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Neuronal and endocrine mechanisms underlying the circadian gating of eclosion: insights from *Drosophila*

Christian Wegener[1,*], Emad Amini[1,#], Javier Cavieres-Lepe[2,$,†] and John Ewer[2,3,‡]

The circadian rhythm of adult emergence (aka eclosion) of the fruit fly *Drosophila* is a classic behavioural read-out that served in the first characterisation of the key features of circadian clocks and was also used for the identification of the first clock genes. Rhythmic eclosion requires the central clock in the brain, as well as a peripheral clock in the steroidogenic prothoracic gland. Here, we review recent findings on the timing and neuroendocrine coupling mechanisms of the two clocks. These findings identify rhythmic prothoracicotropic hormone and downstream ERK signalling as the main coupling pathway and show that the two clocks impose daily rhythmicity to the temporal pattern of eclosion by regulating the timing of the very last steps in metamorphosis.

Addresses
[1] Julius-Maximilians-Universität Würzburg, Biocenter, Theodor-Boveri-Institute, Neurobiology and Genetics, Am Hubland, 97074 Würzburg, Germany
[2] Centro Interdisciplinario de Neurociencias de Valparaíso, Universidad de Valparaíso, Valparaíso, Chile
[3] Instituto de Neurociencias de Valparaíso, Universidad de Valparaíso, Valparaíso, Chile

Corresponding authors: Wegener, Christian (christian.wegener@biozentrum.uni-wuerzburg.de), Ewer, John (john.ewer@uv.cl)
[*] 0000-0003-4481-3567
[#] 0000-0002-4865-0737
[$] 0000-0002-6156-9589
[†] Current address: Departments of Psychiatry and Neuroscience, Chronobiology and Sleep Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA.
[‡] 0000-0002-6806-3628

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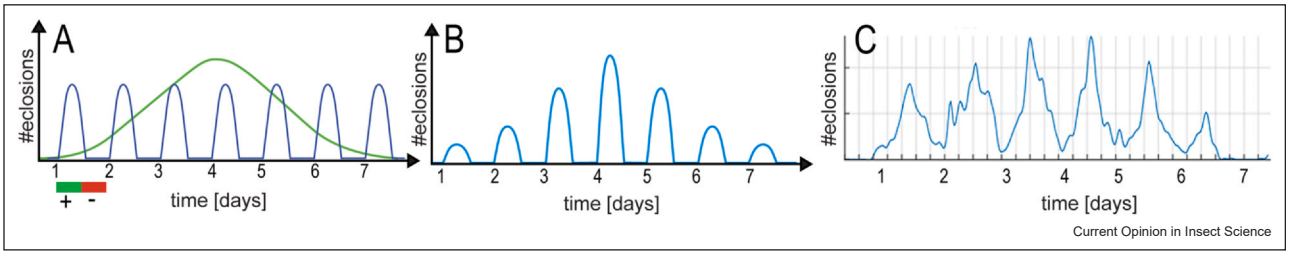
Introduction

The timing of developmental transitions depends on the progress of the underlying developmental processes that occur before the transition. In insects, the steroid hormone ecdysone acts as a master regulator that times development and developmental transitions [1]. This steroid hormone is produced by the prothoracic gland (PG), an endocrine organ that is a key decision-making centre for the timing of the moults [1] that integrates diverse nutritional and integrity inputs via various neuroendocrine signalling pathways as well as autocrine ecdysone feedback [1–3]. The integration of these multiple regulatory signalling pathways provides a mechanism [4] that ensures that developmental transitions are initiated only when the required developmental, metabolic, and environmental conditions are met and, in some cases, that these transitions occur at the right time of day. Importantly, the PG also houses a peripheral circadian clock [5,6].

In insects and other arthropods, a prime example of a developmental transition is the shedding of the old cuticle at the end of the moult (ecdysis). Many ecdysis events (e.g. nymphal ecdyses in the hemipteran bug, *Rhodnius prolixus*, or the adult ecdysis (aka eclosion) of holometabolous insects), are timed by development as well as the circadian clock, which limits ('gates') ecdysis to a specific time of the day (Figure 1).

