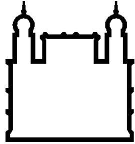

Ministério da Saúde

**FIOCRUZ**
**Fundação Oswaldo Cruz**

**INSTITUTO OSWALDO CRUZ**
**Pós-Graduação em Biologia Celular e Molecular**

Antonio Carlos Alves Meireles Filho

# Análise Funcional Comparativa do Relógio Circadiano de *Drosophila melanogaster* e insetos vetores

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Celular e Molecular.

**Orientador:** Dr. Alexandre Afranio Peixoto

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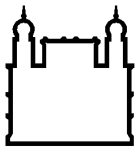

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# Análise Funcional Comparativa do Relógio Circadiano de *Drosophila melanogaster* e insetos vetores

**ORIENTADOR: Dr. Alexandre Afranio Peixoto**

**Aprovada em: 26 / 03 / 2008**

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Rio de Janeiro, 26 de março de 2008

Para Flavia

Este trabalho foi realizado no Laboratório de Biologia Molecular de Insetos do Instituto Oswaldo Cruz, Fundação Oswaldo Cruz e no Instituto de Zoologia da Universidade de Regensburg, Alemanha. Foram utilizados recursos da Fundação Oswaldo Cruz, CNPq, Guggenheim Foundation, Howard Hughes Medical Institute e DAAD (Deutscher Akademischer Austauschdienst).

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# Índice

Lista de Figuras ........................................ ix

Lista de Tabelas ........................................ ix

Índice de Siglas e Abreviaturas ........................................ x

Resumo ........................................ xi

Abstract ........................................ xii

Capítulo 1 - Introdução........................................ 1

1.1 – Ritmos biológicos........................................ 1

1.2 – *Drosophila* como modelo........................................ 4

1.3 – *Drosophila melanogaster* como modelo........................................ 8

1.4 – O controle molecular do relógio biológico de *D. melanogaster*........................................ 11

1.5 –INPUT........................................ 21

1.6 – Um exemplo de OUTPUT: a atividade locomotora........................................ 22

1.7 – Marcapasso central e a autonomia dos relógios periféricos em *D.melanogaster*........................................ 23

1.8 – Diferentes mecanismos para diferentes tecidos........................................ 25

1.9 – O controle molecular do relógio biológico de mamíferos........................................ 27

1.10 – Diferentes mecanismos para diferentes insetos........................................ 30

1.11 – *Lutzomyia longipalpis*........................................ 31

Capítulo 2 – Objetivos........................................ 34

Capítulo 3 – Manuscrito 1........................................ 35

Capítulo 4 – Manuscrito 2........................................ 43

Capítulo 5 – Manuscrito 3........................................ 54

Capítulo 6 – Discussão........................................ 97

6.1 – O relógio biológico de *L. longipalpis*........................................ 98

6.2 – A evolução do relógio biológico de *D. melanogaster*........................................ 101

Capítulo 7 – Referências Bibliográficas........................................ 108

## Lista de Figuras
(com versão simplificada das legendas)

**Figura 1.1:** O experimento de De Mairan, realizado em 1729........................................ 2

**Figura 1.2:** Os três componentes que governam os ritmos circadianos........................... 7

**Figura 1.3:** Atividade locomotora dos três mutantes do gene *period*.............................. 10

**Figura 1.4:** Ritmo circadiano da abundância do RNAm e proteína de *per*...................... 11

**Figura 1.5:** Esquema do modelo da primeira alça regulatória do relógio biológico em *D. melanogaster*........................................................................................... 16

**Figura 1.6:** Expressão dos genes da segunda alça regulatória de *D. melanogaster*.......... 18

**Figura 1.7:** Esquema do modelo corrente do funcionamento do relógio biológico em *D. melanogaster*........................................................................................... 20

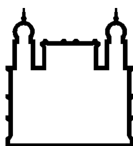
**Figura 1.8:** Fêmea de *Lutzomyia longipalpis* se alimentando através da pele humana.... 31

## Tabela

**Tabela 1:** Genes de relógio descritos em *D. melanogaster*, seu padrão de expressão, modificações pós-traducionais e homólogos em camundongo...................... 28

# Índice de Siglas e Abreviaturas

| | | |
|---|---|---|
| bHLH | = | domínio de ligação ao DNA tipo ***b**asic-**H**elix-**L**oop-**H**elix* |
| *bmal1* | = | gene *brain and muscle Arnt-like protein 1* |
| BMAL1 | = | proteína *brain and muscle Arnt-like protein 1* |
| bZip | = | domínio de ligação ao DNA tipo ***b**asic-leucine-**Zip**per* |
| CCID | = | domínio **C**LK:**C**YC **I**nhibition **D**omain |
| *ck2* | = | gene *casein kinase 2* |
| CK2 | = | proteína CASEIN KINASE 2 |
| *Clk* | = | gene *Clock* |
| CLK | = | proteína CLOCK |
| *cry* | = | gene *cryptochrome* |
| CRY | = | proteína CRYPTOCHROME |
| *cyc* | = | gene *cycle* |
| CYC | = | proteína CYCLE |
| *dbt* | = | gene *doubletime* |
| DBT | = | proteína DOUBLETIME |
| NSQ | = | **N**úcleo **S**upra**Q**uiasmático |
| *pdf* | = | gene *pigment dispersing factor* |
| PDF | = | proteína PIGMENT DISPERSING FACTOR |
| *Pdp1ϵ* | = | gene *PAR domain protein 1 isoforma ϵ* |
| PDP1ϵ | = | proteína PAR domain protein 1 isoforma ϵ |
| PAS | = | domínio de interação proteica **P**ER-**A**RNT-**S**IM |
| PCR | = | *Polimerase Chain Reaction* (Reação em Cadeia da Polimerase) |
| *per* | = | gene *period* |
| PER | = | proteína PERIOD |
| *pp2a* | = | gene *protein phospatase 2a* |
| PP2a | = | proteína PROTEIN PHOSPHATASE 2a |
| s-LNv | = | *small ventrolateral neurons* (neurônios na região ventro-lateral) |
| *sgg* | = | gene *shaggy* |
| SGG | = | proteína SHAGGY |
| *tim* | = | gene *timeless* |
| TIM | = | proteína TIMELESS |
| *vri* | = | gene *vrille* |
| VRI | = | proteína VRILLE |
| ZT | = | *Zeitgeber Time* (Doador de Tempo) |




**INSTITUTO OSWALDO CRUZ**

**Análise Funcional Comparativa do Relógio Circadiano de Drosophila melanogaster e insetos vetores**

**RESUMO**

**TESE DE DOUTORADO**

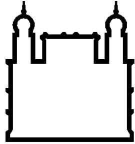
**Antonio Carlos Alves Meireles Filho**

Diversos organismos apresentam variações no comportamento e na fisiologia que são controladas por um relógio biológico interno. O flebotomíneo *Lutzomyia longipalpis* (Diptera: Psychodidae), o principal vetor da leishmaniose visceral nas Américas, é um inseto hematófago com atividade crepuscular/noturna. A hematofagia, crítica na transmissão da doença, está restrita a uma determinada hora do dia, certamente conseqüência do controle do marcapasso circadiano.

Apesar da importância dos ritmos circadianos na dinâmica da transmissão da doença, pouco se sabe sobre seu controle molecular em insetos vetores. Neste trabalho descrevemos algumas propriedades do relógio circadiano de *L. longipalpis*. Comparado a *Drosophila melanogaster*, os genes *period* (*per*) e *timeless* (*tim*), dois elementos negativos da retroalimentação negativa, apresentam padrão similar de expressao de RNAm. Por outro lado, a expressão de *Clock* (*Clk*) e *cycle* (*cyc*), dois elemetos positivos, diferem entre as duas espécies, sugerindo que as diferenças de fase de suas expressões possam estar associadas às diferenças observadas no ritmo de atividade circadiana. Além disso, nós observamos uma redução da atividade locomotora após o repasto sanguíneo, que é correlacionada com uma diminuição dos níveis de expressão de *per* and *tim*.

