Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling

Adam Bajgar[a,b], Marek Jindra[a,c], and David Dolezel[a,b,1]

[a]Biology Center, Academy of Sciences of the Czech Republic, 37005 Ceske Budejovice, Czech Republic; [b]Department of Molecular Biology, Faculty of Sciences, University of South Bohemia, 37005 Ceske Budejovice, Czech Republic; and [c]Animal, Food and Health Sciences Division, Commonwealth Scientific and Industrial Research Organization, North Ryde, NSW 2113, Australia

Edited by David L. Denlinger, The Ohio State University, Columbus, OH, and approved January 31, 2013 (received for review October 3, 2012)

In temperate regions, the shortening day length informs many insect species to prepare for winter by inducing diapause. The adult diapause of the linden bug, *Pyrrhocoris apterus*, involves a reproductive arrest accompanied by energy storage, reduction of metabolic needs, and preparation to withstand low temperatures. By contrast, nondiapause animals direct nutrient energy to muscle activity and reproduction. The photoperiod-dependent switch from diapause to reproduction is systemically transmitted throughout the organism by juvenile hormone (JH). Here, we show that, at the organ-autonomous level of the insect gut, the decision between reproduction and diapause relies on an interaction between JH signaling and circadian clock genes acting independently of the daily cycle. The JH receptor Methoprene-tolerant and the circadian proteins Clock and Cycle are all required in the gut to activate the *Par domain protein 1* gene during reproduction and to simultaneously suppress a mammalian-type *cryptochrome 2* gene that promotes the diapause program. A nonperiodic, organ-autonomous feedback between Par domain protein 1 and Cryptochrome 2 then orchestrates expression of downstream genes that mark the diapause vs. reproductive states of the gut. These results show that hormonal signaling through Methoprene-tolerant and circadian proteins controls gut-specific gene activity that is independent of circadian oscillations but differs between reproductive and diapausing animals.

reproductive diapause | photoperiodism | basic helix-loop-helix protein | oogenesis

To cope with adverse winter conditions, animals either migrate or minimize their metabolism and hibernate or diapause (1). Animals including insects anticipate these annual rhythms by measuring the changes in night or day length (i.e., photoperiod) through a seasonal clock whose mechanism has yet to be elucidated (2, 3). The hallmarks of diapause in insects such as the linden bug, *Pyrrhocoris apterus*, and the bean bug, *Riptortus pedestris*, include cessation of reproduction (4–6) and changes in the physiology of the digestive system (7) and the fat body (8). The arrest is induced by short days and results in small diapause ovaries. Conversely, long days promote ovarian maturation through the action of juvenile hormone (JH), produced by the corpora allata glands (9–11).

JH is an insect sesquiterpenoid that controls reproduction (12) and entry into metamorphosis (13). The connection between JH and reproductive diapause is well documented in various species (14). Application of the JH-mimicking analogue methoprene to diapausing *P. apterus* or *R. pedestris* bugs is sufficient to terminate diapause and induce ovarian growth (6, 15). Endogenous JH or added methoprene act through the Methoprene-tolerant (Met) protein to prevent premature metamorphosis in *P. apterus* juveniles (16). Met is a transcription factor of the basic helix–loop–helix Per-ARNT-Sim (bHLH-PAS) family (17), and it has been characterized as a JH receptor (18, 19). JH-dependent interaction between Met and another bHLH-PAS protein, FISC [synonymous to Taiman (Tai)_in *Drosophila melanogaster*; FlyBase], have been implicated in oogenesis of *Aedes aegypti* mosquitoes (20). Recently, *A. aegypti* Met and the bHLH-PAS circadian clock protein Cycle (Cyc) have been shown to dimerize and activate circadian rhythm-dependent gene expression in response to JH (21).

Whether photoperiodic regulation of seasonal diapause/reproduction timing involves the circadian clock is still debated (2, 22–24). Clinal polymorphism of the circadian gene *timeless* was observed in *D. melanogaster* (25, 26), and diapause in another drosophilid fly, *Chymomyza costata*, is altered by a *timeless* mutation (27, 28). However, whether these *timeless* mutations affect a central "photoperiodic clock" in the brain or compromise the execution of diapause in peripheral tissues remains unknown. A systemic RNAi-mediated knockdown of *cyc* in reproductive *R. pedestris* under long-day (LD) conditions switched the bugs into a diapause mode, whereas *period* (*per*) and *cryptochrome* (*cry*) RNAi terminated diapause and induced reproduction in adults experiencing short days (6, 29, 30). These data led the authors to propose that the three circadian genes, *cyc*, *per*, and *cry*, constituted the photoperiodic clock of *R. pedestris* (6, 29, 30). An alternative explanation is that the circadian clock genes have pleiotropic functions, one of which is to regulate the seasonal physiology by acting downstream of a presently undefined photoperiodic clock (22, 31).

To address the role of circadian genes in the regulation of diapause, we examined expression of circadian genes in the gut of reproductive and diapausing females of *P. apterus*, a species with robust and well characterized diapause biology (8, 32). We discovered an organ-autonomous regulatory feedback between Cryptochrome 2 (Cry2) and another circadian clock component, a basic leucine-zipper transcription factor, Par domain protein 1, isoform 1 ($Pdp1_{iso1}$) (33). We show that *cry2* represses $Pdp1_{iso1}$ and triggers a diapause-specific genetic program in the gut, whereas $Pdp1_{iso1}$ counteracts *cry2* and promotes the reproductive state of this organ independently of the daily cycle. Induction of $Pdp1_{iso1}$ and suppression of *cry2* transcription by JH mimic require the JH receptor Met and the circadian proteins Clock (Clk) and Cyc. Therefore, our data indicate organ-autonomous, yet noncircadian, involvement of clock genes and hormonal signaling in diapause regulation.