The circadian gating of eclosion in *Drosophila* has been instrumental in defining the fundamental characteristic of circadian rhythms [8–10] and depends on the central clock in the brain and the peripheral clock in the PG (see Refs. [6,10,11]). Research, particularly in physiologically and biochemically accessible moths and the kissing bug, *Rhodnius*, has provided strong evidence that the circadian timing of ecdysis/eclosion involves signalling via the prothoracicotropic hormone (PTTH) — ecdysone axis (see Refs. [12,13]). PTTH is a neuroendocrine peptide that stimulates ecdysone production in the PG (see Ref. [14]) and is one of the signals that determine the timing of all moults. In addition to integrating developmental signals, PTTH neurons also receive input from lateral clock neurons (LNs), which are key elements of the central circadian pacemaker [12,13,15]. Ecdysone titres fall at the end of the moult,

Figure 1



Developmental and circadian timing of eclosion. **(a)** The temporal distribution of developmental events depends on the individual variations in the pace of development, resulting in a graded distribution (green curve). A circadian gating process (dark blue curve) allows these developmental events to occur only at certain times (the 'allowed' gate, indicated by a green bar on day 1) but not at other times during the day (indicated by a red bar). **(b)** Temporal pattern of eclosion that results when the gating system is applied to the developmental timing. **(c)** Typical timecourse of eclosion of adult wildtype *Drosophila melanogaster* flies in constant darkness after entrainment in a 12 h light:12 h dark cycle is very close to the pattern shown in part **(b)**. The graph in part **(c)** is adapted from Ref. [7] under a CC BY licence (Sage publications).

which is required for the final maturation of the peptidergic system that triggers ecdysis/eclosion (comprising ecdysis-triggering hormone [ETH] and eclosion hormone [EH]) by activating downstream peptidergic signalling cascades in the ventral nerve cord (see Ref. [16]). In some, and possibly in most insects, the opening and closing of the ecdysis/eclosion gate depend on the daily fall and rise in ecdysone haemolymph titres below and above a certain threshold, respectively.

However, in its simplest form, this model does not apply to *Drosophila*, the insect from which most circadian knowledge has been derived. In contrast to findings in moths and *Rhodnius*, in the fruit fly, i) there is no evidence for a circadian cycling of ecdysone titres [17–19], ii) ecdysone titres are already very low at least 20 h before eclosion [17,18], and iii) PTTH mRNA levels change with an ultradian 8 h, but not circadian 24 h, period [20,21].

In this article, we review recent studies on eclosion timing from *Drosophila* with a focus on the brain–PTTH–PG axis and integrate them with the knowledge from moths and the kissing bug *Rhodnius*.

Rhythmic prothoracicotropic hormone signalling is required for eclosion rhythmicity

Evidence for a role of PTTH in the circadian control of ecdysis/eclosion rhythmicity existed from work in moths and *Rhodnius* [12,13], but it is difficult to manipulate PTTH signalling in these systems. In *Drosophila*, however, available genetic tools allowed to specifically test the role of PTTH signalling in eclosion timing. RNAi-mediated downregulation of *Ptth* in the two PTTH neurons located in the *pars lateralis* of each brain hemisphere was found to render eclosion arrhythmic under conditions of constant darkness and temperature (DD), as was the downregulation of the downstream components of its intracellular transduction pathway in the PG (the PTTH receptor gene *torso*, and its downstream effectors, *ras*, *raf*, and *erk*) [20]. This effect was not specific to light-entrained rhythmicity, as ablation of the PTTH neurons during temperature entrainment also led to arrhythmic eclosion under constant conditions. Importantly, the arrhythmicity caused by *torso* downregulation was not observed when *torso* was knocked down in all clock cells except for the PG. Thus, although *torso* may be expressed in other tissues, its role in circadian timing is likely restricted to actions effected within the PG. Similarly, *ptth* null mutant flies exhibit an arrhythmic pattern of eclosion [22]. Together, this evidence demonstrates that PTTH-to-PG signalling is necessary and sufficient for proper eclosion gating [20].

