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—Review—

Seasonal Time Measurement During Reproduction

Keisuke IKEGAMI[1)] and Takashi YOSHIMURA[1–3)]

[1)]*Laboratory of Animal Physiology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[2)]*Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[3)]*Institute of Transformative Bio-molecules (WPI-ITbM), Nagoya University, Nagoya 464-8601, Japan*

Abstract. Most species living outside the tropical zone undergo physiological adaptations to seasonal environmental changes and changing day length (photoperiod); this phenomenon is called photoperiodism. It is well known that the circadian clock is involved in the regulation of photoperiodism such as seasonal reproduction, but the mechanism underlying circadian clock regulation of photoperiodism remains unclear. Recent molecular analysis have revealed that, in mammals and birds, the pars tuberalis (PT) of the pituitary gland acts as the relay point from light receptors, which receive information about the photoperiod, to the endocrine responses. Long-day (LD)-induced thyroid-stimulating hormone (TSH) in the PT acts as a master regulator of seasonal reproduction in the ependymal cells (ECs) within the mediobasal hypothalamus (MBH) and activates thyroid hormone (TH) by inducing the expression of type 2 deiodinase in both LD and short-day (SD) breeding animals. Furthermore, the circadian clock has been found to be localized in the PT and ECs as well as in the circadian pacemaker(s). This review purposes to summarize the current knowledge concerning the involvement of the neuroendocrine system and circadian clock in seasonal reproduction.

Key words: Melatonin, Pars tuberalis, Photoperiodic time measurement, Thyroid hormone, Thyroid-stimulating hormone

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Seasonal transitions between annual cycle stages force organisms living outside the tropical zone to adapt their physiology and behavior to the environmental changes that occur. Such adaptations include reproduction, migration, hibernation, molting, antler growth, rutting, courting, nesting, and parental behavior. The mechanism responsible for timing such transitions by using environmental cues is critical to understanding how animals adapt to environmental variations. Seasonal breeders adapt their reproductive cycles to specific seasons in order to maximize offspring survival. Hamsters and many birds, which have a gestation period of several weeks and breed during the spring, are called long-day (LD) breeders. Goats, sheep and deer, which have a gestation period of approximately 6 months, breed during the autumn and are called short-day (SD) breeders. For both types of breeders, the offspring are born during spring and summer, when food is abundant. These seasonal breeders use the predictable annual cycle of day length (photoperiod) as a calendar; this phenomenon is called photoperiodism [1, 2]. While photoperiod, temperature, and precipitation all show annual changes, photoperiodic changes are the most reliable seasonal environmental cue because of the stable annual cycle.

Seasonal reproduction is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) synthesized mainly in the preoptic area (POA) of the hypothalamus is secreted from the median eminence (ME) into the hypophyseal portal vessels. Secreted GnRH activates the secretion of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) from the anterior pituitary, regulating a seasonal development and regression of the gonads. In vertebrates, birds show drastic seasonal changes in gonad size, more than 100-fold, thereby contributing largely to our understanding of photoperiodic mechanisms. Among mammals, hamsters and sheep have often been used in studies of photoperiodism because of their drastic photoperiodic responses, although their seasonal gonadal changes are less dramatic than those of birds. The robust seasonal responses of birds may be related to their limited breeding season and the adaptations of birds to flight. In this review, we discuss the current understanding of the mechanisms regulating seasonal reproduction in birds and mammals and their relationship to the circadian clock.