Apesar de muitos aspectos do marcapasso molecular serem conservados em animais, algumas diferencas entre *L. longipalpis* e *D. melanogaster* sugeriram que o relógio circadiano de moscas de fruta divergiu bastante durante a evolução. Por exemplo, enquanto em moscas o domínio de transativação do elemento positivo reside em CLK, em *L. longipalpis* e todos outros animais analisados até o momento ele fica em CYC. Dessa forma, parece que durante o processo evolutivo houve uma transferência funcional do domínio de transativação de CLK para CYC na linhagem de *Drosophila*.

Para elucidar a evolução funcional do relógio circadiano de *Drosophila* nós testamos a hipótese de que CLK e CYC tenham trocado o domínio de transativação durante a evolução. Nossos estudos revelaram que o relógio de *Drosophila* pode funcionar da mesma maneira que o de mamíferos e que CRYPTOCHROME, além do seu papel bem descrito na fotorecepção, pode ter tido um papel ancestral no mecanismo molecular do marcapasso de *Drosophila*.

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**ABSTRACT**

**TESE DE DOUTORADO**

**Antonio Carlos Alves Meireles Filho**

A diversity of organisms has circadian variations of behavior and physiology that are controlled by an internal biological clock. The sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae) is a crepuscular/nocturnal blood-sucking insect that is the main vector of visceral leishmaniasis in the Americas. Blood feeding, which is critical to disease transmission, is tightly adjusted to a specific time of day and it is therefore certainly controlled by the circadian pacemaker.

Despite the importance of circadian rhythms in the dynamics of disease transmission, very little is known about its molecular control in insect vectors. In this work we describe some features of the circadian clock of *L. longipalpis*. Compared to *Drosophila melanogaster*, sandfly *period* (*per*) and *timeless* (*tim*), two negative elements of the feedback loop, show similar peaks of mRNA abundance. On the other hand, the expression of *Clock* (*Clk*) and *cycle* (*cyc*), two positive elements, differs between the two species, raising the possibility that the different phases of their expression could be associated with the observed differences in circadian activity rhythms. In addition, we show a reduction in locomotor activity after a blood meal, which is correlated with downregulation of *per* and *tim* expression levels.

Although many aspects of the molecular pacemaker are conserved in animals, some differences among *L. longipalpis* and *D. melanogaster* suggested that the fruit fly circadian clock have strongly diversified during the course of evolution. For example, while in flies the transactivation domain of the positive element resides in CLK, in *L. longipalpis* and all other animals analyzed so far it is in CYC. Therefore, it seems that during the course of evolution a functional transference of the transactivation domain from CYC to CLK occurred in the *Drosophila* lineage.

To shed light into the functional evolution of the *Drosophila* circadian clock we tested the hypothesis that CLK and CYC have swapped the transactivation domain during the course of evolution. Our studies revealed that *Drosophila* can sustain a mammalian-like pacemaker and that CRYPTOCHROME, besides its well described role in *Drosophila* photoreception, might have had an ancient role in the fruit fly clockwork mechanism.

## Capítulo 1 – Introdução

### 1.1 Ritmos biológicos

O planeta Terra apresenta basicamente dois tipos de movimento: translação e rotação. A translação da Terra ao redor do Sol e a sua rotação ao redor do seu próprio eixo têm duração aproximada de 365 dias e 24hs, respectivamente. Esses movimentos periódicos determinam diferenças nos padrões de luminosidade e temperatura a que ela e todos os seus habitantes são expostos. A inclinação do eixo de rotação da Terra em relação ao seu plano de translação determina que os raios solares cheguem aos hemisférios com diferentes ângulos em relação à superfície terrestre, sendo isto determinante para a retenção de calor. É isso que causa as estações do ano com suas diferenças de temperatura e fotoperíodo (número de horas com luminosidade durante o dia).

Já a rotação da Terra ao redor do seu próprio eixo determina o ciclo de claridade/escuridão que se estende por 24hs. Esse ambiente tão previsível e constante impôs uma forte pressão seletiva nos organismos que ali viviam. Dessa forma, não é surpreendente que a grande maioria desses organismos, de bactérias a humanos, tenha desenvolvido atividades comportamentais e metabólicas com periodicidade circadiana (latim: *circa* = cerca de, *dies* = dia) (Moore-Ede et al, 1982).

A explicação mais simples para esse comportamento seria a existência de uma resposta passiva dos organismos a essas variações.

Porém, em 1729 o astrônomo francês Jean Jacques d´Ortous de Mairan realizou um experimento que demonstrou a existência de um ritmo circadiano independente de fatores externos. De Mairan colocou uma planta “sensível” (*Mimosa pudica*) em uma caixa fechada, de maneira que ela não captasse nenhuma luz exterior. Após observá-la ele notou que ela continuava a abrir suas folhas no mesmo horário que as abria quando em contato com a luz, com uma periodicidade em torno de 24hs (Fig 1.1). Ele comparou seus resultados com dados mostrando que pacientes hospitalizados, mesmo sem ter noção da hora, continuavam a apresentar sinais de fome e sono no horário habitual (Moore-Ede et al, 1982). Com isso ele propôs a existência de um oscilador interno, independente do meio ambiente, que governava tais ritmos. De fato, essa mais tarde veio a ser confirmada como a principal característica dos relógios biológicos: auto-sustentabilidade.

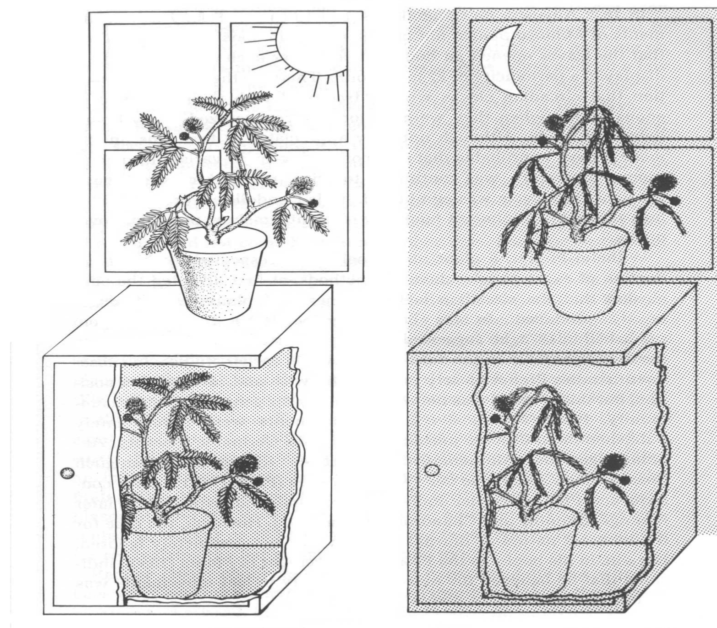

Fig 1.1: O experimento de De Mairan, realizado em 1729 (retirado de Moore-Ede et al, 1982).

