectomies can have differential effects, depending on the photoperiod, species, and reproductive state preceding the pinealectomy. There is no universal answer on whether or not to pinealectomize, but there are observable patterns in the studies reviewed here. Typically, the technique of melatonin administration that follows pinealectomy is timed-infusion, mimicking the nocturnal melatonin signal (see Table 1). Pinealectomies preceding beeswax pellet implants, oral administration, and injections did not measure changes in endogenous melatonin concentrations. Lastly, the variability in post-pinealectomy recovery times, preceding exogenous melatonin administration, lacks any methodological justification. Consistency in post-pinealectomy recovery times would better facilitate the comparability of results from future studies that pinealectomize animals prior to exogenous melatonin administration.

Common Techniques of Melatonin Administration

Once endogenous melatonin production falls under experimental control through changes in lighting regimes and/or pinealectomies, the mode of administration of exogenous melatonin may be determined. The most common modes of administration include injections and oral delivery. Melatonin administration can have variable effects on reproduction or entrainment based on subspecies and age of the model organism. For instance, regularly timed melatonin administration may entrain Siberian hamsters but has no effect on Syrian hamsters beyond what is observed with regularly timed saline injections or scheduled arousal (for review see Hastings et al., 1997). Citing precedence as justification for a given method used in a specific species is not sufficient. Instead, it is useful to determine how these methods were validated in a given

species, age, sex, lighting condition, and photoperiodic history. The efficacy of a particular administration regime in one study is not easily transferable to another.

Techniques that are often used to administer melatonin across vertebrates include oral administration, injections, and SILASTIC® capsules. The affordability of these methods and prevalence of previous research using these methods likely reinforces their current use. These methods are used to answer questions from fields in circadian rhythms, seasonality, and reproduction. Beyond simply achieving the intended outcome of elevating circulating melatonin levels, these administration techniques offer an array of controls that fine-tune the temporal pattern and amplitude of the increase in melatonin concentration. Affordability, time-investment demanded of the researcher, frequency of stressful tampering with animals, and physiological relevance can collectively factor into the experimental design. This section serves to inform methodology in melatonin administration based on research topic and species.

Infusion pumps. (see Table 1 for summary) Infusion pumps, or microinfusion pumps, infuse fluids into the circulatory system with rates as low as 500 nanoliters per hour. Small-volume pumps may include a programmable system that rotates a screw and slowly depresses a syringe. The syringe may be connected to tubing that leads to a gauged needle inserted subcutaneously, intraperitoneally, or intravenously. The opportunity to administer a drug continuously and limit administration to a specific time frame is made possible with infusion pumps. Daily exposure to exogenous melatonin administered with microinfusion pumps over the course of multiple hours may mimic the endogenous circadian rhythm of melatonin previously described. This method has been accomplished by alternating a water-soluble solution containing dissolved melatonin with an oil-based solution within the tubing. Due to the physiological relevance of this administration regime, most of the studies reviewed here using microinfusion pumps to administer melatonin focus on circadian and circannual rhythms. The duration of the melatonin signal is well-established in photoperiodic, seasonally breeding mammals to be the critical parameter for driving reproductive development (for review, see Bartness et al., 1993). Furthermore, a daily period of time with low to absent circulating melatonin (the inter-pulse interval, or IPI) appears to be necessary for reinstating sensitivity to the melatonin signal (Bartness et al., 1993). With the capacity to temporally constrain the melatonin signal, the microinfusion pump has contributed significantly to our understanding of circadian rhythms and seasonality.

Challenges emerge with timed-infusion of melatonin via infusion pumps. One challenge is the locomotor limitations of the model organisms confined by microinfusion pumps. To date, microinfusion pumps and microdialysis (e.g. Ravault et al., 1996) are more commonly used in caged, quadruped animals. Animals caught in the wild must be brought into a lab environment to enable monitored use of microinfusion pumps (e.g. Hyde & Underwood, 1995), but stress-induced physiological shifts can occur when transporting animals from the wild into different settings (Dickens et al., 2009a & 2009b). Microdialysis has been adapted to measure pineal melatonin in free-moving birds (Hasegawa et al., 1994), and a comparable system for microinfusion pumps may be investigated. Options for programmable, implantable infusion pumps are becoming available (see section of this review on mini-osmotic pumps); however, they are typically single-use. Over time, as these options become more affordable and mainstream, we can imagine that the intricacies of microinfusion pumps may be taken outside the caged lab environment for administration studies in the wild.

Another challenge is calculating whether or not the microinfusion pump will supplement or replace the endogenous melatonin rhythm. As previously described in this review, the

endogenous rhythm may be measured through plasma samples, and this rhythm is diminished when the pineal organ is removed or when a photoreceptive animal is exposed to constant light to inhibit pineal melatonin production (as observed in Lynch & Wurtman, 1979 and Lynch et al., 1980). Although plasma concentrations of circulating melatonin are frequently used as a determinant of circadian rhythms, they do not quantifiably represent localized synthesis of melatonin in non-photoreceptive tissues. Maintaining the phototransduction pathway and pineal intact allows experiments using microinfusion pumps to artificially extend the endogenous melatonin signal by administering exogenous melatonin during subjective dawn or dusk (e.g. Romanowicz et al., 2001).

Microinfusion pumps may also supplement the endogenous rhythm of pineal-intact animals housed in constant dim light. Dim light allows endogenous melatonin fluctuations to persist during exogenous administration (as observed in Stanton et al., 1987 and Slotten et al., 2002a & 2002b). The absence of a photic *Zeitgeber* may obscure subjective time in constant dim light. It is important to note in methodologies whether or not the extended period of melatonin administration coincides with the endogenous rhythm or occurs during the inter-pulse interval (IPI) of low to absent circulating melatonin. Microinfusion pumps may replace circulating levels of melatonin in pinealectomized animals housed in constant dim light (as observed in Hyde & Underwood, 1995 and Schuhler et al., 2002). In these studies, pinealectomies led to arrhythmia in circadian locomotor activity, and exogenous melatonin administration of an extended duration restored rhythmic locomotor activity.

Subtle variations across methodologies in studies using microinfusion pumps may address fundamentally different questions in the fields of circadian and circannual research. For example, studies addressing circadian rhythms in locomotor activity typically house animals in constant darkness and may or may not pinealectomize a subgroup. Animals are typically gonadectomized in studies related to seasonality (e.g. reproduction, immune state) prior to exogenous melatonin administration. Gonadectomies isolate the effects and melatonin and light (steroid-independent) from negative feedback of gonadal steroids (steroid-dependent). In experiments that leave the gonads intact, volumetric changes are measured to determine how exogenous melatonin administration affects gonadal reproductive state. Different species of photoperiodic breeding mammals vary in the rate of gonadal growth or regression, so the overall length of the administration period must be long enough to enable these physiological changes (4-8 weeks for adult male Syrian hamsters in Pitrosky et al., 1991 and 2 weeks for Polish Lowland ewes in Romanowicz et al., 2002). It is necessary to recall how lighting conditions (e.g. brightness, photoperiodic history) and/or the timing of pinealectomies can alter the rate of gonadal development (see previous sections), thereby affecting the overall length of the administration period. Careful attention to these details enables optimal replicability of studies using infusion pumps to temporally constrain melatonin administration. For continuous administration of melatonin, beeswax pellets provide an option and are reviewed in the subsequent section.

Beeswax Pellets. (see Table 2 for summary) Beeswax mixed with melatonin crystalline powder is one mode of continuous melatonin administration. The compound is mixed, shaped, and implanted. A common theme across studies using this form of administration is the focus on reproductive development. There are no rationales for concentration, though one study measured the rate of release *in vitro* and assumed a similar rate *in vivo* (Glass & Lynch, 1982). The rate of release *in vitro* from a larger pellet (0.8mm Outer Diameter, OD) on days 1-6 was over 20 times higher than the rate of release on days 7-56, while the smaller pellet (0.4mm OD) rate of release

on the first week was 6-7 times higher than the rate of release during the subsequent seven weeks (Glass & Lynch, 1982).

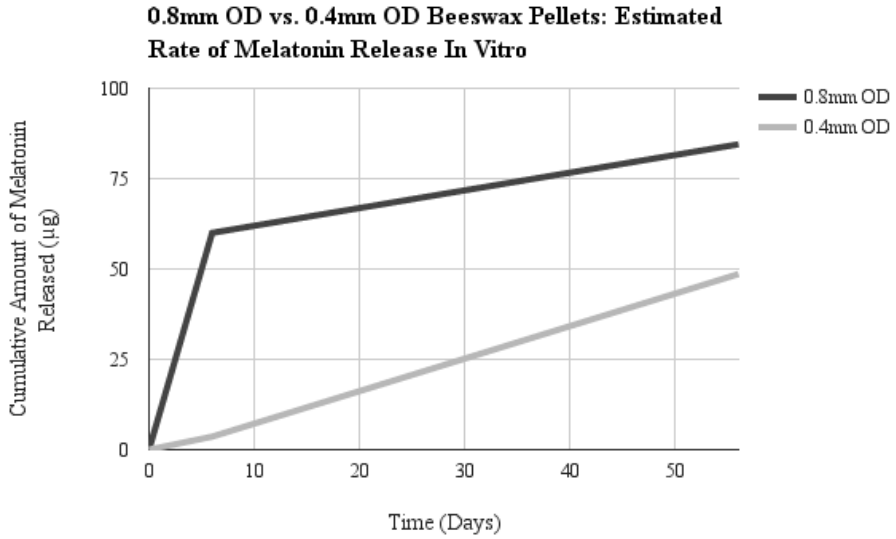

Fig. 1 0.8mm OD vs. 0.4mm OD Beeswax Pellets: Estimated Rate of Melatonin Release In Vitro. Samples were collected on Day 6 and Day 56 and the rate of release from the preceding days were extrapolated from these collection times. Note that the rate of release is less consistent and the overall amount of melatonin released is higher in the 0.8mm OD beeswax pellet. Adapted from Glass & Lynch, 1982.

This pattern demonstrates that the size of the pellet not only affects the *concentration* of melatonin that is diffusing but also the *rate* of melatonin diffusion (see Fig. 1). An earlier study varied the dose and the frequency of pellet replacement, showing that bi-weekly replacements and pellets of concentrations greater than 100µg of melatonin per 24g of beeswax reversed the effects of bilateral enucleation on reproductive development in male Syrian hamsters (Reiter et al., 1975); however, the surface area and volume of the pellet, which would affect the rate of melatonin diffusion, were not specified.

Not all studies using beeswax pellets implant them subcutaneously. An adaptive feature of this mode of administration permits continuous release in specific nuclei of the hypothalamus. For species with a detailed brain atlas with stereotaxic coordinates, this method provides a useful alternative to microinfusion pumps if the desired outcome is continuous and targeted melatonin administration. Although subcutaneously implanted melatonin-beeswax pellets had little effect on reproductive development, microimplants of melatonin-beeswax pellets in the anterior hypothalamus (AH) and the suprachiasmatic nucleus (SCN) had significant physiological and behavioral effects in adult female white-footed mice (Glass & Lynch, 1982). Melatonin beeswax implanted in the AH corresponded to decreased testis volume in male white-footed mice, while subcutaneous implants did not have a significant effect (Glass & Knotts, 1987). Freeman and Zucker tested the responsiveness of pinealectomized Syrian hamsters' reproductive axis to timed daily melatonin infusions, after leaving melatonin-beeswax implants in melatonin-binding sites for 40 weeks (2001). Hamsters with melatonin-beeswax implants in the SCN, PVN, and nucleus reunions (NRE) responded to timed-daily melatonin infusions while hamsters with s.c. SILASTIC® capsules did not respond to the timed-daily melatonin infusions (Freeman & Zucker, 2001). As with any localized administration technique, there remains the possibility for diffusion into adjacent neural sites. Because melatonin-beeswax implants in the third ventricle did not have effects comparable to implants in the SCN, PVN, or NRE, it is unlikely that

melatonin diffuses from beeswax implants to adjacent neural sites (Freeman & Zucker, 2001). Regardless, it is clear that subcutaneous implants are not sufficiently localized to enable targeted, physiological changes made possible through microimplants in the brain.

In addition to how continuous-release melatonin beeswax implants affect reproductive development, how tissue sensitivity to melatonin is altered through this mode of administration is also important. One experiment investigated this possibility by injecting melatonin daily in female Syrian hamsters subcutaneously implanted with melatonin beeswax pellets. The injections, which would have typically inhibited female Syrian hamster reproductive development, were ineffective in hamsters with beeswax pellets within the range of 50µg to 10mg of melatonin (Trakulrungsi et al., 1979), thus demonstrating a decreased sensitivity to the exogenous melatonin signal. Neural sites with extended melatonin exposure may desensitize the tissue to the melatonin signal by downregulating melatonin receptors in the region (Gerdin et al., 2004a & 2004b). Furthermore, blocking melatonin receptors with the antagonist luzindole prevents the pre-dawn decrease in melatonin concentrations, suggesting that the nocturnal elevation of melatonin concentrations is downregulated by a homeostatic feedback mechanism mediated by melatonin receptors (Bedrosian et al., 2013). We can synthesize the results of these experiments to predict that melatonin-beeswax implants, or any continuous-release administration device, decreases sensitivity to the endogenous melatonin signal over time.

Additionally, based on the change in rate of melatonin release *in vitro* previously discussed (summarized in Fig. 1), the frequency that the pellets are replaced also affects melatonin concentration over time. The overall length of the administration period of studies using beeswax implants was 4-12 weeks, typically with weekly replacements of beeswax pellets. While the concentration was usually 1mg melatonin crystalline powder per 24mg beeswax, other variables such as the volume and surface area of the implanted pellet were inconsistently reported. Variation in the weight and size of the model organism must also be considered in justifying the size and concentration of subcutaneously implanted beeswax pellets in future studies. An alternative implant method for continuous melatonin administration, SILASTIC® capsules, are discussed later in this review. One advantage that SILASTIC® capsules has over beeswax pellets is their clear, visible indication that the contents have diffused, due to the transparent structure.

Beeswax microimplants in specific neural sites offer continuous melatonin administration, though it may be difficult to replace the implants past the first week. There are no studies that have used SILASTIC® capsules for this purpose, and the pliancy of beeswax may be better suited for continuous-release microimplants in the brain. However, the relative change in the rate of melatonin release from beeswax pellets is a difficult variable to control. For greater spatiotemporal resolution, localized microinjections have fewer uncontrolled variables than beeswax microimplants. Another administration technique, by far the least invasive technique included in this review, is oral melatonin administration.

Oral Administration: Food and Water. (see Table 3 for summary) Absorption through the digestive tract is one of the various paths of exogenous melatonin administration. A variety of plants and plant-based products have high levels of melatonin, including cherries (Burkhardt et al., 2001), walnuts (Reiter et al., 2005), wine (Fernández-Mar et al., 2012) and several others (for reviews see Reiter et al., 2001 & 2006; Garcia-Parrilla et al., 2009). Adult male humans consuming different fruits with detectable levels of phytomelatonin showed a dose-dependent increase in serum melatonin (Sae-Teaw et al., 2013). An important consideration for animal studies is that the list of sources of phytomelatonin and the ingredients of various chow or feed

provided *ad libitum* are typically not described in the methods of studies focusing on melatonin. Although there are exciting prospects for comparing different sources of phytomelatonin and their influence on circadian rhythms and circannual patterns, this review focuses on experiments that control for the concentration of melatonin by dissolving a measured amount to be orally-administered through the vehicle of water or food.

Most studies reviewed here dissolved melatonin in ethanol and diluted the solution in drinking water (Heigl & Gwinner, 1995; Guyomarc'h et al., 2001; Kumari et al., 2015) or mixed it with the diet and let the ethanol evaporate (Lewis et al., 2006; Chen et al., 2016). Ethanol consumption rapidly and robustly suppress plasma melatonin (Ekman et al., 1993) and salivary melatonin (Rupp et al., 2007) in a dose-dependent manner, however. These studies administered relatively higher levels of ethanol and did not mix melatonin with ethanol directly. Presumably the concentration of ethanol used to dissolve crystalline melatonin powder was too low to affect the endogenous melatonin signal. Not all melatonin needs to be dissolved prior to oral administration. Melatonin has also been fed to deer (*Odocoileus virginianus*) in gelatin capsules stuffed into apple slices (Bubenik & Smith, 1987).

The dosage of orally-administered melatonin corresponds to increases in plasma melatonin detectable through RIA and ELISA, which suggests that dosage has a linear relationship with absorption through the digestive tract; however, only Chen et al., 2016 in the studies reviewed here administered more than one dose and measured circulating levels.

Oral administration of melatonin has several confounding variables that are absent from injection and implant studies. To begin, molecular circadian oscillators regulate everything from food intake, appetite, metabolism, and digestion (for review, see Waterhouse et al., 2007). Circadian and seasonal changes in metabolism (as observed in deer in Freudenberger et al., 1994) may affect how orally-administered melatonin is absorbed. The effects of seasonal fluctuations of melatonin duration on feeding are observed in European sea bass (*Dicentrarchus labrax*), in which orally-administered melatonin affects both the proportion of macronutrients and total amount of food consumed (Rubio et al., 2004). Usually, a study administering melatonin via animal feed weighs the animal and/or the food tray to check for intra-group consistency in food consumption and body weight. Molecular circadian oscillators likely affect the breakdown of melatonin administered through injections and implants, but this has not been studied as extensively as orally-administered melatonin. Secondly, melatonin is subject to a high hepatic extraction ratio (Lane & Moss, 1985). Furthermore, the gastrointestinal (GI) tract synthesizes melatonin at concentrations up to 500 times higher than the nocturnal levels of melatonin (Bubenik et al., 2001). Melatonin in the upper GI tract acts as a free-radical scavenger and has been shown to protect the mucosa layer from irritation caused by GI tract lesions (for review, see Konturek et al., 2007). Orally-administered melatonin may affect endogenous GI melatonin synthesis, along with how melatonin may be catabolized in the GI tract prior to reaching detectable levels in plasma. These factors may be considered in studies using an oral administration technique. Because melatonin changes gastrophysiology (Motilva et al., 2001), the effectiveness of a given dose and the sensitivity of the GI tract likely change with frequent melatonin administration over a longer period of time. Finally, with absorption highest in the ileum and rectum and lowest in the stomach, melatonin itself is classified as a low-permeability drug (Tran et al., 2009). Due to the impressive variation across vertebrate digestive systems (Stevens & Hume, 2004), melatonin absorption may vary based on the anatomical and physiological properties of the GI tract of a given species. A comparative study looking at

melatonin GI absorption across vertebrates would enrich our understanding of previous studies using food or water as the administration method.

The relevance of melatonin administration in food and water may be connected to its pharmaceutical use in humans. Melatonin pills are available over-the-counter in the United States and are unregulated. Research on melatonin pills may relate to effects on circadian rhythms (Lewy et al., 1992), depression (Dalton et al., 2000), senescent-induced insomnia (Rikkert et al., 2001), jet-lag (Arendt et al., 1986; Suhner et al., 1998; Herxheimer et al., 2002), shift-work and blindness (Arendt et al., 1997). Several of these factors have physiological connections that interplay with both sleep quality in the inactive period as well as focus and attention in the active period. Fine-tuning the timing of ingestion, the dose, and the rate of release of melatonin to facilitate optimal sleep without interfering with productivity are the foci of several studies investigating melatonin and its pharmaceutical applications in humans. These techniques may be investigated with metrics appropriate to animal studies, e.g. conducting cognitive behavioral tests in addition to taking physiological measurements to determine the optimal timing and dosage of orally-administered melatonin.

The rate of release of melatonin varies based on the mode and vehicle of oral delivery. For instance, melatonin orally-administered in a gelatin capsule, in corn oil, or in a slow-release pill corresponded to plasma concentrations of melatonin that peaked at different times, depending on the vehicle of administration (Aldhous et al., 1985). The duration of the elevated amplitude of circulating melatonin also varied, suggesting that the mean elimination half-life of melatonin is impacted by its vehicle (Aldhous et al., 1985). The pharmacokinetics of oral melatonin delivery can be altered by how the capsule is coated as well (Lee et al., 1995), and this consideration applies even to non-oral administration routes. As several of the other methods listed in this review dissolve melatonin in ethanol or oil prior to injection, the effects that these different solvents and vehicles have on elimination half-life may affect the duration of the melatonergic effects post-injection. Investigations of the differences between solvents and vehicles on melatonin half-life would help address these potential methodological discrepancies. These considerations for melatonin solvents and vehicles carry into the next section of this review on melatonin injections.

