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Avian Circadian Organization: A Chorus of Clocks

Vincent M Cassone, Ph.D.
Department of Biology, University of Kentucky, Lexington, KY 40506, Phone: 1-(859)257-6766

Vincent M Cassone: Vincent.Cassone@uky.edu

Abstract

In birds, biological clock function pervades all aspects of biology, controlling daily changes in sleep: wake, visual function, song, migratory patterns and orientation, as well as seasonal patterns of reproduction, song and migration. The molecular bases for circadian clocks are highly conserved, and it is likely the avian molecular mechanisms are similar to those expressed in mammals, including humans. The central pacemakers in the avian pineal gland, retinae and SCN dynamically interact to maintain stable phase relationships and then influence downstream rhythms through entrainment of peripheral oscillators in the brain controlling behavior and peripheral tissues. Birds represent an excellent model for the role played by biological clocks in human neurobiology; unlike most rodent models, they are diurnal, they exhibit cognitively complex social interactions, and their circadian clocks are more sensitive to the hormone melatonin than are those of nocturnal rodents.

Each morning, and especially in the spring, we are greeted by a cacophony of small birds singing a dawn chorus. In eastern North America, spring mornings are sometimes defined by the merry roundelay of the American robin, *Turdus migratorius*, the varied staccato whistles of the Northern cardinal, *Cardinalis cardinalis*, the *hey-hey* of the white-breasted nuthatch, *Sitta carolinensis*, or even the cheery chirping of the introduced house sparrow, *Passer domesticus.* In the backdrop, we may hear the doleful *ooh-wah-hoo-hoo* of the aptly named mourning dove, *Zenaida macroura*, as the bass section above croons with the honking of migrating Canada geese, *Branta canadensis.* There is no particular order of who sings or who calls first, and the orchestration is peripatetic at best, seemingly random, although many of these garden songsters are reacting to each other's songs. And yet, there is a coordination of the rhythm and timbre of this dawn chorus. These birds all possess an internal biological clock that is coincidentally entrained to the identical environmental signal, the rising of the morning sun, and, in turn, these internal clocks are tuned to the expression of clocks by their intraspecific and extra-specific neighbors.

While these appear to be the melodious embrace of the warming sun, they are, in fact, a cold war, defining territory for breeding and foraging in anticipation of reproductive success [98,151]. In no other group of animals are the seasonal changes in reproductive function so obvious to the casual observer. We hear them stake their claims. We see them build their nests, incubate the eggs, and raise and fledge their young.

At certain times of year, small songbirds fatten for their annual migrations and, at certain times of day, dusk usually, become increasingly agitated as they gather for their vernal and

autumnal treks to breeding and wintering grounds [67,68]. These birds typically eschew their nightly drifts into slumber during this time, sleeping little or not at all, a phenomenon called *Zugunruhe*, as they migrate during the night, avoiding the gauntlet of diurnal predators as they cross vast areas of our continent.

Each of these processes and more are strictly timed to a time of day and to a time of year [31]. They are not restricted to eastern North America, either, as these processes are repeated time and time again throughout the world, albeit at different times of year, depending on the latitude and local environment [89,90,91]. The question that arises is, "Why do birds so strictly time so many of their behavioral and physiological functions, and how do they accomplish it?" In essence, the child-like question, "Why does the sparrow sing on spring mornings?", is also a scientific question that is beginning to be answered, and these likely entail an understanding of the biological clock or clocks that underlie all rhythmic processes. Specifically, understanding of the molecular, physiological and behavioral mechanisms underlying the temporal coordination of these complex processes and behaviors in birds will tell us more about human chronobiology as well, because like humans and unlike the standard laboratory rodent models for biological clocks, birds exhibit a complex orchestration of circadian behavior that controls daily patterns of sleep: wake, visual sensitivity, cognition and social behavior. Further, study of the mechanisms underlying annual cycles of reproduction, migration and metabolism in birds will provide clues to anticipated ecological changes due to climatic disruption. In essence, birds are images in our own mirrors, and we should pay attention to them more than current biomedical science might prefer.

Biological Rhythms and the Clocks that Control Them

Biological rhythms and the endogenous clocks that control them are fundamental properties of nearly all living organisms, ranging from cyanobacteria to humans [12]. As diverse as the organisms that express biological rhythms, the formal properties of these rhythms are remarkably conserved [124]. These biological rhythms are functionally tied to environmental cycles they estimate; of these, we will concentrate in this review on two- *circadian rhythms* and *circannual cycles*.

The daily 24-hour cycle of day and night imposes a rhythmic cascade of positive and negative selective pressures on nearly all organisms on Earth [124]. Daily light cycles provide the energy for photosynthesis, to warm water to a consistently liquid state and to maintain ambient temperatures for life. It also provides daily cycles in deleterious teratogenic, carcinogenic and desiccating wavelengths. It is therefore no surprise that most free-living organisms, if not all, have adapted to these cycles through the expression of endogenously generated circadian (*circa=approximately; dian = a day)* oscillations that entrain to local time through the process of *entrainment.* Rhythmic processes cannot be identified as *circadian* unless they are experimentally observed to persist for at least 1 or 2 cycles, preferably more, when the organism in question is experimentally placed in constant environmental conditions of either constant darkness (DD) or constant dim light (dimLL) (Constant high light, LL, may have other effects, frequently abolishing circadian rhythms altogether and/or damaging photoreceptive elements in the system [3]). In fact, many circadian rhythms persist for weeks, months or years in constant environmental conditions.

In this scenario, organisms will repeatedly express patterns of behavior, physiology or biochemical processes with a period, τ, of close to but rarely exactly 24 hrs (Figure 1). These endogenously driven rhythms must then be *entrained* to the relevant environmental cycle, typically the light: dark cycle (LD) of day and night, such that the internal phase, ϕ_i, of the organism's clock corresponds appropriately to the external phase, ϕ_e, of the LD cycle.

Thus, a diurnal bird's locomotor activity pattern entrains to the LD cycle so that activity onset ϕ_i corresponds approximately to dawn ϕ_e, maintaining a stable phase relationship, ψ_{ie}.

Similarly, annual environmental cycles correspond to *circannual rhythms* expressed by many organisms when placed experimentally in constant photoperiods of 12hrs of light and 12 hrs of dark (LD12:12) or another constant photoperiod [67,68]. Under these conditions many organisms, including birds, will express cycles of approximately 365 days. These in turn are believed to be entrained to the annual cycle by changes in photoperiod [67] and/or a physiological proxy, such as the duration of the hormone melatonin (see below). Circannual cycles are not as well understood as are circadian rhythms, but they are likely linked physiologically [69, 71].