Este forte indício, contudo, não foi suficiente para que a comunidade científica aceitasse a existência de um mecanismo interno gerador de atividade circadiana. Alguns experimentos foram feitos no século seguinte, mas foi somente durante o século XX que a confirmação da existência e caracterização de osciladores internos em diferentes organismos modelo, notadamente bactérias (*Synechococcus elongatus*, revisado em Iwasaki & Kondo, 2004), fungo (*Neurospora crassa*, revisado em Dunlap & Loros, 2006), planta (*Arabidopsis thaliana*, revisado em Gardner et al, 2006), mosca de fruta (*Drosophila melanogaster*, revisado em Hardin 2005) e camundongo (*Mus musculus*, revisado em Ko & Takahashi, 2006) impulsionaram o estudo do que mais tarde veio a ser chamado de Cronobiologia (grego: *chronos* = tempo).

Dentre as espécies que não são consideradas modelo, os mosquitos provavelmente foram os de maior valia para a Cronobiologia, devido à grande variedade de eventos em seu ciclo de vida que apresentam periodicidade circadiana, como por exemplo oviposição, atividade locomotora e alimentação sanguínea (Clements, 1999). Como conseqüência disto, várias espécies de mosquitos foram estudadas do ponto de vista comportamental durante o século XX. O primeiro relato acerca dos relógios biológicos destes insetos foi publicado em 1918 por Roubaud, numa análise do ritmo de vôo em *Anopheles maculipennis* (revisado em Clements, 1999). Roubaud observou que esta espécie, se mantida em um regime de 12hs de luminosidade seguidas de 12hs de escuridão, sempre voava nas duas primeiras horas após o desligamento

das luzes, permanecendo em repouso nas outras 22hs. Quando transferidos para um regime de escuridão absoluta, o horário em que começavam a voar adiantava alguns minutos a cada dia, mas sempre com o mesmo período. Roubaud inferiu esse ritmo como sendo gerado pelo próprio mosquito, auto-sustentável (ou seja, independente da influência da luz), com uma duração constante e pouco diferente do período de luminosidade e escuridão de um dia (Clements, 1999).

Além da importância dos mosquitos para o desenvolvimento das bases da Cronobiologia, a relação entre os ritmos circadianos e a transmissão de doenças (nos casos de mosquitos com importância médica) torna o estudo dessas espécies de grande importância para a sua biologia. Este é também o caso do flebotomíneo *Lutzomyia longipalpis*, o principal vetor da Leishmaniose visceral nas Américas (ver seção 1.11).

## 1.2 *Drosophila* como modelo

No entanto, foram as moscas do gênero *Drosophila* que mais contribuíram para o estudo dos relógios biológicos. Colin Pittendrigh, um dos fundadores da Cronobiologia, começou por volta de 1950 a trabalhar com moscas da espécie *Drosophila pseudoobscura* que, assim como os mosquitos, apresentavam uma série de eventos controlados pelo relógio biológico. Ele escolheu insetos desse gênero devido a diversos fatores, mas principalmente à facilidade de criação em laboratório e o curto tempo de desenvolvimento. Dessa forma, eventos

como a emergência da fase pupal puderam ser analisados em larga escala em experimentos com grande número de indivíduos.

A emergência do estádio de pupa é um acontecimento único na vida de uma mosca e ocorre preferencialmente nas primeiras horas do dia. Se a mosca não emerge neste horário ela espera a próxima "janela temporal", evitando eclodir no meio do dia onde as altas temperaturas e a exposição a predadores poderiam abreviar sua sobrevivência. Observando-se o padrão de emergência de uma população nota-se claramente que este evento apresenta ritmicidade circadiana. Pittendrigh mostrou que este ritmo era controlado por um relógio biológico, já que persistia na ausência de ciclos de luminosidade e temperatura (com um padrão próprio, pouco diferente de 24hs). Além disso, estabelecendo a emergência da pupa como um evento controlado por um relógio biológico, ele manipulou as moscas de diversas formas e definiu as principais propriedades dos marcapassos endógenos como veremos a seguir.

Em um primeiro momento Pittendrigh notou que pulsos de luz em determinados horários da noite eram capazes de adiantar ou atrasar o relógio, fazendo com que as moscas eclodissem mais cedo ou mais tarde no dia seguinte. Ele concluiu que o relógio era capaz de ser ajustado a um novo ciclo de luminosidade/escuridão, fato que mais tarde foi estendido a todos os organismos modelo já estudados (Pittendrigh, 1954; Dunlap, 1999). Essa propriedade é de vital importância porque os organismos; apesar de possuírem um relógio biológico autônomo e auto-sustentável, devem ser capazes de ajustá-lo de acordo com

mudanças no ambiente externo como, por exemplo, quando mudamos de estação (dias ficam mais curtos ou mais longos) ou quando somos submetidos a uma mudança de fuso horário (como em uma viagem aérea transatlântica).

Outra pergunta feita por Pittendrigh foi se variações de temperatura influenciariam o ritmo circadiano gerado pelo relógio. Por se tratar de organismos pecilotérmicos, as drosófilas têm seu metabolismo alterado de acordo com as variações de temperatura do meio. Porém, um relógio que tem o propósito de fornecer informações para o controle de importantes funções do organismo deve ser confiável, independentemente das condições a que ele esteja exposto. Disposto a provar que isto era o que ocorria, Pittendrigh analisou a emergência de drosófilas submetidas a variações de temperatura e constatou que isso não influenciava o período do ritmo de emergência em escuridão e temperatura constantes (Pittendrigh, 1954).

Essas duas propriedades descobertas por Pittendrigh, em conjunto com a primeira descrita por de Mairan e por ele também confirmada, formam as três principais características dos relógios biológicos, que mais tarde foram estendidas para vários organismos, de bactérias a humanos: auto-sustentabilidade, adaptabilidade ou sincronização a fatores externos e compensação térmica (Moore-Ede et al, 1982).

Atualmente um dos modelos centrais da Cronobiologia propõe a existência de três componentes dirigindo a ritmicidade circadiana (Fig 1.2): o "INPUT ou ZEITGEBER" é qualquer estímulo externo que seja

transmitido ao marcapasso interno para ajustá-lo com o meio ambiente. O MARCAPASSO OU RELÓGIO BIOLÓGICO é um mecanismo oscilador endógeno, auto-sustentável e com mecanismo de compensação térmica gerador do ritmo circadiano. O "OUTPUT" congrega as alterações comportamentais e fisiológicas geradas pela interação entre o relógio e o "INPUT" (Moore-Ede et al, 1982).

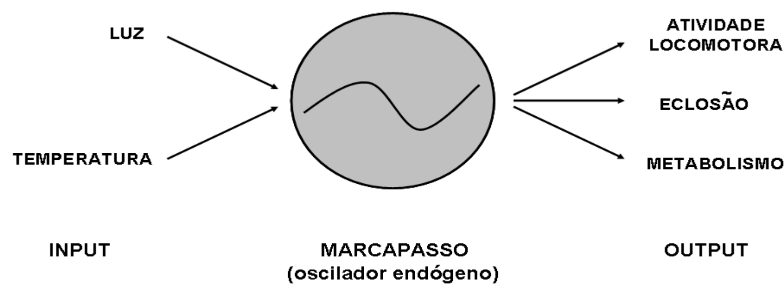


Fig 1.2: Os três componentes que governam os ritmos circadianos.

Apesar de todos os avanços na descrição dos mecanismos envolvidos nos relógios biológicos do ponto de vista fisiológico, a Cronobiologia ainda ressentia de provas cabais que ligassem o controle destes ritmos a um oscilador interno, endógeno. Apesar das evidências experimentais em diferentes organismos (em escuridão e temperatura constantes) provarem que o mecanismo era interno, alguns grupos ainda acreditavam que fatores que naquela época não podiam ser controlados em laboratório, como por exemplo o campo magnético terrestre, poderiam estar sincronizando a atividade desses organismos. Sendo assim, os cronobiologistas começaram a buscar uma prova que mostrasse de maneira irrefutável a existência do relógio biológico.