Injections. (see Table 4 for summary) Concerns from variations in vertebrate digestive systems arising through oral administration may be bypassed with the administration technique of subcutaneous or intracerebroventricular injections. The delay between the time of administration and the subsequent increase in plasma melatonin is shorter from an injection than from oral administration (Kennaway & Seamark, 1980). However, the duration of the elevated signal remaining after the injection is administered is shorter than orally-administered melatonin (Kennaway & Seamark, 1980), for orally-administered melatonin is absorbed through the GI tract in a more distributed fashion. Subcutaneous melatonin injections in sheep and goats caused a transient peak in melatonin, and oral administration via saline or foodstuff maintained elevated levels of detectable, circulating melatonin up to 7 hours after administration (see Fig. 2 adapted from Kennaway & Seamark, 1980). Although the sample size included two sheep and four goats, and the results may not be easily interpreted to apply broadly, replication and expansion on this type of study in different species would serve as an additional foundation to base methodological decisions on administration techniques. One commonality between the studies in which melatonin is injected or administered orally is how the crystalline powder is dissolved prior to its delivery. The concerns, previously described in this review, of the effects of solvents on the

elimination half-life of melatonin administered orally (Aldhous et al., 1985) also applies with solvents used for injections in Kennaway & Seamark (1980) as described in Fig. 2.

A subset of studies using injections as the mode of melatonin administration investigate how dose and concentration affect observable physiological factors (e.g. Cassone et al., 1986a; Juss et al., 1993; Rozenboim et al., 2002; Karuppagounder et al., 2007). The frequency of injections may also affect experimental results (Juss et al., 1993; Rozenboim et al., 2002). In general, there are not dose-dependent, graded effects of melatonin on reproduction state or circadian entrainment, but rather, the effects of exogenous melatonin on reproductive development and circadian entrainment are binary and non-linear. This observation of all-or-nothing physiological responses to properly timed injections is consistent with the duration hypothesis supported in infusion studies (Bartness et al., 1993), but not all injection studies support this. Injections of melatonin at supraphysiological concentrations may alter the sensitivity of target tissue to nocturnal increases in endogenous melatonin by downregulating melatonin receptors (e.g. Gerdin et al., 2004a & 2004b). If melatonin receptors are downregulated as a result of these injections, there may be an artificially induced inter-pulse interval (IPI), simulating physiological responses that equate to low daytime levels of circulating melatonin. However, additional research on the effects of melatonin injections on melatonin receptor transcription, affinity, or function has yet to be conducted.

Prior to administering melatonin injections, only two of studies reviewed here have animals pinealectomized (Reiter et al., 1977; Marumoto et al., 1996a) and bilaterally enucleated (Marumoto et al., 1996a). However, the overall length of the administration period of daily injections in both of these studies may allow for the development of compensatory circadian melatonin production from non-photic tissues (Huether, 1993), as described in the section of this review on pinealectomies. Thus, the removal of pineal melatonin and the recovery time that precedes and includes melatonin injections may have altered endogenous rhythms and sensitivity in a manner that cannot be assumed to be physiologically relevant in these studies.

The majority of injection studies reviewed here neither pinealectomized nor bilaterally enucleated animals. Instead, injection protocols have been synchronized in relation to endogenous melatonin rhythms in the following ways:

1. Artificially extending the subjective night and the corresponding nocturnal increase in endogenous melatonin by administering the injection(s) before the transition between lights on/off (Bartness & Wade, 1985; Juss et al., 1993; Champney et al., 1997; Rozenboim et al., 2002).
2. Housing the animals in constant lighting conditions, either constant darkness (Cassone et al., 1986a) or dim light (Cassone et al., 1986b), and administer the injection out-of-phase with circadian oscillations of melatonin production that persist independently of a photic *Zeitgeber* (Cassone et al., 1986a; Cassone et al., 1986b).

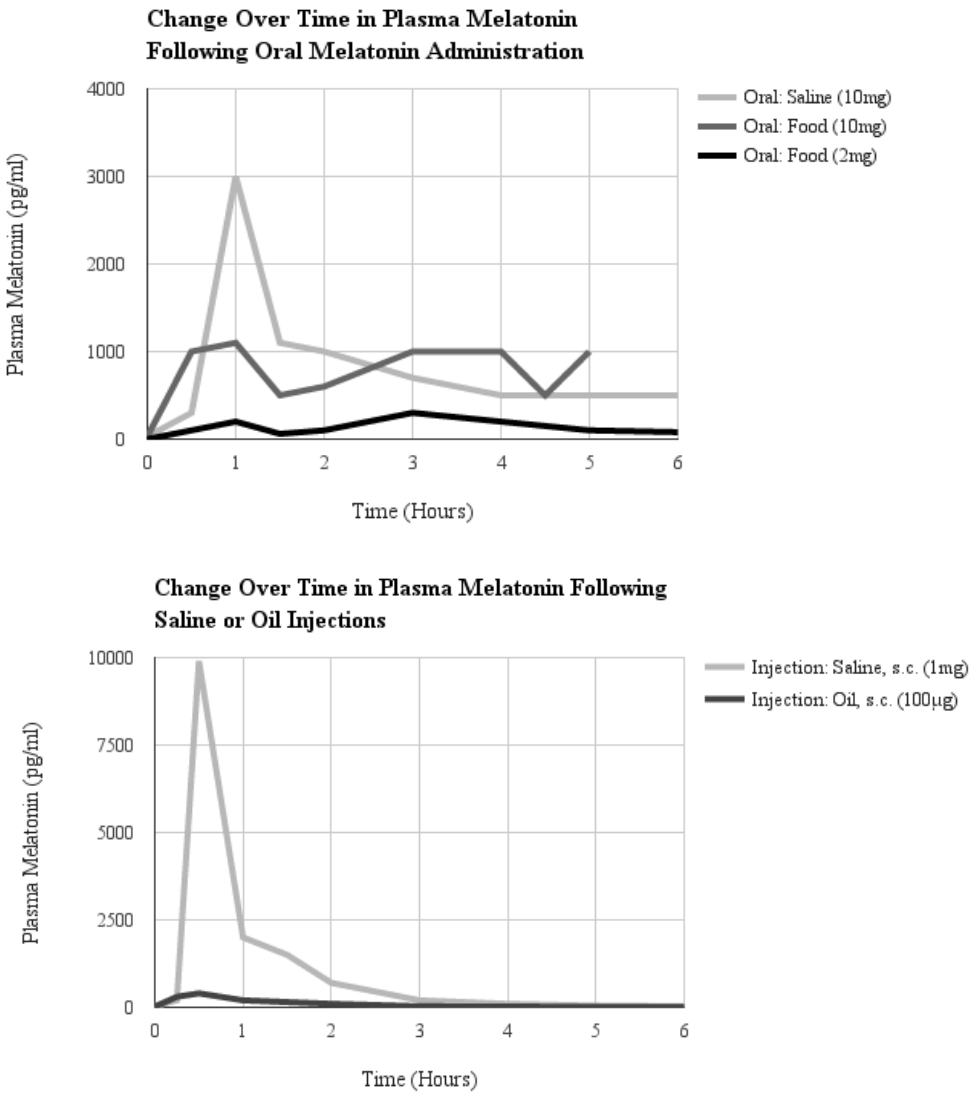

Fig. 2 Plasma melatonin concentrations following melatonin administration to two Merino cross-bred sheep. Note the duration of the elevated peak in plasma melatonin is shorter for melatonin administered via subcutaneous injections relative to orally-administered melatonin regardless of the vehicle (saline or food). The amplitude of the peak in plasma melatonin is lower for melatonin injected with the oil vehicle relative to the saline vehicle. While the concentration of melatonin in the saline injection is 10 times higher than the concentration dissolved in oil, the peak in plasma melatonin is 33 times higher following the saline injection. Adapted from Kennaway & Seamark, 1980.

Lighting conditions that animals were reared in prior to the experiment must also be considered in how animals are primed for melatonin injections, especially if the lighting conditions when animals are reared is different than experimental lighting. For instance, 8 weeks of properly timed melatonin injections (15 µg) administered to pineal-intact hamsters housed in long-days (16L:8D) and subsequently transferred to a relatively shorter photoperiod (12L:12D) stimulates reproductive development in hamsters (Karp et al., 1990). The photoperiodic history and the corresponding duration of the melatonin signal prime the hamster reproductive system to respond to relative changes in the duration of the artificially-induced melatonin signal. Photic input does not override the exogenously administered melatonin signal. Furthermore, there is not an absolute duration of the melatonin signal that guarantees a particular reproductive state in

these species. Because some photoperiodic breeders use relative changes in the duration of the melatonin signal as a cue for reproductive responses, experiments that alter the lighting and/or the timing of melatonin injections prior to or in the middle of an experiment must document these changes and their potential effects on the results.

In summary, injection studies include how melatonin was dissolved and diluted, the lighting condition and photoperiodic history of animals used in the study, and the subjective time-of-day that the injection was administered. It is useful to contextualize how the timing of the injection corresponds to subjective night or, for animals housed in constant darkness, how the injection corresponds to the internal, circadian oscillation of melatonin production. Not all studies provide this information (Karuppagounder et al., 2007; Rojas et al., 2008), obfuscating the implications of the findings in the broader context of melatonin research. Injection studies with a rationale for dose and/or frequency of melatonin injection in a given species provide a strong precedence for subsequent studies in the field. This rationale may include a description of the relationship between (1) the dose and its physiological relevance and (2) the frequency of administration and over what period of time a seasonal physiological change is observed in the wild for a particular species. However, it is important to note the physiological differences observed in laboratory and wild animals (Calisi et al., 2009). A pilot study that measures circadian fluctuations in melatonin would inform experimental design of injection studies by providing physiologically-relevant doses catered to a specific context.

SILASTIC® Capsules. (see Table 5 for summary) Since the late-1960s, crystalline melatonin has been inserted and sealed inside SILASTIC® tubing to be implanted in animals. SILASTIC® is a flexible, inert elastomer composed of silicone rubber and polydimethyl siloxane, produced by Dow Corning Corp. The material is rigid and stable enough to maintain its geometrical shape throughout the length of these experiments. Although a depleted capsule leaves a foreign body embedded in the tissue, there is no evidence of inflammation resulting from the implantation of this material (Kind & Rudel, 1970). The first documented study investigating the potential for SILASTIC® to be used in drug delivery was by Folkman and Long in 1964.

SILASTIC® implants are described as continuous release, but certain variables affect diffusion rate. Over time, the diminishing amount of dissolved powder inside the capsule decreases the overall rate of diffusion (Kind & Rudel, 1970). Furthermore, temperature affects the permeability of the SILASTIC® membrane and the solubility of its contents (Zhang & Cloud, 2006), so diffusion rate may be mildly affected by fluctuations in core body temperature or by more extreme fluctuations of environmental temperature in studies conducted in the wild (such as Greives et al., 2012 and Koresh et al., 2016). Similarly to beeswax implants, SILASTIC® implants would need to be replaced on a regular basis to maintain continuously elevated levels of circulating melatonin. However, all the studies in this review implanted the SILASTIC® capsule once and left it for the duration of the experiment. The change in weight of the SILASTIC® capsule before and after implantation was used to calculate the rate of diffusion in Juss et al., 1993, posited to be 24µg/day, but this calculation assumes a constant amount diffuses daily over the course of 20 days. Future studies may be conducted *in vivo* and *in vitro* to assess the diffusion rate over an extended period of time for the more frequently used volumes of SILASTIC® capsules (e.g. 1.47mm ID x 1.96mm OD x 25.0mm length). These studies would be foundational to determining if SILASTIC® capsules should be replaced, like beeswax pellets, on a weekly or bi-weekly basis for experiments with longer administration periods.

There are no studies to date that address the specific chemical dynamics of melatonin diffusion through SILASTIC®. However, several studies validate that melatonin-implanted groups are left with an empty capsule of SILASTIC® at the end of the experiment and have elevated circulating levels of melatonin relative to blank-implanted groups (Marumoto et al., 1996b; Kennaway & Rowe, 1997; Bentley et al., 1999; Ubuka et al., 2005; Walton et al., 2011). Studies on the passage of progestogens (Lifchez & Scommegna, 1970) or steroids (Dzuik & Cook, 1966) through SILASTIC® material, including when used in wildlife (Koresh et al., 2016), may be referenced to develop methodologies for similar studies using melatonin.

SILASTIC® capsules have been adapted for a range of melatonin research topics. Studies in circadian rhythms (Hau & Gwinner, 1994 & 1995; Marumoto et al., 1996b), photoperiodism and reproduction (Juss et al., 1993; Bentley et al., 1998; Ubuka et al., 2005; Greives et al., 2012), and neuroplasticity (Bentley et al., 1999) each use different types of protocols for lighting, the volume of the SILASTIC® capsule, and the duration the capsule was left implanted. One study used animals subcutaneously implanted with SILASTIC® capsules, with or without melatonin, as control groups to compare to animals with stereotaxically placed beeswax implants (Freeman & Zucker, 2001). Most studies inserted the implant subcutaneously, and only one study reviewed here inserted the SILASTIC® tubing intravaginally (Nowak & Rodway, 1985). In only a few studies were the main sources of melatonin removed (Px in rats Marumoto et al., 1996b; Px and Ex in quail Ubuka et al., 2005), and none of the reviewed studies housed animals in constant lighting conditions, even in circadian studies. Continuous melatonin supplementation via SILASTIC® capsules decreased the time it took for animals to resynchronize circadian locomotor activity to a photic phase-shift (Hau & Gwinner, 1994 & 1995; Marumoto et al., 1996b). Results in reproductive studies, however, are not consistent enough to draw broad conclusions about how continuous melatonin supplementation via SILASTIC® capsules affect seasonal reproduction. In the wild, female great tits (*Parus major*) with melatonin-filled SILASTIC® capsules delay the timing of first-clutch (Greives et al., 2012) and male great tits are more susceptible to cuckoldry (Greives et al., 2015). These observations would be impossible to make in a lab environment where natural breeding behavior is compromised by housing conditions. SILASTIC® capsules are stable enough to tolerate conditions in the wild without risking the onset of adverse inflammatory responses other invasive administration techniques may have, so animals implanted with SILASTIC® capsules may require less stringent monitoring. While SILASTIC® capsules are more convenient and interfere less with animals than daily injections, the change in diffusion over time and the effects of continuous administration on tissue sensitivity to melatonin cannot be disregarded in long-term studies using SILASTIC® capsules. Regardless, SILASTIC® is one of the most commonly used continuous-release implantable devices for hormone administration across vertebrates.

Less Common Techniques for Melatonin Administration

Some techniques have not been as used as commonly for melatonin administration as the previously described techniques. This section serves to provide an overview of other technologies that have been used or are being developed for exogenous melatonin administration: osmotic pumps, transdermal creams, melatonin-loaded beads, and sponges.

Osmotic Pumps. There are numerous types of osmotic pumps on the market used in drug delivery in animal research (for review, see Urquhart et al., 1984 and Verma et al., 2004). There is an internal drug reservoir surrounded by a osmotic sleeve and a semi-permeable membrane (Theeuwus, 1987). The outer diameter of osmostic pumps is larger than SILASTIC® capsules. When implanted, they may cause relatively more local tissue damage than other administration methods (Sallinen et al., 2008). Mini-osmotic pumps may be programmed to administer at a constant volumetric delivery rate (Theeuwus, 1987), so the variability of diffusion rates previously described in SILASTIC® capsules is controlled for with this technique. Furthermore, the release rates *in vivo* and *in vitro* have no significant differences (Theeuwus, 1987). Prevo et al. (2000) used ALZET® osmotic pumps to administer increasing concentrations of melatonin (0.03%, 0.3%, and 3%) to Sprague-Dawley rats and observed a dose-dependent trend in plasma melatonin and 6-sulphatoxymelatonin (a melatonin metabolite) in urine. The constant mass and volumetric delivery of osmotic pumps ensures a controlled release of the same concentration throughout the duration of an experiment, which is not guaranteed with any other method in this review.

Experimental procedures have several options for delivery with osmotic pumps. One option allows for intermittent drug delivery by alternating a water-soluble solution with oil, spacing out the subcutaneous delivery of the drug of interest (with nicotine, Brynildsen et al., 2016). How intermittent delivery enabled by alternating the lipid-water within the coiled reservoir would work for an amphipathic molecule like melatonin is yet to be determined. Injection and infusion studies dissolve melatonin in solvents ranging from water-soluble ethanol (e.g. Bartness & Wade, 1985) to lipids (e.g. peanut oil, Reiter et al., 1977). This technique of intermittent delivery has been used with timed-infusion (e.g. Lynch et al., 1980 administers 6hr of melatonin dissolved in phenolsulfonphthalein solution alternating with 18hr mineral oil), but chemical properties potentially enabling melatonin's lipid-solubility and diffusion through the coiled reservoir were not discussed. Future work attempting intermittent melatonin delivery would benefit from testing these amphipathic properties in different solvents. The alternation between lipid- and water-soluble solutions may appear to stay separated, but it may not prevent the passive diffusion of melatonin between layers.

Another option for melatonin administration available with mini-osmotic pumps is co-infusion. The effects of a drug may be enhanced when administered with chemicals that improve its stability and/or permeability (see also the section on Transdermal Delivery in this review). Also, since melatonin is an antioxidant, research on its role in preventing oxidative damage has been studied through co-infusion via osmotic pumps. In one study, melatonin infused alone did not significantly affect dopaminergic transmission, but when it was co-infused with iron, the oxidative damage caused by iron was reduced (Lin & Ho, 2000). Co-infusion would be challenging to conduct accurately and precisely through the membrane of SILASTIC® capsules, unless the rate of diffusion of the different chemicals used is calculated ahead of time and the concentration of its contents corrected for differences in diffusion rates.

Overall, studies identified using melatonin in osmotic pumps tend to focus on the protective, antioxidant, free-radical properties of melatonin. This includes the protective qualities of melatonin administered implanted in mice post-cardial infarction (programmed at 4.5 mg/kg body weight per day in Sallinen et al., 2008), through senescence to test its role in neuroprotection (4 mg/kg body weight of bolus followed by continuous infusion of 8 mg/kg body weight per day in Kilic et al., 2002), and through the stalling neurodegenerative damage induced by oxidative toxicity (Dabbeni-Sala et al., 2001).

Whether or not these protective properties of melatonin are through its role as an antioxidant or through melatonin-receptor activation can be assessed by administering a melatonin-receptor antagonist. Lemley et al. (2013) administered melatonin and a receptor-antagonist, luzindole, in pregnant sheep using ALZET® osmotic minipumps. The catheter of the minipump was placed next to the gravid uterine vascular network, and results indicated that the increases in arterial blood flow observed from melatonin-enriched feed were also observed from localized melatonin administration via osmotic pumps. This increase in arterial blood flow was not observed in sheep administered luzindole (Lemley et al., 2013), which suggests that these physiological changes were mediated through melatonin's G-protein coupled receptor and not through its role as an antioxidant.

The application of osmotic pumps to study the effects of melatonin in other physiological functions (e.g. circadian rhythms and photoperiodism) is not as prevalent. However, studies using osmotic pumps to administer other neuropeptides involved in reproduction, such as gonadotropin-releasing hormone (GnRH, Ronayne et al., 1993; Robinson et al., 1993) and gonadotropin-inhibitory hormone (GnIH, Ubuka et al., 2006). These methods may be referenced by future studies testing the effects of melatonin administration via osmotic pumps. Although osmotic pumps are designed for single-use, they offer fine-tuning control over the rate and timing of drug release in an unprecedented way, especially when taken into consideration with other laboratory and field factors: day length, temperature, endogenous melatonin rhythms, and concentrations of melatonin in feed.