Author contributions: D.D. designed research; A.B. and D.D. performed research; M.J. contributed new reagents/analytic tools; A.B. and D.D. analyzed data; and M.J. and D.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. 1563012 ($Pdp1_{iso1}$), 1563013 ($Pdp1_{iso2}$), 1562940 (*cyc*), 1562939 (*Clk*), 1563010 (*cwo*), 1563014 (*tgo*), 1562945 (*tai*), 1563016 (*lip*), 1563019 (*def*), 1563020 (*est*), 1563024 (*sod*), and 1563021 (*tf*)].

[1]To whom correspondence should be addressed. E-mail: david.dolezel@entu.cas.cz.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217060110/-/DCSupplemental.

Results

Differential Response of Circadian Clock Genes *cry2* and $Pdp1_{iso1}$ to Diapause. Short day-length (SD) conditions induce reproductive diapause in *P. apterus* females (4), and the ovaries remain small without maturing oocytes (Fig. 1*A*). To examine whether circadian clock genes are involved in diapause regulation, we isolated selected *P. apterus* orthologs including *cyc*, *Clk*, *Pdp1*, and *cry* genes (*SI Methods*). Our phylogenetic analysis showed that, like some other insects (34), *P. apterus* possesses type 2 Cryptochrome (Cry2) that is similar to mammalian Cry (35) rather than to the light-sensitive type 1 Cryptochrome of *D. melanogaster* (Fig. S1). We then compared expression of the circadian clock genes in the gut between reproductive and diapausing adult females. We focused on the gut for two main reasons: (*i*) the gut is the major organ of nutrient uptake that is prerequisite to oogenesis and (*ii*) transcripts of clock genes such as *cry2* do not show circadian oscillation in *P. apterus* guts, making comparisons of gene expression levels practical.

We found, on average, a sixfold enrichment of *cry2* mRNA in the guts of diapause females exposed to the SD condition relative to reproductive females experiencing long-day (LD) conditions (Fig. 1*B*). This difference persisted for at least 4 wk of monitoring and was unaffected by mild daily fluctuations in *cry2* expression. Northern hybridization confirmed this result (Fig. S2). Accordingly, the Cry2 protein was abundant in the gut cells of diapause but not of reproductive females (Fig. 1 *C* and *G*). Because *cry2* expression correlated to the day length and the reproductive status, it was important to determine which of the two factors was causal. We therefore measured *cry2* mRNA in females kept under a critical day length (CDL), i.e., conditions allowing approximately 50% of individuals to enter diapause while leaving the other half reproductive. This experiment showed that up-regulation of *cry2* in the gut depended on diapause, not on the photoperiod (Fig. 1*D*).

In a striking contrast to *cry2*, expression of isoform 1 of *Pdp1* ($Pdp1_{iso1}$) followed a pattern exactly opposite, being high in the guts of reproductive females and minimal under diapause during the 4-wk testing period (Fig. 1*E*). A second *Pdp1* isoform, originating from an alternative transcriptional start, was not expressed differentially between reproductive and diapause animals (Fig. S3). As was the case with *cry2*, daily changes in $Pdp1_{iso1}$ mRNA levels were negligible relative to the major difference associated with the reproductive status (Fig. 1 *B* and *E*).

Finally, we tested whether an experimental switch from reproduction to diapause, achieved by shortening the day length, was sufficient to invert the patterns of *cry2* and $Pdp1_{iso1}$ expression in the gut. Remarkably, 5 to 7 d after transferring reproductive females from LD to SD conditions, $Pdp1_{iso1}$ mRNA declined and *cry2* transcription increased to match the diapause pattern (Fig. 1*F*). These results show that *Pdp1* and *cry2* in the gut respond to the reproductive/diapause status in opposite manner.

Circadian Genes and JH Signaling Regulate *Pdp1* and *cry2* Expression. To explore how $Pdp1_{iso1}$ and *cry2* are regulated, we subjected *P. apterus* females to RNAi against selected transcription factors that are known to act within the circadian clock or in JH signaling. Fig. S4 shows that RNAi knockdown of each individual transcript in the gut was highly efficient. Genes encoding the bHLH-PAS proteins Clk (36) and Cyc (37) and the JH receptor Met all proved to be important for $Pdp1_{iso1}$ and *cry2* expression. In contrast, RNAi targeting two other members of the bHLH-PAS family, Tai and Tango (Tgo), or the circadian protein Clockwork orange (Cwo), affected neither $Pdp1_{iso1}$ nor *cry2* expression (Fig. 2 *A* and *B*). Tai (also known as FISC or steroid receptor coactivator) has been implicated as a partner of Met in JH reception (20, 38) and in JH-dependent oogenesis of *A. aegypti* mosquitoes (20). Tgo is homologous to the aryl hydrocarbon

