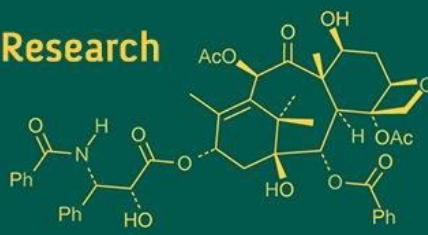


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Ovulatory cycle regulation in hens: The role of circadian rhythms

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Abstract

In hens, the reproductive cycle is governed by a complex interplay of circadian rhythms, hormonal regulation, and follicular development. The ovary exhibits a hierarchical structure, with daily ovulation and oviposition displaying well-characterized rhythms. Studies in chickens and quails reveal that gonadotropins, cell signaling, and the ovarian clock regulate preovulatory follicle development. Clock genes, such as *Per2*, *Cry1*, and *Bmal1*, play crucial roles in this process. The decentralized circadian timing system in birds, involving multiple pacemakers, ensures coordinated rhythmicity. Follicular development and maturation are controlled by follicle-stimulating hormone (FSH) and other factors, with granulosa cells exhibiting daily rhythmicity regulated by the suprachiasmatic nucleus (SCN). Progesterone induction of the pre-ovulatory luteinizing hormone (LH) surge requires estrogen presence. The oviposition-ovulation interval is sequence-length dependent, with shorter sequences exhibiting prolonged intervals. Melatonin, thyroid hormones, and gonadotropin inhibitory hormone (GnIH) also play significant roles in avian reproductive physiology. Melatonin receptors are expressed in ovarian follicles, and their administration increases receptor expression. Thyroid hormones influence egg laying, with decreases in triiodothyronine (T_3) and thyroxine (T_4) before ovulation. GnIH depresses LH and FSH release and expression. Adrenal cortical hormones, such as glucocorticoids, can influence ovulation, with dexamethasone blocking ovulation and premature ovulation induced by deoxycorticosterone, progesterone, and corticosterone. Understanding the intricate relationships between circadian rhythms, hormonal regulation, and follicular development is essential for elucidating the mechanisms governing avian reproductive physiology. Further research is needed to uncover the specific roles of individual pacemakers and hormones in regulating the reproductive cycle.

Keywords: Hens, ovulation, oviposition, circadian rhythms, follicular development

Introduction

Hens typically lay eggs in consecutive sequences, separated by a one-day pause. However, highly productive hens can lay eggs daily, potentially reaching 365 eggs per year. Most hens lay eggs during morning hours, with few exceptions after 3:00 PM. This process is accompanied by either a shortened interval between oviposition and subsequent ovulation or reduced egg residency time in the shell gland (18-25 hours). Egg formation requires 25 to 26 hours, and ovulation of the next egg in the clutch occurs 5 to 60 minutes after the previous egg has been laid. Research has demonstrated that extending the light-dark cycle from 23 to 25 hour enhances egg production (Foster 1968; Foster 1969) ^[11, 12]. This ovulatory cycle arises from intricate interactions within the hypothalamic-pituitary-gonadal axis, where the hypothalamus and pituitary collaborate to generate an LH surge (Fraps 1954) ^[14]. The oviposition (egg-laying) event serves as a reliable temporal indicator, allowing for retrospective prediction of the preovulatory luteinizing hormone (LH) surge and ovulation, which occur 15-30 minutes before egg-laying. The ovary plays a crucial role in regulating follicular maturation, with a mature ovum being essential for ovulation (Yoshimura *et al.* 1994) ^[42]. As follicular maturation nears completion, the pre-ovulatory F1 follicle increases progesterone production, triggering the luteinizing hormone (LH) surge. As a result, ovum release from the follicle depends on the LH surge. Investigations into the hen's ovulatory cycle have focused on the rhythmic oviposition patterns. The Collective opinion suggests that failed LH release underlies missed oviposition. However, hypotheses diverge regarding the locus of dysfunction within the hypothalamic-pituitary-gonadal axis, with proposed sites including the - Hypothalamic regulation (Fraps 1954; Fraps 1955) ^[14, 13] *et al*, ovarian

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follicular dysfunction (Bastian and Zarrow 1955) [4], oviductal influences (Nalbandov 1959) [31].