Transdermal Delivery. Transdermal delivery of melatonin has received more attention recently. The variance of vasculature of different corporeal regions spurs a range of modes for transdermal delivery, including transbuccal (Dawson et al., 1998), which had no significant effect on sleep quality, and intranasal (with polyethylene glycol 300 in Bechgaard et al., 1999; with starch microspheres in Mao et al., 2004). The rate of absorption in nasal passages and the persistence of bioavailability after administration suggest that intranasal is a reasonable alternative to melatonin pills in humans. Nasal administration in animal research appears to be limited to rabbit and rodent model organisms, and applications tend towards pharmacology and not circadian and reproductive research.

Transdermal delivery in other areas that are less vascularized than nasal passages has its challenges. With skin as the primary defense against pathogens, vehicles and enhancers must be combined with melatonin to increase skin permeability (Oh et al., 2001). Franz diffusion cells are used to determine the lag time and permeability for transdermal delivery (hairless mouse skin in Oh et al., 2001; human cadaver skin in Dubey et al., 2006 & 2007). As suggested in the section of this review on oral administration, special consideration must be given to comparative studies across vertebrates addressing permeability of epidermis, dermis, and subcutaneous layers. Dermal permeability can vary at different times of the day (with temperature) and year (e.g. pre-migratory fattening). Melatonin was initially discovered through its lightening effects on skin isolated from *Rana pipiens* (Lerner et al., 1958). In frogs (e.g. *Xenopus laevis*) melanophores

bind melatonin with a high affinity (Ebisawa et al., 1994), so the effects of melatonin on skin pigmentation may be receptor mediated (as observed in fish melanophores, Aspengren et al., 2003). Parsing out the effects of transdermal melatonin delivery from the melatonin receptor-mediated changes in the skin itself may be done through concurrent delivery with a melatonin-receptor antagonist such as luzindole.

Transdermal melatonin delivery has been very important in ornithological field research. Free-living birds pose prohibitive challenges to repeated capture for injections, consistent oral administration via food or water across individuals, and continuous-release implants that may require invasive surgery and must be removed. Melatonin dissolved in Eucerin cream and applied to naked skin of European stonechats (*Saxicola torquata rubicola*) significantly increased plasma melatonin concentrations (Goymann et al., 2008). The temporal architecture of plasma melatonin concentrations varied with the different concentrations of melatonin applied. The application of cream with 6 μg melatonin led to an increase in plasma melatonin concentrations (0.2 - 0.4 ng / ml) that was maintained across timepoints (30, 60, 120, and 180 minutes) after application (Goymann et al., 2008). However, after the application of cream with 20 μg of melatonin, plasma concentrations of melatonin linearly increased from less than 0.2 ng / ml at 30 min post-application to up to an approximate average of 0.7 ng / ml at 180 min post-application (Goymann et al., 2008). In garden warblers (*Sylvia borin*), melatonin cream can increase plasma melatonin concentrations during both the day and night (Goymann et al., 2008). When the concentration of melatonin in cream applied to warblers was doubled (40 μg) relative to the cream applied to stonechats (20 μg), the plasma melatonin concentrations were 7 to 12 times higher, which suggests that the concentration of melatonin in the cream has a non-linear relationship with the amount absorbed and circulated (Goymann et al., 2008). Melatonin dissolved in Eucerin cream also has been applied to *Sylvia* warblers during migration (Fusani et al., 2011 & 2013). There was a lack of melatonergic effects on nocturnal migratory restlessness, or *Zugunruhe*, during the spring migration (Fusani et al., 2011). However, melatonin cream was shown to significantly decrease *Zugunruhe* associated with autumnal migration (Fusani et al., 2013). The validation of transdermal melatonin application enables countless possibilities for future studies in the field and in the laboratory.

Although the discovery of melatonin spurred from its effect on *Rana* skin, research applications related to its transdermal delivery are incipient. Transdermal delivery is less invasive than implants or injections, and additional research would need to be conducted to validate the vehicles and enhancers used. The studies cited here may be resources for accessing melatonin prepared for transdermal delivery. Regardless of the research questions this administration technique is used to answer, methodologies should include information on lighting, temperature, and time of administration. Transdermal delivery of melatonin has the advantage of being less invasive than injections and implants. However, as with any vehicle used to deliver melatonin, the effects of emulsifiers and enhancers on skin permeability in conjunction with melatonin elimination half-life have yet to be confirmed. Melatonin stability may be preserved or even enhanced with carefully selected transdermal vehicles and enhancers.

Beads. Spherification procures small beads with a higher surface area which may be administered orally, intravaginally, or intrarectally. Properties frequently measured in melatonin-loaded beads, such as *trapping efficiencies* (how much melatonin can be loaded) and *release profiles* (rate over time at which melatonin diffuses from the microspheres), can vary from the solution in which the beads are placed (e.g. simulated gastric and intestinal (GI) fluids vs. saline). Several materials have been studied for their trapping efficiencies and release profiles

with melatonin. Sodium alginate, derived from brown algae, is the main component of beads reviewed here that have been studied for melatonin administration. The preparation and release profile of melatonin-loaded alginate beads has been studied *in vitro* (Lee et al., 1996), in GI fluids (Lee & Min, 1996), and in simulated GI fluids (Lee et al., 1999). Another material used to study melatonin-loaded beads is stearyl alcohol, a fatty acid alcohol that forms white microspheres insoluble in water (Lee et al., 1997). Lastly, sugar spheres coated in Aquacoat®, enabling sustained release of water insoluble components to aqueous solutions, have been studied as another means of melatonin administration via beads. Sugar beads were coated with 20% Aquacoat®, loaded with melatonin, and orally administered in humans to monitor the rate of controlled release (Lee et al., 1996).

The costs and availability of these materials is not explicit, and beads as a mode of melatonin administration have not been validated in many species other than humans. Most of the research on melatonin-loaded beads reviewed here is conducted *in vitro*. How the trapping efficiencies and release profiles of melatonin work with other materials reviewed here, whether it is animal feed, beeswax, or SILASTIC® tubing, is worth considering. The methodologies used to assess trapping efficiencies and release profiles of melatonin-loaded materials may be found from the aforementioned work in melatonin-loaded beads.

Sponges. After pre-absorbing melatonin, sponges are typically inserted intravaginally. Sponges as a mode of melatonin administration have most often been used in large livestock such as mares (Thompson et al., 1983) or ewes (Rajkumar et al., 1989; Buffoni et al., 2014; Luridiana et al., 2015). Sponges are relatively large so have unique challenges with insertion. More recently, however, microsponges monitored by a polymer plug have been developed for pulsatile administration (Amer & Elosaily, 2016; Shukla et al., 2016), and because this method has been tested *in vitro*, a novel mode of melatonin administration may become available in the near future.

Models for Personalized Exogenous Melatonin Administration

This review investigates the variability of melatonin administration techniques across fields. Before an experimenter appeals to precedence to decide on a methodology, several other resources in addition to this review may be considered. Mathematical models on the physiological production, anatomical distribution, and metabolism of melatonin (Blumenthal et al., 1997; Breslow et al., 2013) may be referenced to inform mode of administration. Additionally, physiologically based pharmacokinetic (PBPK) models simulate the effects of lighting intensities on endogenous melatonin rhythms, aiding optimization of dose and route of exogenous melatonin administration (Peng et al., 2013). Just as PBPK models may be used in the advancement of personalized medicine (Hartmanshenn et al., 2016), the concept of personalized melatonin administration in animal research has untapped potential. The early stages of personalized melatonin administration are detected in volumetric modifications or changes in dose/concentration relative to body weight of individual animals (for examples see Cassone et al., 1986b; Marumoto et al., 1996a; Rozenboim et al., 2002). PBPK models may also include physiological differences due to age, sex, disease state, and temporal state (Hartmanshenn et al., 2016). The variability of animals captured in the wild for research may be assessed to have personalized doses of physiologically relevant melatonin.

Conclusion

Modes of testosterone administration and the relationships between release rate, dosage, and other advantages/disadvantages have been extensively reviewed (Fusani, 2008; Quispe et al., 2015). This review investigated different modes of administration of the amphipathic molecule, melatonin. Each melatonin administration technique has variables that affect the duration and amplitude of elevated plasma melatonin. Preliminary research on different doses for a specific technique can be used to determine which concentration is physiologically relevant. Additionally, the timing and frequency of administration and effects on tissue sensitivity can be ascertained by quantifying changes in melatonin receptor expression and binding over time. Because melatonin may downregulate its own synthesis in a receptor-mediated fashion (Bedrosian et al., 2013), studies that administer exogenous melatonin for extended periods of time should consider such endogenous effects. Even how plasma samples are collected, stored, and preserved can significantly affect absolute concentrations of melatonin measured (Goymann et al., 2007). A pilot study that factors these variables into their experiment may empirically determine how the administration technique of choice is affecting the responsiveness of the animal and how preservation of relevant samples affects absolute melatonin concentration quantified.

Implants provide continuous diffusion of melatonin, and the diffusion rate decreases over an extended period of time. For smaller sizes and concentrations, implants should be replaced every seven days in experiments that last more than a week to maintain elevated plasma melatonin. For beeswax and SILASTIC® implants, the surface area and volume should be reported in the methods since these factors affect diffusion rate and concentration. Depending on the location of the beeswax pellet, it may deform through the period of its implantation, and this has not been reported in any experiments using beeswax pellets. SILASTIC® implants have relatively more durable structural integrity. The difference between the inner and outer diameters of SILASTIC® tubing, which corresponds to the thickness of the tube, must be considered. Tubing with thicker walls may have slower diffusion rates, and these should be calculated *in vitro* to approximate diffusion rates *in vivo* within the appropriate temperature range. The anatomical location of subcutaneous implants should also be described because there is a clear relationship between diffusion, concentration, and proximity to melatonin-receptor sites. Tissues in the immediate vicinity of the implant will be subjected to relatively higher concentrations than distal tissues and organs.

Acute elevations for short periods of time may be accomplished with microinfusion pumps, oral administration, injections, osmotic pumps, and transdermal delivery. Unlike implants, which maintain melatonin in its crystalline form, these techniques require a vehicle to dissolve and dilute melatonin to the appropriate concentrations prior to administration. How these different vehicles affect mean elimination half-life of melatonin will also affect how long circulating melatonin levels stay elevated following administration. Future research can adopt methods in pharmacology to determine different vehicle combinations that can alter how long circulating melatonin levels stay elevated by intentionally varying the mean elimination half-life of melatonin.

Unless the fluctuations of endogenous melatonin rhythms are charted in a given species, the mode of exogenous administration may render capricious results. Investigations that measure circadian rhythms of plasma and pineal melatonin synthesis in a given species housed in specific

experimental lighting conditions may supplement the research examining localized melatonin synthesis, undetectable through measurements of plasma melatonin levels. Results of the experiments described in subsequent chapters of this dissertation suggest hypothalamic melatonin synthesis in European starlings and zebra finches that vary based on time of day. Furthermore, the testes of Syrian hamsters may respond to its own localized melatonin synthesis in an autocrine or paracrine manner. These studies unveil endogenous melatonergic systems that may be more directly targeted in future experiments administering melatonin.

Table 1. Timed-Infusion Melatonin Administration Experiments

Infusion Pumps (for review in relation to photoperiodic control of seasonal responses, see Bartness et al., 1993)												
Animal Model and Housing	Male Sprague-Dawley rats, weighing 150-200g, 12L:12D (L is 'cool white' fluorescent tubes yielding 100µW/cm²)	Male and Female golden-mantled squirrels trapped east of Redding, CA (time of year not specified). Transferred to cold room (±5°C) housed in individual cages, kept in dark to conditions of hibernacula. Dim light (1.9-3.4 lux) on 10:14h schedule.	Male and Female pinealectomized Western Fence Lizard (*Sceloporus occidentalis*) caught in the wild, housed individually in clear plastic cages kept in light-tight wooden boxes, exposed to constant dim light (LL) at 1 lux at level of lizard	Male Syrian hamsters kept in 14L:10D (L approx. 300 lux; D < 3 lux dim red light); 4-5 weeks old, weighed ~50g	Male Long Evans rats, kept 3 weeks (200lux) 12L:12D (3 lux, red light) prior to expt	Breeding colony of Male Sundian grass rats (*Arvicanthis ansorgei*) raised in lab (weight 119-200g) kept under (200 lux) 12L:12D (<3 lux red light) 22±1°C ambient temp	Adult male Syrian hamsters, kept in 14L:10D	Adult male Syrian hamsters, kept in 14L:10D	Adult male Syrian hamsters, kept in 14L:10D	Young male Syrian hamsters, raised and kept in 14L:10D, sexually active	Mature ewes (Suffolk), in Ann Arbor, MI (42°N latitude). Housed in long days (16L:8D) or short days (8L:16D), with continuous dim red light at night (<3 lux)	2-3 year old Polish Lowland ewes. Used in experiments that lasted over 2 years. Spend first year indoors under natural lighting condition (52°N, 21°E) then spent summer grazing on pasture.
Dose and/or Concentration	0-10µg/6hr to assess amount infused subcutaneously and recovered in urine. Mini-pump designed to discharge at 1µl/hr	200 or 400 ng/0.5µl or CSF was infused intracerebroventricularly (i.c.v) using Alzet osmotic mini-pump attached to injection cannula.	3 mg/mL MEL dissolved in EtOH, added to distilled water. Expt 1: 36.9 µg/mL for 5µg/day infusion OR Expt 2: 0.740 µg/mL for 1µg/day infusions	Expt 1: 10µg/hr for 5hrs Expt 2: 30µg/hr for 5 hrs, or Expt 3: 50µg/hr for 1hr	100µg /hr	100µg/hr	Flow rate: 0.153 ml/hr; Different doses: 20 ng/hr; 30; 40; and 80 ng/hr	Flow rate: 0.153 ml/hr; Different doses: 20 ng/hr; 30; 40; and 80 ng/hr	40ng/hr	80 ng/hr	44µg/hr, pulses every 3.75min	Expt 1: Total dose of 32µg administered over 4 hrs, Expt 2: 300µg over 3hrs (ovary-intact) or Expt 3: 400µg over 4hrs
Rationale for Dose and/or Concentration	None	None	None	Defining conditions of MEL infusion to affect locomotor activity (Kirsch et al., 1993)	In Kirsch et al., 1993--MEL admin in hamster	In Kirsch et al., 1993--MEL admin in hamster	Physio. significant	Physio. significant	Physio. significant	Dose ~nocturnal plasma [MEL] in intact animals (Pitrosky et al., 1991)	None	Expt 1: None Expt 2: Dose stimulated prolactin in anestrous ewes (Misztal et al.1997) Expt 3 = Expt 2
Frequency of Admin	6hr intervals of MEL (dissolved in phenolsulfonphthalein solution) alternating with 18hr of MEL-free mineral oil	Continuous	12hr/day	Daily	15 cycles (T cycles at 23-25hr days)	1 hr/day	12hr/day	12hr/day	6hr/day or 8hr/day	Expt 1: 10hr cont. admin; Expt 2: 5hr cont. admin; Expt 3: Two sets of 2.5hr separated by 3hr inter-pulse interval (IPI); Expt 4: Two sets of 2.5hr with 5.5hr IPI	16hr cont. admin, 8hr inter- pulse interval (IPI)	Expt 1: Once Expt 2: Twice over 2 week period Expt 3: Same as Expt 2
Time of Admin	Housed in constant light for 105 days, time of start of administration not specified	Not specified	N/A	N/A	N/A	Near activity/ rest transition points	2hr after lights off	2hr after lights off	2hr after lights off	9hr after lights off	Not specified	Expt 1: 15.00-19.00 hr Expt 2: 14.00-17.00hr (3.5-5.5hr before sunset) Expt 3: Same as Expt 2
Overall Length of Admin Period	6 cycles of MEL admin (6 days)	14 days	65 days	Constant dim light until free-running. MEL admin until entrainment. MEL admin withdrawn until free-running.	15 days	Until stimulus scanned an entire circadian cycle	4 weeks	8 weeks	4 weeks	8 weeks	120 days	2 weeks
Lighting and/or Temperature Experimental Condition	Continuous light to abolish daily rhythm of melatonin secretion (Lynch & Wurtman, 1979)	Housed in cold room (±5°C) in individual cages, kept in dark to approximate conditions of hernacula. Dim light (1.9-3.4 lux) provided on 10:14h schedule.	Constant dim light (LL) at approximately 1 lux at level of lizard in cage	Constant dim light (<3 lux dim red light)	DD, 3 lux red light	DD, 3 lux red light	8L:16D at 7°C	8L:16D at 18°C	8L:16D at 7°C	After adapting to lab conditions for 6 weeks, divided into short-photoperiod (SP) 10L:14D or long-photoperiod (LP) 14L:10D	After short days (8L:16D), a subset of Px and pineal - intact ewes moved to long-days (16L:8D), melatonin infusion (16hrs)	Natural lighting (52°N, 21°E)

Pinealectomy (Px) and/or Bilateral Enucleation (Ex)?	Px, recovery time not specified	N/A	Px with 2 weeks recovery (as described in Underwood, 1973)	Px with 1wk recovery	N/A	N/A	Px with 2 weeks recovery before infusion	Px with 2 weeks recovery before infusion	Px with 2 weeks recovery before infusion	Px with 2 weeks recovery before infusion	Px, 1-3 years recovery before infusion (only those without nocturnal rise in melatonin included)	N/A
How MEL was measured after/during admin	Sequential 1hr urine samples collected for 10 days, RIA of urinary melatonin	N/A	Eyes, Pineal, and Trunk Blood collected mid-light and mid-day	N/A	RIA assay of plasma collected at 6 time points	N/A	Plasma [MEL] measured in the middle of infusion period (did not differ significantly from [MEL] in intact animals)	Plasma [MEL] measured in the middle of infusion period (did not differ significantly from [MEL] in intact animals)	Plasma [MEL] measured in the middle of infusion period (did not differ significantly from [MEL] in intact animals)	Measurement of plasma MEL during infusion. (MEL reached physiological nighttime values during the infusion, fell to daytime values 1 h after end of infusion period)	Measureplasma [MEL] at 2hr intervals as per Rollag et al., 1978	N/A
Main Findings	In 3 Px and 3 sham-operated rats housed in constant light, programmed micro-infusions can induce melatonin rhythms, and output in urine is consistent with dose	MEL plays a role in hibernation bout cycle. Spontaneous arousal during study attributed to the possible development of receptor sensitivity or downregulation resulting from cont. MEL infusion.	Entrainment of locomotor activity achieved with daily 12hr infusions of 0.1µg/day and 5µg/day in Px lizards and 5µg/day in pineal-intact lizards	Out of 64 animals, 37 entrained and 20 showed transient entrainment with MEL infusions (occurring for several months). All animals in Expt 1 entrained or transiently entrained.	Entrainment of wheel-running to MEL signal procures effect comparable to light as zeitgeber	Entrainment of wheel-running to exogenous MEL observed when MEL is administered near (<3 hr) activity/rest transition points	40ng/hr was minimal effective concentration to induce gonadal atrophy at 7°C	20ng/hr was minimal effective concentration to induce gonadal atrophy at 18°C	8hr/day inhibited sexual activity, while 6hr/day was insufficient	Expt 1: 10hr MEL infusion inhibit testes in Px hamsters in LP, Expt 2: 5hr MEL infusion, no effect. Expt 3: Two sets of 2.5hr MEL w/ 3hr IPI, complete regression. Expt 4: Two sets of 2.5hr MEL w/ 5.5hr IPI, no regression	Infusion of short-day MEL signal (16hr) in ewes housed in long days (16L:8D) kept LH at levels like ewes housed in short days (8L:16D)	Neither GnRH or LH were affected by extended MEL admin in ovary- intact sheep. Pulsatility of LH release decreased in frequency with MEL admin in ovariectomized sheep.
Citation	Lynch et al., 1980	Stanton et al., 1987	Hyde & Underwood, 1995	Schuhler et al., 2002	Slotten et al., 2002a	Slotten et al., 2002b	Pitrosky et al., 1991*	Pitrosky et al., 1991*	Pitrosky et al., 1991*	Pitrosky et al., 1995	Bittman & Karsch, 1984	Romanowicz et al., 2001