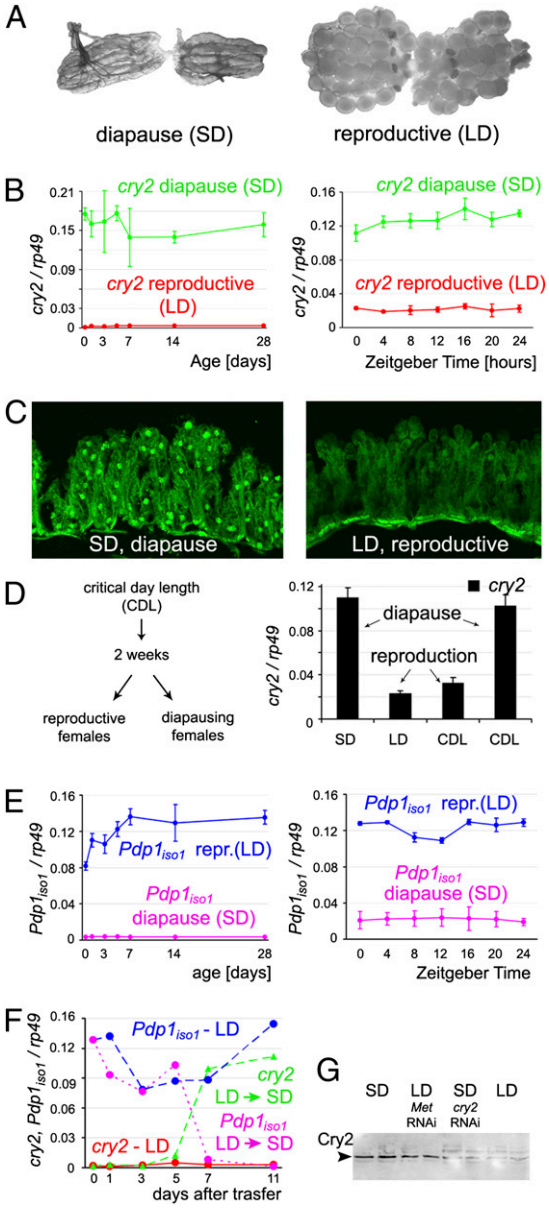

Fig. 1. The *cry2* and $Pdp1_{iso1}$ genes are inversely regulated under diapause and reproductive conditions in the gut of *P. apterus* females. (*A*) Reproductive diapause in *P. apterus* females. Ovaries are small and contain no maturing oocytes under SD condition (*Left*) in which females naturally lack JH. Oogenesis commences upon extending the photoperiod (i.e., LD; *Right*) or after JH mimic treatment. (*B*) Levels of *cry2* mRNA in the gut remain high under diapause and low during the reproductive phase (*Left*) irrespective of daily fluctuations (*Right*). (*C*) The Cry2 protein is detected in cell nuclei of the gut epithelium of diapause females (*Left*) but not in reproductive females. (*D*) CDL experiment (*Methods*) shows that *cry2* expression in the gut depends on the reproductive state rather than on the photoperiod. Females that remained nonreproductive (i.e., diapause) after 2 wk of exposure to CDL (16.5 h light, 7.5 h dark) expressed high *cry2* levels, whereas those that became reproductive under the same CDL conditions showed down-regulation of *cry2*. (*E*) Expression of $Pdp1_{iso1}$ follows a pattern opposite to that of *cry2*. (*F*) Expression of *cry2* and $Pdp1_{iso1}$ transcripts switched to the diapause mode between 5 and 7 d after transfer from LD to SD photoperiod. (*G*) Immunoblot shows that expression of Cry2 protein in the gut of females experiencing diapause was depleted by *cry2* RNAi. Low Cry2 levels occurring in reproductive females increased upon *Met* RNAi. Levels of mRNAs in *B*, *D*, *E*, and *F* were determined by using qRT-PCR and were normalized to *rp49* expression; data are mean ± SEM from three independent experiments (error bars omitted in *F* for clarity).

PHYSIOLOGY

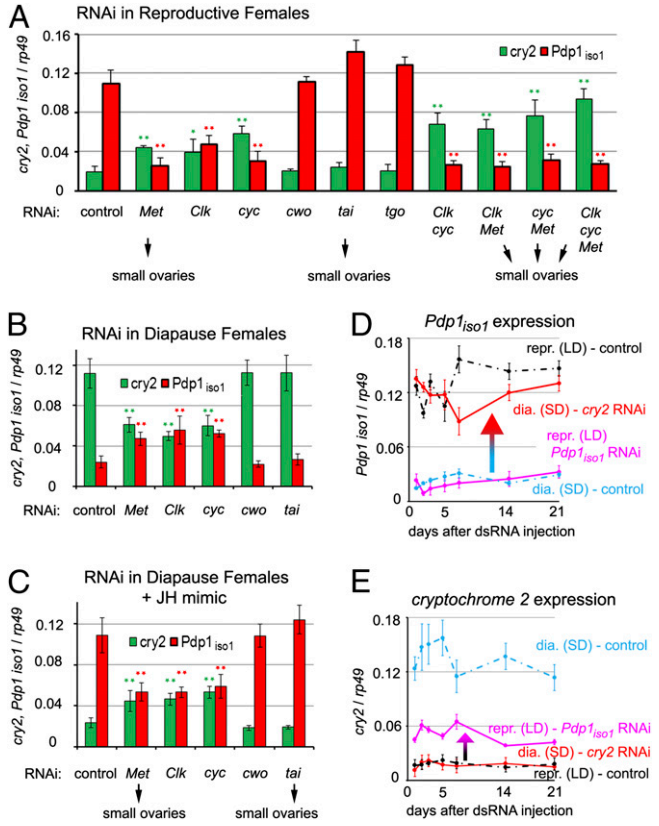

Fig. 2. Factors required for the reciprocal *cry2* and $Pdp1_{iso1}$ regulation in the gut. (*A*) Reproductive females that naturally produce JH were injected with dsRNA to silence the indicated genes, and the levels of *cry2* and $Pdp1_{iso1}$ mRNAs in their guts were determined 4 d later, together with ovarian morphology. *lacZ* dsRNA served as a control. Depletion of Met, Clk, and Cyc (individually or in combinations) altered both transcripts toward their diapause mode; depletion of Met or Tai prevented oogenesis. (*B*) RNAi depletion of Met, Clk, or Cyc in diapause females partially reduced *cry2* and increased $Pdp1_{iso1}$ mRNAs, equalizing their levels. (*C*) Met, Clk, and Cyc are necessary for the JH mimic methoprene to revert the levels of *cry2* and $Pdp1_{iso1}$ mRNAs to the reproductive mode, as both transcripts became approximately equalized in the guts of *Met*, *Clk*, or *cyc* RNAi females that were given methoprene. (*D* and *E*) $Pdp1_{iso1}$ and *cry2* form a feedback loop of mutual repressors in the gut. dsRNAs were injected to females 1 d after adult ecdysis, and transcript levels in their guts were monitored 1, 2, 3, 5, 7, 14, and 21 d later. Knockdown of either gene remained effective throughout this period. Removal of Cry2 in diapause females caused $Pdp1_{iso1}$ mRNA to increase near levels normally occurring in guts of reproductive females (*D*). Conversely, expression of *cry2* was enhanced when reproductive females were subjected to $Pdp1_{iso1}$, although to a lesser extent than in diapause controls. Values are mean ± SEM from three independent experiments. (*$P <$ 0.05 and **$P < 0.001$ vs. *lacZ* controls as assessed, Tukey honestly significant difference test).

receptor nuclear translocator and it is currently unrelated to insect circadian clock or JH signaling. dsRNA derived from the heterologous β-gal (*lacZ*) gene served as a control in all RNAi experiments.

