

**Table 1.** Effect of mixing pyrite with urea on ammonia volatilization (AV) loss (mg N day<sup>-1</sup>) (urea applied at 56.6 mg N 100 g<sup>-1</sup> soil)

Prilled urea : pyrite	Days after incubation												Total in 12 days	AV loss as percentage of total added N
	1	2	3	4	5	6	7	8	9	10	11	12		
Urea (no pyrite)	1.28	7.75	2.43	1.26	1.00	0.39	0.42	0.42	0.44	0.37	0.37	0.35	16.48	29.34
1 : 1	0.65	7.30	2.31	1.26	1.07	0.35	0.35	0.35	0.30	0.25	0.25	0.23	14.67	25.93
1 : 2	0.21	5.27	2.17	1.09	1.00	0.32	0.25	0.25	0.28	0.21	0.18	0.21	11.44	20.22
1 : 4	0.07	3.43	1.98	0.82	0.53	0.28	0.21	0.18	0.18	0.16	0.18	0.18	8.20	14.49
LSD ( <i>P</i> = 0.05)	0.40	1.15	NS	0.19	0.32	NS	0.09	0.16	0.16	0.12	NS	0.12	3.46	–

increased and the differences between the successive levels of pyrites were significant after 1, 2, 4 and 5 days after incubation. From 7 days after incubation all the urea : pyrite ratios (1 : 1, 1 : 2 and 1 : 4) were at par and resulted in a significant reduction in AV loss compared to urea without pyrite. No differences among the treatments were noted after 3, 6 and 11 days of incubation, which is difficult to explain.

Total N loss through AV after 12 days of incubation was 16.48, 14.67, 11.44 and 8.2 mg N with urea (without pyrite) and 1 : 1, 1 : 2 and 1 : 4 urea : pyrite mixtures respectively. Reduction in AV loss due to 1 : 1 urea : pyrite mixture was not significant, but with urea : pyrite mixtures of 1 : 2 and 1 : 3, there was significant reduction in AV loss compared to urea without pyrite; the difference between 1 : 2 and 1 : 3 urea : pyrite mixtures was not significant. Expressed as percentage of total urea-N lost due to AV, it was 29.14 with urea without pyrite and the least (14.49) with 1 : 4 urea : pyrite mixture. Blaise and Prasad<sup>12</sup> also reported that AV with urea after 8 days of incubation was reduced from 27.5% with

urea to 8.9% when urea was mixed with pyrite in 1 : 2 ratio.

The results of the present study show that iron pyrites can be usefully employed for reducing AV losses from the fertilizer urea applied to farm fields. It will also be acceptable to the proponents of organic farming.

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## Vertebrate circadian clock research: Do experiments on birds and lower vertebrates promise better insights?

Most biological processes exhibit daily rhythmic changes. In the absence of environmental cues (e.g. day–night cycle), many of these changes persist with periods ( $\tau$ ,  $\tau$ ) close to 24 h – hence referred to as ‘circadian’ (*circa* – about; *dies* – daily) – reflecting the presence of an endogenous clock mechanism. A major focus of the vertebrate circadian clock research field over three decades has been to identify specific structures that contain the ‘circadian clock’. This includes identification of the suprachiasmatic nucleus (SCN) in the mammalian

hypothalamus (for summary and review see Rusak and Zucker<sup>1</sup>). Identification of additional clock structures included independent clocks in the eye and the pineal gland of fish, reptiles and birds, the eyes of marine molluscs (e.g. *Aplysia* and *Bulla*), and the lateral neurons of *Drosophila*. Thus, circadian clocks appeared to be highly localized cellular processes, and usually found in neural or neuronal structures.