Table 2. Beeswax and Melatonin Administration Experiments

Beeswax							
Animal Model and Housing	Adult Male white-footed mice *(Peromyscus leucopus)*, laboratory-reared, ambient temperature 23±1.0°C	Adult Female white-footed mice, laboratory-reared under long photoperiod (16L:8D), 8 weeks of age (sexually mature)	Adult Male Sprague- Dawley rats weighing 200-250g maintained on 14L:10D, castrasted.	Young adult Male Syrian hamsters (*Mesocricetus auratus*) obtained from colony. Housed 4-5 per cage in room illuminated 14L:10D, ambient temperature 22±2.0°C	Adult Male Hamsters, species not specified (60-80 grams), housed 4-6 per cage, 14L:10D (lights on 06.00hr, lights off 20.00hr)	Male Siberian hamsters (*Phodopus sungorus*), housed in 16L:8D, ambient temperature 22±1°C	Adult Male Blue rock pigeon (*Columbia livia*) from local bird supplier, acclimated to 14L:10D, 27±1.0°C
Dose and/or Concentration	1mg MEL/24mg beeswax	50mg MEL/200mg beeswax, pellets punched out from slab with 0.8mm OD or 0.4mm OD, stereotaxically implanted, unilaterally in the AH or in the SCN	1mg MEL/24mg beeswax, each rat implanted with 25mg	Experiment 1: 1mg MEL/24mg beeswax Experiment 2: 1mg MEL/24mg beeswax; 500µg MEL/24mg beeswax; 100µg MEL/24mg beeswax; 50µg MEL/24mg beeswax; or 1µg MEL/24mg beeswax	1mg MEL/24mg beeswax	28 gauge inner cannula of needle contain MEL mixed with beeswax (1:4 ratio of MEL:beeswax) or just beeswax, preceding timed daily MEL infusion, 100ng/12hr infusion for 6 weeks (Control groups: s.c. silastic implant, Dow-Corning, i.d. 1.47 mm; o.d. 1.96 mm, length 15mm with 10mm crystalline MEL powder or empty capsules)	4mg MEL/30mg beeswax
Rationale for Dose and/or Concentration	No rationale for concentration, method as described in Reiter et al., 1974	No rationale for concentration, rate of release of MEL tested in buffered saline in daily increments.	No rationale for concentration	No rationale for concentration, method as described in Reiter et al., 1974	None	None	No rationale for concentration
Frequency of Admin	One implant, replaced every 2 weeks	Implanted once. Rate of release determined for days 1-6 (10.0µg/day for large & 0.6µg/day for small) and days 7-56 (490ng/day for large & 90ng/day for small) *in vitro*.	One implant, replaced weekly.	Experiment 1: One implant replaced weekly or every 2, 3, 4, 6, or 12 weeks (frequency varies) Experiment 2: One implant replaced every 2 weeks (same frequency)	One implant replaced weekly	One implant, targeting specific hypothalamic region	Implanted once
Overall Length of Admin Period	6 weeks	56 days	28 days	12 weeks	50 days	40 weeks	2 weeks
Lighting and/or Temperature Experimental Condition	16L:8D (MEL administered) or 8L:16D (control)	16L:8D at 23°C	14L:10D	14L:10D, 22±2.0°C	14L:10D	16L:8D	14L:10D
Pinealectomy (Px) and/or Bilateral Enucleation (Ex)?	N/A	N/A	Px as described in Hoffman & Reiter, 1965	Ex	N/A	Px	N/A
How MEL was measured	N/A	MEL not measured, rate of release determined using RIA in vitro	N/A	N/A	N/A	N/A	N/A
Main Findings	Mice in short photoperiod OR long photoperiod with MEL implants had significantly reduced testicular volume and circulating testosterone relative to control mice housed in a long photoperiod.	Subcutaneous MEL beeswax implants had little effect on repro development; however, MEL beeswax microimplants implanted in AH and SCN had a significant increase in imperforate vagina development and a decrease in repro tract weight.	No significant differences in pituitary content nor plasma concentrations of LH, FSH, and prolactin across groups. Pituitary weight was significantly less in MEL beeswax relative to control group.	Experiment 1: MEL beeswax 1mg/24mg replaced weekly or bi-weekly prevented the inhibitory effects of light deprivation (from Ex) on gonadal growth. Experiment 2: Pellets of concentrations >100µg MEL reversed effects of Ex on reproductive organ growth.	Hamsters with MEL beeswax pellets did not respond to daily MEL injections (i.e. unable to depress pituitary LH and prolactin values).	MEL beeswax intracranial implants in the SCN, PVN, and nucleus reunions (NRE) induce testicular regression but eventually lose ability to maintain gonadotropin suppression, enabling testicular recrudescence. Subsequent timed daily melatonin infusions (TDMI) significantly reduced testicular volume in hamsters with MEL beeswax in SCN, PVN, and NRE, hamster w/MEL s.c.. silastic capsules unresponsive to TDMI.	MEL implanted birds show significantly higher glucose, lower T3 & T4, and a higher T3/T4 ratio.
Citation	Petterborg & Reiter, 1981	Glass & Lynch, 1982	Talbot & Reiter, 1973	Reiter et al., 1975	Reiter et al., 1977*	Zucker & Freeman, 2001	Prakash et al., 1998

Table 3. Melatonin Oral Administration Experiments

Oral administration						
Animal Model and Housing	Male White-tailed deer (*Odocoileus virginianus*) born and raised in captivity housed outdoors (housed at 43°North), ambient environmental light only and natural fluctuations in seasonal temperature	Adult Male House sparrows (*Passer domesticus*) transferred from outdoor aviaries into single-housed cages, maintained (20 lux) 12L:12D (0.2 lux) for 17 days then transferred to constant dim light (0.2 lux) for remainder of experiment	Male European quail (*Corturnix corturnix*) from breeding stock originated from south-west France and Portugal. Experiment began in February, when birds were photosensitive. Birds were moved from outdoor aviaries (10.5L:13.5D) into individual cages in the laboratory (12L:12D).	Hyline brown egg-type pullets (Female), 1 day old, continuous light for 2 days followed by 5 days of 18L:6D. Separated into two groups: 1st group on 7L:17L and 2nd group on 14L:10D. Light at 60W incandescent (33±1.1 lux).	Hy-Line Gray commercial pullets (Female), 6 weeks of age, kept in 8L:16D for 70 days. Birds were split into two groups: 1st group kept on 8L:16D, 2nd group shifted to 16L:8D.	Adult Male Indian weaver (*Ploceus philippinus*) birds captured 21°N, 81°E and kept in outdoor aviary under natural light and temperature, then brought indoors and maintained under (25 lux) 12L:12D (0.3 lux) at 22±0 2°C ambient temperature for a week before start of experiment, then placed in individual cages.
Dose and/or Concentration	5mg of MEL fed to deer in No. 4 gelatin capsule	200µg MEL/mL drinking water	15µg MEL/mL in 10mL water	25mg/kg of diet (mixed MEL dissolved in EtOH with commercial chick starter)	20mg or 200mg MEL/kg of diet	200µg MEL/mL in drinking water
Rationale for Dose and/or Concentration	"Orally administered [melatonin] had physiological action centrally, since there are not digestive enzymes for indoles." (no citation)	None	None	No rationale for dose, left EtOH to dissolve as described in Diekman et al., 1991	No rationale for dose, left EtOH to dissolve as described in Lewis et al., 2006	None
Frequency of Admin	Single administration	Rhythms of different periods (T), shifting birds from either 24 to 21hr days or 24 to 27hr days. Melatonin water available for 8hr, regardless of T.	Daily	Daily access to MEL feed during 7.25hr window	Daily access to MEL feed during 8hr window	Daily for 8hr or 16hr duration
Time of Admin	13.00hr	N/A (constant dim light)	During the last 3 hrs of the photophase (14.00hr to 17.00hr)	Between 07.30hr and 14.45hr for birds in 14L:10D	Between 09.00hr and 17.00hr for birds in 16L:8D	N/A (constant bright light)
Overall Length of Admin Period	N/A	14 days	28 days	70 days	220 days	30 days
Lighting and/or Temperature Experimental Condition	Natural photoperiod/Temperature fluctuations	Constant dim light (0.2 lux)	12L:12D with ambient temperature 19±1°C	14L:10D (33±1.1 lux) for MEL admin group	16L:8D (15±2.4 lux) for MEL admin groups	Constant bright light (25 lux) for pineal intact group. Constant dim light (0.5 lux) for Px
Pinealectomy (Px) and/or Bilateral Enucleation (Ex)?	N/A	Px, 3 weeks recovery	N/A	N/A	N/A	Px with 2hr recovery
How MEL was measured after/during admin	Plasma melatonin detected using RIA	N/A	Plasma MEL was extracted from dichloromethane according to Brown et al., 1985 (in hamsters). MEL was measured using RIA.	Chloroform extracted sera and used iodinated tracer modified from Fraser et al., 1983	MEL concentration determined using enzyme-linked immunosorbent assay (as per Cheung et al., 2003)	N/A
Main Findings	Mid-scotophase peak in MEL not observed in deer administered exogenous MEL, which caused a rapid elevation in blood levels followed by a depression in nighttime peak.	MEL in drinking water administered to arrhythmic Px birds can induce synchronized rhythm of feeding and locomotor activity (as observed in Heigl & Gwinner, 1992, 1994)	MEL in drinking water reached physiologically relevant elevations (compared to Underwood & Siopes, 1984; Meyer & Milliam, 1991). Sexual development of the treated birds slowed down (length of cloacal vent).	Birds with MEL diet had detectably high circulating MEL. The experimental groups matured 6-11 days later than controls.	Dose-dependent effect on maturation, with increasing MEL dose delaying sexual maturation more than 16L:8D control but earlier than 8L:16D control. No significant differences in FSH, LH, oestrogen, and insulin across groups.	Px birds with 16hr access to MEL water significantly higher GnIH-ir cells than Px birds with 8hr access to MEL water. Pineal intact birds with 16hr access to MEL had significantly smaller song nuclei (high vocal center and robust nucleus of the arcopallium) relative to other groups.
Citation	Bubenik & Smith, 1987	Heigl & Gwinner, 1995	Guyomarc'h et al., 2001	Lewis et al., 2006	Chen et al., 2016	Kumari et al., 2015

Table 4. Experiments Administering Melatonin via Injection

	Injection									
Animal Model and Housing	Adult Male hooded Long-Evans rats, housed in 14L:10D (lights on at 14.00hr, lights off at 06.00hr) for 12 days then moved to constant darkness for 25 days	Adult Male Long-Evans hooded rats, housed in (250-300 lux) 14L:10D (0.1 lux dim red light) with lights on at 06.00 and lights off at 20.00, ambient temperature 21±1°C	Male Sprague-Dawley (250-300 g), laboratory bred and reared, housed 12L:12D (light on at 0700 h) ambient temperature 23±2°C	Adult Male Hamsters, species not specified (60-80 grams), housed 4-6 per cage, 14L:10D (lights on 06.00hr, lights off 20.00hr)	Male Japanese quail (*Corturnix japonica*) of a highly photoperiodic strain were reared under non- stimulatory short days (8L:16D), 6-10 weeks of age, weight 80-120g, singly housed	Adult Male White Leghorn Roosters, surgically castrated, reared under 16L:8D, light intensity at 0.1W/m², housed individually	Adult Male Syrian Hamsters purchased from supplier, maintained according to NIH Care and Use of Laboratory Animals, ambient temperature 21-22°C	Adult Male Siberian Hamsters (*Phodopus sungorus*) derived from breeding stock, housed singly, 16L:8D (lights on 01.00hr), ambient temperature 22±1°	Male Sprague-Dawley rats, weighed 250-300g, 12L:12D	Male Sprague-Dawley (290-395 g), kept on warming blanket for operation 37±0.5°C
Dose and/or Concentration	1mg, 500µg, 250µg, 100µg, 50µg, 10µg, 7.5µg, 2.5µg, 1.0µg, 500ng, 100ng, or 10ng per kg weight, s.c. injections 0.6-0.8ml MEL dissolved in EtOH and Saline	1mg MEL/kg body weight	1mg/kg body weight	25µg per 0.1cc peanut oil	Experiment 1: 10µg MEL/100µL vehicle (dissolved in EtOH, diluted with saline) Experiment 2: 20µg MEL/100µL vehicle (same) Experiment 3: 10µg MEL/100µL vehicle (same)	Experiment 1: 5mg, 20mg, or 80mg MEL per kg body weight. Experiment 2 & 3: 80mg MEL/kg body weight	Experiment 1: 25µg, s.c., dissolved in EtOH and diluted 1:10 in Saline. Experiment 2: Same dose as Experiment 1	12.5µg/day in 0.05ml 5% EtOH-0.15M NaCl vehicle	10, 20, or 30mg/kg i.p. every 12hrs	15mg/kg (low dose) or 150mg/kg (high dose), i.p.
Rationale for Dose and/or Concentration	To determine dose-dependent entrainment	None	None	None	None	None	None	None	None	None
Frequency of Admin	Daily	Daily	Daily	Daily	Experiment 1: Four injections every 24 hours Experiment 2: Two injections every 24 hours	Experiment 1: Once daily. Experiment 2: Twice daily (2nd 140 min after 1st). Experiment 3: Once daily.	Experiment 1: Once daily. Experiment 2: Once daily.	Daily	Every 12 hrs	Once
Time of Admin	12.00hr	10.00hr	12.30hr	19.00hr	Experiment 1: 2hr before dusk, at dusk, 2hr before dawn, & at dawn Experiment 2: 2hr before dawn	Experiment 1: 11.00hr Experiment 2: 11.00hr and 13.20hr Experiment 3: 11.00hr	Experiment 1: 16.45hr Experiment 2: 17.00hr	3 hrs before lights out	Not specified	15min (low dose) or 3hr (low or high dose) after induced ICH
Overall Length of Admin Period	30 days	90 days	56-68 days	50 days	Experiment 1: 17 days Experiment 2: 34 days Experiment 3: 17 days	Experiment 1: 3 days Experiment 2: 1 day Experiment 3: 10 days	Experiment 1: 8 weeks Experiment 2: 10 weeks	14 weeks	4 days	1 day
Lighting and/or Temperature Experimental Condition	Constant darkness	Constant dim light (0.1 lux red)	12L:12D (light on at 0700 h), 23±2°C OR Bright constant light (300 lux) OR Dim constant light (3 lux)	14L:10D	Experiment 1: 2L:12D Experiment 2: 12.25L:11.75D Experiment 3: Sorted in 11L:13D, 12L:12D, or 13L:11D	16L:8D, light intensity at 0.1W/m²	14L:10D, lights on at 5.00hr, 21-22°C	16L:8D, ambient temperature 22±1°	12L:12D, 22 ± 1°C	Not specified
Pinealectomy (Px) and/or Bilateral Enucleation (Ex)?	N/A	N/A (there were SCN and neurotoxic lesions)	Px, 7 days recovery, then Ex with 20 days recovery before first injection	Px with 7 days recovery	N/A	N/A	N/A	N/A	N/A	N/A
How MEL was measured after/during admin	Chloroform-extracted plasma, RIA	N/A	N/A	N/A	RIA as per Follet et al., 1972	Expt 1: Plasma collected 30min before and 10, 30, 60, & 120 min after MEL injection. Expt 2: 30min before 1st injection & 30, 60, 120,170, 200, & 240 min after 2nd injection. Expt 3: 240 min after injection	N/A	N/A	N/A	N/A
Main Findings	1mg-100µg per kg	Rats with	Entrainment to	Daily MEL	MEL injections	Significant	MEL	Daily MEL	Dose-	MEL

	(body weight) were doses that were sufficient to induce locomotor entrainment in constant darkness. 50µg-1µg per kg had mixed results. <1µg per kg did not entrain locomotor activity.	sham-lesions of neurotoxic lesions entrained to daily MEL injections and not to vehicle. Rats with SCN lesion did not entrain.	MEL injections when injections coincided with activity onset in rats housed in constant dim light and in blinded rats (Px and Ex), but not for rats housed LL.	injections significantly decreased LH and prolactin.	extended the duration of nocturnal, endogenous MEL signal yet resulted in a significant stimulation in reproductive state as determined by paired testes mass, circulating LH, and cloacal gland area.	decrease in plasma LH observed 240min after last MEL injection (80mg/kg), and attenuated LH persisted 3-10 days of daily injections.	treatment in hamsters for 8-10 weeks increased spleen size & splenic lympho-proliferative response to a polyclonal T-cell mitogen and decreased testosterone and testes weight.	injections decreased food intake and carcass lipid stores.	dependent decrease in oxidative stress	reduced oxidative stress but no sig.t effect on brain edema nor neuro. deficits
Citation	Cassone et al., 1986a	Cassone et al., 1986b	Marumoto et al., 1996	Reiter et al., 1977*	Juss et al., 1993*	Rozenboim et al., 2002	Champney et al., 1997	Bartness & Wade, 1985	Karuppag-ounder et al., 2007	Rojas et al., 2008

Table 5. Melatonin Administration Experiments Using SILASTIC®

SILASTIC® Capsules										
Animal Model and Housing	House sparrows, (*Passer domesticus*) (13 lux) 12L:12D (0.3 lux) to synchronize birds	Female House sparrows *(Passer domesticus)* housed individual, exposed to 2 X incandescent lamps	Male Sprague-Dawley rats, 3 mo. of age, housed in (200 lux) 12L:12D at 23±2°C ambient temp	Male Japanese quail (*Corturnix japonica*) of a highly photoperiodic strain reared under non-stimulatory short days (8L:16D), 6-10 weeks of age, weight 80-120g, singly housed	Male Wistar rats, albino, 20 days old	Photorefractory Male European starlings (*Sturnus vulgaris*) held at 18L:6D, castrated	Adult Male Japanese quail (*Corturnix japonica*), 3 mo. of age, 18L:8D, individually caged at 25±2°C ambient temp	Adult Female Great tits *(Parus major)* captured in the wild (between March and April 2010 and 2011) at 47°44'24" and 8°58'48", implanted, and released/studied as free-living	Photorefractory Male European starlings (*Sturnus vulgaris*) held at 18L:6D, castrated	Male white-footed mice (*Peromyscus leucopus*) housed with same-sex littermates until adulthood (60-90 days), clear plastic cages, 21±4°C ambient temp
Dose and/or Concentration	1cm crystalline melatonin, subcutaneous implant	Silastic tubing (0.058in ID x 0.077in OD), 11mm long, filled with crystalline MEL powder	Silastic tubing (2.5mm ID x 3.0mm OD x 55mm length) containing MEL (additional properties not described)	Silastic tubing (1.47mm ID x 1.96mm OD), 25mm length, of which crystalline MEL filled 22mm length	Silastic (0.040inch ID x 0.085inch OD) inserted into another silastic (0.132inch ID x 0.183inch OD x 0.64inch length)	Silastic tubing (1.47mm ID x 1.96mm OD), 60mm	Silastic plate (1.5 × 15 × 2.0 mm), containing low dose 2.5mg/plate, medium dose 10mg/plate, and high dose 40mg/plate	Silastic tubing, 10mm long	Silastic tubing (1.47mm ID x 1.96mm OD), 60mm	Silastic tubing (1.47mm ID x 1.96mm OD x 15mm length) packed with 10mm melatonin powder
Rationale for Dose and/or Concentration	In European starling, same size capsule increases plasma [MEL] 100-fold and abolishes internal rhythm (Beldhuis et al., 1988)	None	None	None, though capsules were weighed before and after implantation to calculate a possible rate of release (posited to be 24µg per day)	Protocol adapted from Staples et al., 1992	Calculated for "high dose" as per Fuchs et al., 1983 (in House finch)	References Tsutsui et al., 1998 and Ubuka et al., 2001 for use of plate for oestradiol-17β and progesterone at 10mg, but does not justify dose for MEL	Implants of this type mask circadian rhythms (Beldhuis et al., 1988, in European starlings) and circadian melatonin secretion (Hau & Gwinner, 1994, in sparrows)	Calculated for "high dose" as per Fuchs et al., 1983 (in House finch)	As described in Turek et al., 1976 (MEL admin in hamsters)
Overall Length of Admin Period	4-7 weeks	Implants removed 2-3 weeks after phase shift	24 days	20 days	21 days	58 days on 18L:6D	7 days after beginning of treatment	Implanted 9-26 days before first egg lay, date of removal not specified	58 days on 18L:6D	10 weeks to induce adaptive responses to daylength (as per Pyter et al., 2005; Walton et al., 2011)
Lighting and/or Temperature Experimental Condition	LD decreased 2:1 lux with two groups: 11.25L:11.25D (22.5hr/day) and two groups in 12.25L:12.25D (24.5hr/day) for 6-9 weeks prior to implant	4 expts, advanced OR delayed phase shifts.Light intensity 13:0.3 lux 12L:12D; after 3-7 days, switched to L:D cycles (4:1 lux)	Rats were subjected to 5, 6, or 8hr phase advance or delay	Transferred to 12L:12D	12L:12D (lights off at 18.00)	1 group kept on long-days (16L:8D, photorefractory) throughout experiment, 2 groups transferred to short-days (8L:16D) to regain sensitivity to long-days for 24 days, 1 group kept on short-days	Birds with implants kept in 16L:8D	Natural photoperiod/Temperature fluctuations	2 groups transferred to short-days (8L:16D) to regain sensitivity to long-days (16L:8D) for subsequent photostimulation, 1 group kept on short-days	Two groups: 16L:8D (long-day) and 8L:16D (short-day)
Pinealectomy (Px) and/or Bilateral Enucleation (Ex)?	N/A	N/A	Px with MEL implants compared to Sham operated with blanks	N/A	N/A	N/A	Px plus Ex, two days recovery	N/A	N/A	N/A
How MEL was measured after/during admin	N/A	N/A	Plasma collected every 3 days for 24 days, mid-day/ mid-night	RIA as per Follet et al., 1972	RIA assay of plasma and pineal MEL	N/A	MEL extracted from diencephalic and also measured in plasma.	N/A	Plasma MEL measured with RIA with prebleed and terminal bleed	Terminal blood samples through retro-orbital sinus for plasma RIA
Main Findings	MEL facilitates synchronization to non-24hr cycles (that are close to 24hr)	Cont. MEL admin increased rate of resynchronization to phase shifts with low-light cues	MEL accelerates entrainment to a new light-dark cycle as reflected by locomotor activity	Heterogeneous response in MEL group, but a significant increase in mean cloacal gland area and mean testicular mass in birds with MEL implant. 4 of the 8 birds were unaffected by	Laboratory rats exposed to cont release melatonin mimic short-day or timed melatonin admin. MEL delays onset of	Birds in photorefractory state had higher immune function based on splenocyte proliferation assay. In photostimulated birds, MEL attenuated the photoperiodic	Melatonin produced a dose-dependent increase in GnIH precursor and peptide in Px and Ex birds	First-clutch was significantly delayed in MEL implanted birds without affecting clutch size, onset of activity, or body mass	MEL attenuated long-day (photostimulated) increase of volume of nuclei in the song control pathway, the high vocal center and area X	MEL implants reproduce neurological changes of short days (impaired Schaffer collateral LTP in hippocampal region CA1, impaired performance on behavioral tests