Individual or combinatorial RNAi silencing of *Clk*, *cyc*, or *Met* reduced the $Pdp1_{iso1}$ transcript in the guts of reproductive (LD condition) females (Fig. 2*A*), in which it was normally abundant (Fig. 1*E*). Unexpectedly, reduction in $Pdp1_{iso1}$ mRNA levels upon depletion of *Clk*, *cyc*, or *Met* coincided with up-regulation of the *cry2* transcript (Fig. 2*A*) and, at least in the case of *Met* RNAi, of the Cry2 protein (Fig. 1*G*). This reciprocal $Pdp1_{iso1}$ and *cry2* regulation was even more prominent in the double and triple knockdown experiments combining *Clk*, *cyc*, and *Met* dsRNAs (Fig. 2*A*). Silencing of *Met* or *tai* reduced the ovaries in reproductive females to a size found during diapause (SD condition; Fig. 1*A*), confirming the expected necessity of JH signaling for oogenesis (6, 20). Therefore, high expression of $Pdp1_{iso1}$ in the gut of reproductive females required Clk, Cyc, and Met, whereas oogenesis itself required the JH/Met/Tai signaling but was independent of Clk and Cyc. Conversely to the situation in reproductive females, removal of *Clk*, *cyc*, or *Met* increased $Pdp1_{iso1}$ and reduced *cry2* mRNA levels in the gut of diapause females (Fig. 2*B*). All three genes (*Clk*, *cyc*, and *Met*) that were important for the differential regulation of $Pdp1_{iso1}$ and *cry2* were themselves expressed in the guts of reproductive and diapausing females, with levels slightly higher in the latter (Fig. S5). Therefore, the switch from the reproductive to the diapause mode was not caused by the absence of Cyc, Clk, or the JH receptor Met.

The requirement of *Met* for $Pdp1_{iso1}$ expression suggested a role for JH in regulating the circadian genes. Indeed, topical application of the JH mimic methoprene on diapausing females, which causes them to exit diapause, resulted in up-regulation of $Pdp1_{iso1}$ and a simultaneous decrease of *cry2* mRNA (Fig. 2*C*) to levels seen in the guts of reproductive females (compare Fig. 2 *A* vs. *C*). Therefore, the exogenous JH mimic was able to override the gut diapause status, imposed on the animals by SD conditions, by acting upon circadian gene expression. Importantly, application of methoprene could no longer enhance $Pdp1_{iso1}$ or reduce *cry2* mRNA levels in diapause females deficient for *Met*, *Clk*, or *cyc* function (Fig. 2*C*), indicating that the JH receptor Met as well as the two circadian proteins were required for JH-dependent regulation of $Pdp1_{iso1}$ and *cry2* in the gut. Consistent with the lack of effect of *cwo* and *tai* RNAi on $Pdp1_{iso1}$ and *cry2* expression (Fig. 2 *A* and *B*), methoprene still induced $Pdp1_{iso1}$ and suppressed *cry2* when applied to diapause females upon *cwo* or *tai* silencing (Fig. 2*C*).

Our next goal was to verify whether JH signaling acted autonomously in the gut. As expected, methoprene turned on $Pdp1_{iso1}$ and suppressed *cry2* even in guts that had been removed from diapause females and cultured in vitro (Fig. 3*B* and Fig. S6). However, subjecting animals to *Met* RNAi before gut dissection prevented this effect of the JH mimic on both $Pdp1_{iso1}$ and *cry2* expression (Fig. 3*B*). Taken together, these experiments show that the function of *Clk*, *cyc*, and *Met* in the gut is necessary for JH-dependent regulation of $Pdp1_{iso1}$ and *cry2* in this organ.

Reciprocal Regulation Between $Pdp1_{iso1}$ and *cry2*. High expression of $Pdp1_{iso1}$ coincides with suppression of *cry2* and vice versa, depending on whether females experience reproduction or diapause, respectively (Fig. 1). This inverse relationship suggests a negative feedback between the two circadian genes. Indeed, experimental depletion of *cry2* itself led to elevated expression of $Pdp1_{iso1}$ in the guts of diapause females (Figs. 2*D* and 3*A*, *Upper*). Conversely, low levels of *cry2* mRNA in the guts of reproductive females increased upon $Pdp1_{iso1}$ RNAi, albeit to lower levels than normally observed under diapause (Figs. 2*E* and 3*A*, *Lower*). These changes then persisted for at least 3 wk (Fig. 2 *D* and *E*), suggesting that manipulation of *cry2* or $Pdp1_{iso1}$ reprogrammed expression of the other gene for as long as the RNAi was effective. Nevertheless, as *cry2* RNAi females in SD conditions still retained small diapause ovaries, the loss of *cry2* and the resulting ectopic expression of $Pdp1_{iso1}$ alone were not sufficient to render the entire animal reproductive.

To exclude the possibility that removal of *cry2* function might have induced $Pdp1_{iso1}$ by increasing JH production in diapause females, we examined transcript levels of the *Krüppel-homolog 1* gene (*Kr-h1*), whose expression strictly depends on the presence of JH and Met in *P. apterus* (16). As expected, *Kr-h1* mRNA was low during diapause (a JH-free state), and application of methoprene induced it near the levels occurring in the guts of reproductive females (Fig. S7). The expression of *Kr-h1* was also