The role of the circadian clock in annual cycles has been known for some time [24, 51,86,106, 130]. In many species of birds, exposure to photoperiods of longer than 11.5 hrs/ day results in the rapid induction of the hypothalamo-hypophysial-gonad axis, causing development and growth of testes and ovarian follicles. Although there are differences among species of birds and between birds and other taxa, neither the absolute length of the photoperiod, length of the scotoperiod (the duration of the dark phase) or their ratio is the proximal causes of gonadal induction. Rather, it is the circadian ϕ at which light impinges on photoreceptive elements that causes reproductive changes. For example, male Japanese quail, *Coturnix japonica*, and white-crowned sparrows, *Zonotrichia leucophys*, which are maintained in LD 6:18 will exhibit regressed testes. However, if the last hr. of the 6 hr. photoperiod is extended each long night to a specific "photoinducible phase", ϕ_{pi}, usually 11–12hrs following the onset of the short photoperiod, reproductive activity is commenced. Thus, birds exposed to LD 6:18 or L5: D1: L1: D17 (a single 1hr light pulse interrupting the night) will retain regressed gonads, but if the 1hr pulse occurs 5hrs later (L5:D6:L1:D12), gonads will recrudesce [48, 106, 131,132,161]. The same total amount of light (and dark) is present for each 24hrs, but the effect is dramatically different. It is the *timing* of light coinciding with an internal process that induces or prevents reproductive activity, suggesting a circadian clock underlies photoperiodic time measurement. Nanda and Hamner [116] had shown this to be the case in plants through an elegant series of experiments in which soy bean plants exposed to a 6hr photoperiod coupled with scotoperiods of varying lengths that were multiples of a 24hr cycle (e.g. LD6:18; LD6:42 or LD6:66) did not flower. However, when plants were exposed to 6hr photoperiods followed by scotoperiods of lengths that did not resonate with 24hrs (e.g. LD6:6; LD6:30 or LD6:54) flowering was observed. Similar studies in several species of birds showed that seasonal changes in both gonadal recrudescence and regression are regulated by a circadian clock [50,51,89].

These and other observations have led to two competing models for a role of circadian clocks in photoperiodic time measurement. In one, the "external coincidence model" proposed by Bünning [24] suggests that light has two complementary roles. First, light entrains the circadian clock with a stable ψ_{ie} such that the photoinducible phase, ϕ_{pi}, is also maintained with a approximately 11.5 hrs following the beginning of the photoperiod (lights on). When stable ψ light coincides with the ϕ_{pi} either because the photoperiod is long under natural conditions or through exposure to an experimental light pulse, the reproductive axis is induced. The competing notion, the "internal coincidence model" proposed by Pittendrigh [cf. 124], stems from the observation that many circadian systems behave as if they are composed of at least two oscillators, one entrained to dawn and the other to dusk. In the internal coincidence model the phase relationship between the dawn oscillator to the dusk oscillator, $\psi_{dawndusk}$, induces seasonal changes in reproduction. As we will see below, each of these models has support from different experimental systems in birds. However, until specific structures and/or molecules become associated with these models, they are essentially untestable at the physiological level.

Molecular Genetics of Circadian Clock Function

Circadian rhythms are regulated by a highly conserved set of genes, collectively called "clock genes", whose products are believed to dynamically interact to elicit rhythmic patterns of transcription, translation, biochemical and physiological processes, and behavior (Figure 2) [12,46]. In animals ranging from *Drosophila* to humans, the central core of this gene network can be broadly characterized as "positive elements" *clock* and *bmal1* and "negative elements" *Period 1 (Per1), period 2 (per2), period 3 (per3)* and the cryptochromes *cryptochrome 1 (cry1)* and *cryptochrome 2 (cry2).* In contrast to mammals, birds do not express a *per1* and have been shown to only express only *per2* and *per3* [5, 6, 156,157,163]. *Clock* and *bmal1* are transcribed and then translated in the cytoplasm, where they dimerize and reenter the nucleus and activate transcription of the negative elements through the activation of E-box promoter elements. The *pers* and *crys* in turn are transcribed and translated in the cytoplasm, where the PER proteins are targeted for proteosomal proteolysis by a series of protein kinases, most notably casein kinase 1ε (CK1ε) and CK1δ. This process slows the accumulation of the cytoplasmic PER and thereby increases the period of the molecular cycle. In the cytoplasm, PER and CRY proteins form oligomers that reenter the nucleus and interfere with the CLOCK/BMAL1-mediated activation. A secondary cycle involving two genes containing E-box promoters, *ReverbA* and *rorA*, amplify the cycle by activating and inhibiting *bmal1* transcription respectively. Disruption and/or knock-out of these genes' action has profound effects on the expression of circadian rhythms in animals in which these technologies are possible (i.e. mice and *Drosophila*) ranging from changes in period to arrhythmicity.

Unfortunately, these technologies are not routinely available in birds just yet. However, there are several observations that tentatively link clock gene expression with avian rhythmic behavior. Noting population studies in human populations of single nucleotide polymorphisms (SNP) in the *clock* gene's 3′-UTR have revealed differences in times of sleep and sleep duration, Steinmeyer et al. [137] have shown weak (5.9%) to moderate (46.7%) associations between awakening time of blue tits, *Cyanistes caeruleus*, with single nucleotide polymorphisms (SNPs) in *period2* and *CK1ε.* However, these studies were conducted in free-living tits using radio frequency transponders under semi-natural conditions, not under DD or dimLL, so that these effects cannot distinguish circadian clock effects from light-induced effects or effects on homeostatic regulation of sleep. Other studies have focused on allelic differences in C-terminal polyglutamine repeats in the CLOCK protein in breeding and migratory patterns of barn swallows, *Hirundo rustica*, suggesting negative selection on "deviant" genotypes [27]. However, other studies in several different swallow species in the genus *Tachycineta* failed to show similar associations [42].

Extraocular Photoreception

In addition to photoreceptors in the lateral eyes shared by all vertebrate classes, it has been known for some time that non-mammalian vertebrates express functional photopigments within the brain that are critical for entrainment of both circadian rhythms and circannual cycles. In birds, these reside in the pineal gland, the preoptic area, the lateral septum, and the tuberal hypothalamus. Early studies by Benoit in the 1930's showed that domestic ducks, *Anas platyrhynchos*, that had been blinded (enucleated) continued to exhibit reproductive responses to changing photoperiod [13], but work by Menaker and colleagues in the 1960's and 70's in passerine birds clearly showed that the eyes are not necessary for circadian entrainment [99,102] or seasonal control of reproduction [105,107,108,142]. In a classic series of experiments, Menaker's group demonstrated that enucleated house sparrows could entrain to a series of LD cycles of dimmer and dimmer illuminances. Once birds were no longer capable of entrainment, they showed that the responsible photoreceptor resided inside

the head by simply plucking feathers, and entrainment was reinstated. They then blocked entrainment by injecting India ink beneath the scalp [105].

Subsequent research has now identified at least 4 distinct structures within the brain that are functionally photoreceptive, containing several opsin-based photopigments and photoisomerases [4,53,94,99]. These include the pineal gland, which expresses a pineal-specific opsin, pinopsin [5, 99,100,120], as well as melanopsin (OPN4) [4, 5, 36] and iodopsin (OPN1) [5, 99] and whose photoreceptive function will be discussed further below. In addition, neurons within the preoptic area express VA (vertebrate ancient) opsin [38,39,135], and project to the tuberal hypothalamus, while the tuberal hypothalamus expresses a plethora of photoreceptive cells that appear to be divergent among avian species. For example, in domestic turkeys, *Meleagris gallopavo*, melanopsin-expressing cells in the premammillary nucleus (PMM) in the dorsal tuberal hypothalamus project directly to the median eminence [79,80,87]. In Japanese quail, *Coturnix coturnix*, CSF-contacting neurons in the mediobasal hypothalamus (MBH), which may be homologous to the PMM, express both OPN4 and neuropsin (OPN5) [111,112]. In house sparrows, neurons within the arcuate nucleus express rhodopsin (OPN2) itself, in addition to OPN4 and OPN5 [52,149]. While it is not clear whether or which opsin-based photopigments are expressed in the lateral septal organ, illumination of this area of cerebrospinal fluid contacting neurons elicits a physiological response [93,94]. Further, it is not clear whether each of these photoreceptive organs and/or their photopigments subserve mutually exclusive physiological processes or whether these overlap in their functions. In addition to opsin-based photopigments, all animals express flavin-based cryptochromes [145]. While cryptochrome is the major photopigment responsible for photoentrainment in *Drosophila* [47], the multiple cryptochromes expressed by vertebrates have not been established as photoresponsive molecules. Even so, in birds, the role of cryptochromes in celestial orientation is light-dependent (see below).