				MEL implant.	puberty.	decrease in splenocyte proliferation.				for spatial learning and memory)
Citation	Hau & Gwinner, 1994	Hau & Gwinner, 1995	Marumoto et al., 1996	Juss et al., 1993*	Kennaway & Rowe, 1997	Bentley et al., 1998	Ubuka et al., 2005	Greives et al., 2012	Bentley et al., 1999	Walton et al., 2013

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Chapter 2: Melatonin Synthesizing Enzyme Expression in the Hypothalamus of Photoperiodic and Opportunistic Songbirds

Abstract

Melatonin is well established in photoperiodic breeding mammals to drive reproductive state, demonstrated indirectly from the effects of light exposure and directly from the impact of exogenous administration. There is less consistency in experiments looking at how melatonin affects different breeding types of birds. This study investigates a novel mechanism that may be considered for previously observed variability in the efficacy of melatonin to drive reproductive state. Specifically, we focus on how the avian hypothalamus may integrate seasonal changes in photoperiod through melatonin synthesis by quantifying expression levels of melatonin-synthesizing enzymes. In both an absolute photoperiodic breeder, European starling (*Sturnus vulgaris*), and an opportunistic breeder, Zebra finch (*Taeniopygia guttata*), all four enzymes directly involved in melatonin synthesis from its amino acid precursor, tryptophan, have been sequenced. Furthermore, through quantitative Real-Time PCR analysis, we determined the mRNA expression levels of the penultimate enzyme in the biosynthetic pathway, arylalkylamine-N-acetyltransferase (AANAT), and the final melatonin-synthesizing enzyme, hydroxyindole-O-methyltransferase (HIOMT). Results show the expression levels of AANAT mRNA in the photoperiodic breeder to be significantly higher in the positive control, the pineal, and the hypothalamus in birds that were collected at night relative to day, but only for birds collected in the fall. HIOMT mRNA only showed significant differences between day and nighttime expression levels in the pineal but not in the hypothalamus of the opportunistic breeder. This is the first study to demonstrate that in addition to the pineal as a melatonin-synthesizing photoreceptive site, the avian hypothalamus has the capabilities to regulate melatonin synthesis in response to changes in photic information in photoperiodic breeding songbirds.

Introduction

In seasonally breeding animals, the neurological mechanisms detecting proximate cues of the environment and synchronizing reproductive activity with favorable conditions share some underlying similarities, and yet these mechanisms contain notable distinctions across species of a given class. Given how photoperiod, or day length, plays a predictive role in the annual fluctuation of resource availability and conditions outside of the tropics, it is regarded to be the primary proximate cue for many seasonal breeders. (Karsch et al., 2013) Within various animal models, melatonin is researched as the chemical transducer of photic information because its synthesis and secretion peaks in darkness and is influenced by light pulses. (Ralph et al., 1967; Lynch, 1971; Panke et al., 1979; Gwinner et al. 1997). It is well-established how seasonally breeding mammals, short-day and long-day breeders alike, can have their reproductive physiological state impacted by properly timed light pulses or administration of exogenous melatonin. (Bittman, 1983; Barness et al., 1993; Goldman, 2001)

For birds, our understanding of how melatonin interacts with reproductive state is mostly limited to what is synthesized by the pineal. (Saylor & Wolfson, 1968) It has been formerly established that retinal phototransduction is not independently sufficient to transduce information regarding the duration of light exposure necessary for photoperiodic responses. (Menaker et al., 1970) Even subjecting tree sparrows (*Spizella arborea*) to both pinealectomy and bilateral

enucleation, removing all of the known photoreceptive sites capable of synthesizing melatonin, has been shown to not prevent changes in gonadal development with changing photoperiod. (Wilson, 1990) However, we must also consider the presence of deep-brain photoreceptors that have been detected in various bird species (Nakane et al., 2010).

Given that melatonin is the chemical transducer of photic information in other photoreceptive tissue in birds, it is likely serving a similar, possibly compensatory, role in the avian hypothalamus. In fact, dopamine-melatonergic neurons in the turkey (*Melleagris gallopavo*) hypothalamus have been shown to track changes in long-term exposure to different light cycles, potentially contributing to the internal photoperiodic clock and controlling seasonal reproduction. (Kang et al., 2007; El Halawani et al., 2009) The premammilary nucleus (PMM) of the turkey hypothalamus has been shown to be rhythmically activated (c-Fos) by light pulses and can induce gonadotropin releasing hormone type-I (GnRH-I) expression and activation. (Thayananuphat et al., 2007) This is potentially enabled by the photoreceptive capabilities of melanopsin co-localized with dopamine-melatonergic neurons in the turkey PMM. (Kang et al., 2010)

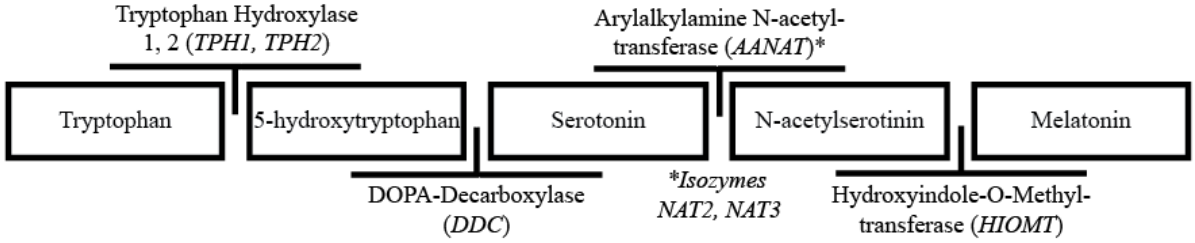
Melatonin has a stimulatory effect on gonadotropin inhibitory hormone (GnIH) expression in the brain of Japanese quail, *Coturnix japanica* (Ubuka et al., 2005) and a dose-dependent effect on GnIH release in the quail hypothalamus in vitro. (Chowdhury et al., 2010) However, whether or not the hypothalamus is independently capable of transducing photic information and synthesizing melatonin de novo prior to affecting the cascade of the hypothalamic-pituitary gonadal (HPG) axis has not been definitively demonstrated in any avian species. While quail and turkey have relatively weak photoperiodic responsiveness, the potential for the effects of melatonin synthesis on other breeding types of birds, such as opportunistic and absolute photoperiodic species, have not been investigated to date. This study considers two different breeding types, the Zebra finch (*Taeniopygia guttata*) as an opportunistic breeder and European starling (*Sturnus vulgaris*) as an absolute photoperiodic breeder when investigating the potential of the avian hypothalamus to differentially synthesize melatonin in a light-dependent manner, focusing on melatonin-synthesizing enzyme expression.

There are four key enzymes involved in melatonin-synthesis from its amino acid precursor, tryptophan (see Figure 1). Two isoforms of the first enzyme of the pathway, trytophan hydoxylase, have been sequenced in various species including Zebra finch (TPH1, NC_011469.1 and TPH2, NC_011463.1). Since TPH1 has been cloned from the pineal and TPH2 from brainstem raphe somata in turkey, and since TPH1 and AANAT but not TPH2 were expressed in melatonergic neurons of the hypothalamus (Kang et al., 2007), TPH1 continues to be associated with melatonin synthesis while TPH2 with serotonin synthesis. (Walther et al., 2003; in mice Zhang et al., 2004; in rat Patel et al., 2004; in pigeon Meneghelli et al., 2009) However, knockout mice TPH1(-/-) have shown no significant differences in behavior or serotonin synthesis relative to the wildtype, (Veenstra-VanderWeele & Cooke, 2003) suggesting that TPH2 has the potential to fulfill a compensatory role in these models. The second enzyme in the melatonin-biosynthetic pathway is DOPA-decarboxylase (DDC, in starling NW_014650505.1). While expressed in almost all organisms from bacteria to vertebrates, DDC isoforms have not been documented in NCBI to date. Expression of both Tph1 and Ddc transcripts has been shown in postembryonic chicken pineal to change seasonally. (Piesiewicz et al., 2014) Seasonal regulation of these enzymes has not yet been researched in exta-pineal tissue. The resulting product of decarboxylation, serotonin (5-hydroxytryptamine, 5-HT), is converted by arylalkylamine N-acetyltransferase (AANAT, in Zebra finch NC_011482.1 and in European

starling NW_014650741.1) to N-acetylserotonin (NAS), the immediate precursor to melatonin. AANAT expression is commonly regarded as the rate-determining enzyme in the melatonin biosynthetic pathway (Klein et al., 1997; Ganguly et al., 2002) with more than a 150-fold increase in mRNA expression measured in rat pineal. (Rosebloom et al., 1996) Its circadian and photic-dependent regulation has also been detected in chicken (*Gallus gallus*) pineal and retina. (Bernard et al., 1997) Two genes of AANAT have been shown to regulate melatonin synthesis in fish. (Coon et al., 1999)

More recently, AANAT isozymes have been sequenced from the avian pineal (NAT-2 in chicken, NC_006098.2; NAT-3 in Zebra finch, NW_002226445.1) and potential seasonal differences in these variants have not yet been researched. AANAT has also been suggested to not be the rate-determining enzyme, but rather, the last enzyme in the pathway is. (Liu & Borjigin, 2005) Hydroxyindole- or acetylserotonin-O-methyltransferase (HIOMT or ASMT, in chicken NC_006088.3) makes the final conversion from NAS into melatonin. Observed differences in the nocturnal peak of melatonin synthesis in short-photoperiod compared to long-photoperiod correspond to changes in HIOMT activity in the Siberian hamster. (Ribelayga et al., 2000) Antibody directed against HIOMT showed labelling in photoreceptive sites of the pineal of various avian species. (Guerlotte et al., 1988) The study described here takes a closer look at the differential expression of the enzymes involved in this pathway at night- and day-time during different seasonal timepoints. Furthermore, by focusing on the Zebra finch as an opportunistic breeder and the European starling as an absolute photoperiodic breeder, we can consider the potential underlying function of hypothalamic melatonin synthesis on reproductive timing in different breeding types of birds.

Figure 1: Melatonin biosynthetic pathway, including variants of enzymes described in study



Materials and Methods

All procedures were performed in accordance with federal and state laws and with appropriate agreements from the UC Berkeley Office of Laboratory Animal Care.

European starlings (*Sturnus vulgaris*) were kept in outdoor aviaries at the Field Station for the Study of Behavior, Ecology, and Reproduction (FSSBER) in Berkeley, CA, where they were exposed to natural photoperiods. They were caught with hand nets on November 7, 2012 (day length = 10:00)* and February 1, 2013 (day length = 9:59)* at 12:30PST (n = 7 males, 5 females) and 21:30PST (n = 9 females, 7 males) on both days. Nighttime collections took place under red light from headlamps (Energizer). The birds were anesthetized with isoflurane and decapitated within 30min of capture. Trunk blood was collected and stored on wet ice. Brain, heads with eyes intact, and gonadal tissue were collected and stored on dry ice. Blood was spun down 1500rcf at 4°C within the hour and plasma collected and stored at -80°C for ELISA. Tissue was stored at -80°C until sectioned.

Zebra finches (*Taeniopygia guttata*) were housed in buildings with large windows, also exposed to natural photoperiods. Tissue was collected at 22:00PST (*n* =6 males) and 9:00PST (*n*=6 males) in September 2014 and October 2014, respectively. Tissue was prepared and stored in the same procedure as the starlings.

RNA Extraction and Reverse-Transcription. RNA was extracted from hypothalamic punches and cerebellum cut from the brain, eyes extracted from the frozen heads, and from gonadal tissue. Tissue was homogenized and stored in PureZOL RNA isolation reagent (Biorad #7326890) until extracted in accordance with the manufacturer's protocol. NanoDrop was used to assess RNA concentration and contamination, and RNA samples with >1.6 260/280 ratio were excluded from further analysis. 3ug of total RNA was reverse transcribed with iScript™ Reverse Transcription Mix (Biorad #1708841) and stored in -20°C until performing quantitative, Real-Time PCR (qRT-PCR).

Cloning and Sequencing. Primers were designed from sequences from NCBI GenBank (AANAT Gene ID: 100228432, HIOMT Gene ID: 396286), and the properties were calculated by Primer3 (Untergrasser et al., 2012; Koressaar & Remm, 2007) to have relatively low-likelihood to form hairpins or primer dimers. Primers were tested with cDNA from each tissue through PCR amplification with ExTaq Polymerase Kit (TaKaRa Ex Taq™; Takara Bio Inc., Shiga, Japan) and run with a 94°C hot start for 5min followed by 45 cycles of 94°C for 30sec, 60°C for 30sec, and 72°C for 1min. PCR products were run on 1.5% Agarose gel, and products with only a single band detected under ethidium bromide fluorescence in a UV Transilluminator (UVP Inc., Upland, CA) were ligated with the pGEM®-T Easy Vector System (Promega #A1360) for two days in -4°C. Ligated products were transformed in JM109 Competent Cells (Promega #L1001) according to the manufacturer's protocol and stored in 30°C for 1-2 days. Colonies were blue-white screened and run 35 cycles with M13F/R primers using ExTaq Polymerase Kit. Colony PCR products were selected, purified with ExoSAP-IT (Affymetrix #78200), and sequenced at UC Berkeley DNA Sequencing Facility. Returned sequences were determined with NCBI nucleotide BLAST tool to be genes of interest. Table 1 shows pairwise sequence alignments with sequences derived from the hypothalamus of European starling with top-matched results from the NCBI nBLAST searches.

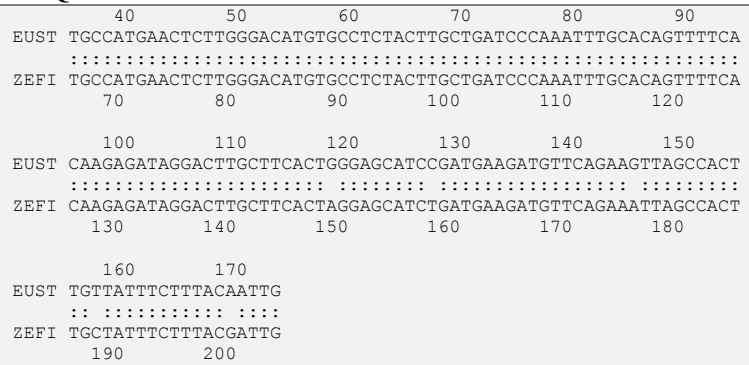
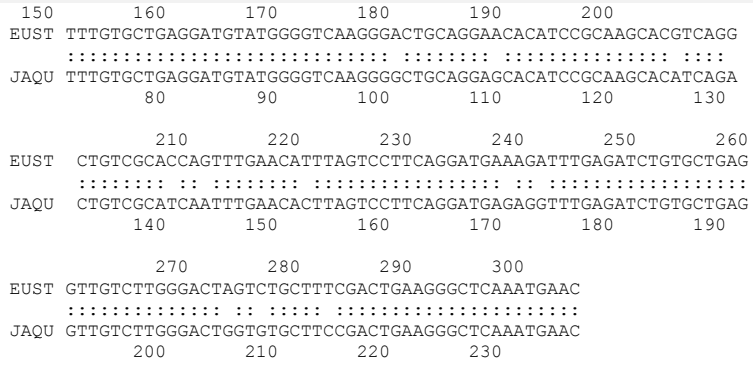
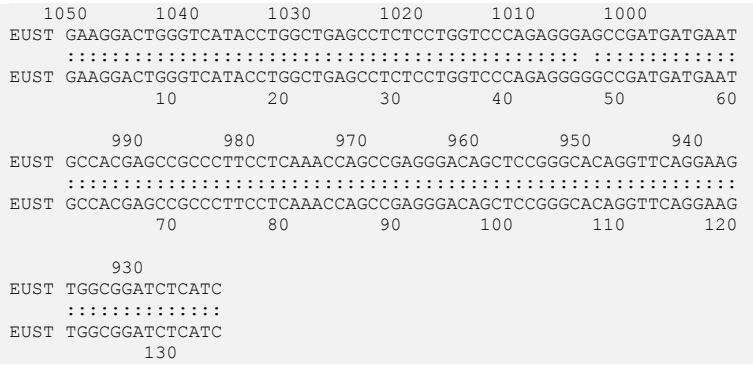
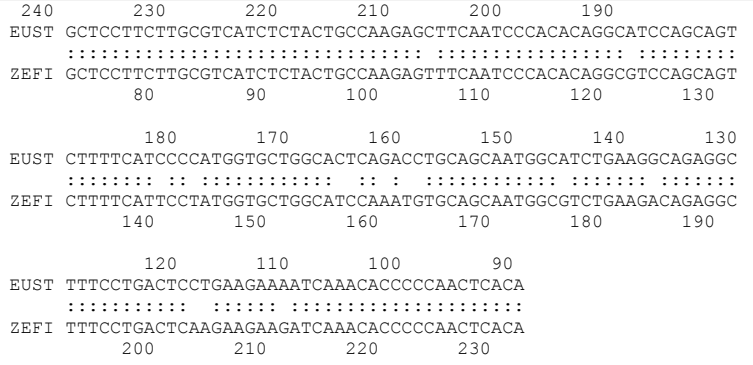
GENE	SEQUENCE	PAIRWISE ALIGNMENT
TPH2		**96%** NC011463
DDC/AADC		**93%** AB533266.1
AANAT		**99%** AY255681.1
HIOMT/ ASMT		**91%** XM_002195277

Table 1: Pairwise sequence alignment between European starling hypothalamus-derived sequences and NCBI nucleotide BLAST sequences using *Clustal W and Clustal X version 2.0.* (2007 November 01) *Bioinformatics* (Oxford, England) 23 (21): 2947-8. PMID: 17846036

quantitative, Real-Time Polymerase Chain Reaction (qRT-PCR). Relative expression of genes of interest was determined by qRT-PCR. cDNA of different tissues across individuals of consistent concentrations were mixed with Bio-Rad Sso Advanced SYBR Green Supermix (no. 172-5261) and primer combinations in line with the protocol for the Supermix, rendering 15μL per well. Each 96-well plate consisted of individuals from nighttime and daytime collections, blanks substituting cDNA with nuclease-free water, and pools of cDNA combining all individuals of each tissue to check for consistency across plate runs. Blanks were used to check for primer dimers, and a melt curve analysis was used to check the specificity of the primers used at 5μM concentration. Samples were run in duplicates. Raw data was collected and analyzed using Real-time PCR Miner (Zhao & Fernald, 2005), which determined primer efficiency and Ct (cycle threshold) values. Products that did not amplify or had levels of expression outside of two σ of the average were re-run, and if the results persisted, they were excluded from the PCR Miner analysis. Data from PCR Miner is exported into Microsoft Excel and expression values are calculated by $1/[(1+E)^{Ct}]$, where E is the average efficiency of a certain primer pair raised to the Ct of an individual well. Ct values of the control genes were used to determine stability of the control genes, GAPDH and β-Actin, to test reference genes with four different algorithms: Delta Ct, BestKeeper, GeNorm, and Normfinder (Xie et al., 2012). Intra- and inter-control gene variance was the geometric mean of expression values of both GAPDH and β-Actin was used to normalize the expression data of the gene of interest. If one control amplified but the other did not, the individual was excluded from the gene expression analysis. On these parameters, one individual was excluded from the day collection in Fall 2012 (11 individuals remaining), and four individuals were excluded from the eye data (7 individuals remain from day collection, 11 individuals from the night collection).