Foi então que Pittendrigh propôs que se este mecanismo fosse controlado pelo próprio organismo, ele deveria ser controlado por genes

e, portanto, ser regido pelas leis da genética. Partindo deste princípio ele começou a observar os ritmos naturais de uma população de moscas. Ele notou que, dentro de uma mesma população, era possível notar a existência de certa variabilidade quanto ao período de eclosão das moscas. Se essas variações fossem controladas por genes, por cruzamentos controlados seria possível isolar populações com indivíduos com períodos cada vez mais próximos entre si. Partindo deste princípio ele selecionou moscas de acordo com suas características temporais, realizou cruzamentos controlados entre indivíduos com períodos semelhantes e observou que as características temporais paternas foram transmitidas para as próximas gerações (Pittendrigh, 1967). Com isso ficou definitivamente provado que o relógio estava sob controle genético.

### 1.3 *Drosophila melanogaster* como modelo

Os estudos de Colin Pittendrigh foram feitos em *Drosophila pseudoobscura* que, apesar de ter proporcionado avanços no entendimento das bases da Cronobiologia, teve seu uso limitado devido ao seu pequeno poder de manipulação genética. Então *Drosophila melanogaster* passou a ser mais estudada, justamente por ter em seu acervo técnicas mais refinadas que permitiram dissecar as bases moleculares do relógio.

No final da década de 1960, no Instituto de Tecnologia da Califórnia nos EUA, o laboratório de Seymour Benzer iniciava um

projeto que se tornou um marco no desenvolvimento da Neurogenética de *Drosophila*. Benzer propôs que genes específicos controlariam diferentes aspectos do comportamento e que, por meio de mutações nesses genes, seria possível estudar o sistema nervoso e a regulação do comportamento animal. Desta maneira seu laboratório induzia mutações pontuais (em apenas um gene) e buscava moscas que apresentavam alterações no sistema nervoso. Dessa forma foram isolados vários genes que afetavam fenômenos diversos como gravidade, aprendizado, comportamento sexual e neurodegeneração, dentre outros (revisado em Greenspan, 2008).

Um dos alunos de Benzer, Ronald Konopka, que conhecia o trabalho de Pittendrigh, provocou mutações no cromossomo X de *D. melanogaster* e depois separou as moscas adultas que emergiam das pupas em horários diferentes do observado em moscas não-mutantes. Assim, ele isolou três linhagens com mutações que alteravam o ritmo circadiano de emergência dos adultos: uma com ciclos de 19 horas, uma com ciclos de 29 horas e uma arrítmica (sem ciclos de emergência). Com técnicas de genética clássica, Konopka e Benzer demonstraram que as três mutações ocorriam no mesmo gene, batizado de *period*. Eles testaram o padrão de atividade locomotora das três linhagens mutantes em escuridão e temperatura constantes, e a duração dos ciclos repetiu a do padrão de emergência. Tais mutações ficaram conhecidas como $per^{S}$ ("short", período curto – 19hs), $per^{L}$ ("long", período longo – 29hs) e $per^{01}$ (arrítmico) (Konopka & Benzer, 1971). O isolamento desses mutantes por Konopka e Benzer veio a corroborar de maneira indubitável os

dados de Pittendrigh acerca dos cruzamentos controlados com *D. pseudoobscura*, inferindo um caráter endógeno (e genético) a produção dos ritmos circadianos. Além disso, esse trabalho tem importância ímpar na Neurobiologia, visto que *period* foi o primeiro gene de comportamento isolado em qualquer ser vivo.

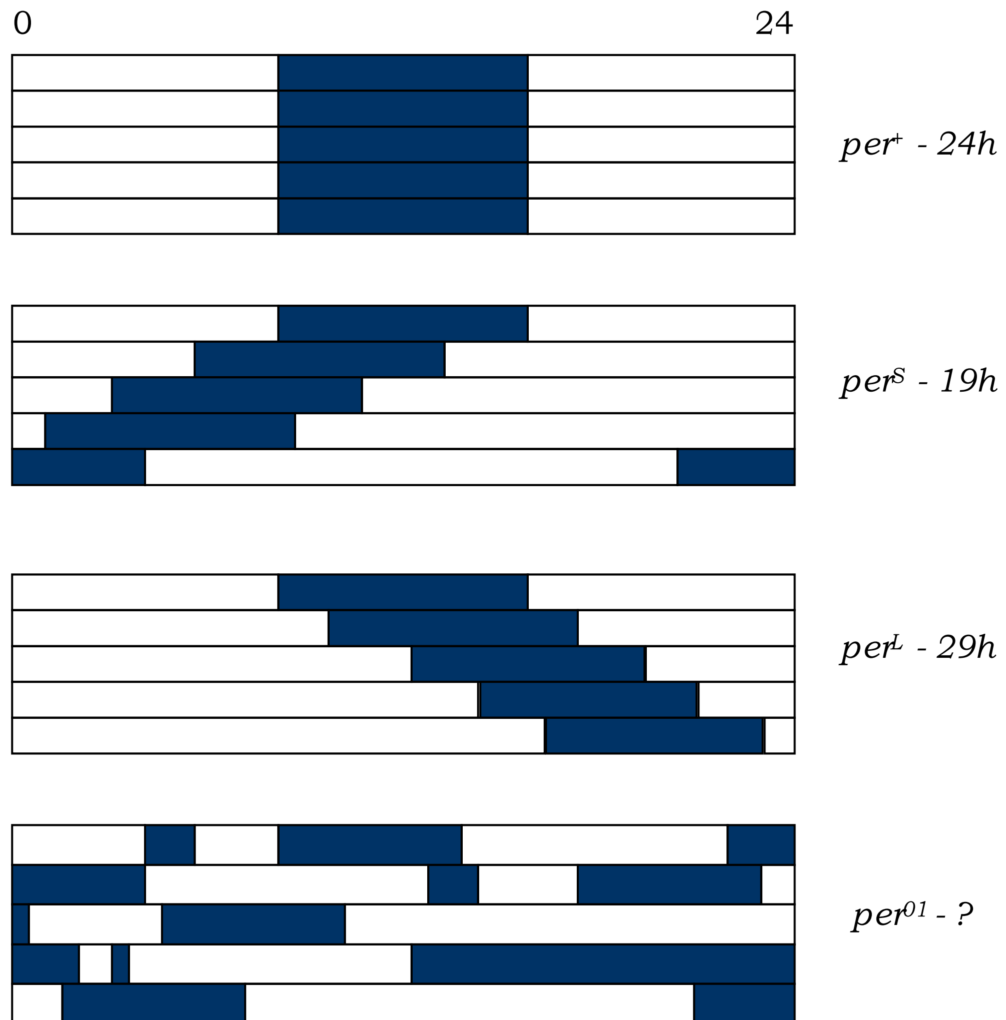


Fig 1.3: Padrão de atividade locomotora em escuridão e temperatura constantes de moscas selvagem e mutantes do gene *period* de *D. melanogaster*. As barras horizontais simbolizam períodos de 24 horas em dias consecutivos. Área em branco, período de repouso; em azul, período de atividade. Enquanto uma mosca normal (*per*$^{+}$) inicia e termina sua atividade aproximadamente na mesma hora todo dia, o mutante *per*$^{S}$ inicia sua atividade cerca de 5 horas mais cedo. O oposto ocorre com o mutante *per*$^{L}$. Já o mutante *per*$^{01}$ é essencialmente arrítimico (adaptado de Konopka & Benzer, 1971).