The central clock modulates ecdysone production via inhibition of prothoracicotropic hormone signalling

Based on the proximity of the terminals of the LNs and PTTH neuron arborisations in *Rhodnius* [23] and larval *Drosophila* [24,25], it was expected that the LNs expressing the neuropeptide pigment-dispersing factor (PDF) would transmit the time signal from the circadian pacemaker to the PTTH neurons [13,15]. The existence of this connection was also consistent with the fact that the larval LNs provide light input to the PTTH neurons, which is important for the switch from negative to positive phototaxis that occurs at the start of wandering in the third larval instar [25,26]. Recent work showed that the larval LN-to-PTTH neuron signalling is maintained in pharate adults and is required for rhythmic eclosion. During metamorphosis, the larval LNs persist and become the so-called small ventrolateral neurons (sLNvs) of adult flies [27]. In the pharate adult brain, the sLNv terminals in the superior protocerebrum are in close contact with the dendritic arborisations of the PTTH neurons [20], and trans-synaptic tracing techniques showed that all four PTTH neurons are downstream of the sLNvs [22]. Functional imaging revealed that the sLNvs inhibit the Ca^{2+} activity of the PTTH neurons signal via short neuropeptide F (sNPF)

but not PDF [20]. There is no direct evidence for the cyclical release of sNPF, although this is likely given the rhythmic calcium and electric activity of the sLNvs [28,29]. This opens the possibility that the clock times the required decrease of the ecdysone titre via the sLNvs and subsequent peptidergic inhibition of the PTTH neurons. However, a direct effect of sNPF-mediated inhibition of PTTH neuron activity on the ecdysone titre has not been demonstrated. Nevertheless, the functionality of this peptidergic connection is supported by the finding that PTTH neurons express receptors for sNPF but not for PDF [22]. Moreover, RNAi-mediated downregulation of the sNPF receptor in the PTTH neurons renders eclosion arrhythmic under constant conditions [20]. Recent work demonstrated that larval PTTH neurons are postsynaptic to two pairs of dorsal neurons (DN1 and DN2) [30] in addition to the LNs. Importantly, dorsal neurons modulate the temperature-entrained locomotor rhythmicity in flies [31–34]; however, their role in the rhythm of adult eclosion has not been evaluated. Considering that PTTH neurons are required to gate adult emergence following temperature entrainment [20], future studies should investigate whether the input from DNs to the PTTH neurons is involved in temperature-entrained eclosion rhythmicity.

The clock drives the daily rhythmicity of prothoracicotropic hormone/*torso* signalling

In *Rhodnius*, PTTH release is under clock control, with a peak during the night [35]. This circadian release appears to be relevant for imposing a rhythm in ecdysteroidogenic gene expression in the PG by causing an increase in the activity of the PG and in ecdysone biosynthesis during the night [13,36]. In *Drosophila*, live imaging using the Ca^{2+} sensor, GCaMP6, recently showed that Ca^{2+} levels in the soma of PTTH neurons in experimentally accessible prepupal fruit flies cycle in a daily manner, with a maximum at ZT12 (under LD12:12 conditions) or CT 18 (under DD conditions), and a minimum around ZT4 or CT6 [22]. This daily cycling of the free intracellular Ca^{2+} levels in PTTH neurons appears to depend on intracellular Ca^{2+} release from the endoplasmic reticulum and is required for rhythmic eclosion in DD [22]. In addition, PTTH levels cycle in a daily manner, as measured by immunofluorescence in the terminals of the PTTH neurons in the PG at the prepupal stage [22]. *torso* mRNA in the PG also cycles, but surprisingly, it does so in antiphase relative to the PTTH cycling observed at the terminals of the PTTH neurons [22]. Importantly, the daily cycling of Ca^{2+} and PTTH levels in the PTTH neurons and of *torso* expression in the PG was lost in arrhythmic per^{01} flies, indicating that these rhythms require a functional clock. Rhythmic expression of *torso* was also reported in a previous RNAseq study, which showed that *torso* expression in the larval PG is regulated downstream of TIMELESS [37]. Interestingly, *torso* levels do not cycle in *Ptth* null mutants, which suggests a potential feedback mechanism modulating the PTTH signal transduction in the PG [22]. These findings suggest that central clock-regulated PTTH signalling drives rhythmic *torso* expression [22].