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Correspondence: T Yoshimura (e-mail: takashiy@agr.nagoya-u.ac.jp)

Regulation of Photoperiodism in Birds

Among birds, the Japanese quail (*Coturnix japonica*) is a good model animal for understanding photoperiodism, because it shows a rapid response to changing day length [3]. Some studies have indicated that in quail, the mediobasal hypothalamus (MBH) is the center of seasonal reproduction for the following reasons: (1) lesions in the MBH suppressed photo-induced LH release [4, 5]; (2) local illumination in the brain induced testicular growth [6]; (3) electrical stimulation of the MBH led to LH secretion [7]; and (4) induction of c-Fos was observed under LD conditions [8]. Brief light pulses interrupting the long nights of SD conditions have also been demonstrated to induce a photoperiodic response [9]. The sensitive phase begins from 11 to 16 h after dawn, and therefore, we hypothesized that some molecular events must occur within the MBH in response to light stimuli. Using the MBH of a quail that

received a light pulse and no light pulse in the photo-inducible phase, differential subtractive hybridization identified induction of the type 2 deiodinase gene (*DIO2*) [10] encoding the thyroid hormone (TH)-activating enzyme that converts the prohormone thyroxine (T_4) to bioactive T_3 (Fig. 1) [11]. *DIO2* expression is upregulated under LD conditions and downregulated under SD conditions in the tanycytes of the ependymal cells (ECs) lining the ventrolateral walls of the third ventricle within the MBH [10]. In contrast, it was later found that expression of *DIO3* (TH-inactivating enzyme type 3 deiodinase), which converts T_4 and T_3 to inactive T_3 (reverse T_3: rT_3) and 3,3'-diiodothyronine (T_2), respectively, is upregulated under SD conditions and downregulated under LD conditions [12]. This switching may be responsible for the 10-fold higher concentration of TH in the MBH under LD conditions than under SD conditions, in spite of the constant level of plasma TH during both photoperiods. Intracerebroventricular (i.c.v.) T_3 administration under SD conditions induced testicular growth in a dose-dependent manner, while the administration of a DIO2 inhibitor under LD conditions blocked testicular growth [10], indicating the functional significance of locally activated TH.

It has been demonstrated that TH is important not only for the metabolism but also for the development and plasticity of the central nervous system [11]. In immunoelectron microscopy of the median eminence (ME) in quail kept under both SD and LD conditions, we found morphological changes between GnRH neurons and glial end feet [13] (Fig. 1). Under SD conditions, nerve terminals of the GnRH neurons were encased by the end feet of glial processes and did not contact the basal lamina, while under LD conditions, they seemed to be in close proximity to the basal lamina [13]. The administration of T_3 into the brain under SD conditions mimicked LD-induced morphological changes [14]. Neuroendocrine terminals must be in direct contact with the pericapillary space (i.e., the basal lamina) in order to secrete the neurohormone into the hypophyseal portal vessel [15]. It is also noteworthy that seasonal plasticity within the GnRH system is reported in the ewe [16]. These reports appear to support the hypothesis that the T_3-induced morphological changes between GnRH neurons and glial cells regulate seasonal GnRH secretion. In addition to the photoperiodic regulation of GnRH secretion by the morphological changes in the ME, seasonal changes in GnRH synthesis, mainly in the POA, are important for seasonal reproduction among birds such as starlings. However, because it has been reported that GnRH synthesis in quail is not seasonal [17], secretion of GnRH may be a more important event for seasonal reproduction than photo-induced GnRH synthesis in quail.

To clarify the mechanism involved in regulating the photoperiodic switching of *DIO2*/*DIO3* in the quail MBH, we used a chicken high-density oligonucleotide microarray (Affymetrix Chicken Genome Array) to perform genome-scale gene expression analysis during the transition from SD conditions to LD conditions in Japanese quail [18]. Analysis of more than 38,000 probes identified induction of thyroid-stimulating hormone β subunit (*TSHB*) and the transcriptional co-activator eyes absent 3 (*EYA3*) in the pars tuberalis (PT) of the pituitary gland at 14 h after dawn on the first LD (Fig. 2). *DIO2* was upregulated and *DIO3* was suppressed 4 h later in the ECs. Since EYA3, a transcriptional co-activator, was expressed in adjacent but different regions of the ECs, EYA3 does not appear to be