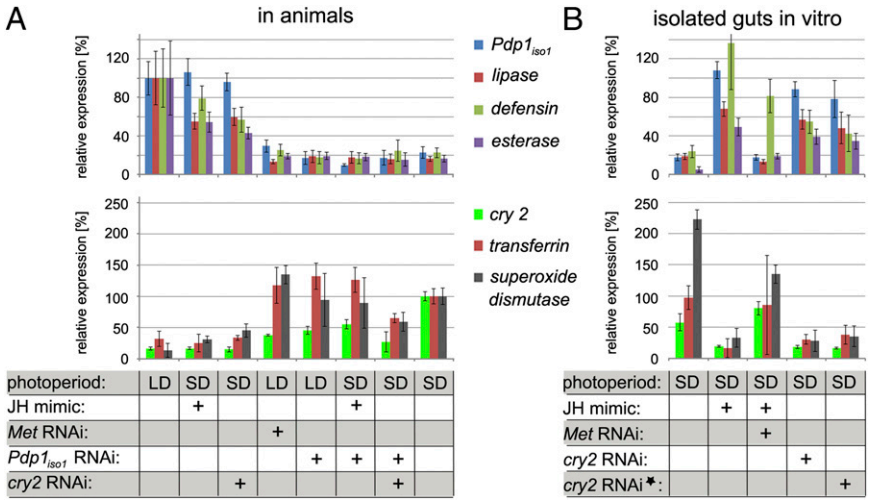

Fig. 3. Regulation of genes downstream of Cry2, Pdp1$_{iso1}$, and JH/Met in the gut. (*A*) Reproductive (i.e., LD) and diapause (i.e., SD) females were injected with dsRNAs and, after 4 d, treated with methoprene as indicated below the columns. Four days later, mRNA levels were measured in their guts for *Pdp1$_{iso1}$*, *lip*, *def*, and *est* genes, whose expression characterizes the reproductive state (*Upper*), and for *cry2*, *tf*, and *sod* genes, which are active under diapause (*Lower*). (*B*) Expression of the reproduction and diapause downstream genes in isolated guts. Guts were dissected from control or from diapause (i.e., SD) females 2 d after dsRNA injection, and were cultured for 2 d with or without methoprene as indicated. Alternatively, isolated guts were exposed to *cry2* dsRNA for 2 d in culture (asterisk). All data have been normalized to *rp49* expression and are shown relative to the levels of reproduction downstream genes in untreated LD females (*Upper*) and to the levels of diapause downstream genes in untreated SD females (*Lower*), respectively, that were set to 100%. Statistical significance of the differences is shown in Table S1.

low in diapause females subjected to *cry2* RNAi (Fig. S7), indicating that JH remained low in these animals. Therefore, although methoprene can induce *Pdp1$_{iso1}$* (Fig. 2*C*), endogenous JH is likely not required for *Pdp1$_{iso1}$* up-regulation that results from *cry2* removal.

To test the autonomy of the feedback between *Pdp1$_{iso1}$* and *cry2*, we cultured guts isolated from diapause females with *cry2* dsRNA in the absence of methoprene. After 48 h, we detected depletion of *cry2* mRNA and up-regulation of *Pdp1$_{iso1}$* in these cultured organs (Fig. 3*B*), confirming that the mutual repression between *cry2* and *Pdp1$_{iso1}$* can operate independently of the systemic JH signal.

Genes Downstream of *Pdp1$_{iso1}$* and *cry2*. Our next question was whether the expression of *Pdp1$_{iso1}$* or *cry2* was important for the gut function. Twenty-four *P. apterus* genes expected to function in the gut were cloned (*SI Methods*), and corresponding mRNA levels were compared between diapause and reproductive female guts. Five of these transcripts were identified as dependent on the reproductive status. These encode digestive enzymes [*lipase* (*lip*) and *esterase* (*est*)], an antimicrobial peptide [*defensin* (*def*)], an oxidative stress response enzyme [*superoxide dismutase* (*sod*)], and *transferrin* (*tf*) whose relationship to diapause has been previously reported (6). Three of these transcripts, *lip*, *est*, and *def*, were preferentially expressed in the gut of reproductive females (we refer to them as reproduction downstream genes), whereas *sod* and *tf* were highly active in diapause guts (diapause downstream genes).

First, we established that application of methoprene to diapause females turned on expression of the reproduction downstream genes while suppressing the diapause downstream genes in the gut (Fig. 3*A*; Table S1 shows statistical analysis). Both the induction and the suppression required *Met*, because all changes were abolished when animals were previously injected with *Met* dsRNA (Fig. 3*A* and Table S1). Importantly, expression of the reproduction downstream genes was also reduced in the guts of reproductive females exposed to RNAi against *Pdp1$_{iso1}$*, in which *lip*, *est*, and *def* remained weakly expressed despite the presence of endogenous JH (in reproductive females) or exogenous JH mimic (applied to diapause females; Fig. 3*A* and Table S1). Therefore, expression of the reproduction-associated genes *lip*, *est*, and *def* in the gut depends on *Pdp1$_{iso1}$* downstream of JH signaling.

The low level of *Pdp1$_{iso1}$* expression, either natural under diapause or upon RNAi in reproductive females, resulted in activation of *cry2* and the diapause downstream genes *sod* and *tf* (Fig. 3*A* and Table S1). To discriminate whether it was the absence of *Pdp1$_{iso1}$* or the presence of *cry2* activity that induced the diapause downstream genes *sod* and *tf*, we exposed diapause females under SD conditions to *cry2* and *Pdp1$_{iso1}$* double RNAi. Guts of these females expressed low levels of *sod* and *tf* (Fig. 3*A* and Table S1), indicating that the function of *cry2* was required for expression of the diapause-specific transcripts.

Finally, we examined regulation of the downstream genes by JH/Met and Cry2 in cultured guts isolated from diapause females. The levels of the reproduction and diapause downstream genes switched to the reproductive pattern after methoprene application in vitro, and the JH mimic had no such effect on guts obtained from diapausing females that were subjected to *Met* RNAi (Fig. 3*B* and Table S1). Depletion of Cry2 in the gut, whether deployed before dissection in intact diapause females or in culture, resulted in up-regulation of *Pdp1$_{iso1}$* and the reproduction downstream genes, whereas the diapause downstream transcripts decreased (Fig. 3*B* and Table S1).