Once the expression value of the gene of interest (AANAT, AANAT2, or HIOMT) was divided by the geometric mean of the expression values of GADPH and β-Actin, the output yielded the normalized expression value (NEV). 'Fold Change' was calculated by grouping individual NEVs in a given tissue and collection time (e.g. Pineal Night) and, subsequently, dividing the individual NEVs by the minimum NEV of the group, yielding fold change above the minimum NEV.

Statistical Analyses. qRT-PCR data was analyzed using an unpaired, two-tailed T-test with Welch's correction in GraphPad Prism version 7.0a for MacOS (GraphPad Software, La Jolla California USA). Only relative gene expression within the same tissue (e.g. pineal collected in daytime and nighttime, Fall 2012) is statistically compared.

Results

Identified sequences of melatonin-synthesizing enzymes in the hypothalamus of Zebra finch and European starling. We identified and partially sequenced all four of the enzymes necessary to synthesize melatonin from its amino acid precursor, tryptophan in the hypothalamus of European starling: TPH2, DDC, AANAT, and HIOMT/ASMT (See Table 1). The variants of these enzymes were also sequenced (TPH1, TPH2, AANAT2, AANAT3) from RNA extracted from hypothalamic tissue punches of the zebra finch.

qRT-PCR expression of AANAT and HIOMT in photoreceptive tissue collected at night compared to daytime in European starling. With February and November collections of European starling, the absolute photoperiodic breeder, taking place at times of the year when daylength is practically identical (10:00 hours and 9:59 hours respectively), we may expect to see the last two enzymes of the melatonin-biosynthetic pathway being expressed at comparable levels in tissue known to play a critical role in circadian melatonin synthesis and release in birds, the pineal and the eyes. From the qRT-PCR study conducted here (Figures 2 and 3), we observed a significant difference between night and daytime expression levels of AANAT in the pineal ($p=0.0451^*$) and the hypothalamus ($p=0.031^*$) in the group collected in November. In the February collection, AANAT expression levels were significantly greater in the pineal collected at nighttime compared to the daytime ($p<0.05$). There were no significant differences between daytime and nighttime expression levels of HIOMT across tissues.

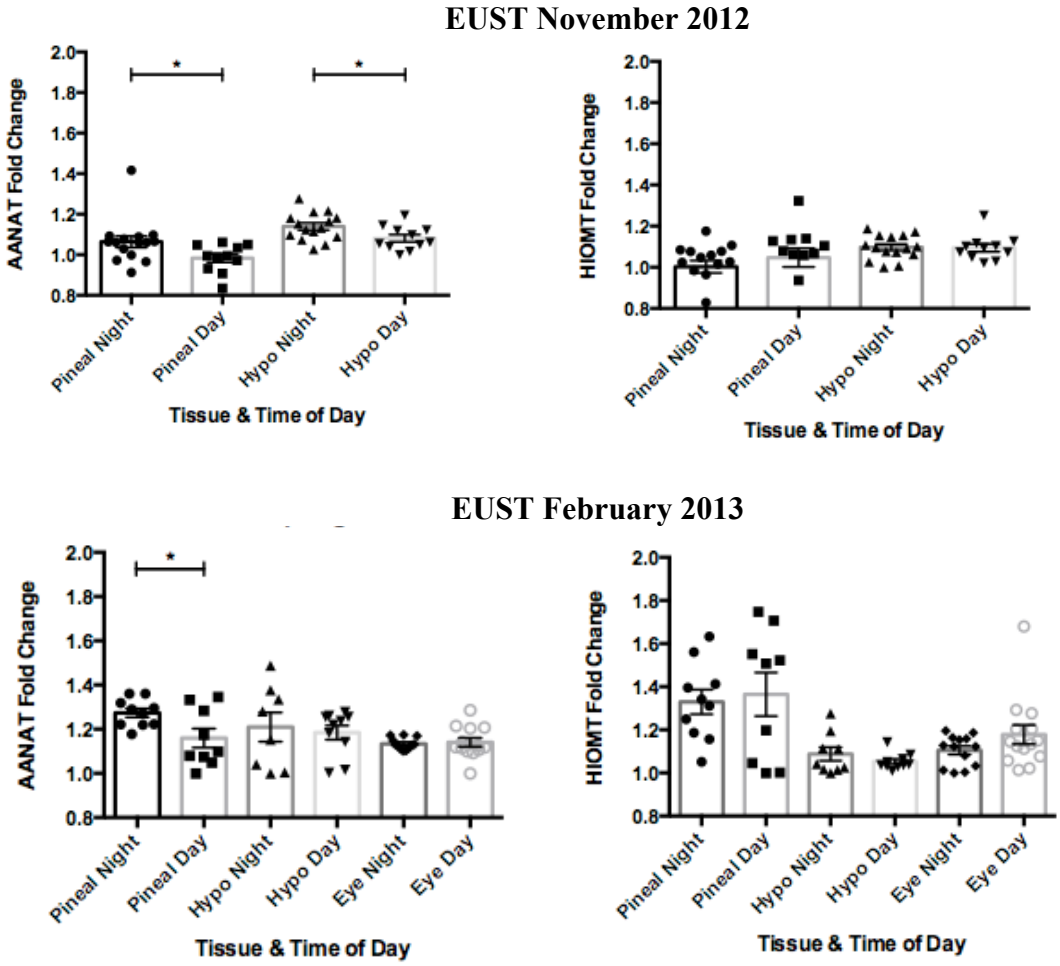


Figure 2: European starlings (EUST) collected in November 2012 and February 2013, fold change in mRNA expression of AANAT and HIOMT in the pineal, hypothalamus, and eyes. Error bars represent mean fold change ± *SEM*, statistical difference determined at $p < 0.05$ by unpaired two-tailed *t-test* with Welch's correction.

qRT-PCR expression of AANAT2 and HIOMT in photoreceptive tissue collected at night compared to daytime in zebra finch. Meanwhile, in the opportunistic breeder housed indoors but exposed to natural photoperiods, we observe no significant difference between nighttime and daytime collections in the *Aanat* variant, AANAT2, nor in the last enzyme in the melatonin biosynthetic pathway, HIOMT, in neither the hypothalamus nor the negative control, the cerebellum (Figure 3). The only significant difference was observed in the fold change of HIOMT in the positive control, the pineal tissue.

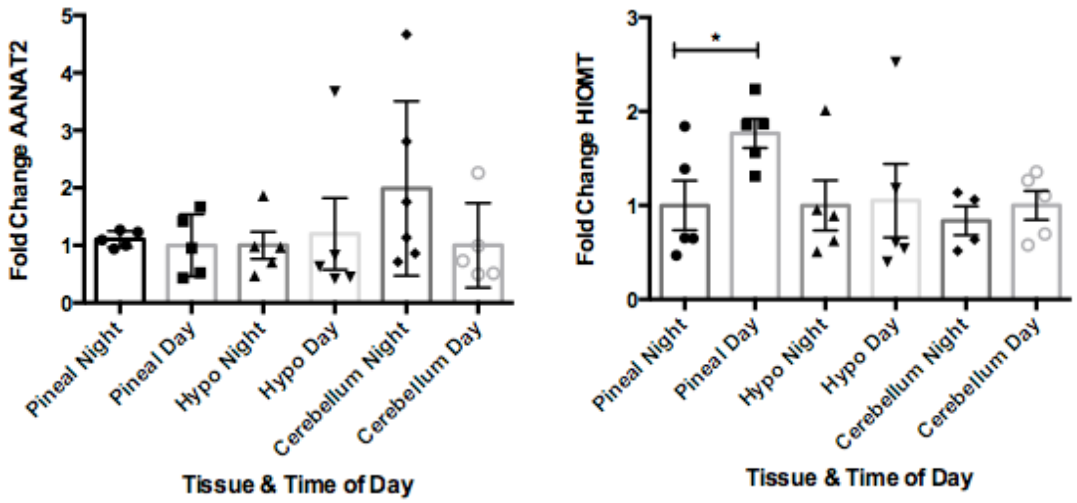


Figure 3: Zebra finches collected in September and October 2014, fold change in mRNA expression of AANAT2 and *HIOMT* in pineal, hypothalamus, and cerebellum. Error bars represent mean fold change ± *SEM*, statistical difference determined at $p < 0.05$ by unpaired two-tailed *t-test* with Welch's correction.

Discussion

Time and time again, melatonin has been studied for its potential effect on the reproductive state of non-mammalian vertebrates (for review, Mayer et al., 1997). These studies in birds are limited by the manner in which melatonin is administered, either with hyperphysiological-level injections (e.g. Juss et al., 1993) or with continuous-release implants (e.g. Hau et al., 2012). This study focuses upstream of these by researching the differential expression of enzymes of the melatonin-biosynthetic pathway in both an opportunistic and an absolute photoperiodic seasonally-breeding species of bird. The results localize the enzymes necessary for melatonin synthesis in the hypothalamic region of the avian brain in both of these breeding types.

The question of what mechanisms have evolved to render the observed diversity of breeding strategies in birds must continue to be addressed. The differences in the capacities of photoreceptive neural sites to express enzymes that synthesize melatonin suggest a novel compensatory role that the hypothalamus fills in the absence of the pineal and the retina in previous studies on photoperiodic birds. The absolute photoperiodic breeder, the European starling, shows a significance difference in the expression of the penultimate enzyme, AANAT, in both the hypothalamus and pineal collected in the Fall photorefractory phase (November), but only the pineal shows a significant different in AANAT expression during early phases of

photostimulation (February). Meanwhile, the opportunistic species of this study, the zebra finch, appears to be lacking a comparable hypothalamic melatonin regulation system, expressing non-significant differences in the levels of melatonin-synthesizing enzymes regardless of time-of-day or lighting condition.

Such findings align and contribute to former research on these opportunistic breeders. Perfito has shown how long days play a permissive role in gonadal development but is only supplementary to food availability, the primary cue. (Perfito et al., 2008) At the mechanistic level, given that the second subtype of gonadotropin-releasing hormone (GnRH-II) varies across reproductive state in wild male Zebra finches, with more and larger immunolabeling in breeding males, but irGnRH-I and irGnIH labeling and mRNA expression do not vary, (Perfito et al., 2011) a different integration system may exist. Previous work showing GnRH-II fibers extend to the pineal gland of European Sea Bass *Dicentrarchus labrax*, which express GnRH-II subtype receptors, and both in vitro and in vivo studies show a stimulatory role on melatonin release. (Servelli et al., 2010) However, melatonin has been shown to have no effect on GnRH-II release in the goldfish, *Carassius auratus*. (Trudeau, 1997) Consideration of comparative studies such as these pose additional questions of what regulates melatonin synthesis secretion and how melatonin regulates other aspects of the reproductive system in non-photoperiodic species.

The role melatonin plays in the reproductive state of photoperiodic species appears to be more direct than opportunistic or non-photoperiodic species. Removing the main source of melatonin in starlings, the pineal, prevents a second testicular recrudescence when housed in 12h days, on the cusp of their critical daylength that induces photorefractoriness. (Gwinner & Dittami, 1980) Melatonin has been recently reviewed to stimulate the synthesis and release of GnIH in quail (Chowdhury et al., 2013). Pinealectomized subtropical Indian weaver birds, *Ploceus philippinus*, administered varying durations of melatonin showed, with longer durations, (16h) significantly higher GnIH-ir labeling compared to shorter durations (8h) of melatonin administration, but no significant changes were observed in GnRH-I. (Kumari et al., 2015) The seasonal variation and sex differences of melatonin binding in the song control system in starlings suggest a role for modulating neuroplasticity. (Bentley et al., 2013) Ecological implications of the interaction between melatonin and reproduction must consider the consequences of urban light pollution, which has been shown to reduce melatonin release in another songbird, the European blackbird (*Turdus merula*). (Dominoni et al., 2013)

While this study investigates the regulation of melatonin synthesis, the responsiveness of different neural sites and tissues to melatonin synthesis is an important part of its effect on reproductive state. We have previously shown how different subtype receptors of melatonin (Mel1B and Mel1C) are expressed in the gonads of starlings, and their expression corresponds to both GnIH expression and reproductive state. (McGuire et al., 2010) These downstream effects show that there is more than what meets the eye, hypothalamus, or pineal for the role of melatonin in regulating reproductive timing in birds.

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Chapter 3: Melatonin and Steroidogenesis in Syrian Hamster Testes

Abstract

Syrian hamsters completely alter reproductive state in response to specific durations of circulating melatonin. This change in reproductive state manifests in changes in testicular volume. The acute changes in testicular steroidogenesis in response to extended melatonin exposure are not well understood. The study presented shows preliminary findings of melatonin synthesis at the level of the testes through immunohistochemistry. Additionally, testes collected from photostimulated Syrian hamsters were cultured *in vitro* with melatonin, and changes in testosterone production were measured over time. Testes incubated with melatonin for 12 hours did not show an increase in testosterone production, as observed in control groups cultured without melatonin. To determine the mechanism that underpinned this observation, expression levels of Steroidogenic Acute Regulatory protein (*StAR*) and melatonin receptor subtype 1B (*Mel1B*) were quantified. There were no significant changes in expression levels of these genes, implying that the differences in testosterone production are regulated downstream in the steroidogenic pathway. Furthermore, either another melatonin receptor subtype or post-transcriptional regulation of melatonin receptors may correspond to changes in testicular sensitivity to the exogenous melatonin signal.

Introduction

A fundamental observation in the field of seasonal reproductive endocrinology is the annual fluctuation of gonadal volume in photoperiodic animals exposed to a certain duration and wavelength of light (Benoît, 1964). As described in previous chapters, the chemical transducer of photic information, melatonin, is a tryptophan-derived product synthesized in the pineal primarily (Wurtman et al., 1963; Axelrod et al., 1964). Melatonin synthesis and release is detected in plasma at higher levels in darkness (for review see Reiter, 1991). In mammals, light is transduced via the eye and hypothalamus to the pineal. The absence of light prevents the breakdown of a key enzyme in the melatonin biosynthetic pathway (arylalkylamine-N-acetyltransferase, AANAT or NAT), enabling melatonin synthesis to occur (Klein & Weller, 1972; Klein et al., 1997). Endogenous rhythms of melatonin vary based on circannual fluctuations of day length, or photoperiod, experienced by organisms exposed to daylight outside of the tropics. Reproductive state of a male animal is observed in part through testicular development and regression. The extent to which reproductive state is driven by photoperiod, food, mate availability, or other external cues varies depending on the species and the environment. The duration of melatonin administration, mirroring the length of the night of a particular season, can overhaul the reproductive physiology of photoperiodic, seasonally breeding mammals (Bartness et al., 1993). How pineal-derived or exogenously administered melatonin transmits a seasonal signal to the gonads to affect reproductive state varies across species.

Mammals and birds express melatonin subtype receptors in the gonads (Frungieri et al., 2005; Sundaresan et al., 2009; McGuire et al., 2011) and in peripheral reproductive tissue such as the epididymis (Shiu et al., 2000). The fluctuation of melatonin subtype receptor expression in the gonads serves as an indicator for gonadal sensitivity to the melatonin signal. In European starlings, *Sturnus vulgaris*, the annual fluctuation of relative expression of melatonin receptor

subtypes in the testes fluctuates in relation to photoperiod. Exogenous melatonin induces the synthesis and secretion of gonadotropin inhibitory hormone (GnIH) in testis culture (McGuire et al., 2011). GnIH inhibits testicular development in Japanese quail, *Corturnix japonica* (Ubuka et al., 2006). Seasonal changes in the melatonin receptor subtype expression in the testes coincides with annual fluctuations in testicular volume and reproductive physiology (McGuire et al., 2011). Therefore, the duration of pineal melatonin synthesis and secretion, corresponding to the length of the night, is capable of transducing photic information peripherally by binding to melatonin receptors in the gonads to inform seasonality.

The effects of melatonin synthesis and binding in the testis vary across species. Melatonin is synthesized in the testis of rats as demonstrated in experiments using radiolabeled precursor, *S*-adenosyl-l-[methyl-^{14}C]methionine (Tijmes et al., 1996). The potential for autocrine and paracrine regulation of reproductive function is likely mediated through melatonin-subtype receptors MT1, MT2, and other melatonin-related receptors in rat testes, which change expression levels through stages of development (Izzo et al., 2010). Additionally, duck gonads bind [I-125] iodomelatonin (Ayre et al., 1994), and frog testes contain cDNA encoding the *Mel1b* subtype receptor (Serino et al., 2011). Activation of MT1 and MT2 melatonin subtype receptors regulates the development and function of bovine Sertoli cells and mediates the expression of spermatogenesis-related genes (Yang et al., 2014). Melatonin-synthesizing enzyme expression in ram testes and accessory glands, corresponding to high concentrations of melatonin in seminal plasma during the day, indicate potential for reproductive organs to locally synthesize and secrete melatonin (Gonzalez-Arto et al., 2015). Testes of reproductively active Syrian hamsters also bind melatonin, and activation of the Mel1a subtype receptor appears to inhibit androgen production through the testicular corticotropin-releasing hormone (CRH) system (Frungieri et al, 2005; Rossi et al., 2012). In humans, melatonin has anti-proliferative and anti-oxidative properties in the testes (Rossi et al., 2014). The diverse effects of the local synthesis and binding of melatonin on testicular development and function, however, do not include mechanisms of upstream melatonin binding within neural sites integrating photic information. Melatonin binding in these specific neural sites indirectly affects testicular development and regression by regulating reproductive neuropeptides in seasonal photoperiodic mammals.