Studies have shown that the ovary possesses a circadian clock (Fahrenkrug *et al.* 2006; Karman and Tischkau 2006; Nakao *et al.* 2007; Yoshikawa *et al.* 2009) [10, 21, 30]. Additionally, the ovulatory cycle in both avian and mammalian species is subject to circadian regulation. The circadian clock regulates physiology and metabolism harmoniously with the 24-hour day-night cycle (Bass *et al.* 2010) [3]. Circadian rhythms, characterized by their persistence in the absence of external stimuli, typically have free-running periods approximating 24 hours under constant environmental conditions, such as continuous illumination. Under constant environmental conditions, these rhythms typically have periods close to, but often slightly less than, 24 hours (Bass *et al.* 2010) [3]. In birds, the central circadian system comprises three distinct oscillators: the pineal gland, retinae, and a hypothalamic region analogous to the mammalian suprachiasmatic nucleus (SCN) (Underwood *et al.* 2001; Kirsch *et al.* 2011; Karaganis *et al.* 2008) [36, 22, 20]. The precise impact of each pacemaker on the body's circadian rhythm remains unclear, but shared opinion indicates that master pacemakers, like the SCN and avian pacemakers, synchronize oscillators in peripheral tissues.

Circadian clock in the ovary

The ovary of chickens is a highly organized, multi-compartmental structure, displaying a strict hierarchical ranking of follicles (F1-F6), with one follicle ovulating daily (Karman *et al.* 2006; Fahrenkrug *et al.* 2006; Tischkau *et al.* 2011; Nakamura *et al.* 2010) [21, 10, 35, 28]. Nakao's study in quails showed that StAR mRNA expression exhibits a daily rhythm, paralleling Per2 expression, particularly in the largest follicle (Nakao *et al.* 2007) [30]. This suggests that gonadotropins, in conjunction with cell signaling and the ovarian clock, establish a hierarchical framework for preovulatory follicle development. A detailed investigation of clock gene expression in preovulatory follicles is essential to understanding the ovarian clock's function during follicular development, verifying circadian periodicity, and uncovering specific expression patterns across follicular subpopulations (Ball *et al.* 2007) [2]. Two significant factors make birds an exceptional model organism for studying the impact of biological clocks on female reproductive processes, they are 1. The ovulation-oviposition cycle in adult female birds exhibits well-characterized daily rhythms that can be readily monitored (Sharp *et al.* 1984, Silver *et al.* 1986) [33, 34]. 2. The anatomical structure and morphological properties of preovulatory follicles are established, and follicle size is used to classify developmental stages. Zhang *et al.* (2017) stated the physiological importance of rhythmic clock (Jasim HH *et al.* 2024) [44] gene expression in ovarian follicles, where FSH and LH play crucial roles. Specifically, FSH initiates clock development in immature granulosa cells (GCs), while LH synchronizes clocks in mature GCs. Notably, their findings revealed the spatial specificity in the localization of clock cells within hen preovulatory follicles, supporting the hypothesis that gonadotropins cue the development of functional cellular clocks in immature GCs.