The Pineal Gland is a Master Pacemaker for Avian Circadian Clocks

Searching for the location of the intracranial, extraretinal photoreceptors, Gaston and Menaker [60] surgically removed the pineal gland (PINX) from house sparrows. While the birds retained their ability to entrain to LD, they became arrhythmic when placed in DD, demonstrating that the pineal gland is necessary for self-sustained circadian rhythmicity. However, the data also showed that the pineal gland is part of a system of circadian clock components, since PINX sparrows could anticipate the time of lights on in an LD cycle and because birds only gradually became arrhythmic over 5–15 days following transfer from LD to DD. Further, the effect of PINX is not universal among avian species. PINX of European starlings, *Sturnus vulgaris*, results in a range of behavioral changes ranging from arrhythmicity akin to those seen in house sparrows to slight disruption of behavioral locomotor rhythmicity [69]. Circadian rhythms of locomotor behavior in columbiform and galliform birds are little or not affected at all by PINX [45,143].

Even so, the pineal gland represents both the capacity for rhythmicity and time of day. In an elegant experiment, Zimmerman and Menaker [167] transplanted pineal glands from two groups of house sparrows into the anterior chambers of the eye of PINX, arrhythmic sparrows maintained in DD. The first group of donor birds were entrained to an early LD cycle, with lights on at midnight, while the second set of donors were entrained to a late LD cycle, with lights on at 11 AM. Transplantation restored circadian rhythms to both groups of recipients within one day. Moreover, birds that received pineal glands from early donors, exhibited an early ϕ_i while the recipients of late donor pineal glands exhibited a late ϕ_i. Thus, the pineal gland is not only necessary for circadian rhythmicity in these birds, but it contains a correlate that confers time of day to recipient birds.

Importantly, the data also suggested that the pineal gland must affect behavior through the secretion of a hormone, because one day is not thought to be sufficient for re-innervation of target tissues, wherever they are. That hormone was known even then to be the indoleamine melatonin from earlier work of Lerner and later of Axelrod, Klein and their co-workers [cf. 85], who explored the biochemical basis for melatonin biosynthesis in the pineal gland of the chick, *Gallus gallus domesticus.* Research from a large number of investigators have shown that pinealocytes, the photoreceptive, secretory cells of the avian pineal gland, take up the amino acid tryptophan, which is converted to 5-hydroxytryptophan by tryptophan hydroxylase (TrH; EC 1.14.16.4) [37] and then decarboxylated to produce serotonin (5HT) by aromatic L-amino acid decarboxylase (AAADC; EC 4.1.1.28). During the night in LD and subjective night in DD, 5HT is converted to N-acetylserotonin (NAS) by arylalkylamine (or serotonin)-N-acetyltransferase (AANAT; EC 2.3.1.87) [19]. NAS is then converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT; EC 2.1.1.4) [146]. The genes encoding each of these enzymes have been isolated, cloned and sequenced in several avian species. In chick, at least, TrH, AANAT and HIOMT are regulated by both the molecular clockworks within the pinealocytes and directly by light at the transcriptional, translational and post-translational levels, so that the enzymatic regulation of pineal melatonin is a dynamic, rhythmic process [85].

Avian pineal glands contain the circadian clockworks and photoreceptors to generate circadian patterns of melatonin biosynthesis *in vitro* as well as *in vivo*, which can be entrained to LD cycles directly [20]. Pineal tissue and pinealocyte cultures express circadian patterns of AANAT activity [20,83,148] and melatonin efflux [139] such that levels are high during the night and low during the day in LD. These rhythms persist for 4–10 days in DD before damping to arrhythmicity. Exposure to light has three effects on cultured pineal rhythms: 1) Light inhibits melatonin biosynthesis. 2) Light increases amplitude and decreases damping, and 3) light phase-shifts the clock within pineal cells [165]. *In vivo*, the avian pineal gland is innervated by post-ganglionic sympathetic nerves, and receives daily and circadian input through release of norepinephrine (NE) during the day and subjective day. Administration of NE to chick pineal glands *in vivo* and *in vitro* has two effects on pineal melatonin rhythms: 1) NE inhibits melatonin biosynthesis. 2) NE increases amplitude and decreases damping, but does *not* phase-shift the pineal circadian clock [28,32,166].

Using cDNA microarrays of chick pineal gland *in vivo* and *in vitro* and retinae *in vivo,* we have shown that many clock genes are expressed rhythmically in a fashion consistent with circadian patterns of clock gene expression in other model systems, such as *Drosophila* and mice [5,6,81]. *In vivo*, *bmal1*, *bmal2* and *clock* are expressed predominantly during late subjective day/early subjective night, while putative negative elements *per2* and *per3* (*per1* is not present avian genomes) are expressed during the subjective night to early subjective day. Interestingly, *cry1* and *cry2* are expressed during the early to mid-subjective day. In addition, transcripts associated with melatonin biosynthesis and photoreception are expressed rhythmically in patterns that are consistent with previous studies described above. Importantly, the 5′-flanking region of the chicken *aanat* gene contains an E-box [37], indicating the CLOCK-BMAL1 dimer may regulate *aanat* expression in the late subjective day/early subjective night. Transfection of COS cells with a reporter construct expressing luciferase and the chicken *aanat* promoter region in the presence of either chicken or human BMAL1 and CLOCK increases luciferase activity [37]. Mutation of the E-box region dramatically decreases luciferase activity, suggesting that binding of the *aanat* E-box by CLOCK/BMAL1 heterodimers is a critical component of the regulation of melatonin biosynthesis [37, 83]. Interestingly, in pineal glands, but not retinae, many transcripts associated with cytokine biosynthesis, immune function and lymphopoeisis are both highly and rhythmically expressed in chick pineal gland. These data are similar to those obtained by *in situ* hybridization in Japanese quail [156,157].

Although *in situ* hybridization for clock genes suggest that these genes are enriched in the pineal gland, retinae and other structures associated with clock function [5,6,152], quantitative real-time PCR for these transcripts indicate they are widely expressed in other parts of the brain and in the periphery. Daily and circadian patterns of *cry1, per3* and *bmal1* expression were observed in chick telencephalon, diencephalon and optic tectum in the brain, as well as in heart and liver. PINX and EX of chicks decreases the amplitude of these clock gene rhythms but does not completely abolish them [82].