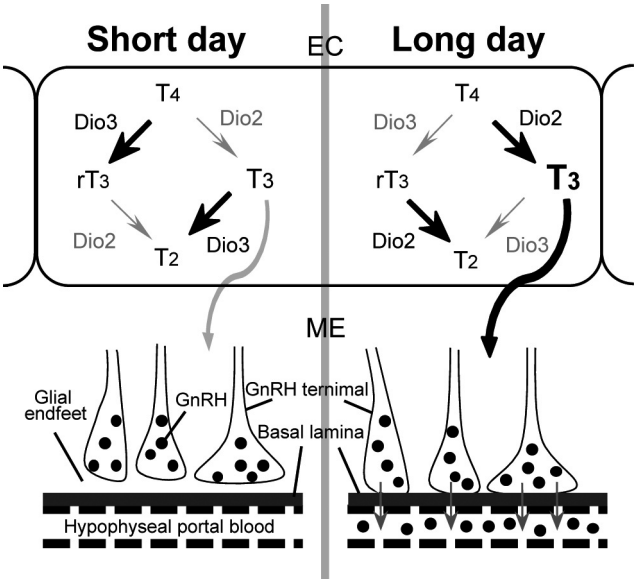

Fig. 1. DIO2 converts the prohormone thyroxine (T_4) to bioactive triiodothyronine (T_3) under LD conditions, while DIO3 metabolizes THs under SD conditions in birds and mammals. In quail, LD-induced T_3 appears to induce morphological changes in the GnRH nerve terminals and glial processes, thereby causing GnRH secretion into the hypophyseal portal blood. GnRH, gonadotropin-releasing hormone; LD, long-day; SD, short-day.

involved in *DIO2*/*DIO3* expression. Therefore, we predicted that PT-derived TSH (PT-TSH) may regulate the seasonal *DIO2*/*DIO3* switching. We also found the expression of TSH receptor (TSHR) and binding of ^{125}I-labeled TSH in the ECs. Administration of i.c.v. bovine TSH induced *DIO2* expression and testicular growth under SD conditions, while passive immunization by administration of anti-TSHβ antibodies suppressed LD-induced *DIO2* expression in the ECs. These data suggest that PT-TSH is a master regulator of seasonal reproduction in birds. Promoter analysis supported the involvement of the TSHR-Gsα-cAMP signaling pathway in this TSH-DIO2 process.

Regulation of Photoperiodism in Mammals

Photoperiodic regulation of *DIO2* and/or *DIO3* has also been demonstrated in mammals, including the Siberian hamster [19, 20], Syrian hamster [21, 22], rat [23, 24], goat [25] and sheep [26], as well as birds (e.g., tree sparrow [27] and chicken [28]). Local activation of TH by *DIO2*/*DIO3* switching within the MBH is also important for seasonal reproduction in both LD breeding birds and mammals, as daily subcutaneous (s.c.) T_3 injection induced testicular growth [29] and chronic infusion of T_3 into the brain prevented testicular regression [30] in Siberian hamsters. In sheep, T_4 administration suppressed the breeding activity via a decrease in serum LH [31, 32] and LD stimulation activated the TSH-DIO2 pathway [26]. It has been known for several decades that thyroidectomy blocks the photoperiodic response in a number of species, such as the sparrow [33], starling [34], and sheep [35]. The involvement of TH in