Key molecular components governing circadian rhythm: PER, CRY, CLOCK, and BMAL

Although birds and mammals share similar molecular

clockwork components, their systemic circadian timing systems display notable dissimilarities. Mammal rhythmicity is predominantly regulated by a single central pacemaker located within the hypothalamic suprachiasmatic nucleus (SCN). Unlike mammals, birds have a decentralized circadian timing system, controlled by multiple pacemakers in the retina, suprachiasmatic nucleus (SCN), and pineal gland (Gwinner and Brandstatter 2001; Bell-Pedersen *et al.* 2005) [17, 5]. The exact contribution of individual pacemakers to overall circadian organization remains unknown. However, master pacemakers are recognized to synchronize oscillators in peripheral tissues, ensuring coordinated rhythmicity. At the heart of circadian physiology lies a molecular clock, characterized by interconnected transcriptional-translational feedback loops, which has been comprehensively reviewed in (Karman *et al.* 2006; Gras *et al.* 2012) [21, 16]. The transcription factors CLOCK (or its paralog NPAS2) and BMAL1 form a heterodimer, initiating rhythmic transcription of the period (*per1*, *per2*) and cryptochrome (*cry1*, *cry2*) gene families. Following translation in the cytoplasm, PER and CRY proteins associate, undergo phosphorylation by casein kinases (CK1 ϵ , δ), and translocate to the nucleus as modified PER:CRY complexes. These complexes then inhibit the activity of the CLOCK:BMAL1 heterodimer, completing the negative feedback loop. Over several hours, PER:CRY complexes are degraded, and eventually the CLOCK:BMAL1 complex is released from feedback inhibition. The precise timing of individual reactions is not well understood, but the complete process unfolds over a 24-hour period, setting the oscillator's cycle length. The 24-hour oscillatory period is conserved across tissues, but the timing of peak clock gene expression differs substantially between cell types and tissues with notable examples including early night peaks in lung tissue and midnight peaks in liver tissue (Johnson *et al.* 2002) [18].

Circadian rhythms in follicular development and maturation

There is a hierarchy of yellow (yolk-filled) follicles with follicles increasing in size to a maximum diameter of 2.5 cm due to their filling with yolk. There is also maturation of the granular and thecal cells. The largest follicle will be the first to be ovulated and then the next largest. A new follicle recruited on a daily basis. The recruitment of small (pre-hierarchical) follicles involves follicle-stimulating hormone (FSH) and other factors such as growth factors increasing FSH receptors by granulosa cells from pre-hierarchical follicles. Primordial follicles mature into primary follicles through a transformation of granulosa cells from flat to cuboidal. Viable primary follicles can remain dormant for months or years, then activate and form a thecal layer. Unlike mammals, the granulosa layer stays single-celled throughout development. The mechanisms triggering the transition from dormant to slowly growing follicles in birds remain poorly understood. Birds and mammals share a similar follicular selection process, where one follicle is chosen from a few growing follicles, dependent on FSH for continued growth. The preovulatory follicle cohort in laying hens consists of 6-12 follicles (6-8 mm diameter). In contrast to mammals, these follicles do not atrophy after selection, largely due to sustained FSH support. The mechanism by which photoperiodic signals from retinal and hypothalamic photoreceptors regulate avian ovarian activity

remains unknown (Kosonsiriluk *et al.* 2013; Nakane *et al.* 2010) [23, 29]. Photoperiodic signals synchronize ovarian rhythms with daily and seasonal cycles. Most body tissues, including ovarian cells (Chu *et al.* 2011) [9], exhibit circadian rhythms controlled by the suprachiasmatic nuclei (SCN) in the hypothalamus. Granulosa cells from preovulatory follicles in birds (quail, chicken) show a daily rhythmicity controlled by SCN signals and synchronized with the external photoperiod (Nakao *et al.* 2007, Tischkau *et al.* 2007) [30]. Research by Nakao *et al.* (2007) [30] found that BMAL1-CLOCK binding regulates STAR mRNA expression in chicken granulosa cells. They proposed that the timing of progesterone surge and ovulation relies on an LH/LHR/cAMP-responsive clock within the largest preovulatory follicle. Notably, a secondary rhythm governing follicular growth and maturation of the preovulatory (F1) follicle operates independently of the circadian clock, exhibiting a periodicity greater than 24 hours. Consequently, ovulation of the largest F1 follicle requires coincidence of both rhythms, resulting in a daily 6-10 hour 'open window'. The cellular mechanisms controlling the 24 hour rhythm of follicle maturation remain unknown. In contrast, research shows that undifferentiated granulosa cells in rats (Chu *et al.* 2012) [8] and quails (Nakao *et al.* 2007) [30] don't exhibit internal clock rhythms until they differentiate. Furthermore, FSH regulates clock gene expression in differentiating mouse ovarian cells (Chu *et al.* 2012) [8].