Interestingly, the photoreceptors in the retinae of the lateral eyes also synthesize and release melatonin in many vertebrate species. In fact, in Japanese quail and domestic pigeon, *Columba livia*, the retinae release almost as much melatonin into the systemic circulation as does the pineal gland and removal of this source by enucleation (EX) or retinectomy in addition to PINX results in arrhythmic circadian locomotor behavior, similar to the effects of PINX alone in passerine birds [35,45,143]. Thus, the variability of the effects of PINX among birds may in part be due to this retinal component in some species and that it is not the pineal *per se* but rhythmic melatonin that is important for circadian locomotor behavior. To punctuate this view, rhythmic administration of melatonin to PINX house sparrows and European starlings or to EX/PINX pigeons [35, 71,74,96,150] restores a daily pattern of locomotor behavior. This synchronization of locomotor behavior by rhythmic melatonin administration represents entrainment of circadian clockworks in the PINX bird, since melatonin administration in a T-cycle different from 24hrs results in systematic changes in the phase relationship (ψ) of melatonin to the onset of locomotor activity [71,74].

Sites of Melatonin Action in Birds

In the 1980's and 90's, high affinity melatonin receptor binding using the radiolabeled agonist 2[^{125}I]-iodomelatonin (IMEL) [144] revealed high densities of IMEL binding in retinal, retinorecipient structures and visual integrative structures in the avian brain as well as peripheral tissues [43,129] (Figure 3). Binding affinity studies indicated kD's in the pM range with high specificity for melatonin itself. Brain structures that bind IMEL included retinorecipient structures in the visual suprachiasmatic nucleus (vSCN) of the circadian system, the ventrolateral and dorsal geniculate nuclei of the thalamofugal visual pathway, the optic tectum of the tectofugal pathway, and the nucleus of the basal optic root (nBOR) or the accessory optic pathway. In all species, integrative structures of the tectofugal pathway such as nucleus rotundus (Rt) and the ectopallium (Ep) also bind IMEL [33,129]. In some but not all species, hyperpallial structures, including the visual Wulst are sites of IMEL binding. In male passerine birds but not females, structures associated with bird song learning and control also revealed high affinity IMEL binding [58,153]. These will be discussed in more detail below.

Reppert and colleagues were able to isolate and clone genes encoding two high affinity melatonin receptors; these were designated the Mel_{1A} and Mel_{1C} receptors [128]. Independent work isolated partial sequences encoding an ortholog of the Mel_{1B} receptor in the same year [95]. Subsequent work has confirmed in birds that there are at least three melatonin receptors, the $Mel_{1A,}$ Mel_{1B} and the Mel_{1C} receptors [126]. Pharmacologists working with mammals have named the Mel_{1A} and Mel_{1B} receptors MT1 and MT2 receptors respectively [44]. However, mammals do not express the Mel_{1C} receptor sub-type, and the Mel_{1A} and Mel_{1B} receptors have not been fully characterized pharmacologically in birds. In this review, we will employ the original nomenclature, the $Mel_{1A,}$ Mel_{1B} and the Mel_{1C} receptors. All three melatonin receptor sub-types represent 7-transmembrane domain, GTP-binding protein structures and all three are in the G_i GTP-binding protein category, although some cross-talk with G_q has been documented [126]. The distributions of these three receptor sub-types are not uniform in chicks, zebra finches and house sparrows. The

Mel_{1A} receptor predominates in central nervous neurons and peripheral tissues [82,117], while the Mel_{1B} receptor is expressed in inner retinal neurons and photoreceptors as well as other central nervous neurons [117]. In passerines, the Mel_{1B} receptor is the major receptor sub-type in song control nuclei, but the other 2 are expressed as well [17,78]. The Mel_{1C} receptor, on the other hand, predominates in non-neuronal elements of the central nervous system [128]. Culture studies with chick astrocytes [2] show 95–100% diencephalic astrocytes express the Mel_{1C} receptor, while an overlapping 5–10% expresses Mel_{1A}. Astrocytes do not appear to express $Mel_{1B.}$ Intriguingly, neither IMEL binding nor strong melatonin receptor expression is present in the tuberal hypothalamus and/or hypophysis [33,128,129].

Avian circadian organization

Elimination of the pineal gland's photic and neural inputs results in damped circadian patterns of melatonin release, which also can be restored by rhythmic administration of light and/or NE [28, 139, 165, 166]. Conversely, removal of the pineal pacemaker in passerine birds at least results in damped rhythmicity in behavioral and physiological output, suggesting whatever is left represents a damped circadian oscillator capable of entraining to light [60]. Whatever is left is likely at least in part the avian homologue for the mammalian hypothalamic nucleus (SCN) [25,26], which is a master pacemaker for mammalian circadian organization [cf. 110].

In birds, two sets of structures have been associated with SCN function: the medial suprachiasmatic nuclei (mSCN) and the visual suprachiasmatic nuclei (vSCN) [25,26,30, 162]. These structures are connected via neuronal projections and are contiguous in terms of their cellular populations, especially in the distribution of astrocytes. The vSCN, but not the mSCN, expresses metabolic and electrical rhythmicity and receives retinohypothalamic (RHT) input. Further, the vSCN, but not the mSCN, contains melatonin receptor binding [33,96,128,129], and exogenous melatonin inhibits metabolic activity in the vSCN [96] but not in the mSCN. Finally, light activates *c*-fos expression in the vSCN, but not in the mSCN [84]. In quail, only the mSCN expresses clock gene rhythmicity [157,158], while in the house sparrow, both structures rhythmically express *per2* rhythms [1]. Lesions directed at the mSCN result in arrhythmicity similar to that observed following PINX [138]. The most likely scenario is that the functions subsumed by the mammalian SCN are regulated through the integration of both mSCN and vSCN.

Each component, the pineal gland, retinae and SCN, is integrated dynamically such that overt circadian organization is synchronized to environmental light cycles (LD) and such that internal processes are adaptively orchestrated [29,69]. Pineal (and retinal) melatonin, synchronized to LD cycles via endogenous photopigments, is secreted during the night and inhibits rhythmic metabolism and electrical activity of the vSCN. In turn, as the day approaches, oscillators within the pineal gland and retinae wane in their output, disinhibiting SCN activity. Oscillators within the mSCN and vSCN are active during the day, synchronized by LD via RHT input to the vSCN and possibly extraretinal input to the mSCN. One of the outputs of the vSCN at least is the rhythmic regulation of sympathetic activity, releasing norepinephrine (NE) within many peripheral targets. Among these is the pineal gland, where NE inhibits melatonin biosynthesis and release. It is not completely clear whether sympathetic NE synchronize circadian clockworks within the pineal gland, nor is it clear whether pineal melatonin affects clock gene expression in the SCN *in vivo*. However, *in vitro*, rhythmic melatonin administration synchronizes rhythms of both metabolic activity and the expression of both *per2* and *per3* [122].