Prothoracicotropic hormone signalling may adjust the circadian phase of prothoracic gland activity

Although *torso* cycling in the PG depends on the time signal transmitted from the brain clock via PTTH, both the central clock and the peripheral clock in the PG are required for rhythmic eclosion. Indeed, stopping the molecular clock in either the clock neurons in the brain or in the PG renders eclosion arrhythmic [6,20]. In addition, 'speeding up' or 'slowing down' the molecular clock using $doubletime^{short}$ or $doubletime^{long}$ expression in the brain clock, respectively, reduced or increased the period τ of eclosion rhythmicity [20]. In contrast, the same manipulations in the PG had no significant effect on τ [20]. This suggests that the brain clock acts as a 'dominant (master) clock', which transmits time information to the peripheral PG clock. While direct evidence for this hypothesis at the level of the molecular clock is still missing, recent imaging of the activity of the PTTH neurons and the PG based on activity-related gene-luciferase (ARG-Luc) imaging [38] in intact flies under constant conditions provides initial correlative evidence that at least the phase of PG activity might be controlled by PTTH signalling [22]. This work has revealed that whereas no ARG-Luc activity was detectable in PTTH neurons and in the PG during the initial phase of metamorphosis, a first peak of activity was recorded from the PG about two days before eclosion, which was maximal 40 h before eclosion. In PTTH neurons, the first peak of activity occurred around 30 h before emergence and was then followed 6 h later by a peak of activity in the PG. The day before eclosion, the activity of PTTH neurons peaked around 7 h before eclosion, again followed by a peak of PG activity 6 h later, at the time of eclosion [22]. While the exact role of each activity peak in the PTTH neurons and the PG has not been established, these findings may have several biological implications. First, in the PTTH neurons, the ARG-Luc activity pattern nicely matches the changes in Ca^{2+} levels observed in these neurons at the prepupal stage, suggesting a potential association between transcriptional activity and Ca^{2+} activity. In addition, PTTH signalling may be relevant for setting the phase of activity of the PG close to the time of emergence. In this regard, a study on organotypic cultures showed that in the PG, *per* transcriptional and translational rhythms, as well as Ca^{2+} dynamics, are modulated by brain inputs [39], suggesting that PTTH may be relevant to the functioning of the molecular clock in the PG.

ERK signalling in the prothoracic gland is a key intracellular pathway for rhythmic eclosion

In addition to PTTH, insulin is a major developmental timer and regulator of ecdysone biosynthesis by the PG

(see Ref. [2]). Moreover, insulin signalling influences the expression of the clock gene, *timeless*, in the PG [37]. Yet, although insulin/PI3K signalling plays an important role in coupling growth to the nutritional state and regulates ecdysone production in the PG (see Ref. [2]), it appears not to be involved in eclosion timing from the non-feeding pupa. Indeed, circadian rhythmicity of eclosion was normal in various insulin-like peptide (DILP) and Lgr3 receptor null mutants, as well as in flies bearing RNAi-based downregulation of the insulin receptor or its downstream targets PI3K and PTEN in the PG [22].