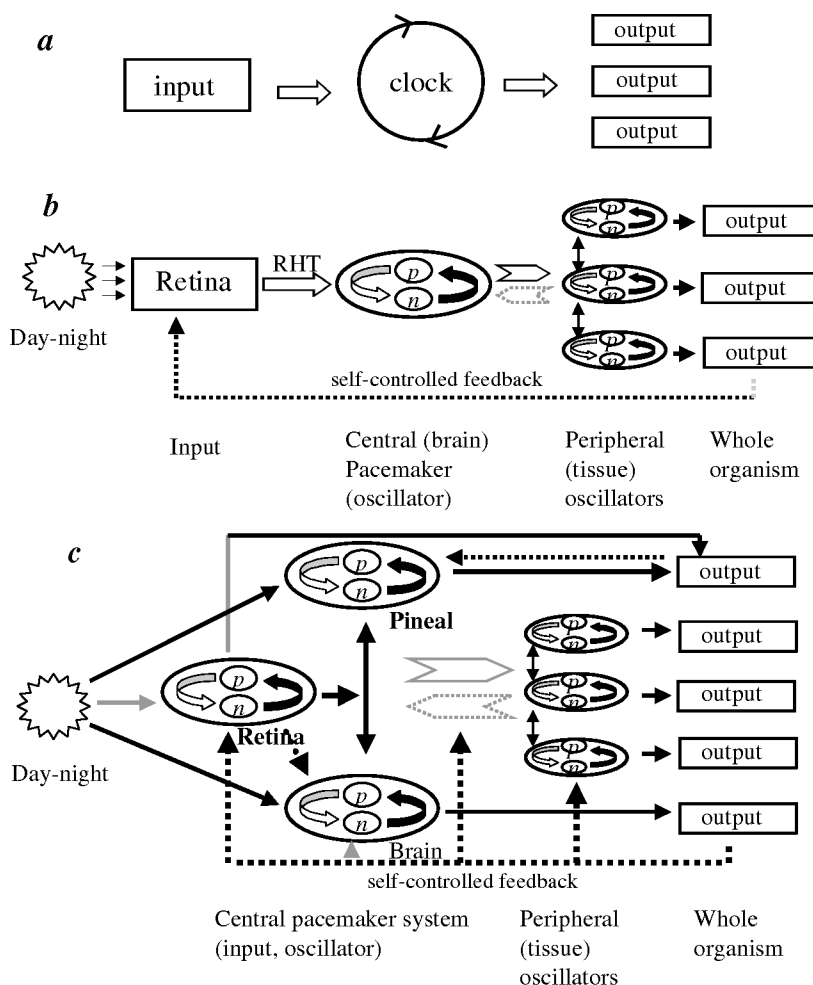
In the conceptualized framework, a clock system is shown with the oscillator at the centre, with afferents receiving input from

the environment, and with efferents to several outputs (Figure 1 a), although we now know that multiple input and output pathways as well as feedback loops exist in this circadian clock circuitry<sup>2</sup>. Relentless research efforts in the last 30 years on vertebrate clocks were directed to designate SCN as the central or ‘master’ clock. The ‘master’ SCN clock is supposed to set the ‘pace’ of several other clocks located elsewhere; this thinking gave rise to the ‘master–slave’ oscillatory concept of the circadian clock system. A refined version

of the ‘master–slave’ system was called the hierarchical organization of the circadian system wherein, for example, oscillator A affects oscillator B that in turn controls an overt rhythm. In both these concepts, the common theme is that temporal events of the organism are synchronized with the environment by zeitgeber (*zeit* = time; *geber* = giver; time cue from the environment – for example, light–dark [LD] cycle) inputs to the ‘central or master’ clock (Figure 1 *b*), which in turn controls the activity of the other clocks. For instance, in a mammalian clock system the self-sustained oscillations generated within the SCN are entrained by light via retina and neuronal pathways, and the entrained SCN oscillators set the phase and synchronize the timing of peripheral functions by regulated release of some humoral or hormonal factor<sup>3,4</sup>.