Circadian Regulation of Visual System Function

The presence of dense, high affinity melatonin receptors within the retina, retinorecipient structures of all 4 visual pathways and visual integrative structures in the brains of multiple avian species [33], strongly suggests visual sensitivity, accommodation and more complex aspects of visual perception may be regulated on a circadian basis by melatonin. Circadian patterns in electroretinogram (ERG) and visually evoked potentials (VEP) recorded within the TeO show greater response amplitude during the day in LD and during subjective day in DD in both domestic chicks and pigeons [97,123,155]. In chicks and pigeons, the implicit time of the A-wave, which reflects photoreceptor activity, and B-wave, which reflects inner retinal activation, are greater during the night and subjective night than during the subjective day, while light sensitivity is greatest during the subjective night, reflecting the activity of dark-sensitive rods [123,155]. PINX of chicks reduces the amplitude of the circadian rhythm in ERG amplitude [101], and exogenous melatonin injections decreases ERG b-wave during the subjective day to levels similar to those described at night [101,123]. At this stage, it is not clear whether the effects of melatonin on avian visual system function directly influence physiological processes within visual system structures and/or regulate circadian clocks within the brain. The avian TeO expresses rhythms in clock gene expression in areas [156] superimposed with high affinity melatonin receptors [33], but the physiological link has yet to be made. Further, it is not clear how higher order visual function is affected by the circadian clock and melatonin.

Interestingly, this pattern of circadian regulation of visual system function, at least at the ERG level is nearly identical in humans with high implicit times during the night and high b-wave amplitudes during the day [72,119]. Further, administration of exogenous melatonin to human volunteers decreases b-wave amplitude during the day [59], similar to the situation in birds [101,123]. The role of melatonin in retinal physiology and pathophysiology is an emerging area of research [141], and birds provide an excellent model for human visual physiology in this regard.

Seasonal Cycles and Photoperiodism in Birds

Birds living in temperate zone latitudes generally restrict breeding to the spring and summer, maximizing the likelihood that young will be hatched during times at which food is plentiful [67,71,124]. As such, many primary and secondary sexual characteristics in birds undergo dramatic changes in both form and function. In the short days of winter, gonadal activity and gonad size regress and become inactive, while gonads recrudesce, becoming more active, in response to increased photoperiod. If birds are maintained in long photoperiod, their reproductive systems become insensitive to the photostimulatory effects of long photoperiod and spontaneous regress. This process is called photorefractoriness, and birds remain photorefractory until they are placed in short days for some time to make them photosensitive again [9,64,89,124].

In seasonally reproducing mammals such as hamsters and deer-mice, the nocturnal secretion of pineal melatonin is a critical signal that transduces time of year to the hypothalamo-hypophysial-gonadal axis to control reproductive function [11,63]. The duration of pineal melatonin biosynthesis faithfully reflects the duration of the scotoperiod; the duration is long during the long nights of winter and short during summer. These animals also exhibit a seasonal cycle of reproductive activity in which testes in males and estrus cyclicity in females increase as photoperiod increases in the spring. When photoperiod decreases, testes regress and females become anestrus. PINX in several species of hamsters prevents regression of gonads when the hamsters are transferred from long photoperiods to short photoperiods; their reproductive systems become blind to photoperiodic changes. When

melatonin is infused into PINX Djungarian hamsters, *Phodopus sungorus*, long durations of melatonin, simulating winter, induce gonadal regression, while short durations, simulating summer, enable recrudescence. The sites for melatonin's activity in this process are a combination of melatonin receptors in the SCN and in the *pars tuberalis* of the hypophysis. Therefore, the *circadian* control of pineal melatonin regulates the *annual* control of reproduction. This brief summary of mammalian seasonality is necessarily short in a review about birds; extensive reviews of these mechanisms can be found elsewhere [cf. 63,73].

Intriguingly, in spite of the fact that the rhythmic production of melatonin is critical for the expression of circadian locomotor rhythms in birds, melatonin does not affect seasonal changes in primary reproductive function in these species. As in mammals, pineal melatonin levels faithfully reflect the length of the scotoperiod both *in vivo* and *in vitro* [21, 23]. However, PINX and/or EX of several species of birds has little effect on seasonal changes in gonad size or activity [14, 90,131,132,133]. Moreover, administration of exogenous melatonin of different durations has little effect on primary reproductive function [34,104]. This corresponds to the relative absence of melatonin receptor activity in the tuberal hypothalamus and hypophysis [33], in stark contrast to the situation in seasonally reproducing mammals, where IMEL binding and melatonin receptor expression in *pars tuberalis* is a major site of melatonin action [63,73].

Yoshimura and colleagues have instead pointed to circadian clock function within the mediobasal hypothalamus (MBH) itself of Japanese quail controlling photoperiodic time measurement for reproductive function [161]. Earlier studies had shown that lesion of the MBH blocked testicular recrudescence in response to lengthening photoperiods and illumination of this area had resulted in in excitation of the tuberal hypothalamus and testicular growth [52, 91,113]. As stated above, the MBH of quail and the PMM of turkeys have been shown to express both OPN4 and OPN5 in cerebrospinal fluid (CSF) contacting neurons [80,112,113]. Noting that PINX or EX or even SCN lesion had little effect on photoperiodic regulation of gonadal function, Yoshimura's group identified rhythmic expression of the clock genes in the MBH and hypothesized that this structure contained the circadian pacemaker associated with photoperiodic time measurement [114,121]. Using differential subtractive hybridization, they found type 2 iodothyronine deiodinase (*Dio2*) to be induced in the MBH by a light pulse associated with long-day induction. *Dio2* encodes an enzyme that catalyzes the conversion of inactive thyroxine (T4) to active triiodothyroine (T3) [114,115,157,160]. Later, they showed that type 3 iodothyronine deiodinase (*Dio3*), which inactivates T3, was induced in the MBH by exposure to short days. The scenario they envision is that photoperiod is perceived by photopigments in the MBH that entrain a circadian oscillator within the MBH. In long days the circadian clock induces *Dio2*, while short days induce *Dio3*. Indeed, thyroidectomy induces gonadal recrudescence in starlings and Japanese quail [40, 49], and injection of T3 into the MBH induces quail gonadal growth [49,164]. These authors envision an external coincidence model in which circadian oscillators within the MBH are entrained by photoperiod co-localized in that structure. When the length of the photoperiod coincides with a ϕ_{pi}, *Dio2* is induced, enabling a metabolic cascade in response to T3 hormone, and gonadal induction occurs. It is not clear, at this stage, what molecular components link the circadian clock to *Dio2* or *Dio3*. The MBH of quail and the PMM of turkeys rhythmically express clock genes [76,77,92]. In humans, *Dio2* is regulated by CCAAT/enhancer-binding proteins, and *per2* is known to be a target of these transcription factors as well [61,140]. Perhaps, this link may provide clues to an analogous mechanism in birds.

Avian song production and perception

In parallel to primary reproductive processes, both the probability of a male bird to sing in response to a given stimulus as well as the size and complexity of the song control nuclei within its brain vary depending upon the time of year [40, 98,154]. The song control system of oscine passeriform birds is a specialized network of brain nuclei involved in singing and song learning. This system receives auditory input from ascending, primary auditory pathways beginning in the cochlear nuclei (Co), which project to the lateral dorsal mesencephalic nuclei (MLd). MLd in turn projects to the thalamic *nucleus ovoidalis* (Ov), which in turn projects to Field L in the forebrain. Song processing begins in secondary auditory areas in the caudal mesopallium (cM) and caudomedial nidopallium (NCM), which interact with the anterior forebrain pathway for song plasticity and learning. This pathway includes the HVC in the dorsal forebrain, which projects to Area X, whose projections form a loop between the dorsolateral thalamus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Then, both HVC and LMAN project to the robust nucleus of the archipallium (RA), which forms the song motor output pathway [7,8,118].