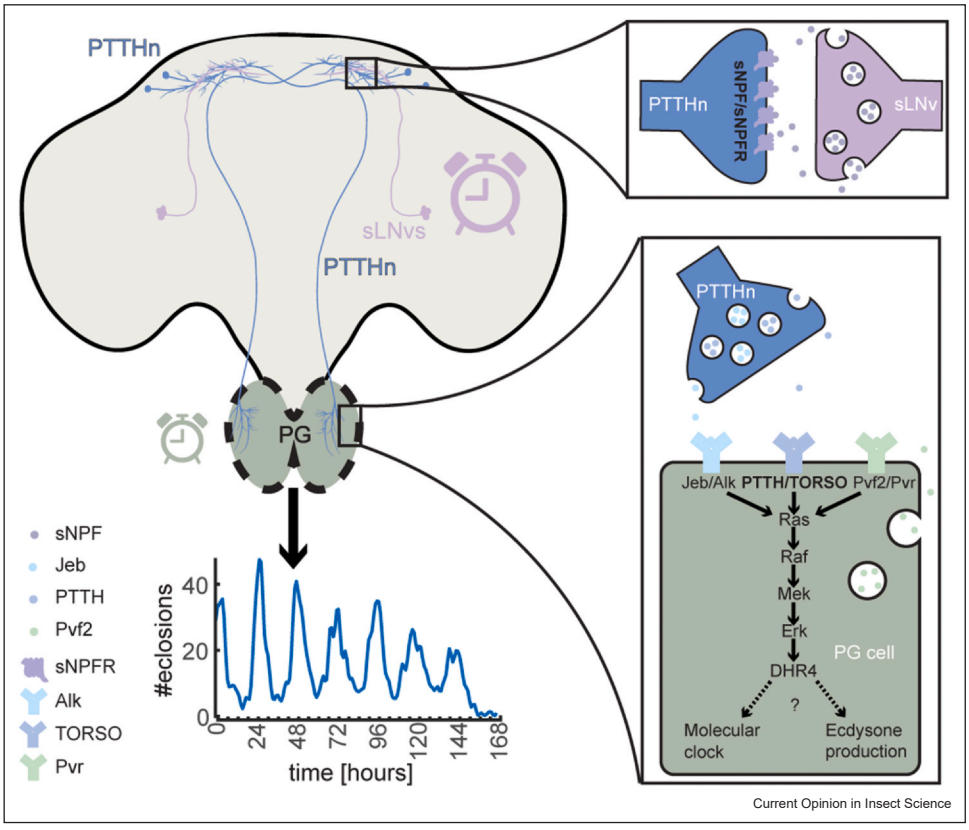
In contrast, several results suggest that the pathway activated in the PG by the PTTH receptor tyrosine kinase (RTK), TORSO, regulates rhythmic eclosion. Downregulation of *ras*, *raf*, and *erk* in the PG renders eclosion arrhythmic [20]. Moreover, ERK (a MAP kinase) activity cycles in a circadian and TORSO-dependent manner in the prepupal PG [22]. Recently, two additional RTK signalling pathways, anaplastic lymphoma kinase (Alk) and platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) receptor related (Pvr), which converge on the Ras/ERK intracellular transduction cascade in the PG, have been shown to co-regulate developmental timing and facilitate ecdysone production in the PG together with PTTH/TORSO [3]. Interestingly, reducing Alk and Pvr signalling in the PG weakened but did not completely eliminate the circadian rhythmicity of eclosion [22]. This suggests that, similar to their role in modulating developmental timing [3], Alk and Pvr can act to strengthen TORSO transduction. This notion is supported by results from ARG-Luc imaging of PGs expressing a dominant negative version of Pvr, which showed that PG activity is greatly diminished, while the phase of its transcriptional activity was not affected [22]. The Alk ligand Jelly belly (Jeb) is expressed by PTTH neurons [3], and downregulation of *Jeb* in the PTTH neurons weakened eclosion rhythmicity similar to downregulation of *Alk* in the PG [22]. In contrast, the Pvr ligand Pvf3, which is also expressed by PTTH neurons [3], seems to not be involved in regulating eclosion timing [22]. Instead, downregulation of the Pvr ligand Pvf2, which is expressed by the PG [3], significantly weakened eclosion rhythmicity when it was knocked down in the PG. This autocrine role for Pvf2 may explain why a functional clock is required both in the central brain and in the PG for the expression of a robust circadian rhythm of eclosion. Finally, in the PG, EGFR also acts via the Ras/ERK pathway during larval stages (see Ref. [2]); however, EGFR overexpression leads to a failure in adult eclosion [22]. Thus, it would be interesting to test whether EGFR ligands (Vein and Spitz), which are expressed in the PG, act in an autocrine manner to modulate Ras/ERK transduction (see Ref. [2]) in the context of circadian eclosion rhythmicity.

How does the rhythmic activity of the central clock-prothoracicotropic hormone-prothoracic gland axis gate eclosion?