Timing of oviposition and ovulation

Normally, ovulation is triggered by a surge in LH and progesterone levels. However, some studies in turkeys found rare instances where LH and progesterone surges didn't result in egg laying (Liu *et al.* 2001a; Liu *et al.* 2001b) [24, 25]. The inter-surge interval for LH/progesterone increases during the late reproductive phase (Liu *et al.* 2002) [26]. A positive feedback mechanism operates wherein LH stimulates granulosa cells, particularly those in the largest follicle, to produce progesterone, which in turn enhances LH secretion. Progesterone induction of the pre-ovulatory LH surge is well-established (Rothchild and Fraps 1949; Wilson and Sharp 1975) [32, 38]. Progesterone triggers the pre-ovulatory LH surge, but only with estrogen present (Rothchild and Fraps 1949; Wilson and Sharp 1975) [32, 38]. In fact, progesterone only stimulates LH release in hens without ovaries if they've received daily estradiol doses beforehand (Wilson and Sharp 1976) [39]. The oviposition interval ranges from 24 to 27 hours in galliform species, specifically: chickens 24-27 hours (Attwood 1929) [1], and turkeys (26.8 hours Liu *et al.* 2001a; 26 hours Brady *et al.* 2019) [24, 7]. The inter-oviposition interval in laying hens is sequence-length dependent (Attwood 1929) [1], with shorter sequences exhibiting prolonged intervals. This interval encompasses the oviductal transit time and the period between oviposition and subsequent ovulation (Biellier and Ostmann 1960) [6]. Ovulation in laying hens is precisely timed, occurring approximately 30 minutes post-oviposition (Warren and Scott 1935; Melek *et al.* 1973) [37, 27]. Notably, the oviposition-ovulation interval exhibits photoperiodic independence, with durations of 24 minutes under 14L:10D and 36 minutes under ahemeral 14L:13D lighting conditions (Melek *et al.* 1973) [27]. Ovulation is induced 30 minutes post-oviposition, preceded by a surge in luteinizing hormone (LH) and progesterone levels approximately 6 hours prior.

Selective breeding under continuous illumination (24L:0D) has resulted in reduced inter-oviposition intervals in laying hens (Gow *et al.* 1985) [15]. At the same time, these hens exhibit decreased intervals between LH pulses. The initial LH surge of a sequence is precisely timed to coincide with scotophase onset in hens under 14L:10D photoperiod (Johnson and van Tienhoven 1984) [19]. Consistently, the pre-ovulatory LH surge in chickens occurs at scotophase initiation (Wilson *et al.* 1985) [40]. Phase-advancing scotophase triggers a moderate increase in circulating LH, though not sufficient to elicit a full surge (Wilson *et al.*, 1985) [40]. Oviposition and ovulation in chickens and turkeys (14L:10D) are restricted to an 8-10 hour diurnal interval, termed the 'open period'. The initial ovulation within a sequence commences 8-9 hours post-subjective dusk, with the final ovulation occurring 18-19 hours later. Notably, the LH/progesterone surge precipitating the first ovulation arises 3 hours into scotophase, while subsequent surges exhibit progressive delays, culminating 12 hours post-dusk.

Conclusion

The reproductive cycle of hens is a complex process regulated by the hypothalamic-pituitary-gonadal axis, involving intricate interactions between the hypothalamus, pituitary gland, and ovary. The ovulatory cycle is characterized by a daily rhythm, with oviposition (egg-laying) serving as a reliable temporal indicator of the preovulatory luteinizing hormone (LH) surge and ovulation. The ovary plays a crucial role in regulating follicular maturation, with a mature ovum essential for ovulation. The circadian clock governs physiology and metabolism harmoniously with the 24-hour day-night cycle. The ovary possesses a circadian clock, with clock gene expression exhibiting daily rhythms. Gonadotropins (FSH and LH) cue the development of functional cellular clocks in immature granulosa cells. The timing of progesterone surge and ovulation relies on an LH/LHR/cAMP-responsive clock within the largest preovulatory follicle.

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