Giebultowicz *et al.*<sup>5</sup> challenged the above concept by providing evidence for an autonomous circadian clock in insect testis controlling the timing of sperm release. This pioneering discovery was not much appreciated then by many circadian biologists. In fact, early descriptions of circadian rhythms in isolated structures other than those connected with the brain were also forgotten<sup>6</sup>. The thinking that the circadian system was not centralized emanated from three research reports showing (i) an endogenous circadian clock residing within the cultured rodent retina<sup>7</sup>, (ii) a circadian rhythm in gene expression in a variety of isolated body parts of *Drosophila* using a *period-luciferase* model system<sup>8</sup>, and (iii) independent tissue clocks in rat organs using a *period-luciferase* transgenic animal<sup>9</sup>. Nonetheless, these findings did not challenge the core of central dogma that a ‘master’ clock is required for the expression of circadian rhythms in peripheral tissues. For example (i) the circadian oscillations measured in the rat liver, lung and skeleton muscle dampened out more rapidly than the SCN cell oscillations<sup>9</sup>, (ii) the peripheral clocks shifted less rapidly than the SCN clock<sup>8</sup>, and (iii) gene expression in peripheral tissues lagged by 3–9 h compared to that in the SCN<sup>10,11</sup>.

The findings of Yoo *et al.*<sup>12</sup> on mouse circadian rhythms using period 2 :: luciferase real-time reporting techniques clearly speak against the existence of ‘master–slave’ oscillatory concept or hierarchical organization of the circadian system. Their results indicated that isolated peripheral tissues of mouse show self-sustained circadian oscillations for at least 20 cycles, and the



**Figure 1.** *a*, Oversimplified conceptualized framework of a clock system with oscillator in the centre. *b*, A model of the mammalian circadian system showing hierarchical organization. *c*, A model of non-mammalian vertebrate circadian system showing multiple clock components. In both (*a*) and (*b*), there are feedback loops regulating circadian timing. *p* and *n* represent, respectively, positive and negative limbs of molecular feedback loops.

circadian oscillations retain tissue-specific differences both in the period and phase. Two most significant results of this study were: (i) Peripheral tissues continued showing circadian rhythms in *mPer2<sup>Luciferase</sup>* knockin mice in which SCN was lesioned. (ii) There was desynchrony of circadian oscillations among different peripheral tissues of an individual animal and from animal to animal, which suggested existence of synchronizers of circadian rhythms at the cell and tissue level.

Inadvertent omissions are natural, and science is no exception. One pertinent question is why discovery of the peripheral clocks became so much widespread only in recent years? First, there was general move to a greater ‘neurocentric’ view of physiology in the 1970s, and so early data on peripheral clocks ‘fell from favour’. Secondly,

before the discovery of the clock genes, clock-function traditionally could only be inferred by measuring oscillations in clock outputs or downstream clock-regulated processes. As one would now appreciate, the wheel running can be an excellent assay for the circadian function at organism level, but is not a relevant clock-output for tissues. Clearly, therefore, discovery of genes involved in the clock mechanism provided tools to look for common clock elements in a variety of neural and non-neural tissues. The identification and cloning of ‘clock genes’ made possible to look for expression patterns of clock components in isolated tissues directly<sup>13</sup>. The wide tissue distribution of these genes and oscillations in their transcript levels led to the discovery of autonomous peripheral clocks. Molecular and genetic attempts

to understand the clockwork (mechanism by which the clock produces rhythmicity), suggest that circadian rhythmicity is generated by autoregulatory feedback loops involving transcription of the 'clock genes' and post-translation activities of the 'clock proteins'. Nearly a dozen clock genes have been identified. Based mainly on the results from the mammalian models, the vertebrate pacemaker genes are thought to interact with each other in a complex circuit formed by at least two interlocked (Figure 1) autoregulatory negative feedback loops. Three *period* genes (*per1*, *per2* and *per3*) and two *cryptochrome* genes (*cry1* and *cry2*) comprise the negative limb of the loop. A *clock* gene (*clk*) and the gene-encoded brain-muscle Arnt-like protein 1 (*bmall*) form the positive limb of the loop. The positive and negative limbs of this molecular feedback loop are indicated by *p* and *n*, respectively in Figure 1 *b* and *c*.