## 1.4 O controle molecular do relógio biológico de *D. melanogaster*

O gene *period* foi posteriormente clonado (Bargiello et al, 1984; Reddy et al, 1984) e desde então muito do trabalho desenvolvido com a genética dos ritmos biológicos se baseou no estudo desse gene (revisado em Hardin 2005).

Tanto a proteína PER quanto seu RNAm são expressos de maneira circadiana, porém em fases diferentes (Hardin et al, 1990; Zerr et al, 1990) (Fig 1.4). Esta diferença de fase sugeriu que PER poderia estar fazendo uma auto-regulação negativa já que quando a proteína estava na sua máxima concentração do dia sua transcrição era reprimida (Hardin et al, 1990; Zerr et al, 1990).

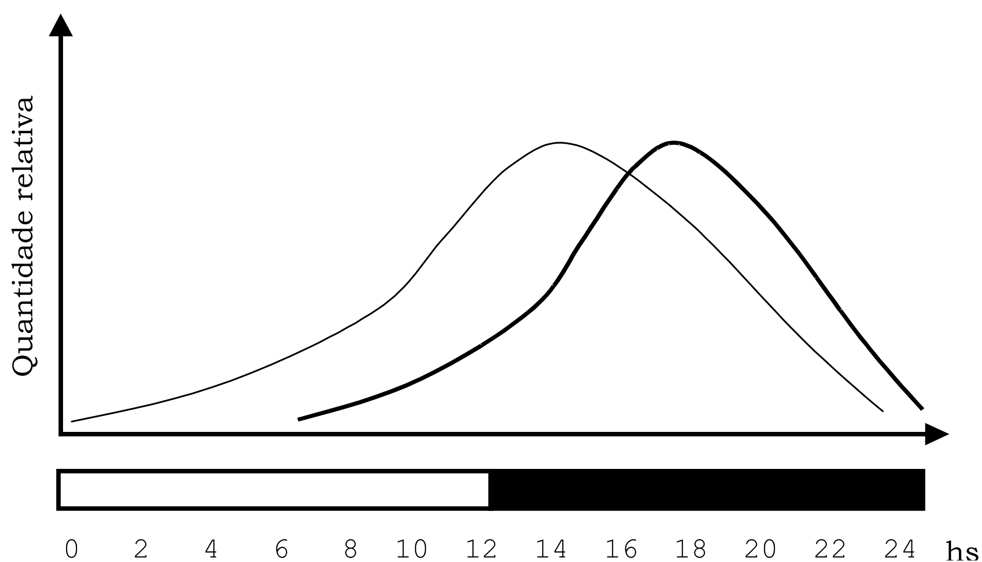


Fig 1.4: Ritmo circadiano da abundância do RNAm e proteína de *per* durante um ciclo de 24hs em 12:12 LD (12 “light” : 12 “dark”; 12hs de luz seguidas de 12hs de escuridão). Em linha fina: expressão do RNAm; em linha grossa: a proteína PER. Barra abaixo do gráfico: em branco, dia; em preto, noite.

A seqüência de aminoácidos codificada por *period* inclui um domínio de dimerização entre proteínas chamado PAS (de **P**ER-**A**RNT-**S**IM, proteínas que fundaram esta família) comum a vários fatores de transcrição da família bHLH ("basic-helix-loop-helix", região na qual esses fatores se ligam ao DNA) (Huang et al, 1993). Entretanto *period* não possuía tal domínio de ligação ao DNA. Deste modo deveria inibir sua própria transcrição indiretamente, inibindo um fator de transcrição que estivesse ativando-o. Isso deixou em aberto a possibilidade da existência de outros genes controlando o relógio biológico em *D. melanogaster*.

Foi quando em 1994 Sehgal e colaboradores identificaram uma outra mutação que alterava o padrão de atividade circadiana de *D. melanogaster* e a denominaram de *timeless (tim)* (Sehgal et al, 1994). A clonagem e caracterização do padrão de expressão de *tim* mostraram que ele oscila na mesma fase de *per*, tanto ao nível do RNAm quanto da proteína (Sehgal et al, 1995; Myers et al, 1995). Por estar presente nos mesmos horários que PER, cogitou-se que TIM poderia ser o fator de transcrição que se ligava a ele. De fato, TIM se liga a PER e a formação deste dímero é necessária para a estabilização do último. Isto porque apesar do RNAm de *per* ainda apresentar alguma oscilação, a proteína por ele codificada quase não é detectada em horário algum no mutante $tim^0$ (Price et al, 1995). Além disso, TIM também é importante para a localização nuclear de PER e a entrada deste dímero no núcleo é necessária para que PER exerça sua função inibitória (Saez & Young, 1996) (Fig 1.5).

Um passo importante na manutenção da periodicidade do relógio é o "atraso" entre o pico de abundância do RNAm de *per* e sua proteína (Fig 1.4). Logo após o início de sua transcrição a proteína PER não se acumula no citoplasma graças à ação de uma quinase chamada DOUBLETIME (DBT) (Kloss et al, 1998; Price et al, 1998). Esta quinase adiciona grupamentos fosfato ao PER monomérico marcando-o para degradação, imprimindo o atraso necessário na formação do dímero PER:TIM. Em alguns mutantes de *dbt* a dimerização de PER e TIM é antecipada, fazendo com que eles entrem no núcleo mais cedo e conseqüentemente inibam sua própria transcrição antes do horário habitual, adiantando todo o relógio (Kloss et al, 1998; Price et al, 1998).

Uma outra quinase envolvida no controle da passagem do complexo PER:TIM para o núcleo é a proteína traduzida do gene *shaggy (sgg)* (Martinek et al, 2001). SGG fosforila TIM, mas diferentemente de PER isto não é um sinal para sua degradação. Mutantes que superexpressam *sgg* apresentam TIM hiperfosforilado, fazendo com que o dímero PER:TIM entre no núcleo mais cedo, enquanto que mutantes que apresentam baixa expressão de SGG tem TIM hipofosforilado e entram no núcleo tardiamente, em ambos os casos levando consigo PER (Martinek et al, 2001). Ainda há a ação de outra quinase, CASEIN KINASE 2 (CK2), que atua também desestabilizando PER e controlando sua entrada no núcleo (Lin et al, 2005). Além de uma fosfatase, PROTEIN PHOSPHATASE 2a (PP2a), que atua de maneira contrária às quinases, estabilizando PER (Sathyanarayanan et al, 2004). A fina

regulação de ambas dita a estabilidade e concentração tanto de PER quanto de TIM.

A clonagem de *per* e *tim* e a demonstração de que seus RNAms variavam em abundância de forma rítmica deixou uma questão central em aberto: quem estaria ativando a transcrição destes dois genes? Hao e colaboradores identificaram na região promotora de *per* uma seqüência de 69 pares de base que promovia a transcrição circadiana de um gene repórter (Hao et al, 1997). Além disso, nesta região havia uma seqüência E-box, que é sítio de ligação de fatores de transcrição da família bHLH-PAS. Isto reforçou a idéia que componentes desta família estariam envolvidos na ativação de *per* (Hao et al, 1997).

Em 1998, *Clock (Clk)* e *cycle (cyc)* foram isolados a partir de mutantes ($Clk^{Jrk}$ e $cyc^{01}$ respectivamente) que alteravam tanto o padrão de atividade locomotora quanto o de eclosão (Allada et al, 1998; Rutila et al, 1998). As proteínas por eles codificadas (CLK e CYC) são fatores de transcrição da família bHLH-PAS que, dimerizados, se ligam aos E-box nas regiões promotoras de *per* e *tim* ativando suas transcrições (Darlington et al, 1998). *Clk* também é regulado pelo relógio e seu RNAm cicla em antifase a *per* e *tim*, enquanto seus níveis protéicos de mantém inalterados ao longo do dia (Darlington et al, 1998; Bae et al, 1998; Lee et al, 1998; Houl et al, 2006; Yu et al, 2006). Já *cyc* é constitutivo tanto ao nível transcricional quanto traducional e seus níveis de proteína são mais altos que as demais proteínas do relógio durante todo o dia (Rutila et al, 1998; Darlington et al, 1998; Bae et al, 2000).