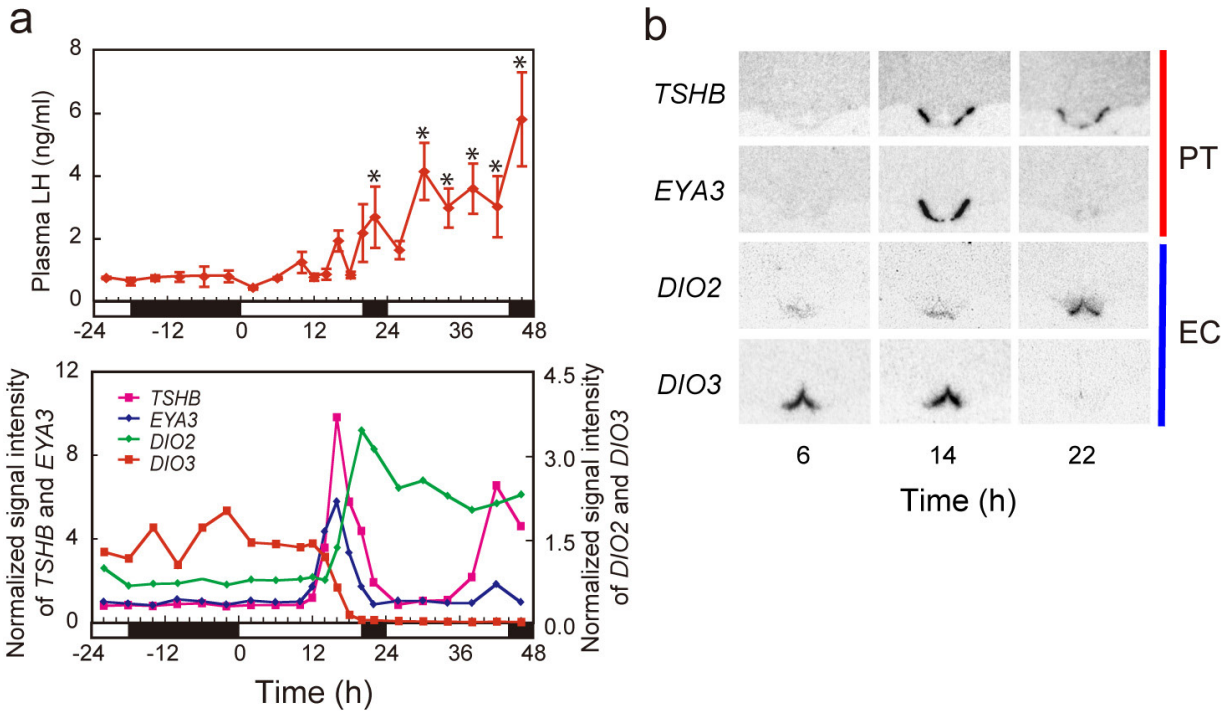

Fig. 2. Temporal expression profile of photoperiodic genes during the photo-induction process. (a) Long day-induced plasma LH concentration. Expression of first-wave genes (*TSHB* and *EYA3*) was induced within 14 h of the first LD after dawn, and expression of second-wave genes (*DIO2* and *DIO3*) occurred 4 h later. (b) The first-wave genes were expressed in the pars tuberalis (PT), whereas the second-wave genes were expressed in the ependymal cells (ECs). The time 0 h corresponds to dawn on the first long day. Modified from previous data [18]. LH, luteinizing hormone.

photoperiodism has also been suggested [36].