Specific neural sites in the hypothalamus, such as the suprachiasmatic nucleus (SCN), coordinate photic cues and circadian responses and also modulate pineal melatonin synthesis via the mammalian photic transduction pathway (Stephan and Zucker, 1972; Rusak & Morin, 1976). In some species, the SCN directly binds melatonin (in rats in Gauer et al., 1993; Laitinen et al., 1989; and Vaněček, 1988; in ewes Bittman & Weaver, 1990; in humans Reppert et al., 1988; in Syrian hamsters Gauer et al., 1998). Additionally, mammalian hypothalamic sites peripheral to the phototransduction pathway also bind melatonin. For instance, the dorsomedial hypothalamus (DMH) in Syrian hamsters uniquely hosts both melatonin and androgen receptors, enabling interaction between steroidal negative feedback and the photoperiodic timing system. Ablation of the adult male Syrian hamster DMH prevents gonadal response to exogenous melatonin infusions (Maywood et al., 1996). Furthermore, DMH ablation prevents the short-day induced testicular regression (Jarjisian et al., 2013). These studies suggest that the DMH is necessary for testicular regression in response to both an artificially extended exogenous melatonin signal and to truncated light exposure. Given that RF-amide Related Peptide (RFRP, the mammalian homolog of avian GnIH) has differential expression in the DMH depending on the reproductive state of the Syrian hamster (Mason et al., 2010), it is a likely intermediate between the photochemical signal of melatonin and gonadal steroid negative feedback contingent upon

reproductive state. Although extended subcutaneous administration of melatonin initiates testicular regression for sham-operated controls, it fails to initiate testicular regression in DMH-ablated hamsters (Maywood et al., 1996). How the testes receive and respond to the indirect melatonin signal mediated by the Syrian hamster DMH to induce testicular regression is not fully understood.

The gonadal reproductive effects of neural melatonergic signal transduction may be understood through studying the sensitivity and responsiveness of gonads to melatonin. For example, Syrian hamsters housed for 10 weeks at the critical day length (12.5L:11.5D) showed similar levels of plasma melatonin concentrations and *MT1* expression in the testes compared to those housed 10 weeks in long days (16L:8D) (Mukherjee & Haldar, 2014). However, hamsters housed in short days for 10 weeks showed significantly higher plasma melatonin and testicular *MT1* expression (Mukherjee & Haldar, 2014), suggesting seasonality in circulating melatonin levels and also testicular sensitivity to the melatonin signal. The physiological consequences of melatonergic activity at the level of the gonads include correlations between testicular steroidogenic processes and melatonin. Androgen receptor (*AR*) and Steroidogenic acute regulatory protein (*StAR*) expression positively correlated with plasma testosterone concentrations, which varied based on lighting condition. Testes collected from Syrian hamsters housed in long days (photostimulatory conditions, corresponding to increased reproductive activity) had significantly higher *AR* and *StAR* expression relative to testes collected during photoinhibitory short days. Additionally, *AR* and *StAR* expression across different photoperiodic conditions were inversely related to plasma melatonin and *MT1* expression (Mukherjee et al., 2014). The study conducted here focuses on testes collected at night and cultured with chronic exposure (12 hours) to melatonin. The testes, despite the absence of direct photoreception, are capable of receiving external input of lighting condition via melatonin receptor binding. This chapter investigates whether there are differences in steroidogenic enzyme and melatonin receptor expression in Syrian hamster testes cultured with melatonin. Furthermore, I address the time-dependent effects of extended melatonin exposure *in vitro* on testosterone production.

Methods

Tissue Collection for Immunohistochemistry, RNA Extraction, and cDNA Synthesis. Male Syrian hamsters were purchased from vendor Charles River (Wilmington, MA) and individually housed in translucent propylene cages in a temperature-controlled colony room (23 ± 1°C) until use. Access to food and water was provided *ad libitum* for the duration of the study. All procedures were conducted in accordance to the protocol (R295) approved by the Institutional Animal Care and Use Committee of the University of California, Berkeley.

Tissues were collected for immunohistochemistry from hamsters (*n=4*) that were 11 months old in the light period (ZT12.0-12.5) and dark period (ZT16.5-17.0). Testes were flash frozen and stored in -80°C freezer until coronally cut (40µm thickness) in -20°C cryostat and mounted on subbed slides. Every other testis slice was collected in nuclease-free tube and stored in -80°C until RNA was extracted using the protocol and materials provided in the Bioline Isolate II RNA Mini Kit (BIO-52073). RNA concentration and purity was measured in a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of the RNA was verified by agarose gel electrophoresis. This RNA was converted to cDNA using the iScript Reverse Transcription Supermix for RT-qPCR (Catalogue No. 170-8841) to be used for PCR and Real Time-quantitative PCR.

Testis Culture. Tissue used for the testes culture was collected from 18-month-old Syrian hamsters that were housed in 14L:10D light cycle for 135 days (lights on at 02:30 and lights off at 16:30). Animals were put under with 0.2 mL euthasol injections per adult male hamster (*n = 4*). Testes were collected and stored in gauze wetted with filtered PBS [with Acrodisc PF Syringe Filters, 25mm (Item #4187)] and kept on wet ice during transport, until cut with sterilized surgical scissors (DNase, ethanol, and NF water) directly into the tissue culture media.

The culture media master mix included 49 mL Eagle's MEM [(+) Hank's Salts, (+) L-Glutamine], 0.50 mL pen / strep, 0.50 mL HEPES (1M) from Sigma (CAS Number 7365-45-9), and 0.05 g bovine serum albumin (BSA). The media was filtered by Acrodisc PF Syringe Filters, 25 mm (Item #4187), into sterile 50mL cylinder tubes then transferred into sterile media containers. 10 mL of the culture media was kept for control. Three other groups were separately mixed as follows:

Group 1. 8 mL of culture media with 1 mL LH (1 IU/μL, Sigma L5269-1VL) and 1 mL FSH (1 IU/μL, Sigma F2293-1VL),
Group 2. 8 mL of culture media with 200 μL melatonin (ThermoFisher Scientific, Stock#J62452-14) dissolved in 100% ethanol (333 mg melatonin/mL ethanol)
Group 3. 4 mL of Group 1 solution with 100 μL melatonin dissolved in 100% ethanol (333 mg melatonin/mL ethanol)

After the culture media was mixed, the different groups of solutions were stored in an incubator oven at 37° C to preheat while the testes were being collected (less than 8 hours). Testes were cut with sterilized surgical scissors directly into the preheated media. The culture experiment started at 23:00 (11:00 PM). 4 hours (3:00 AM) and 6 hours (5:00 AM) after the beginning of the incubation, 250 μL of media was collected and stored immediately on dry ice. The terminal collection of media (1 mL) was collected 12 hours after the start of the culture (11:00 AM), and all media collections were stored in -80° C freezer until the testosterone assay was conducted. The mass of the tissue was recorded at the end of the tissue culture then stored immediately on dry ice then moved into a -80° C freezer until RNA extraction for gene expression analysis.

Testosterone Radioimmunoassay. The culture media was thawed on ice then room temperature. Steroids were extracted from culture media using diethyl ether and dried under N_2 in a dry bath and then reconstituted in phosphate buffered saline with gelatin. The RIA was conducted as described in Licht et al., 1983 using testosterone antisera from PerkinElmer Inc (Testosterone, [1,2,6,7,16,17-^{3}H(N)]).

Primer Design and qPCR. Primers for PCR were custom designed using Primer3 and NCBI Primer Blast then ordered from Sigma Genosys. They were tested in accordance with the MIQE Guidelines (Bustin et al., 2009) and identified for specificity with 45 cycle PCR with 94°C hot start, 60°C annealing temperature, and 72°C extension step. PCR products were run on a 4% agarose gel and single bands were submitted to the UC Berkeley Sequencing Facility to confirm the product to be the gene of interest (Primer sequences are in Table 1).

The same primers were tested for Real Time-quantitative PCR (RT-qPCR) using the SsoAdvanced™ Universal SYBR® Green Supermix (Catalogue No. 172-5271) and Biorad

CFX384™ Real-Time System with a standard curve to test for ideal efficiencies. Once determined, the cDNA of different individuals was used as templates and relative expression was calculated using the geometric mean of reference genes, β-actin and GAPDH, measured to not vary across groups.

Target Gene	**Primer Sequences**	**Accession No.**
Mesocricetus auratus Mel1b melatonin receptor pseudogene, partial sequence	Forward: 5'CCCTCGAGTATGAGCCAAGC Reverse: 5'ACCACAGCGATTGGAAGGAG	gi|24251143|AY145849.1
StAR steroidogenic acute regulatory protein [*Mesocricetus auratus* (golden hamster)]	Forward: 5'GAGTGGAACCCCAATGTC Reverse: 5'GCACCATGCAAGTGGGAC	U66490.1
PREDICTED: Mesocricetus auratus tryptophan hydroxylase 2 (Tph2)	Forward: 5'GCCATGAACTCTTGGGACAT Reverse: 5'AGCACGCAGTTGTCCTTCTT	gi|880943456|ref|XM_005081469.2|
PREDICTED: Mesocricetus auratus dopa decarboxylase (aromatic L-amino acid decarboxylase) (Ddc), mRNA	Forward: 5'GAGGAGTGATCCAGGGGAGT Reverse: 5'CTGAACCTCACTGCACCGAT	gi|880950313|ref|XM_005082971.2|
PREDICTED: Mesocricetus auratus N-acetyltransferase 2 (arylamine N-acetyltransferase) (Nat2)	Forward: 5'CACTCCAGCTAATAAGTACAGCA Reverse: 5'TCTGTCAAACGGAAGATGGC	gi|880909446|ref|XM_013117158.1|
Reference Gene	**Primer Sequences**	**Accession No.**
glyceraldehyde-3-phosphate dehydrogenase [*Mesocricetus auratus* (golden hamster)] (GAPDH)	Forward: 5'ACAGTCAAGGCTGAGAACGG Reverse: 5'TCCACAACATACTCGGCACC	DQ403055.1
Actb actin, beta [*Mesocricetus auratus* (golden hamster)]	Forward: 5'GACCCAGATCATGTTTGAGACCT Reverse: 5'TCCGGAGTCCATCACAATGC	NM_001281595.1

Table 1 Primers. Primer sequences in light gray (Mel1b, StAR, GAPDH, and β-actin) were used in quantitative Real Time-PCR for testes harvested from tissue culture (12-hour incubation with dissection media, melatonin, and/or LH and FSH).

Immunohistochemistry. The testicular tissue thaw mounted on slides was used in immunohistochemistry. Melatonin antibody (antibodies-online, Catalogue No. ABIN1111121) was diluted 1:1000 in 0.1M PBST. This primary antibody was preadsorbed (PMEL) with melatonin (99+% powder, Thermo Fisher Scientific, Stock No. J62452-14) by incubating the melatonin antibody with 0.1g/mL melatonin at room temperature on a magnetic stirrer for 1 hour then storing at -4°C until use (used within two days of dissolving melatonin).

Tissue was exposed to three different regimes: (1) no primary antibody, (2) melatonin primary, or (3) preadsorbed melatonin (PMEL) antibody. A subset of slides was first exposed to a promiscuous melatonin receptor antagonist, Luzindole (abcam, Catalogue No. ab145232), by incubating the slides for 24 hours at 4° C with Luzindole, first dissolved in 200 proof molecular grade ethanol then mixed 1:1 with PBST. Both Luzindole-treated and non-treated slides were fixed with PFA for 15 min, rinsed in four 10 minute washes of PBS, then incubated for 1 hour in Normal Donkey Serum (NDS). The slides are transferred to opaque, vertical slide-holders filled with one of the three regimes (no primary, melatonin, and PMEL) and stored for 1 hour at room temperature then stored for 48 hours at 4° C. After three 5-minute washes in PBST, the tissue was incubated in Cy^{TM} 3-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) secondary antiserum (Jackson ImmunoResearch Laboratories, Inc., Code No. 711-165-152 and Lot No. 126219) diluted 1:25k for 1 hour, washed for three 5 minute PBS washes, then incubated for 10 minutes with VECTASHIELD Antifade Mounting Medium with DAPI (Catalogue No. H-1200) diluted 1:20k in PBST. After two 5 minute washes in PBS, the slides are left to dry at room temperature overnight. The final dehydration step prior to coverslipping with Fluoromount-G (SouthernBiotech, Catalogue No. 0100-01) was completed by submerging the slides in Histo-Clear (Catalogue No., HS-200) for 15 minutes then dried overnight prior to being visualized.

Results

qRT-PCR. The expression pattern of testicular melatonin subtype receptor b (Mel1b) and Steroidogenic Acute Regulatory protein (StAR) showed no strong indication of regulation from 12-hour exposure to melatonin (see Figure 1). All tissues (melatonin only, LH/FSH only, melatonin + LH/FSH) had higher expression levels than tissue cultured only in dissection media and was graphed based on normalized expression levels relative to the dissection media only group.

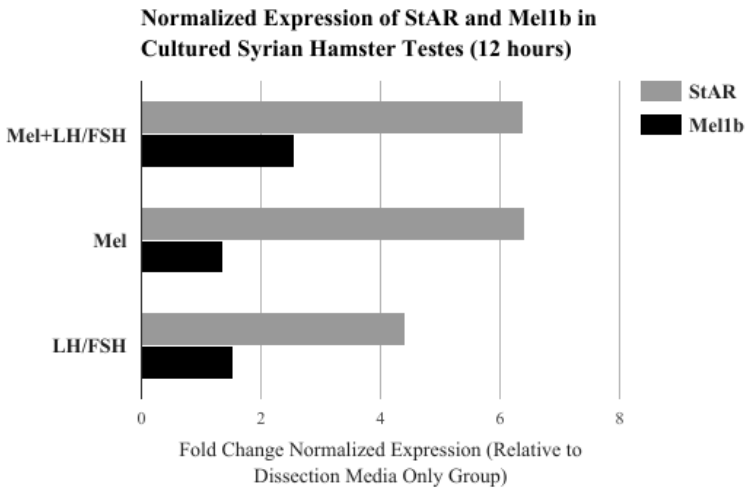
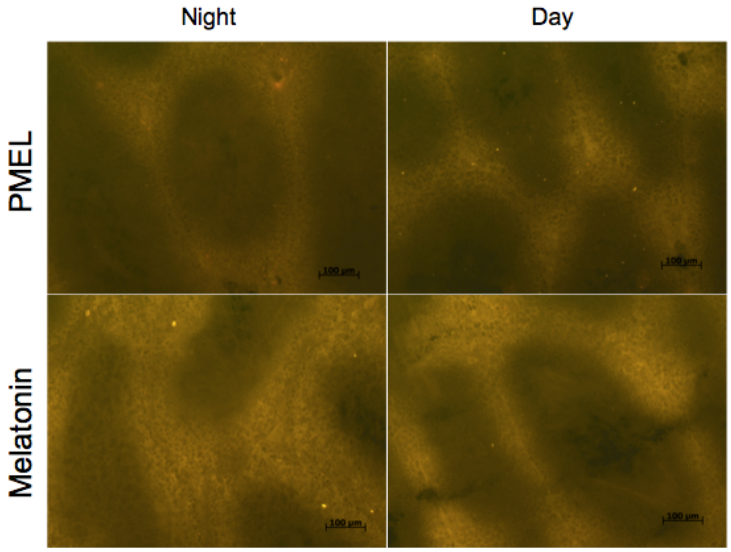


Figure 1 Normalized Expression of StAR and Mel1b in Cultured Syrian Hamster Testes (12 hours). Groups include Melatonin only (Mel), LH/FSH only (LH/FSH), and Melatonin + LH/FSH (Mel + LH/FSH). Gene expression RO values are normalized to the geometric mean of control genes (β-Actin and GAPDH), averaged then divided by the average normalized expression of genes from the dissection media only group.

Immunohistochemistry. Figure 2 shows the results of the immunohistochemistry. This pilot showed differential binding of melatonin and PMEL in hamster testis collected during nighttime relative to hamster testis collected during daytime. Preadsorbed melatonin binding (PMEL) was blocked in tissue that was pre-incubated with luzindole, and the binding of the melatonin antibody was not blocked in slides incubated with luzindole (data not shown). The proposed mechanism for these observations are outlined in Figure 3. Because luzindole appeared to block PMEL binding and not melatonin binding, the melatonin receptor antagonist may block binding to the melatonin receptor. This effect may not be confirmed based on steric effects because the epitope of the melatonin antibody has not yet been determined (email communication with vendor).

A.



B.

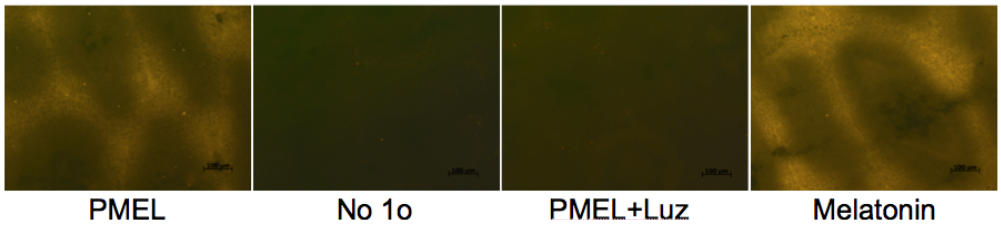


Figure 2 Immunohistochemistry of Syrian Hamster Testes with Melatonin Antibody. Scale bar set at 100um. Photos taken at 2.99 sec exposure, 10x magnification. (A) Labeling of tissue collected at nighttime and daytime (B) Hamster testes collected during the light cycle and exposed to preadsorbed melatonin (PMEL), no primary antibody (No 1°), PMEL + melatonin receptor antagonist Luzindole (PMEL + Luz), and melatonin antibody (Melatonin).

Testosterone RIA. The testicular tissue incubated with melatonin (Mel or Mel + LH/FSH) did not show a time-dependent increase in testosterone as observed in the groups without melatonin [dissection media only (DM) or LH/FSH]. Treatment with LH/FSH did not stimulate testosterone production above basal levels (DM) as predicted. The effects of melatonin on testosterone levels in the media, relative to groups absent of melatonin, are not observable until the 12-hour time point (terminal collection).

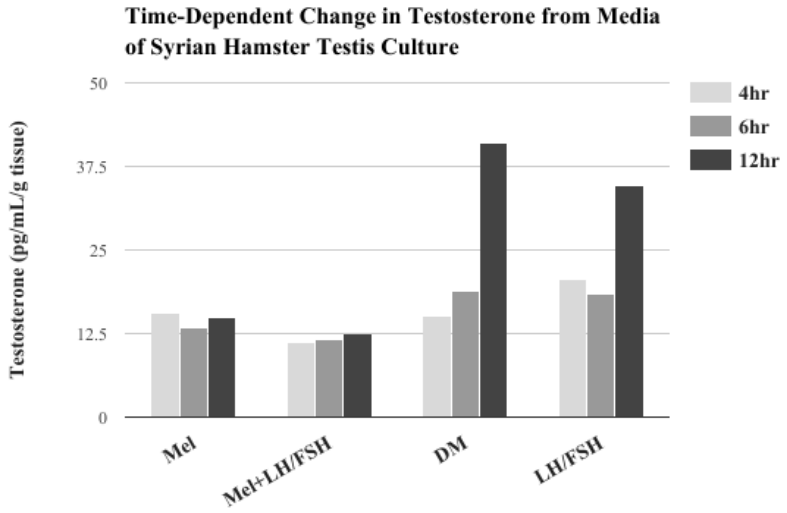


Figure 4 Time-Dependent Change in Testosterone from Media of Syrian Hamster Testis Culture. Testosterone was measured with RIA from media collected at 4 hours (light grey bars), 6 hours (grey bars), and 12 hours (dark grey bars). The groups incubated melatonin do not show the 12-hour increase observed in the dissection media only (DM) and LH/FSH groups.

Discussion

How melatonin affects enzymes involved in steroidogenesis and testosterone production in photoperiodic breeders has been previously studied in male Syrian hamster (Frungieri et al., 2005). In this previous study, testes were collected and incubated 3hr *in vitro* with melatonin. Melatonin blocked hCG-stimulated testosterone production from photostimulated testes, and this melatonin-associated decrease in testosterone production was reverted by the melatonin-receptor antagonist, luzindole (Frungieri et al., 2005). These findings indicated a role for melatonin at the level of Syrian hamster gonads for regulating reproductive steroidogenesis in a receptor-mediated fashion, not just as an antioxidant.