Sumarizando, o modelo geral de funcionamento do que se convencionou chamar de "primeira alça regulatória" funciona da seguinte maneira: no início do dia *per* e *tim* começam a ser transcritos heterodímero CLK:CYC (Bell-Pedersen et al, 2005; Hardin, 2005). CLK é fosforilado ritmicamente, sendo que as formas hipofosforiladas estão presentes na parte da manhã e tem função ativadora, enquanto que as formas hiperfosforiladas estão presentes ao fim do dia e não promovem ativação da transcrição (Yu et al, 2006). Na medida em que *per* e *tim* vão sendo transcritos suas traduções se iniciam. Entretanto a acumulação de PER é retardada graças à ação de DBT, que se liga a PER e o fosforila, sinalizando-o para degradação (Kloss et al, 1998; Price et al, 1998). PER só consegue se acumular quando os níveis de TIM aumentam, já que em sua presença o complexo formado (PER:TIM:DBT) é estável, ainda que fosforilado. A entrada deste trímero no núcleo é regulada pelo grau de fosforilação de TIM por SGG e de PER por CK2 (Martinek et al, 2001; Lin et al, 2005). Já dentro do núcleo PER, por seu domínio CCID ("CLK:CYC inhibition domain"), se liga e remove CLK:CYC do DNA, impedindo assim a sua própria transcrição e a de *tim* (Bae et al, 2000; Chang & Reppert, 2003; Yu et al 2006). Isto acontece com ele ainda ligado a TIM, porém esta inibição é mais consistente após eles dissociarem (Rothenfluh et al, 2000). Apesar de reprimir com mais intensidade CLK:CYC dissociado de TIM, nesta condição PER continua a ser fosforilado por DBT e é degradado, finalizando a repressão e dando início a um novo ciclo com a ativação de *per* e *tim*. Este mecanismo

constitui portanto a primeira alça regulatória que gera transcrição cíclica de *per* e *tim* e dura aproximadamente 24hs (Fig 1.5).

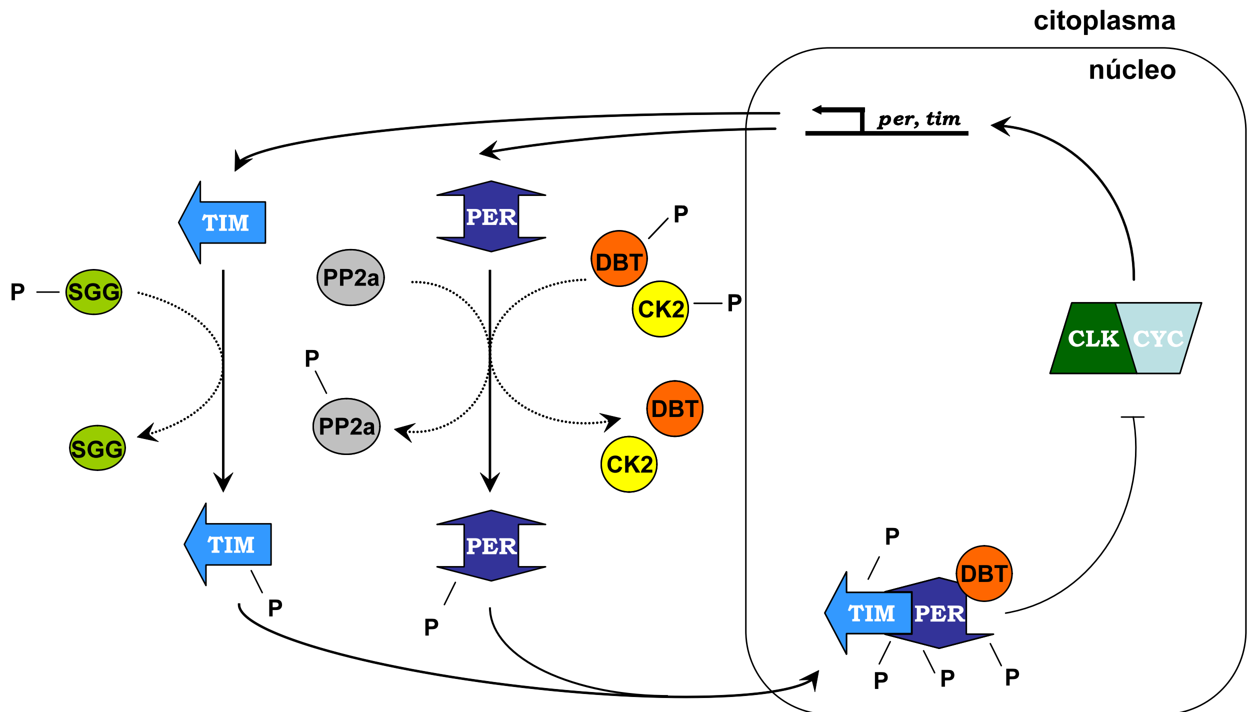


Fig 1.5: Esquema do modelo da primeira alça regulatória do relógio biológico em *D. melanogaster*. Retas terminando em setas, ativação; setas terminando em barras, inibição. *per* e *tim* são transcritos pela manhã pelo dímero CLK:CYC. O acúmulo de PER é retardado pela ação de DBT e CK2 que o fosforila marcando-o para degradação. Sua ação é contrabalanceada pela fosfatase PP2a, que remove fosfatos de PER e mantém sua estabilidade. Na presença de TIM, o complexo PER:TIM torna-se estável. A entrada do complexo no núcleo é regulada por SGG, que fosforila TIM, e CK2, que fosforila PER. No núcleo, o complexo (e também PER monomérico) inibe a ação de CLK:CYC, inibindo sua própria transcrição e iniciando um novo ciclo.

Ainda em 1999, Glossop e colaboradores sugeriram a existência de um outro mecanismo de retro-alimentação negativa, ligado ao primeiro já descrito, que seria responsável pela regulação dos níveis de *Clk*. Entretanto este trabalho não identificou nenhum gene desta segunda alça regulatória (Glossop et al, 1999). Também em 1999, uma

deleção no gene *vrille* (*vri*) foi caracterizada por tornar esses mutantes arrítmicos, mas sua função não pôde ser descrita na época (Blau & Young, 1999). Foi quando em 2003 dois trabalhos elucidaram esta questão, atribuindo a *vri* uma função na nova alça de "feedback" negativo e adicionalmente incriminando *PAR domain protein 1* isoforma $\epsilon$ (*Pdp1*$\epsilon$) como o mais novo componente do relógio biológico em *D. melanogaster* (Glossop et al, 2003; Cyran et al, 2003).

O primeiro trabalho acerca do papel de *vri* no relógio mostrou que ele cicla na mesma fase de *per* e *tim*, sugerindo que era ativado também pelo dímero CLK:CYC. Além disso, quando superexpresso a produção de *per* e *tim* diminui, o que é um indício de que poderia fazê-lo por meio da inibição de CLK:CYC (Blau & Young, 1999). A estrutura de VRI indicou que se tratava de uma proteína com domínio de ligação a DNA (bZip – "basic-leucine-zipper"), portanto tal inibição poderia ser mediada pela sua ligação à regiões promotoras de *Clk*, já que ambos ciclam em antifase. A análise da região promotora de *Clk* revelou vários sítios de ligação de E4BP4 (homólogo de VRI em mamíferos) e tais sítios eram capazes de se ligar a VRI *in vitro*, fazendo com que a expressão de *Clk* fosse diminuída. Isto definiu um papel para *vri* no segundo mecanismo de retro-alimentação proposto anteriormente (Glossop et al, 2003).