Melatonin is a hormone synthesized and secreted from the pineal gland during the night. It is responsible for detecting the length of the night, and it also plays a critical role in seasonal reproduction in mammals. For example, pinealectomy blocks seasonal reproduction, whereas melatonin administration mimics the effect of short days on reproductive function [37–39]. The mechanism of melatonin action on seasonal reproduction has been gradually uncovered. Reppert *et al.* have cloned 2 melatonin receptors (MT1 and MT2) in mammals [40, 41]. Since the MT1 receptor is reported to be expressed in the thyrotroph cells of the PT [42, 43], but not in the ECs [44, 45], melatonin appears to affect *DIO2*/*DIO3* switching via TSH in the PT. Although laboratory mice are insensitive to photoperiod, transgenic and gene-targeted mice are excellent models for understanding the molecular mechanisms underlying photoperiodic response. Arylalkylamine *N*-acetyltransferase (AA-NAT) and hydroxyindole-*O*-methyltransferase (HIOMT) [46] are rate-limiting enzymes in melatonin synthesis in the pineal gland. However, because most laboratory mice do not express these enzymes and cannot produce melatonin [47, 48], the photoperiodic response in melatonin-proficient strains and melatonin-deficient strains was analyzed. *TSHB*, *DIO2*, and *DIO3* expression changed photoperiodically in the melatonin-proficient CBA strain, whereas no response was observed in the melatonin-deficient C57BL strains [24]. Daily intraperitoneal (i.p.) administration of melatonin into C57BL mice induced an SD-like effect in these photoperiodic genes [24]. From these results, we concluded that mice are an excellent model for the study of molecular mechanisms of photoperiodic response at the gene level. Our group also found that TSHR-knockout mice did not respond to melatonin administration, suggesting that TSHR mediates melatonin regulation of the TSH-DIO2 signaling pathway. Examination of the effect of photoperiod and melatonin on mice lacking the MT1 or MT2 receptors identified the MT1 melatonin receptor as the mediator of melatonin effects on photoperiodic signal transduction [49]. Because LD-induction of *TSHB* in the PT has been also reported in SD breeder sheep [26], PT-TSH appears to mediate photoperiodic information in both LD and SD breeders, indicating the different mechanism of T_3 action between LD breeders and SD breeders.

Involvement of the Circadian Clock in Photoperiodism

In Japanese quail, it has been reported that testicular growth can be observed during the transition from SD conditions to photoperiods longer than 11.5 h [50]. In hamsters, day length greater than 12.5 h induces testicular growth [51, 52]. These photoperiods are called the “critical photoperiod” to induce the photoperiodic response. It is also known that light pulses during a limited time at night during SD conditions induce a photoperiodic response; this limited time is called the “photo-inducible phase” [8]. In resonance experiments in which house finch and quail were exposed to a day-length duration

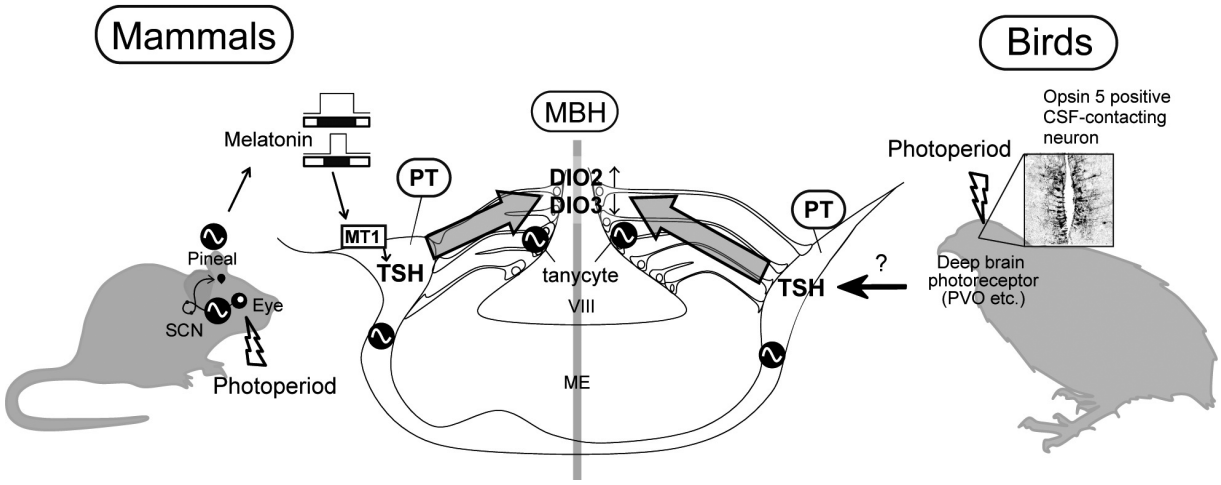

Fig. 3. Mechanism of photoperiodic signal transduction in mammals and birds. Melatonin mediates the transmission of photoperiodic information received by the eyes in mammals, while deep brain photoreceptor(s) (e.g., Opsin 5) directly receive light through the scalp in birds. LD-induced pars tuberalis (PT)-derived TSH acts on the tanycyte of the ependymal cells (ECs) to induce *DIO2* expression and reduce *DIO3* expression in both mammals and birds. TSH, thyroid-stimulating hormone.