When we have evidence<sup>12</sup> challenging the status of SCN as the primary pacemaker, the idea of hierarchical organization of the circadian clock system has no firm grounds. It is then important to consider whether vertebrate circadian system is actually a multiple pacemaker (oscillator) system. The first evidence that the vertebrate circadian system comprises more than one pacemaker comes from studies on birds. In the late sixties and early seventies, Michael Menaker's laboratory showed that the removal of pineal gland disrupts circadian rhythms in the locomotor rhythm<sup>14</sup> and body temperature<sup>15</sup> of house sparrow (*Passer domesticus*). The same laboratory further showed that (i) pinealectomised (pinx) sparrows implanted with pineal gland from another individual exhibit circadian phase of the donor individual<sup>16</sup>, (ii) blind (enucleated) sparrows free-ran under a LD cycle of dim light intensity to which sighted individuals would entrain, implying that eyes amplify the perception of light by the circadian system<sup>17</sup>, and (iii) lesion of a hypothalamic area severely impairs circadian rhythmicity in pineal-intact sparrows<sup>18</sup>. Later studies on house sparrows, measuring other circadian outputs (e.g. feeding<sup>19</sup>), and on other species<sup>19-21</sup> support conclusions drawn from initial sparrow studies<sup>13-17</sup>.

All three avian structures, retina, pineal and hypothalamus (avian equivalent of the SCN, medial SCN [mSCN] and visual SCN [vSCN]), contain self-sustained circadian pacemaker with their own input-oscillator-output systems<sup>22-24</sup>, and hence appear similar to the mammalian SCN<sup>25-30</sup>.

The important point, therefore, is to consider whether these self-sustained clocks constitute together the avian circadian clock system. If yes, then how are the component clocks interconnected? Isolated tissues of all three clocks (retina, pineal, and hypothalamus) show self-sustained cellular and molecular rhythmicity (express all putative clock genes of both positive and negative molecular limbs<sup>25-30</sup>), and so there is no inter-dependency as far as their intrinsic pacemaker properties are concerned<sup>25-31</sup>. However, as shown by the effects of the removal of one of the clocks, circadian outputs (e.g. circadian behavioural rhythms) suggest interdependence among the retinal, pineal and hypothalamic clocks. As noted in the preceding paragraph, removal of the pineal clock leads to arrhythmicity in several functions<sup>14-19</sup>. There were two other important observations in house sparrow studies<sup>14,15,19</sup>. (i) Pinealectomy abolished circadian rhythms when sparrows were kept in constant conditions (e.g. constant darkness, DD or dim light, LL<sub>dim</sub>) and freerun (i.e. expressed their endogenous rhythmicity with  $\tau = \sim 24$  h), but not when they were kept in LD and synchronized. (ii) Pinx sparrows did not become arrhythmic immediately; rather they had residual rhythmicity for 3-7 days. Interestingly, the effects of pinealectomy and enucleation on circadian rhythmicity were found variable in different species. In the European starling (*Sturnus vulgaris*), for example, pinealectomy impairs locomotor, but not feeding rhythms<sup>32</sup>. Pinealectomy has no effect<sup>33</sup>, but enucleation impairs circadian activity rhythms in the Japanese quail (*Coturnix c. japonica*)<sup>22</sup>. In pigeon (*Columba livia*), pinealectomy together with blinding, but neither of these alone, produces arrhythmicity<sup>34</sup>. Further, eye illumination alone entrained circadian melatonin rhythms in the Japanese quail<sup>35</sup>, but failed to do so in pigeon<sup>36</sup>. Also in pigeons, mSCN lesions produce a loss of expression of the circadian free-running rhythm, but not of their photoentrainment capability. The residual rhythmicity in mSCN-lesioned pigeons completely disappears in both LD and LL<sub>dim</sub> conditions, when they are also pinealectomized and enucleated<sup>26</sup>.