Adicionalmente, observou-se que *Pdp1*$\epsilon$ também desempenhava papel no mecanismo de regulação de *Clk* e que pertencia a mesma família de fatores de transcrição de *vri*, a família bZip. Por apresentarem o mesmo domínio de ligação a DNA foi testado se eles competiam pela seqüência na região promotora de *Clk*, e foi confirmado. Experimentos

*in vitro* fusionando a seqüência reguladora de *Clk* a um gene repórter mostraram que tanto PDP1ε quanto VRI competiam pela mesma seqüência. Como esperado, a verificação do padrão de expressão de *Pdp1ε* ao longo do dia revelou que ele cicla na mesma fase de *per*, *tim* e *vri*, já que é também controlado por CLK:CYC. Porém, interessantemente, com um atraso de 3-6hs. Esta diferença de acumulação é essencial para que a ativação (por PDP1ε) aconteça logo após a repressão (por VRI), fazendo com que *Clk* apresente um padrão de expressão oscilatório que se estenda por 24hs (Fig 1.6)(Cyran et al, 2003).

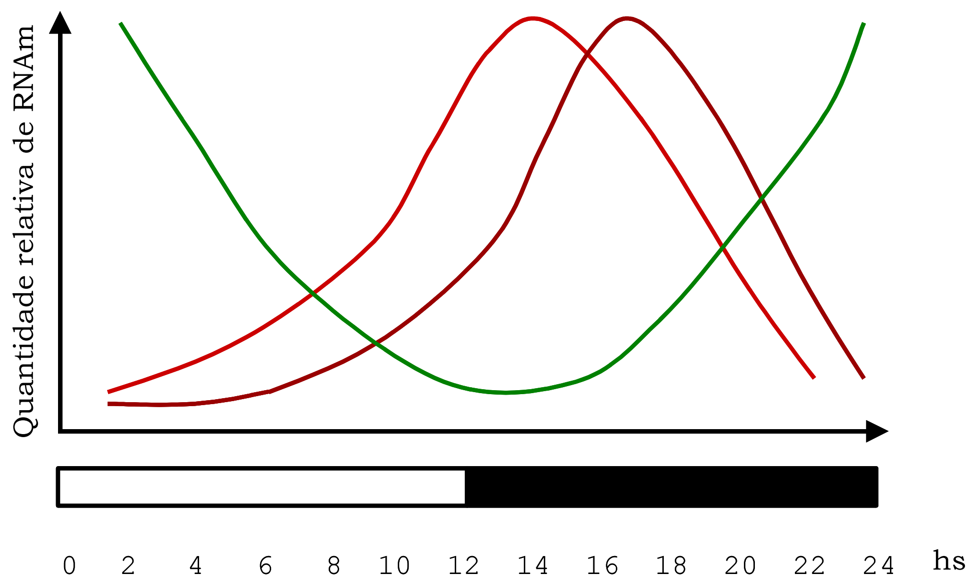


Fig 1.6: Expressão dos genes da segunda alça regulatória. Em verde, *Clk*; em vermelho claro, *vri*; em vermelho escuro *Pdp1ε*. Barra abaixo do gráfico: em branco, dia; em preto, noite.

Esse mecanismo constitui a segunda alça regulatória do relógio biológico de *D. melanogaster*. Sintetizando, no começo da manhã o heterodímero CLK:CYC começa a transcrever *vri* e *Pdp1ε* (Glossop et al,

2003; Cyran et al, 2003). Os produtos destes genes não se acumulam na mesma velocidade: o fator negativo (VRI) atinge níveis altos mais cedo. Com isso ele se liga às regiões regulatórias de *Clk* e inibe sua produção (Glossop et al, 2003). Pouco mais tarde (dentre 3-6hs) os níveis de PDP1ϵ (o fator positivo) sobrepujam os de VRI. Dessa forma PDP1ϵ ativa *Clk* ao mesmo tempo em que causa uma diminuição da inibição causada por VRI, já que ambos competem pelo mesmo sítio de ligação ao DNA (Cyran et al, 2003). Com isso, já no final da noite, *Clk* volta a ser produzido dando início a um novo ciclo na segunda alça (Fig 1.7, Tabela 1).

Dessa forma os produtos dos genes *Clk* e *cyc* funcionam como intercomunicadores entre as duas alças. Se na primeira o heterodímero CLK:CYC controlava a expressão de *per* e *tim* e tinha sua atividade regulada por seus produtos, na segunda *Clk* tem a transcrição regulada por VRI e PDP1ϵ. Estes mecanismos de retroalimentação negativa levam aproximadamente 24hs para completar um ciclo e por isso são chamados de relógio circadiano (Fig 1.7). Vale-se notar que vários fatores de transcrição do marcapasso central apresentam picos de atividade em horários distintos. Como esses fatores de transcrição são capazes de se ligar a regiões promotoras de genes alvo, os alvos do relógio podem apresentar periodicidades distintas, mesmo sendo controlados pelo mesmo mecanismo central. Dessa maneira o marcapasso central controla a fisiologia e o comportamento do organismo ao nível molecular (Fig 1.7).