of 6 h and varying duration of night length in multiples of 12 h (e.g., 6 h light [L], 18 h dark [D] cycle; 6 L 18 D, 6 L 30 D, 6 L 42 D, 6 L 54 D), light stimulus during the photo-inducible phase with a circadian-based rhythm induced testicular development [8, 53]; in even multiples of 12 h (e.g., 6 L 18 D, 6 L 42 D), every other light pulse induced a photoperiodic response, while in odd multiples of 12 h (e.g., 6 L 30 D, 6 L 54 D), the photoperiodic response was not observed because of light exposure in the insensitive phase of the rhythm of photosensitivity. Similar observations were also reported in golden hamsters [54]. These results suggest that 6 h of light pulse during the photo-inducible phase of the day in light cycles that are multiples of 24 h induces the photoperiodic response, indicating the involvement of the circadian clock in photoperiodic time measurement. Involvement of the circadian clock in the regulation of photoperiodism in fish [55] and reptiles [56] has also been reported.

Light Input Pathway for Seasonal Reproduction in Birds and Mammals

The only known photoreceptor in mammals is the eye, and removal of the eyes blocks the photoperiodic response [39]. The master circadian pacemaker is localized in the suprachiasmatic nucleus (SCN) in mammals [57, 58]. Photoperiodic, or light, information received by the eyes is transmitted to the pineal gland via the SCN [39, 46] (Fig. 3). In the pineal gland, melatonin is synthesized and secreted during the night and acts as the night-length signal as described above. Melatonin is secreted into the cerebrospinal fluid (CSF) in the third ventricle through the pineal recess, an evagination of the third ventricle [59]. However, it remains unclear whether melatonin secreted into blood or into the third ventricle CSF is more effective during seasonal reproduction. Because the SCN is required to generate nocturnal melatonin secretion profiles, SCN lesions also disrupt the photoperiodic response in hamsters. Hamsters with SCN lesions do not show gonadal regression regardless of short photoperiods [60, 61].

Functional photoreceptors in birds appear to be localized in the eye, the pineal organ and the deep brain. The master circadian pacemakers are localized not only in the SCN but also in the eyes and the pineal organ [62–65]. In contrast to mammals, disruption of these regions (removal of the eyes in ducks [66], lesions around the SCN [5] or pinealectomy [67] in quail) does not affect the photoperiodic response. In addition, the effect of melatonin on seasonal reproduction in birds differs from that in mammals. Melatonin has little effect on the photoperiodic response in avian gonads regardless of nocturnal secretion of melatonin in birds as well as in mammals [68, 69]. These findings suggest that the mechanism of seasonal reproduction differs between birds and mammals.

In birds, photoreceptors in the deep brain are involved in the reception of photoperiodic information. The injection of India ink under the scalp blocks testicular recrudescence [70], and light stimulation by implantation of an illuminant in the MBH or septal region of the telencephalon induces gonadal growth [6, 66]. Recent studies have shown the expression of several rhodopsin superfamily genes (melanopsin, VA opsin, and Opsin 5) in the avian brain [71–74]. Among these, Opsin 5, which is called neuropsin, is expressed in the CSF-contacting neurons within the paraventricular organ (PVO) in the hypothalamus and appears to respond to short-wavelength light (from UV to blue light), while melanopsin and/or VA opsin responds to longer-wavelength light (480 nm) than Opsin 5. Because Opsin 5-positive neurons project to the external layer of the ME adjacent to the PT in quail, it was predicted that light information received by the Opsin 5-expressing CSF-contacting neurons is transmitted to the PT, where it leads to partial or complete induction of TSH in the PT [73] (Fig. 3). However, the effects of Opsin 5 and other photoreceptors such as melanopsin and VA-opsin on the photoperiodic response in birds remain unclear.