Discussion

In many insects, including *P. apterus*, the seasonal decision between reproduction and diapause involves JH (12). Under extended day length, signals from neurosecretory cells in the pars intercerebralis of the brain stimulate JH secretion from the corpora allata gland (Fig. 4). JH then acts on target organs to induce exit from diapause and to promote oogenesis. By using the gut of *P. apterus* females, we show that, at the organ level, JH achieves the switch from a diapause mode to a reproduction mode through its receptor Met. In the gut of reproductive females, the function of Met is necessary for enhanced expression of *Pdp1$_{iso1}$* and downstream genes that are active during reproduction and for simultaneous suppression of *cry2* and other genes that characterize diapause (Fig. 4). When supplied to diapausing females experiencing SD conditions, methoprene overcomes the lack of

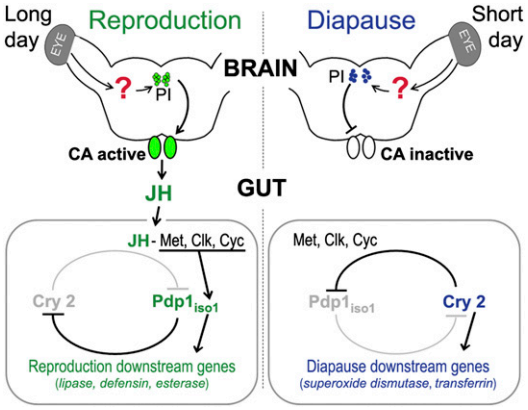

Fig. 4. Regulation of the reproductive/diapause status of the insect gut. Under LD conditions, which favor reproduction (*Left*), the corpora allata (CA) secrete JH in response to signals from the pars intercerebralis (PI) of the brain. In the gut, JH acts through its receptor Met, Clk, and Cyc to stimulate expression of $Pdp1_{iso1}$ and other genes that characterize the reproductive state, whereas expression of *cry2* and diapause downstream genes is suppressed. Although the regulation involves circadian clock genes, it is independent of daily oscillations of the circadian gene expression. In the absence of endogenous JH under short photoperiod (*Right*), expression of *cry2* prevails over $Pdp1_{iso1}$, favoring the diapause-specific program. By acting through Met, Clk, and Cyc, exogenous JH mimic can induce the reproductive program under SD condition.

endogenous JH and terminates diapause. Also in this scenario, Met is needed in the gut for the JH mimic to activate $Pdp1_{iso1}$, suppress *cry2*, and switch expression of the respective downstream genes toward the reproductive mode (Fig. 3*B*).

Unlike Met, its partner Tai was not required for the reciprocal $Pdp1_{iso1}$ and *cry2* regulation in the guts of reproductive and diapause females (Fig. 2). Similarly, *tai* RNAi did not prevent methoprene from inducing $Pdp1_{iso1}$ and repressing *cry2* during diapause. Therefore, Tai is unlikely to be part of a JH receptor complex that contains Met and mediates the effect of methoprene on the diapause gut. Nonetheless, Met and Tai were necessary for ovarian growth at the entire organism level, because either *Met* or *tai* RNAi blocked oogenesis in reproductive females or in methoprene-treated females under SD conditions (Fig. 2*A* and *C*). These results indicate that, in *P. apterus*, JH stimulates oogenesis through Met and Tai and regulates gene expression in the gut through Met, Cyc, and Clk.

The inverse correlation of $Pdp1_{iso1}$ and *cry2* activity with diapause and with the presence of JH suggests that $Pdp1_{iso1}$ and Cry2 direct the gut toward the reproductive or the diapause state by acting as mutual repressors (Fig. 4). Expression of $Pdp1_{iso1}$ in the guts of diapause females increased not only following methoprene treatment but also upon *cry2* RNAi, even when isolated guts were cultured without methoprene. Therefore, although the $Pdp1_{iso1}$/*cry2* regulatory circuit responds to the systemic JH signal, it operates organ-autonomously. Interestingly, although *cry2* RNAi in diapause females enhanced $Pdp1_{iso1}$ mRNA near levels observed during reproduction, *cry2* expression upon $Pdp1_{iso1}$ knockdown reached less than half its diapause level (Figs. 2 *D* and *E* and 3*A*). The difference could be caused by residual $Pdp1_{iso1}$ protein or an additional signal from the brain (9, 10).

RNAi knockdown of *Met*, *cyc*, or *Clk* in reproductive females and in methoprene-treated diapause females prevented expression of $Pdp1_{iso1}$ to prevail over *cry2* in the presence of endogenous JH or its added mimic, and combined silencing of two or all three of these genes in reproductive females inverted the $Pdp1_{iso1}$/*cry2* expression ratio to the diapause mode (Fig. 2*A*). These results show that signaling through the JH receptor Met cooperates with the bHLH-PAS transcription factors Clk and Cyc at the level of regulating the $Pdp1_{iso1}$ and *cry2* genes to promote the reproductive program in the gut (Fig. 4). In agreement with our data, the Clk-Cyc dimer is known to activate the *Pdp1* gene in *D. melanogaster* (33). However, in contrast to the cyclic expression pattern of the fly *Pdp1* orthologue (33) or of the mammalian *cry* gene (35), neither $Pdp1_{iso1}$ nor *cry2* mRNAs displayed daily fluctuations in the gut of *P. apterus*. Instead, expression of both genes depended on whether females experienced oogenesis or diapause, and our critical photoperiod experiments further showed that the reproductive status, rather than the day length, was the decisive cue.