This system enables birds to process a complex species-specific identification of both self and con-specifics as well as other dynamics in birds' acoustic environments (competitors, prey, predators etc.). These acoustic environments as well as their reproductive and survival relevance are not constant. Territories change hands and the available range of mates fluctuates depending on the time of day or time of year. Auditory sensitivities to these signals must be tuned to appropriate con-specific and perhaps inter-specific signals. At the same time, the structure and behavior of song itself must be tuned to reproductively appropriate situations.

Birds from temperate zones in the short days of winter possess small HVC, RA, and depending on the species other structures in the system. When birds are photostimulated as photoperiod increases, the song control nuclei grow in parallel with the growth of the testes, and, as they become photorefractory, these structures regress in both size and complexity. Seasonal fluctuations in androgens and estrogens appear to be critical for changes in song control [7–9]. Song control nuclei contain both androgen receptors (AR) and both estrogen receptor sub-types (ERα and ERβ), as well as aromatase (AA), capable of converting androgens into biologically active estrogens. Further, the rate of male song in several songbird species increases when testosterone levels are high, castration decreases this rate, and hormone replacement reestablishes vernal song patterns. Multiple studies have linked the activity of gonadal steroids to both song behavior and regulation of song control nuclei, which have been reviewed extensively [cf. 9].

Even so, a few studies have indicated gonad-independent regulation of song control nuclei under different photoperiods. In both American tree sparrows and house sparrows, HVC and RA increase in size in response to a change in photoperiod from a short day to long day [18, 66, 154]. Castrated birds in these studies also exhibit photostimulated song control nuclei, although the level of induction is not as great as in the sham-operated birds. In the house sparrow study, castrated birds also showed a blunted photorefractory phase. Together, the data indicate that, while the song system certainly responds to the seasonal changes in gonadal steroids, regulation of song control nuclei comprises a gonad-independent as well as a gonad-dependent aspect.

Role of pineal melatonin in song control

The observation that song control nuclei express melatonin receptors points to a role for melatonin in song behavior and in the growth and regression of song control nuclei [58,

153]. Melatonin binding and Mel_{1b} receptor mRNA is affected by changing photoperiod, but not by castration [17, 154]. Bentley et al. [16] have shown that continuous administration exogenous melatonin attenuated the long-day-induced volumetric increase in HVC and also decreased the volume of another song-control nucleus, area X, in European starlings. This effect was independent of the birds' reproductive state. However, seasonal changes in binding and expression of Mel_{1b} receptor mRNA in Area X was affected by social conditions and laboratory captivity [17]. Further, Jansen et al. [78] have shown that neuronal firing in RA is decreased *in vitro* by administration of melatonin and that administration of the Mel_{1B} antagonist luzindole decreases song behavior in male zebra finches the next day. However, neither of these studies addresses the rhythmic role of melatonin in the control of song control nuclei and/or song behavior.

We have asked whether PINX of house sparrows and subsequent rhythmic administration of summer-like (short duration) or winter-like (long duration) melatonin affected song control nuclei HVC and RA [34]. All birds were PINX and placed in constant darkness (DD), whereupon they all became arrhythmic in locomotor behavior for 30 days. Birds that received no melatonin administration in DD remained arrhythmic, exhibited small, regressed testes and small regressed HVC and RA volumes. Birds that received either the short duration or the long duration melatonin entrained to the melatonin cycle but still exhibited small testes and small song control nuclei. Therefore, we repeated the experiment in intact birds maintained in constant light (LL). In this case, all birds became arrhythmic as above, but all birds exhibited large testes. They also exhibited vocal behavior. Birds that received no melatonin or the short duration melatonin cycle (who entrained to the regime) exhibited large HVC and RA, but birds that received the long duration melatonin entrained to the melatonin regime but showed regressed HVC and RA. This showed that melatonin affects song control nuclei independently of gonadal state, but does melatonin affect song behavior?

As stated above, application of melatonin to brain slices from zebra finches containing RA decreases RA firing rate, and administration of the Mel_{1B} antagonist lucindole affects song duration the next day [78]. Derégnaucourt et al. [41] recently have reported that PINX shortens the duration of song in an LD cycle. Recently, we have asked whether the pineal gland and melatonin cycles influence the daily and circadian pattern of song behavior in zebra finches [156]. In contrast to the Derégnaucourt et al. [41] study, we did not observe a shortening of the duration of song. However, we did observe effects of both PINX and melatonin under constant environmental conditions. Control birds expressed circadian rhythms of locomotion, song and call in constant dim light (dimLL) for up to 30 days. PINX birds' locomotion, song and call in dimLL gradually became arrhythmic, but they became arrhythmic at different rates, such that song became arrhythmic more rapidly than did either locomotor or call rhythms. When PINX birds were placed in a melatonin cycle of 10 hrs melatonin/24 hrs, they entrained all three outputs. However, locomotion entrained in 4 days, song in 15 days and calls took 26 days to entrain. When PINX and control birds were placed in LL, locomotor, song and call rhythms all became arrhythmic, although PINX birds became arrhythmic more rapidly. In sham birds, song became arrhythmic most rapidly, but in PINX birds locomotor behavior damped most rapidly followed by song and then call behavior. Interestingly, all behaviors in both PINX and sham birds were entrained to melatonin cycles in LL almost immediately. The bottom line for these studies is that locomotor behavior, song production and call behavior are all under the regulation of the circadian clock and pineal melatonin. However, they are regulated differentially, perhaps via separable circadian clocks.

Biological clocks, migration and navigation

In migratory birds, circannual and circadian rhythms play integral roles in both the timing of migratory behavior and the capacity for orientation during migration [66,68,88]. Endogenously generated circannual rhythms regulate the initiation of both the vernal and autumnal migrations. Many species of migratory birds, including sparrows, finches and warblers, maintained in captivity under natural photoperiodic conditions spontaneously exhibit two bouts of migratory behavior in which normally diurnal birds express nocturnal activity called *Zugunruhe* or "migratory restlessness" at the same times of year that coincide with natural migration [57,136]. When birds are maintained for more than a year in a constantly equinoctial photoperiod (LD 12:12), they express two bouts of *Zugunruhe* approximately 6 months apart, strongly indicating an internally generated temporal program produces these migratory behaviors.

There is some evidence that a circadian clock and pineal melatonin are important for the expression of *Zugunruhe* [91]. First, when migratory white-throated sparrows, *Zonotrichia albicollis*, *Sylvia* warblers and other migratory birds are placed in DD or dimLL, *Zugunruhe* is expressed periodically with a stable ψ with circadian patterns of diurnal activity (ψ_{zd}) [10, 102,103]. Secondly, PINX of white-crowned sparrows abolishes both the free-running τ of circadian diurnal activity but also the expression of *Zugunruhe* [103]. Thirdly, the pattern of melatonin secretion is altered during *Zugunruhe*. During periods of nocturnal migratory restlessness in the lab, the amplitude of nocturnal melatonin titers is reduced [54,56,70]. If garden warblers, *Sylvia borin*, are disturbed during *Zugunruhe* by feeding, for example, the melatonin amplitude rebounds [55]. If melatonin is administered to bramblings, *Fringilla montifringilla*, during *Zugunruhe*, nocturnal activity is suppressed [54].