Circadian Clock and Photoperiodic Time Measurement

It is well established that the transcription–translation feedback loop of circadian clock genes generate circadian rhythm [75, 76], although the existence of a circadian clock mechanism that lacks a transcription–translation feedback loop was recently suggested in some studies [77–79]. Clock genes are also expressed not only in the pacemaker(s) but also in other regions of the brain and in peripheral tissues [80–82], leading to alterations in physiology and behavior [83]. In birds, because the circadian pacemakers are not essential for the photoperiodic response, the existence of another "photoperiodic clock" has been suggested. Rhythmic expression of clock genes and proteins was observed in the MBH as well as in the master pacemakers [84, 85]. Although the photoperiod affects the temporal expression profiles of clock genes in the SCN and the pineal gland, in the MBH, these genes are stable under various photoperiodic schedules, and perhaps contribute to the stable photo-inducible phase in animals [84].

In mammals and birds, temporal expression patterns of circadian clock genes in the SCN change under different photoperiods [86–91]. It has been suggested that the photoperiod is encoded at the neuronal network level in the SCN [92, 93], and clock genes in the SCN detect seasonal time [89, 90, 94–96]. The "internal coincidence model" for photoperiodic time measurement, which predicts the existence of 2 internal oscillators with alteration of their phase relationship, has also been proposed [97]. Lincoln *et al.* suggested that this internal coincidence timer in the PT provides a potential mechanism for generating the photoperiodic response, because rhythmic expression of circadian clock genes was observed in the ovine PT and the phase relationship between the morning *Period (Per)* peaks and the evening *Cryptochrome (Cry)* peak changed among photoperiods [98, 99]. The expressions of clock genes in the PT are influenced by changing photoperiods in both birds and mammals [88, 91, 98, 100–102]. However, the involvement of the circadian clock genes and the internal coincidence timer within the PT in the photoperiodic responses of *TSHB*, *DIO2* and *DIO3* remains unknown. The circadian clock gene *Per2* is one of the most important clock genes [103–108] and is a component of the internal coincidence timer [98]. To examine whether *Per2* is involved in photoperiodic response, we generated melatonin-proficient *Per2*-deletion mutant mice by using the speed congenic method. Although the amplitude of clock gene (*Per1*, *Cry1*) expression was greatly attenuated in the SCN and the PT of *Per2* mutant mice, the expression profile of *Aanat* was unaffected in the pineal gland, and robust photoperiodic responses of the *TSHB*, *DIO2* and *DIO3* genes were observed. These results indicate that *Per2* is not necessary for photoperiodic responses in mice and that the internal coincidence timer in the PT is not a universal mechanism. Recently, it was also reported that LD-induced EYA3 appears to regulate *TSHB* expression in the PT through the circadian transcription factor thyrotropin embryonic factor (TEF) in mammals [109, 110]. Phase synchronization and direct suppression of *EYA3* expression by melatonin may be linked with the induction of *EYA3* expression in the morning under LD conditions to induce *TSHB* expression. This "external coincidence" timer [111] indicates the possible involvement of the circadian clock in the photoperiodic response in mammals.

Conclusion

Recent studies have uncovered that the PT is the most important regulatory relay point from photoreception to photoperiodic physiological changes in birds and mammals. In addition to birds and mammals, an anatomically distinct PT has been observed in reptiles and amphibians, but not in fish. Therefore, identification of the photoperiodic center in fish is expected in the future.

It is well established that the circadian clock is involved in the photoperiodic response in various vertebrates, including fish [55], reptiles [56], birds [8, 53] and mammals [54]. Recently, molecular mechanisms for the generation of circadian rhythms and photoperiodic signal transduction have gradually been understood. However, the mechanism of measurement of day length by the circadian clock (i.e., definition of the photo-inducible phase or critical photoperiod) remains unclear.

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