Taken together, our data show a pathway in which JH conveys upstream photoperiodic information to downstream target tissues. Intriguingly, the key players mediating the diapause/reproductive state of the gut involve the circadian genes *Clock*, *cycle*, *Pdp1*, and *cry2*. Previous work in *R. pedestris* (6, 39) showed that Cyc, Per, and Cry2 orthologues were all required for a circadian cycle of cuticle deposition. In addition, knockdown of *per* or *cry2* stimulated oogenesis even under diapause-inducing conditions, whereas *cyc* RNAi always prevented it. Based on altered expression of JH-response genes, the authors implicated Per and Cyc in a photoperiodic clock operating upstream of JH secretion, and excluded their involvement in the pleiotropic regulation of ovarian development itself (6). In our *P. apterus* model, oogenesis under long photoperiod could be blocked upon depletion of the JH receptor Met or its partner Tai, but not by RNAi against Cyc, Clk, or $Pdp1_{iso1}$. Likewise, *cry2* RNAi was insufficient to induce oogenesis in diapause females. Consistently, JH-dependent expression of the *Kr-h1* gene did not significantly increase in the gut of these Cry2-deficient females (Fig. S7). Although we cannot rule out effects of systemic RNAi on the brain, we do not suspect that the circadian proteins control the seasonal status of the gut by acting upstream of JH secretion. Instead, our data show that Clock, Cyc, Pdp1, and Cry2 engage in an organ-autonomous regulatory mechanism that is independent of circadian oscillations and that responds to the centrally released hormonal signal. Our results therefore support the idea that circadian clock components can operate pleiotropically in peripheral tissues to execute the reproductive/diapause program without necessarily being connected to their other function as part of the canonical circadian clock (3, 22, 31).

Methods

Animal Rearing Conditions. *P. apterus* bugs (short-winged form) were reared at 25 °C as described previously (40), and were maintained from hatching either under LD conditions (18 h light, 6 h dark) that permit reproduction or under diapause-inducing short photoperiod (12 h light, 12 h dark). For CDL experiments, developing bugs were kept in an intermediate photoperiod of 16.5 h of light and 7.5 h of dark. Females were dissected 2 wk after adult ecdysis, and their diapause phenotype was assessed according to ovarian morphology.

mRNA Quantification. Total RNA was isolated from guts of adult *P. apterus* females with the TRIzol reagent (Invitrogen). After TURBO DNase (Ambion) treatment, 1 μg of total RNA was used for cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen). Relative transcript levels were measured by quantitative RT-PCR (qRT-PCR) by using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (Bio-Rad). All data were normalized to the relative levels of ribosomal protein (Rp49) mRNA as described previously (41). Primer sequences used for qRT-PCR are listed in Table S2.

Antibodies and Immunodetection. A mixture of synthetic peptides, ESSTERTKKTEESIYC and CFAPPASTFRGSLNKK, corresponding to the C-terminal region of *P. apterus* Cry2 was used to generate polyclonal antibodies in guinea pigs (Eurogentec). Western blots were performed with the anti-Cry2 antibody diluted 1:200. For tissue staining, guts were dissected in cold Ringer solution and then fixed for 4 h in 4% paraformaldehyde in PBS solution with 0.03% Triton X-100. After incubating overnight in 30% (wt/vol) sucrose in PBS solution at 4 °C, the tissue was embedded in a Tissue-Tek system (Sakura Finetek). Cryosections 15 μm thick were successively incubated with the Cry2

antibody (1:100) overnight at room temperature and with a FITC-conjugated guinea pig secondary antibody (1:1,000; Jackson Immunoresearch) for 2 h at room temperature. The washed and mounted tissue (Aqua Polymount; Polysciences) was observed with a FluoView 1000 confocal microscope (Olympus).

RNAi. dsRNA was synthesized by using the T3 and T7 MEGAscript kit (Ambion) from plasmids containing the appropriate gene fragments (Table S3 shows primer sequences). Before cloning, these fragments were cross-examined by using dot-plot analysis to avoid interference of the individual dsRNAs with multiple target genes. Experimental animals were injected with 4 μL of 4 μg/μL dsRNA solution. For double and triple knockdown experiments, individual dsRNA were mixed in equal ratio and diluted to the final concentration of 4 μg/μL. The efficiency of RNAi-mediated depletion of each targeted mRNA was verified by qRT-PCR.

Organ Culture Experiments. Guts of intact or RNAi-treated females were dissected and washed twice in Grace medium (G8142; Sigma), supplemented with a twice-concentrated antibiotic/antimycotic mix (A5955; Sigma). Individual guts were incubated in 96-well plates (50 μL media per well) at 22 °C to 23 °C in the dark. For in vitro RNAi experiments, 5 μL of *cry2* dsRNA (4 μg/μL) was added to each well.

JH Mimic Treatments. Five microliters of 0.3 mM methoprene (VUOS) in acetone (or acetone alone for control) were topically applied to the dorsal side of CO_2-anesthetized females. The animals were then kept under SD conditions for 4 d. Guts were then dissected and subjected directly to qRT-PCR or first cultured as described earlier. Five microliters of 0.3 mM methoprene (or acetone alone for control) was applied on individual guts, cultured in 50 μL of Grace media.

ACKNOWLEDGMENTS. We thank Jana Mikešová for insect rearing, Ivan Fiala for phylogenetic analysis, and Ivo Sauman for permanent support. This work was supported by Academy of Sciences Grants IAA500960802 and Z50070508, Grant Agency of the Czech Republic Projects 204/08/P579 and P502-10-1612, and Marie Curie Fellowship Award 276569 from the European Union (to M.J.).