The study outlined here was initially an attempt to replicate the study of Frungieri et al. (2005) using the same qRT-PCR primers for testes cultured for an extended period (12 hours), but the efficiencies of these primers were too high (>200%) to assume with certainty that they were not dimerizing. Furthermore, the blank samples (i.e. nuclease-free water is used instead of cDNA) had comparable *Ct* values as the pooled cDNA samples, providing additional evidence that the amplified product was a result of primer dimerization. The melt curves also reflected the presence of multiple products within temperature ranges associated with primer dimerization.

Nonetheless, Frungieri et al. (2005) offers some points for comparison. Firstly, the hamsters used in Frungieri were 14 weeks old, whereas the current study used 1.5-year-old hamsters. The effect of aging on melatonin production and reception is an important consideration. In young Syrian hamsters (6 weeks), adults (15 weeks), and old (2 years), there is

a significantly lower *MT1* expression in the testes and lower plasma melatonin concentrations in the old group (Mukherjee & Haldar, 2015), which must be considered in relation to the observations of the study described here. Secondly, the testes cultured by Frungieri et al. (2005) were incubated with melatonin for 3 hours; however, the length of the night and the corresponding duration of circulating melatonin exposure can be much longer. In the current study, I incubated the testes in melatonin for 12 hours. Lastly, the time of the killing and tissue collection was not reported in Frungieri et al. (2005). For the study described here, the animals were sacrificed and the testes were collected two hours after lights off, when circulating melatonin begins to peak in Syrian hamsters (Tamarkin et al., 1979; Brainard et al., 1982; Maywood et al., 1993; Gündüz, 2002). These key differences between Frungieri et al. (2005) and the study described here should be kept in mind in the design of future studies assessing the gonadal steroidogenic effects of melatonin in Syrian hamsters.

The results here show melatonin may suppress testosterone production over time. When Leydig cells were isolated from Siberian hamsters (*Phodopus sungorus*) and cultured with varying concentrations of melatonin, melatonin had the most suppressive effects on LH-stimulated Leydig cells (Niedziela et al., 1995). I did not observe notable differences in LH/FSH-stimulated testicular tissue and tissue cultured without LH/FSH. These differences may be attributed to differences in cultured Leydig cells and intact testicular tissue. The testicular tissue used for the culture study conducted here included additional physiologically-relevant structures. For instance, melatonin alters the glycolytic profile of Sertoli cells (Rocha et al., 2014). Melatonin also affects sperm quality (Luboshitzsky et al., 2002), potentially in a receptor-mediated fashion (Fujinoki, 2008; Ortiz et al., 2011). In another study, Leydig tumor cells were isolated from MA-10 mouse and observed after a 12hr incubation with melatonin (at both 1nM and 10μM concentrations) (Wu et al., 2001). StAR expression was decreased (Wu et al., 2001), counter to what I observed in the current study with entire testicular tissue. The confluence of melatonergic effects on testicular substructures are apparently different with entire testicular tissue and arguably more physiologically relevant.

Lastly, the results of immunohistochemistry show a potential difference in nighttime and daytime levels of testicular melatonin. Furthermore, since the melatonin receptor antagonist, luzindole, blocked preadsorbed melatonin antibody (PMEL) binding but not melatonin antibody alone, I may have found a new method for receptor labeling (see Figure 4). This is yet to be confirmed through additional studies with verified receptor labeling, as demonstrated through verifiable methods such as autoradiography.

There are still many specific questions that may be answered to understand how melatonin affects testosterone production at the level of the gonads in Syrian hamsters. Lerchl & Nieschlag (1995) measured testosterone production from cultured Siberian hamster testes in hourly increments, but because it was only over the course of 24hrs, there is no definitive evidence for a circadian regulation. In Lerchl & Nieschlag (1995), testosterone levels were highest at the end of the light period and significantly decreased over the course of the dark period. The findings of the study presented here would support a mechanism involving melatonergic regulation of testosterone production. Future studies should also consider this change in testosterone production over the course of a day. Additionally, differences in gonadal structure (testes and ovaries) and the conversion of androgens into estrogens may also differentially impact reproductive and circadian activity (Widmaier & Campbell, 1980; Krizo & Mintz, 2016; Yan & Silver, 2016). Even social forces can influence these rhythms, such as dominance/submissive behavior observed for cohabiting male Syrian hamsters (Paul et al.,

2014). Although these diverse effects of melatonin on testicular development and function do not directly elucidate circadian differences in melatonin-synthesis and binding, studying the effects of melatonin on photostimulated testes provides insight into melatonergic mechanisms underpinning testicular response to seasonality.

Mounted Testicular Tissue

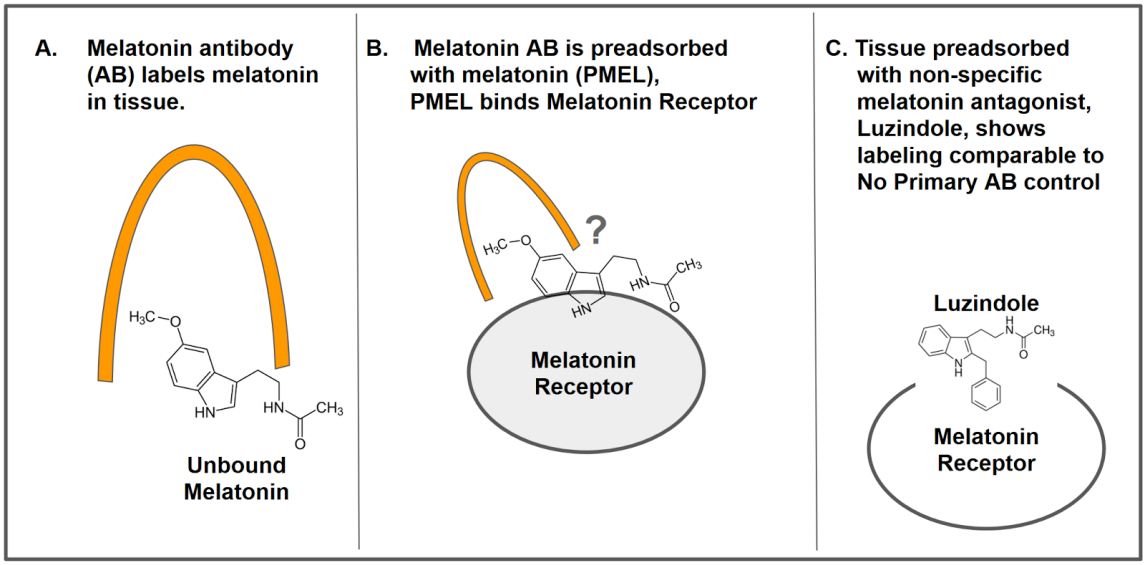


Figure 4 Proposed Mechanism for Melatonin Receptor Labeling via Preadsorbed Melatonin Binding. Because the specific binding epitope for the antibody has not been characterized (e-mail communication with vendor), A is a theoretical model based on these experimental observations. Three-dimensional quantitative structural-affinity analysis conducted by Spadoni & Bedini, 2007 hypothesized a mechanism for how melatonin binds to its receptors (subtypes MT1 and MT2), depicted schematically in B. Column C schematically represents how tissue preadsorption with Luzindole may block labeling as observed in Column B.

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Conclusion

Most of the melatonin research conducted for this dissertation has focused on comparative reproductive endocrinology. In Chapter 1, I investigated how variables of melatonin administration affect circadian rhythms and seasonal reproduction and how exogenous melatonin affects endogenous signaling in animals. In Chapter 2, I researched whether the avian hypothalamus has the capacity to differentially synthesize melatonin at nighttime and daytime in a manner analogous to the pineal. In Chapter 3, I shifted my focus to a photoperiodic breeding mammal, the Syrian hamster, to explore how reproductively active testes may detect and respond to the melatonin signal.

The findings of Chapter 1 are particularly relevant for a country in which melatonin, at various doses and release rates, is unregulated and sold over the counter to another animal species, *Homo sapiens*. In my introduction, I proposed guidelines for research on melatonin in seasonal reproductive endocrinology using Tinbergen's four questions on mechanism, ontogeny, adaptive value, and phylogeny. Here, I propose that another criterion must be considered: biological research in the sociohistorical context. My interest in melatonin as a chemical cannot ignore the role it plays in the ecosystem of the U.S. health market. I use this conclusion of my dissertation as an opportunity to explore how the science behind melatonin is interpreted to inform federal regulation on melatonin sales and distribution in the U.S. Furthermore, I propose means to better integrate scientific research and policy on melatonin, which lessons, hopefully, may be extrapolated to other dietary supplements.

A Brief History of Melatonin Regulation

In 1995, Richard J. Wurtman, the first author on the 1963 paper discovering that light controls melatonin, patented "Methods of Inducing Sleep Using Melatonin." Congress had just passed the Dietary Supplement Health and Education Act (DSHEA), which had amended the Federal Food, Drug, and Cosmetic Act to restrict the Food and Drug Administration's (FDA) authority to take action against manufacturers of dietary supplements (reviewed in Frankos et al., 2009). The law had defined "dietary supplements" to include "vitamins, minerals, botanicals, and amino acids." Melatonin is synthesized in edible plants (for reviews, see Manchester et al., 2000 and Tan et al., 2012), and so melatonin was categorized as a dietary supplement, and melatonin suppliers were subjected to lighter FDA regulation than drug and pharmaceutical distributors. Melatonin rapidly hit the American health store market (for historical overview, see Olcese, 2000) and was accompanied by marketing by scientists, such as Wurtman, and distributors via the media.

Melatonin Media

In the same year Wurtman patented "Methods of Inducing Sleep Using Melatonin," media hype on melatonin was on the rise, including but not limited to being pitched on the cover of *Newsweek* as a cure-all (Cowley, 1995). Although Fred W. Turek, who studied reproductive effects of melatonin in hamsters and birds, wrote an article responding to the melatonin hype in a timely manner (1996), Turek's commentary was published in *Nature*, which has different access, distribution, and readership than *Newsweek*. To address how the "melatonin craze" manifested, Turek wrote: "The short answer is simple: scientists went public…This is exactly what scientists

should be doing in the current climate of 'distrust of science' and at a time when public and government support of science is diminishing." However, Turek placed responsibility on a subset of scientists for "exaggerating the significance of a few selected studies." (1996)

It is difficult to disentangle the role of the scientists who appeared on talk shows and wrote books about melatonin from the uptake, interpretation, and presentation of the facade of "melatonin" by the media. Very much like the market of tangible goods, media has its own supply and demand. Melatonin marketed as a cure-all is incomparable to the melatonin that, under specific conditions, has acute physiological effects that may or may not lead to longevity and/or prevent senescence in the animal models studied. What we demand to watch on television, read online, and purchase at grocery stores supplants the supply of media provided. How can scientists present their findings in melatonin research in a world demanding a story on a cure-all? How can we shift the demand for media that accurately represents findings with thoughtful skepticism and scientific optimism? If Turek was correct in his description of melatonin media in 1996, and if his speculation remains relevant today, we have a lot to learn as scientists about public engagement. The veritas of the media and the gravitas of public health depend on science vis-à-vis education and policy

Considerations & Consequences: Let Sleeping Consumers Lie?

The DSHEA regulates dietary supplement primarily through labeling requirements. But melatonin labeling guarantees neither the safety of melatonin nor health benefits to consumers. Additionally, interactions of melatonin with other pharmaceuticals are not well studied, because melatonin is not regulated as a drug. With the passage of the DSHEA, Congress shifted the burden of proof: instead of distributors needing scientific evidence to demonstrate that a product is safe prior to distribution, it is the FDA's job to establish that a product is unsafe or mislabeled before it can be removed from the market (Harmon, 1996). Furthermore, the DSHEA shifted the criteria for mislabeling. Supplement labels alluding to "cure, mitigation, treatment, or prevention of any disease," previously categorized as drug claims, under the DSHEA, were subject to broader exceptions for informational pamphlets (Harmon, 1996). Therefore, FDA regulation of dietary supplements is limited to specific kinds of labeling. Only products with consumer accounts and/or empirical studies of detectable injury or toxicity may be subjected to FDA scrutiny (Harmon, 1996). Distributors, however, are still liable health risks due to melatonin itself or its preparation of their product. This legal risk may be incentive enough for companies to self-regulate melatonin preparation in order to maintain customer satisfaction.

Melatonin safety with long-term or chronic use remains unclear (Buscemi et al., 2004; Hampton, 2005), but the absence of evidence has not impacted the availability or marketability of melatonin to consumers. As with the media and all dietary supplements, the demand drives the supply. Consumers of melatonin reinforce an unregulated, free market of dietary supplements via purchasing power. The purity of processing and consistency of dosage within a bottle of melatonin will remain unchecked as long as consumers are unconcerned. The argument for increased FDA labeling requirements has been presented (Harmon, 1996), but whether or not consumers make decisions based on labels, reference citations found on labels (if findings are published in journals to which the consumer has access), or even read the labels cannot be ascertained. Harmon (1996) described the support of Congress for DSHEA to be rooted in concern for consumer empowerment, quoting S. Rep. No. 410 *supra* note 42: "consumers should be empowered to make choices about preventative health care programs based on data from

scientific studies of health benefits related to particular dietary supplements." Having additional information on a label, though it may not be read or fully understood by consumers, at least provides consumers a direct opportunity to engage with it. By circumventing FDA regulation and pressure for randomized controlled studies, distributors can offer dietary supplements like melatonin at affordable prices. Consumers participate in the free market, and the products that sufficiently meet consumer demands will flourish. However, the access to studies that require expensive journal subscriptions and the variability of educational opportunities that prepare consumers to evaluate "data from scientific studies" must be considered as federal regulation of dietary supplements changes or declines.

Melatonin Research, Education, and Regulation

How are consumers informed about the efficacy of melatonin based on scientific research? One outlet reviewing scientific research on the effects of melatonin supplements in humans is the Agency for Healthcare Research and Quality (AHRQ) of the U.S. Department of Health and Human Services. AHRQ set clear criteria for literature reviews investigating the effects of melatonin on normal sleepers and people with sleep disorders (Buscemi et al., 2004). The AHRQ meta-analysis concluded that, from the research to date, melatonin was not effective for treating sleep disorders or sleep disturbance resulting from jet lag or shift-work, but, "Melatonin is safe with short-term use." (Buscemi et al., 2004) Although the AHRQ meta-analysis offered a review of scientific literature intended for consumers (and was not itself a scientific review), there are strengths and weakness in the distribution and maintenance of AHRQ material. This particular AHRQ meta-analysis on melatonin is publicly accessible, archived online, and searchable, though it required a lengthy search through the FDA website to discover it. Because the AHRQ scientific literature review was conducted over a decade ago, the website has a notification that the conclusions do not factor in the most recent research. With more new chemicals on the market to review, and the lack of pharmaceutical investment in long-term randomized controlled trials on regular melatonin use, it appears that the recommendations of AHRQ to direct future melatonin research were archived along with the meta-analysis. Despite these challenges, consumers may be informed of the work of AHRQ through labeling practices, which may incentivize public support for both scientific research of dietary supplements like melatonin and the AHRQ services, which critically review and summarize this work.

How does the FDA regulate distributors of products containing melatonin? Currently, the FDA may issue warning letters to companies that misbrand their products. Misbranding includes using language that suggests that the product cures or prevents a disease, reading more like a drug than a supplement. Another form of misbranding includes adding dietary supplements, like melatonin, to products that are marketed as food. Adding melatonin to food categorizes melatonin as a food additive, subject to different regulations than dietary supplements. One may argue, however, that regardless of how we categorize melatonin, people are still ingesting the same chemical with comparable physiological properties (disregarding confounding variables, such as synergism, that may be occurring with other ingredients of the vehicle). What language is on the wrapper should not alter the physiological consequences of melatonin ingestion, but the language does matter to inform consumers about these potential consequences.

Finally, how does scientific research contribute to FDA regulation of melatonin? When melatonin is added to food products, it is evaluated by the FDA using criteria for food additives

to determine if it is "Generally Recognized As Safe" (GRAS). Below is an example of how the FDA referenced scientific research in a warning letter sent to a distributor in 2011 (see Appendix for References):

> "We know of no basis for general recognition of safety for melatonin based either on scientific procedures or common use in food prior to January 1, 1958. Melatonin is a neurohormone that is used for medicinal purposes, primarily as a sleep aid in the treatment of sleep-related disorders. In assessing the GRAS status of melatonin for use in a conventional food such as 'Lazy Larry,' we considered the criteria described above. FDA is not aware of data to establish the safety of melatonin for use as an ingredient in conventional foods. On the contrary, reports in the scientific literature have raised safety concerns about the use of melatonin. Among these are concerns about effects on blood glucose homeostasis (References 1- 4), and effects on the reproductive/developmental (References 5- 11), cardiovascular (References 12- 18), ocular (References 19- 21) and neurological systems (References 22, 23). Therefore, the use of melatonin in your 'Lazy Larry' product does not satisfy the criteria for GRAS status under 21 CFR 170.30."

Much of the same text from this previous letter was copied and pasted in a warning letter for another distributor in 2015, but the references (see Appendix) were not included in the online version:

> "We know of no basis for general recognition of safety for melatonin based either on scientific procedures or common use in food prior to January 1, 1958. Melatonin is a neurohormone that is used for medicinal purposes, primarily as a sleep aid in the treatment of sleep-related disorders. In assessing the GRAS status of melatonin for use in a conventional food such as 'Koma Unwind Liquid Relaxation,' we considered the criteria described above. FDA is not aware of data to establish the safety of melatonin for use as an ingredient in conventional foods. On the contrary, reports in the scientific literature have raised safety concerns about the use of melatonin. Among these are concerns about effects on blood glucose homeostasis (References 1- 4), and effects on the reproductive/developmental (References 5- 11), cardiovascular (References 12- 18), ocular (References 19- 21) and neurological systems (References 22, 23). Therefore, the use of melatonin in your 'Koma Unwind Liquid Relaxation' product does not satisfy the criteria for GRAS status under 21 CFR 170.30."

Upon review of the citations that are provided to substantiate the biological claims, one may notice the language used to describe and contextualize these findings in the warning letters raised concerns about melatonin that implied adverse effects. The papers cited show melatonergic correlations in models and/or methods that are difficult to extrapolate as broadly as depicted. Although the AHRQ meta-analysis previously described appears to inform the broader public of the science behind melatonin use and safety, and despite the fact that AHRQ is referenced on the FDA website, there appears to be a fruitful collaboration that serves to better inform distributors of the safety of their products, so they can better inform their consumers. If the FDA also provided distributors with information gathered by AHRQ, there would be less redundancy in the process. Instead, distributors are asked by the FDA to provide substantiation of their claims, and this substantiation may not even include evidence-based scientific research. The agency that is summarizing scientific research on dietary supplements for consumers, the AHRQ, may also share these summaries with distributors through the FDA to better perpetuate

scientifically informed labeling practices. Under the currently proposed federal budget, however, the U.S. FDA will not have resources to expand oversight or services in the foreseeable future. How may melatonin researchers better engage consumers, distributors, and governmental agencies to address the issue of sleep hygiene overall?

Melatonin affects multiple biological pathways ranging from reproduction to metabolism across vertebrates. Empowering consumers with knowledge about reproducible scientific research on the efficacy of melatonin may help them decide if trying a supplement or turning off the light is the safest approach to fulfilling their sleep needs. Anecdotally, many people are surprised when I tell them that light at night inhibits melatonin production. "I look at my phone and read when I can't fall asleep," some people respond. With increased urbanization and light pollution, blocking light at night is an additional challenge for urban dwellers. Intentional use of light to extend days at extreme latitudes to mitigate symptoms of Seasonal Affective Disorder (SAD) is yet another consideration in how light and melatonin affects our lived experience. While irregular sleep schedules affect individuals across socioeconomic classes, from jet-lagged businesspeople and politicians to shift-workers of countless professions, we cannot disregard other variables of these inequitable working conditions that compound the effects and jeopardize the safety of people who sleep inadequately.

I do not think melatonin researchers need to exaggerate the benefits of exogenous melatonin consumption, as Turek (1996) warned us nearly 20 years ago. We can work on disseminating information through the media with attentive care, contacting governmental agencies to learn more about improving educational and regulatory practices, and even engaging in daily conversations about the various factors that affect sleep hygiene to better understand the international predicament of irregular or unsatisfactory sleep quality. Melatonin research is just the beginning.

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Appendix

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