At this stage, the site for the circadian oscillators responsible for *Zugunruhe* is not known. However, there is evidence that *Zugunruhe* is regulated by circadian oscillators that are separate from, albeit coupled to, the circadian clock system controlling diurnal locomotor behavior. Bartell and Gwinner [10] have shown that garden warblers placed in a skeleton photoperiod of 11.5D: 1L: 10.55D: 1L entrain their diurnal locomotor behavior with a 24 hr period. However, in some birds, *Zugunruhe* free-ran with a long τ, indicating that a separate circadian oscillator must control *Zugunruhe* vs diurnal locomotor behavior. The authors suggest that the internal coincidence model best explains the data. In this scenario, the circadian clock(s) controlling normal diurnal behavior maintain a stable ψ_{ie} with the photoperiod. Another clock or population of clocks associated with *Zugunruhe*, the hypothesis goes, is unexpressed unless and until their phase relationship with the circadian clocks controlling diurnal behavior coincide with the solstices. Then, *Zugunruhe* is induced. It is an intriguing idea that awaits the identification of specific structures or molecules associated with *Zugunruhe*.

Perhaps, one of the greatest mysteries associated with birds is their ability to navigate over great distances with remarkable accuracy during semiannual migrations or even in their day to day navigations within their home ranges [68]. There are many theories about avian orientation and navigation, including sensitivities to barometric pressures, olfactory cues and the Earth's magnetic field [152]. These are neither mutually exclusive nor unrelated [22]. One of the most enduring ideas is the sun compass or time-compensated navigation. Early work by von Frisch in honeybees has shown that bees learn to visit a food source at a particular time of day and convey that information to the hive via its famous dance [147]. Using this information, bees navigate to the food source by interpolating the position of the sun's azimuth with the bees' internal sense of time. The fact that an internal sense of time (*Zeitgedachnis*) was required for this navigation was demonstrated by shifting the light: dark cycles of the hive by 6hrs (or 90° of the 24hrs day) and showing that the bees consistently

make a 90° error in orientation. This capacity has also been demonstrated in several species of birds, including European starlings and domestic pigeons [75,88]. One question that has arisen from these studies was whether the internal sense of time was one and the same as the biological circadian clock. In an elegant series of studies, Hoffmann [75] showed that starlings maintained in constant dim light exhibited gradual shifting in the birds' orientation with a period equivalent to the period of their free-running rhythms in locomotion. These data suggested that the orientation clock and the circadian clock shared clock properties, but the studies were conducted before any neuroscientific or molecular components of the clock had been identified.

An intriguing link between the circadian clock and avian magnetoreception has been made by several physicists in theoretical quantum coherence properties of cryptochromes. As noted above, cryptochromes are both photopigments in circadian entrainment in insects but a component of the negative element arm of the circadian molecular clockworks [47]. In birds, *cry1* and *cry2* are expressed rhythmically in the photoreceptor layer of retina of the lateral eyes [6]. Cryptochrome photosensitivity derives from light activated transfer of an electron from a pterin cofactor to the flavin moiety on the cryptochrome, but this process has also been shown to be modified by changes in magnetic inflection and amplitude. In insect species such as *Drosophila* and monarch butterflies, *Danaus monarchus*, the ability to perceive changes in magnetic field orientation are both dependent on ultraviolet-A/blue light and the expression of *cry* [47, 127]. Intriguingly, if human *cry2* is expressed in *cry*-knockout *Drosophila*, magnetoreception is restored. Thus, it is possible that even humans have the capacity to detect magnetic fields [60, 127]. In European robins, *Erithracus rubecula*, and garden warblers, magnetic sensitivity is light-dependent with maximal sensitivity in the UVA/blue range, consistent with light sensitivity of cryptochromes [48, 122, 127]. At this stage, however, it is not clear that cryptochromes actually confer sensitivity to magnetic fields in birds nor is it clear whether the role of cryptochromes derive from their role as components of the molecular clock or whether the molecular clockworks are involved directly in time-compensated navigation [125]. It is, however, intriguing.

A chorus of clocks

This review began with an allegorical discussion of a dawn chorus of birds singing on a spring morning. In this allegory, some birds sing at their own pace, referring directly to their own biological clock entrained independently to the dawn. Others, the chickadees, perhaps, sing and call according to their own circadian clock but are also coupled to each other, responding to a conspecific just over the hedge. Still others, the jays, perhaps, respond to their clocks, their fellow jays and also to some of the other species of the garden, coupled to the community.

This is one way to envision the avian circadian system and perhaps vertebrate circadian clocks in general [65]. At the heart of it, all rhythmic processes are either entrained directly or indirectly to the photoperiod, the rising of the dawn sun. The central core of the avian circadian system, the pineal gland, the SCN and the retina, express robust molecular clockworks, but each component is not capable of self-sustained circadian rhythms. Instead, the pineal gland and retinae maintain SCN rhythmicity by inhibiting SCN output during subjective night through the actions of melatonin. These structures, being oscillators, wane in their outputs as dawn approaches, disinhibiting SCN output. We do not know if SCN activity affects retinal function, but it inhibits pineal melatonin biosynthesis during the day, and the system is maintained in a yin and yang mutually inhibitory relationship. Each of these core oscillators are also pacemakers and influence downstream processes in the brain and periphery via neuroendocrine (pineal and retinal melatonin) and neural (SCN modulation of sympathetic tone) output pathways. In turn, downstream processes possess

their own circadian clockworks and entrain their tissue-specific rhythms to one, the other or both outputs of the circadian system. In this way, the system orchestrates coordinated physiological processes that maintain efficient circadian activities (Figure 3). This is the core of the neuroendocrine loop model for avian circadian organization [29].

Complementing these processes, other circadian oscillators in the tuberal hypothalamus, song control nuclei, visual system and un-described structures associated with migration and navigation maintain stable ψ with the circadian system, responding to its output in differential fashion. Reproductive function appears to be independent, but song and visual system structures are clearly regulated by melatonin, entraining to its nocturnal signal. And thus, it appears to be a cacophony, but it is not. It is a symphony or a chorus at least, coordinated in some processes but independent in others, each tuned to the rising sun and the dawn.

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Highlights

- Biological clocks are fundamental to biological processes in birds, regulating many behaviors.
- The formal properties and molecular components of clocks are highly conserved.
- The core components of the clock interact to ensure stable phase relationships among them.
- The outputs of this system entrain downstream oscillators and processes.
- These processes include sleep, bird song, vision, migration and reproduction.