1. Hahn DA, Denlinger DL (2011) Energetics of insect diapause. *Annu Rev Entomol* 56: 103–121.
2. Kostál V (2011) Insect photoperiodic calendar and circadian clock: Independence, cooperation, or unity? *J Insect Physiol* 57(5):538–556.
3. Bradshaw WE, Holzapfel CM (2010) What season is it anyway? Circadian tracking vs. photoperiodic anticipation in insects. *J Biol Rhythms* 25(3):155–165.
4. Hodek I (1968) Diapause in females of *Pyrrhocoris apterus* L (Heteroptera). *Acta Entomol Bohemoslov* 65:422–435.
5. Slama K (1964) Hormonal control of respiratory metabolism during growth, reproduction, and diapause in female adults of *Pyrrhocoris apterus* L (Hemiptera). *J Insect Physiol* 10:283–303.
6. Ikeno T, Tanaka SI, Numata H, Goto SG (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. *BMC Biol* 8:116.
7. Socha R, Sula J, Zemek R (1997) Feeding, drinking and digestive enzyme activities in long- and short-day females of *Pyrrhocoris apterus* (Heteroptera). *Physiol Entomol* 22:161–169.
8. Kostál V, Tollarová M, Dolezel D (2008) Dynamism in physiology and gene transcription during reproductive diapause in a heteropteran bug, Pyrrhocoris apterus. *J Insect Physiol* 54(1):77–88.
9. Hodková M (1976) Nervous inhibition of corpora allata by photoperoid in Pyrrhocoris apterus. *Nature* 263(5577):521–523.
10. Hodková M, Okuda T, Wagner RM (2001) Regulation of corpora allata in females of *Pyrrhocoris apterus* (Heteroptera) (a mini-review). *In Vitro Cell Dev Biol Anim* 37(9): 560–563.
11. Shimokawa K, Numata H, Shiga S (2008) Neurons important for the photoperiodic control of diapause in the bean bug, *Riptortus pedestris*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 194(8):751–762.
12. Raikhel AS, Brown MR, Bellés X (2005) Hormonal control of reproductive processes. *Comprehensive Insect Science*, eds Gilbert LI, Iatrou K, Gill SS (Elsevier, Amsterdam), pp 433–491.
13. Jindra M, Palli SR, Riddiford LM (2013) The juvenile hormone signaling pathway in insect development. *Annu Rev Entomol* 58:181–204.
14. Denlinger DL, Yocum GD, Rinehart JP (2012) Hormonal control of diapause. *Insect Endocrinology*, ed Gilbert LI (Elsevier, Amsterdam), pp 430–463.
15. Slama K (1974) Physiological and biochemical effects of juvenoids. *Insect Hormones and Bioanalogues*, eds Slama K, Romanuk M, Sorm F (Springer, New York), pp 236–243.
16. Konopova B, Smykal V, Jindra M (2011) Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PLoS ONE* 6(12):e28728.
17. Ashok M, Turner C, Wilson TG (1998) Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proc Natl Acad Sci USA* 95(6):2761–2766.
18. Miura K, Oda M, Makita S, Chinzei Y (2005) Characterization of the *Drosophila* Methoprene -tolerant gene product. Juvenile hormone binding and ligand-dependent gene regulation. *FEBS J* 272(5):1169–1178.
19. Charles JP, et al. (2011) Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc Natl Acad Sci USA* 108(52):21128–21133.
20. Li M, Mead EA, Zhu JS (2011) Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc Natl Acad Sci USA* 108(2):638–643.
21. Shin SW, Zou Z, Saha TT, Raikhel AS (2012) bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proc Natl Acad Sci USA* 109(41):16576–16581.
22. Bradshaw WE, Holzapfel CM (2010) Circadian clock genes, ovarian development and diapause. *BMC Biol* 8:115.
23. Saunders DS, Bertossa RC (2011) Deciphering time measurement: The role of circadian 'clock' genes and formal experimentation in insect photoperiodism. *J Insect Physiol* 57(5):557–566.
24. Schiesari L, Kyriacou CP, Costa R (2011) The hormonal and circadian basis for insect photoperiodic timing. *FEBS Lett* 585(10):1450–1460.
25. Sandrelli F, et al. (2007) A molecular basis for natural selection at the timeless locus in *Drosophila melanogaster*. *Science* 316(5833):1898–1900.
26. Tauber E, et al. (2007) Natural selection favors a newly derived timeless allele in *Drosophila melanogaster*. *Science* 316(5833):1895–1898.
27. Stehlík J, Závodská R, Shimada K, Sauman I, Kostál V (2008) Photoperiodic induction of diapause requires regulated transcription of timeless in the larval brain of *Chymomyza costata*. *J Biol Rhythms* 23(2):129–139.
28. Kobelková A, Bajgar A, Dolezel D (2010) Functional molecular analysis of a circadian clock gene timeless promoter from the Drosophilid fly Chymomyza costata. *J Biol Rhythms* 25(6):399–409.
29. Ikeno T, Numata H, Goto SG (2011) Circadian clock genes period and cycle regulate photoperiodic diapause in the bean bug Riptortus pedestris males. *J Insect Physiol* 57(7):935–938.
30. Ikeno T, Numata H, Goto SG (2011) Photoperiodic response requires mammalian-type cryptochrome in the bean bug *Riptortus pedestris*. *Biochem Biophys Res Commun* 410(3):394–397.
31. Emerson KJ, Bradshaw WE, Holzapfel CM (2009) Complications of complexity: Integrating environmental, genetic and hormonal control of insect diapause. *Trends Genet* 25(5):217–225.
32. Socha R (1993) *Pyrrhocoris apterus* (Heteroptera) - an experimental model species: A review. *Eur J Entomol* 90:241–286.
33. Cyran SA, et al. (2003) vrille, Pdp1, and dClock form a second feedback loop in the *Drosophila* circadian clock. *Cell* 112(3):329–341.
34. Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol Biol Evol* 24(4):948–955.
35. Kume K, et al. (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98(2):193–205.
36. Allada R, White NE, So WV, Hall JC, Rosbash M (1998) A mutant *Drosophila* homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* 93(5):791–804.
37. Rutila JE, et al. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila* period and timeless. *Cell* 93(5):805–814.
38. Zhang ZL, Xu JJ, Sheng ZT, Sui YP, Palli SR (2011) Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. *J Biol Chem* 286(10):8437–8447.
39. Ikeno T, Katagiri C, Numata H, Goto SG (2011) Causal involvement of mammalian-type cryptochrome in the circadian cuticle deposition rhythm in the bean bug Riptortus pedestris. *Insect Mol Biol* 20(3):409–415.
40. Dolezel D, Sauman I, Kost'ál V, Hodkova M (2007) Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, Pyrrhocoris apterus. *J Biol Rhythms* 22(4):335–342.
41. Dolezel D, Zdechovanova L, Sauman I, Hodkova M (2008) Endocrine-dependent expression of circadian clock genes in insects. *Cell Mol Life Sci* 65(6):964–969.