As reviewed above, recent research in *Drosophila* has refined our understanding of the mechanisms underlying eclosion timing (Figure 2). The basic peptidergic circuitry from the central clock to the PG and its rhythmicity has largely been defined, and Ras/ERK signalling has emerged as a key intracellular pathway in the PG that is required for rhythmic eclosion. In the *Drosophila* larva, ERK shuttling between the cytoplasm and nucleus in the PG is important for the timing of ecdysone pulses via interaction with the nuclear receptor DHR4 [40]. Though not tested, it is possible that ERK maintains this function in the pharate PG, which is consistent with the fact that downregulation of *dhr4* in the PG renders eclosion arrhythmic [41]. Nevertheless, there is no evidence for a circadian cycling of ecdysone titres during *Drosophila* metamorphosis, and ecdysteroid hormone titres are at baseline levels by 20 h before eclosion [17–19]. Thus, although clock genes modulate the expression of ecdysteroidogenic genes in the larval PG [37], it is unlikely that the temporal pattern of ecdysone biosynthesis directly gates eclosion behaviour. Consistent with this hypothesis, injection of ecdysone into late pupae delays eclosion in *Drosophila* [41], flesh flies [42], and moths [43] but does not abolish its gating. Moreover, neither injection of ecdysone [41] nor optogenetic activation of PTTH neurons at times when the gate is open [44] triggers eclosion. This suggests that the ecdysone titre determines which gate is chosen but does not define the gate or trigger eclosion itself.

A recent study showed that the circadian clock controls the timing of the completion of metamorphosis [41]. This control is not directly exerted via a change in the ecdysone titre but instead seems to occur downstream of ecdysone receptor activity since constitutive and conditional expression of a dominant-negative form of the ecdysone receptor or constitutive down-regulation of *dhr4* in the PG leads to arrhythmic eclosion [41]. Importantly, the fraction of pharate adults induced to emerge prematurely by injection of ETH (a key signal triggering eclosion) depends strongly on their developmental proximity to eclosion. Whereas animals in the prefinal 'smooth' stage (about 9 h before eclosion [45]) never responded to ETH injection, eclosion was consistently triggered in flies in the 'grainy' stage [41] when moulting fluid has been resorbed and the pharate head appears rough (3 h before eclosion [45]). Thus, the *Drosophila* clock appears to gate eclosion by setting the time when the animal initiates the final step(s) in metamorphosis [41]. In other words, eclosion gating via the central clock-PTTH-PG-ecdysone axis is essentially a developmental process and is caused by a clock-dependent synchronisation of the last steps in development [41]. In line with this proposal, the circadian clock

Figure 2



Updated model for the signalling pathway underlying eclosion timing. The sLNv clock neurons convey time information to the PTTH neurons (PTTHn) via the neuropeptide sNPF, which inhibits PTTH neuron activity and PTTH release. PTTH binds to its receptor TORSO on the PG and activates the Ras/Erk pathway. Through still uncharacterised downstream targets, this pathway alters the phase of the clock in the PG and/or the rhythmic production of ecdysone. PTTHn activity also activates Jeb release and activation of its receptor Alk on the PG. Clock-driven PG activity leads to the release of Pvf2 from the PG itself, which, in an autocrine fashion, activates its receptor Pvr on the PG. Both Alk and Pvr activation strengthen TORSO signalling via Ras/Erk.

appears to measure the developmental state between lights-off and about 5 h before eclosion [46]. Moreover, the daily eclosion peaks in *Drosophila melanogaster* in DD follow a Gaussian 'developmental' distribution (Figure 1) rather than a skewed distribution that would be expected from gating of eclosion itself. Under LD conditions, the eclosion peak is indeed more skewed to first hours of the day because light itself causes a 'lights-on' surge of eclosion; this surge depends on the endocrine neurons that produce EH, which is the second key hormone that triggers eclosion behaviour [47].

An interesting aspect of the model in Figure 2 is that all communication in the central clock-PTTH-PG axis is based on peptides rather than classic transmitters, resulting in a slow and integrative process. While we now have a better understanding of the timing and gating mechanisms underlying rhythmic eclosion, many exciting questions remain. Perhaps the most important of these is to further understand why both the central clock in the brain and the peripheral clock in the PG are needed for a robust gating of emergence. Interestingly, this scenario parallels what occurs in the mammalian system that controls the circadian rhythm in glucocorticoids [48]. Indeed, this cycling also requires functional clocks in the suprachiasmatic nucleus and in the adrenal gland. Another outstanding issue is how the time signal is transmitted from the PG to the EH/ETH signalling system or other mechanisms that initiate emergence behaviour.

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Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this review.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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