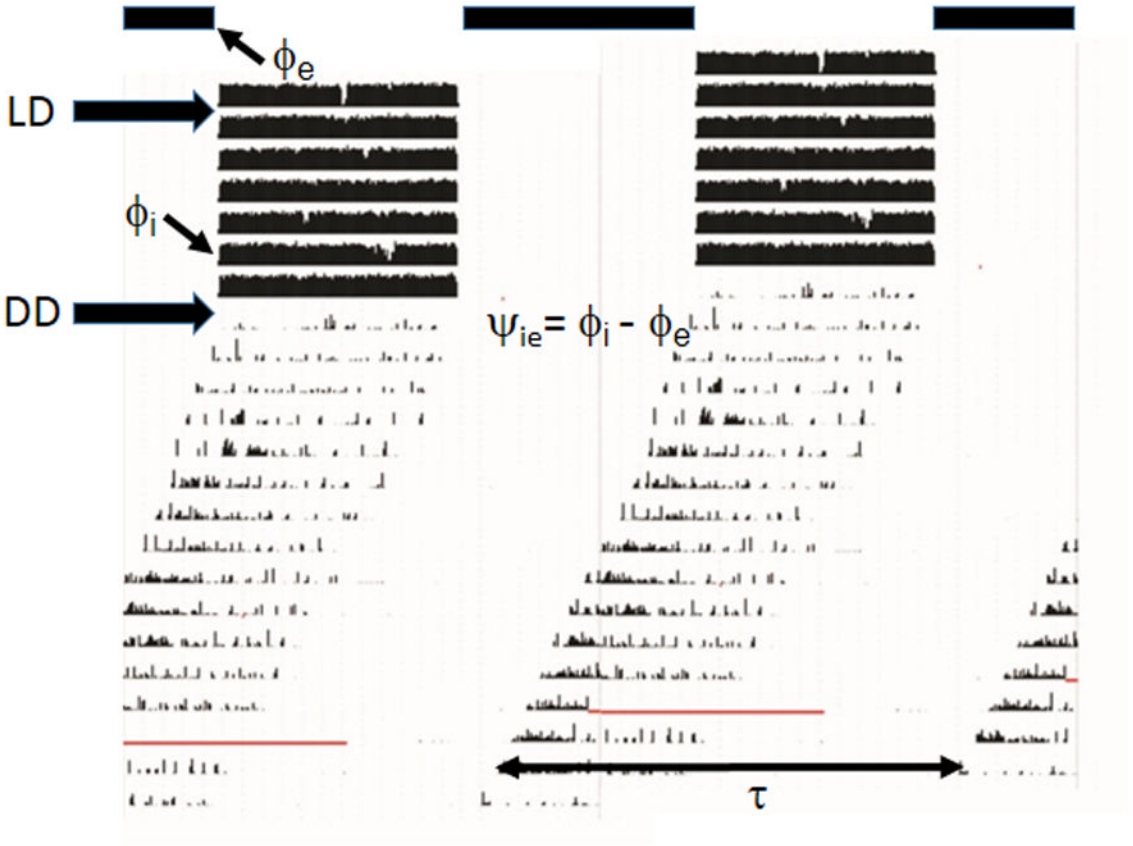

Figure 1.
Actogram of locomotor activity from a single zebra finch, *Taeniopygia guttata*. The top bars indicate the times during which lights are off (black) vs on (white). These are plotted in a 48hr timespan in order to "double-plot" the data. The time off lights on, ϕ_e, indicated by the arrow, is a phase reference determined by the investigator. The internal phase, ϕ_i, is determined to be the activity onset. The relationship between ϕ_i and ϕ_e is called ψ_{ie}. This relationship may change depending on the time of year and physiological condition of the bird. The internal period, τ, is indicated here as the average interval between activity onsets.

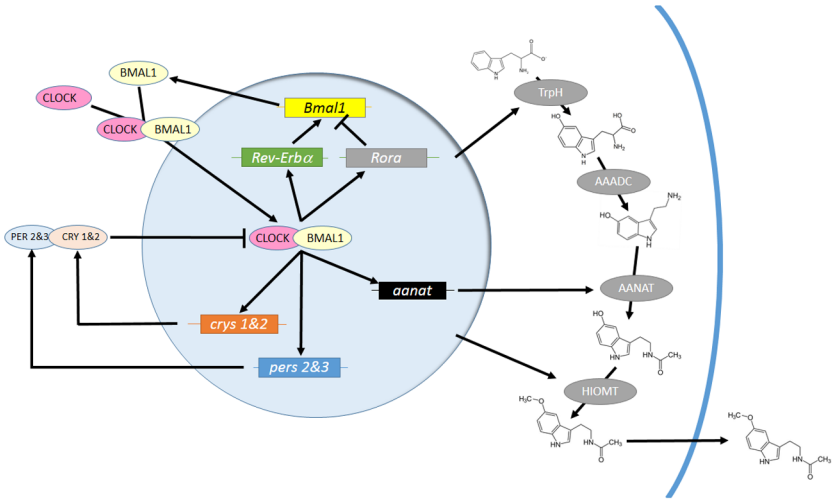

Figure 2.

Schematic of the molecular clockworks regulating circadian patterns of melatonin biosynthesis in a pinealocytes or retinal photoreceptor. Positive elements CLOCK and BMAL1 enter the nucleus and activate expression of genes whose promoters contain an E-Box. Among these are the negative elements *period 2 & 3 (pers 2&3)* and *cryptochromes 1& 2 (crys 1&2)*, Rev-Erbα and *Rora*, which form a secondary loop regulating *Bmal1* transcription, and output, clock-controlled genes such as arylalkylamine-N-actyltransferase (*aanat*). The *pers* and *crys* are translated, form heterodimers with other components, such as the casein kinases, and reenter the nucleus to interfere with CLOCK/BMAL1 activation. Melatonin biosynthesis pathways are indicated on the right. Amino acid tryptophan is converted to 5-hydroxytryptophan by tryptophan hydroxylase (TrpH). Aromatic amino acid decarboxylase (AAADC) then converts 5-hydroxytryptophan to 5-hydroxytryptamine (5HT; serotonin), Then, during the night, AANAT converts 5HT to N-acetylserotonin, a substrate for hydroxyindole-O-methyltransferase (HIOMT), which produces melatonin itself. Presumably, melatonin diffuses out of the cell at this time, although a release mechanism may exist.

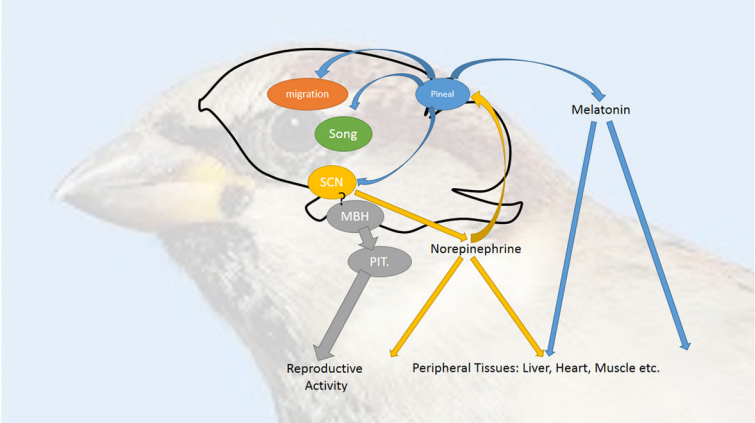

Figure 3.

A chorus of clocks. The coordination of circadian and circannual patterns of behavior and physiology is the composite of multiple circadian oscillators and their interactions. At the core are circadian pacemakers in the pineal gland and suprachiasmatic nuclei (SCN), whose interactions maintain each other's stability and self-sustainment through mutually inhibitory activity. Each of these pacemakers may influence downstream processes through entrainment of circadian oscillators controlling and residing in brain (e.g. song, vision and migration) and peripheral (e.g. liver, heart, muscle) function. In birds, regulation of primary gonadal activity has been separated from this circadian system with circadian oscillators residing in the mediobasal hypothalamus itself. There is little evidence that these oscillators are affected by pineal melatonin, but it is an open question whether SCM oscillators influence MBH function in the circadian and seasonal control of reproduction.