One obvious conclusion from all avian clock studies is that the retinal, pineal and hypothalamic circadian pacemakers closely interact by humoral and neural mechanisms<sup>37,38</sup> to constitute the circadian pacemaker system (Figure 1 *c*). The other conclusion from all the evidence accumu-

lated thus far<sup>20</sup> is that the role of hypothalamic clock is consistent in all birds<sup>33,34,39</sup>, but that of the retinal and pineal clocks is species-specific (e.g. retinae are the critical components of the circadian system of the quail, but not of the house sparrow<sup>17,20,22,33,34</sup>). It is not unlikely that there are other unidentified putative clock centres contributing to the avian circadian organization. Furthermore, there is increasing evidence for the presence of peripheral oscillators in birds, as in mammals<sup>4</sup>. Initial physiological evidence for a bird peripheral oscillator stemmed from the experiments on Japanese quail, suggesting that there was circadian melatonin synthesis in body tissues other than the retina and pineal<sup>40</sup>. This is also true in the case of pigeon<sup>41</sup>. At the molecular level also, Fu *et al.*<sup>42</sup> showed in the Japanese quail that *qcry1* mRNA levels were high in the heart and breast muscles, intermediate in the liver, testis and skin, and low in the lung. Further, *qcry2* mRNA appeared to be relatively abundant in the eye, intermediate in concentration in the lung and skin, and low in other tissues examined.

Besides daily rhythmic functions, many birds exhibit robust long-term (of several months to a year) rhythmicity in many behavioural and physiological functions<sup>20</sup>. These are called circannual rhythms ( $\tau = \sim 1$  yr). Some well-documented circannual rhythms in birds are those of food intake, body mass, gonadal growth and development, moulting, etc. Circannual rhythmicity continues to be expressed under LL or LD conditions<sup>43,44</sup>. Gwinner's laboratory<sup>44</sup> had records extending for many years of circannual rhythms in the testicular and moulting cycles of stonechats (*Saxicola torquata*). Whether generation of the circannual rhythms is a functional consequence of the circadian system, or the circannual timing system is independent of the circadian system, is unclear. The anatomical site of the avian circannual clock is also completely unknown. Furthermore, we have no evidence whether several circannual rhythms are generated from the same set of oscillators or from separate oscillators. Therefore, it is interesting as well as challenging to find the physiological and molecular bases of the circannual clocks, and birds are ideal subjects of research for this, given their distinct circannual physiological functions<sup>44</sup>.

Including the evidence available from lizards<sup>45-47</sup> and other vertebrates<sup>48</sup>, it appears that the central clock system in non-mammalian vertebrates comprises multi-

ple inputs, pacemaker sites, and outputs within the brain and connected to the brain which appear to function as a 'circadian loop'<sup>19,21,40</sup>. It is possible that peripheral clocks in these non-mammalian vertebrates function in the same way, as do the central clocks. Therefore, ignoring much of the clock research on non-mammalian vertebrates, and the research hype for many years on the mammalian system appears to have paid lesser dividends for vertebrate circadian clock research. Should we then consider non-mammalian vertebrates as attractive models to understand the biological clock system in vertebrates? To my mind, experiments on birds equipped with circadian and circannual clock systems (and many diurnal migratory species becoming also intensely active during the night at two different times of the year, crossing several latitudes and, in some cases several time zones) may provide better insights into the biological timing mechanisms in vertebrates. There may be certain disadvantage of not having knock-outs among birds and in many other non-mammalian vertebrates, but the species diversity is so enormous among these groups that we can find enough models to answer relevant questions and understand better the biological timing system.

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