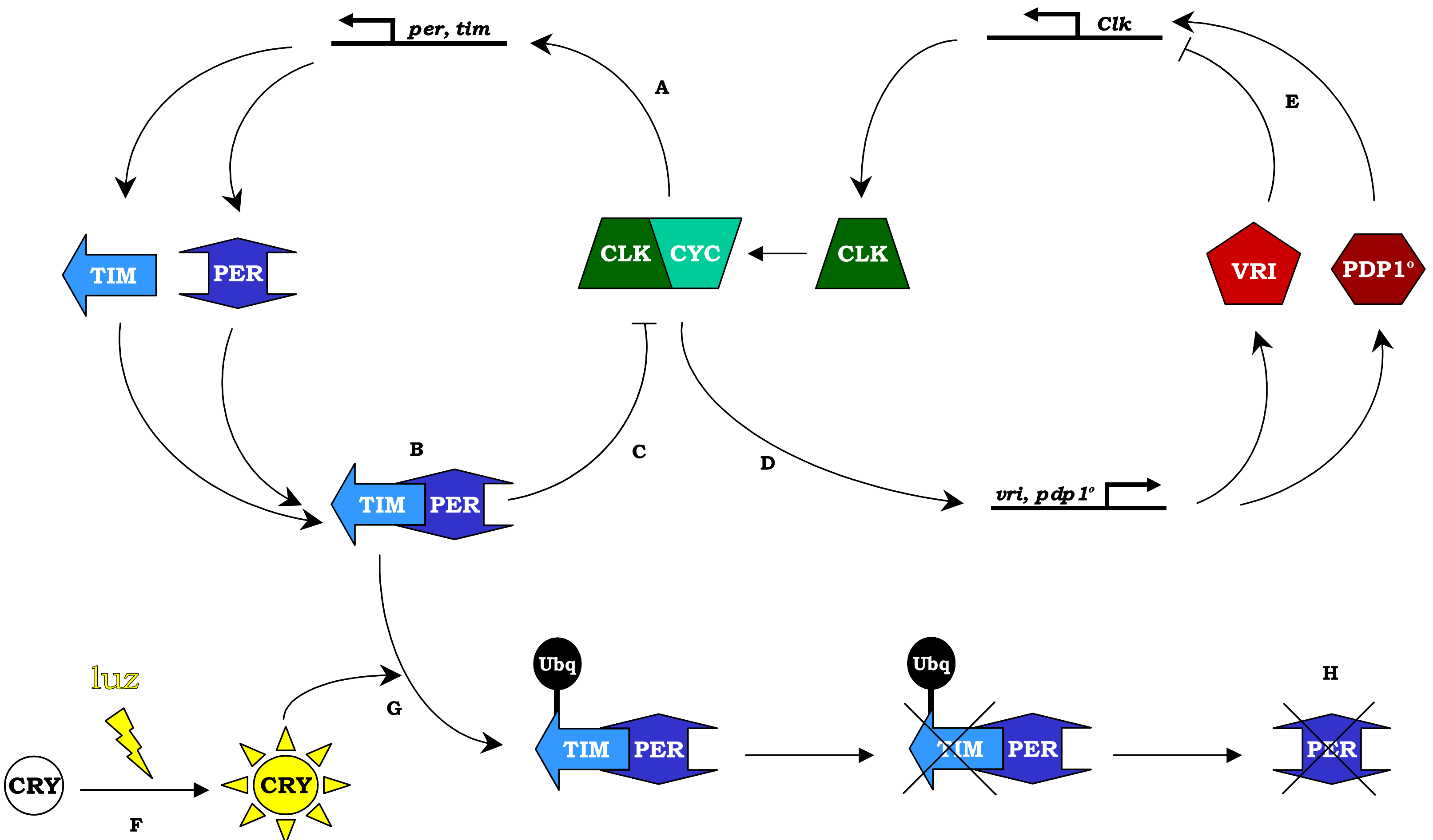


Fig 1.7: Esquema do modelo corrente do funcionamento do relógio biológico em *D. melanogaster*. As quinases foram omitidas para melhor visualização. Retas terminando em setas, ativação; setas terminando em barras, inibição. Ubq, ubiquitina. 1a alça: *per* e *tim* são transcritos pela manhã pelo dímero CLK:CYC (A). PER se liga a TIM, se estabilizando e entrando no núcleo (B). PER se liga a CLK:CYC e os inibe (C). 2a alça: CLK:CYC, no começo da manhã, transcreve *vri* e *Pdp1ϵ* (D). VRI atinge o pico em ZT 13 (inibindo a produção de *Clk*), já PDP1ϵ em ZT 18 (ativando a produção de *Clk*)(E). INPUT: pela manhã a luz sensibiliza CRY (F), fazendo com que ele marque TIM para degradação (G). Sem TIM para estabiliza-lo, PER é degradado (H). Ver detalhes do funcionamento de CRY na Secção 1.5 – INPUT.

## 1.5 INPUT

Como foi dito anteriormente, os principais atores moleculares na geração do mecanismo auto-regulatório estão descritos. Mas existem ainda mais dois parâmetros fundamentais para os ritmos observados: a interação entre o marcapasso e os fatores externos (INPUT) e a ação resultante desta interação (OUTPUT) que gera os ritmos observados no comportamento e na fisiologia.

O INPUT começou a ser desvendado logo após a clonagem de *tim*. Verificou-se que no início do dia TIM era degradado pela ação da luz (Zeng et al, 1996). O mecanismo pelo qual isto acontecia era desconhecido até o isolamento do mutante $cry^{baby}$ ($cry^{b}$) (Stanewsky et al, 1998). Neste mutante a ritmicidade é mantida mesmo quando as moscas são submetidas à iluminação constante, situação em que moscas selvagens se tornam arrítmicas. A proteína selvagem CRYPTOCHROME (CRY) apresenta homologia com fotoliases bacterianas que utilizam energia luminosa para reparar erros em seu DNA, de onde se partiu para o estudo que a caracterizou como um fotoreceptor em *D. melanogaster* (Emery et al, 1998). A análise funcional de CRY demonstrou que ela se liga a TIM e o degrada (após a adição de uma ubiquitina, que é um marcador para degradação via proteossomo), numa reação dependente de luminosidade, que não ocorre em $cry^{b}$ (Stanewsky et al, 1998; Ceriani et al, 1999; Naidoo et al, 1999). Como PER fosforilado é instável na ausência de TIM, ele também acaba sendo degradado. Então, no início da manhã, quando os níveis de *per* e *tim*

estão diminuindo graças a repressão de CLK:CYC, o complexo PER:TIM residual é degradado pela ação da luz via CRY. É desta maneira que a natureza "acerta" o relógio quando há mudanças na duração do período luminoso do dia ou na mudança de fuso horário (Fig 1.7).

## 1.6 Um exemplo de OUTPUT: a atividade locomotora

Em mamíferos o controle celular do relógio reside no NSQ (núcleo supraquiasmático, do inglês suprachiasmatic nucleus - SCN), localizado na região anterior do hipotálamo (revisado em Reppert & Weaver, 2002). A busca de uma região com função similar em *D. melanogaster* revelou pequenos neurônios na região ventro-lateral do cérebro (s-$LN_v$ – "small ventrolateral neurons") como controladores da atividade locomotora circadiana nesta espécie (revisado em Jackson et al, 2001). Estes neurônios expressam PER ciclicamente e estão ausentes no mutante *disconnected* (*disco*), o que o torna arrítmico (Helfrich-Förster, 1995; Helfrich-Förster, 1998). Usando o hormônio PDF ("PIGMENT DISPERSING FACTOR") como sonda era possível marcar os s-$LN_v$ e suas projeções, notadamente suas extremidades axonais, além de outros neurônios do cérebro que não apresentavam PER ciclando. Este peptídeo foi escolhido por estar relacionado a regiões marcapasso em outros insetos (Helfrich-Förster, 1995). O conjunto destes dados sugeriu que os s-$LN_v$ eram o centro celular do marcapasso e controlavam a atividade locomotora através da secreção de hormônios (provavelmente PDF) em diferentes neurônios receptores.

Esta hipótese foi corroborada após a análise dos mutantes *pdf* $^{01}$, que não expressavam a proteína. Eles eram arrítmicos em escuridão constante somente após três dias, o que está de acordo com a hipótese secretora. Caso PDF fosse um componente do marcapasso central, os mutantes deveriam perder ritmicidade logo após o apagar das luzes (Renn et al, 1999). Apesar de PDF ciclar dependentemente de *per*, *tim*, *Clk* e *cyc*, sua transcrição é constitutiva, o que sugere que seu controle se dá ao nível pós-traducional (Park et al, 2000). Além disso, é observado um ritmo circadiano na localização celular de PDF, estando presente nas terminações axonais dos s-$LN_v$ principalmente nas primeiras horas do dia, ausentando-se no início da noite (Park et al, 2000). Estes trabalhos confirmam a hipótese de que o relógio biológico localizado nos s-$LN_v$ controla o ritmo da atividade locomotora em *D. melanogaster* através da liberação circadiana de PDF (Jackson et al, 2001).

### 1.7 Marcapasso central e a autonomia dos relógios periféricos em *D. melanogaster*

Em 2003 foi descrito que a glândula prototorácica, estrutura que controla a eclosão, possui um mecanismo de relógio próprio. Contudo este relógio não é capaz de sozinho controlar o padrão circadiano de eclosão, para tal ele necessita ser sincronizado através da liberação de PDF pelos s-$LN_v$ (Myers et al, 2003). Isto está de acordo com o observado em mamíferos, onde o NSQ, que faz parte do Sistema

Nervoso Central, controla os relógios dos diferentes órgãos (Reppert & Weaver, 2002).

Entretanto diversos trabalhos mostram que órgãos periféricos de *D. melanogaster* possuem relógios que podem funcionar de maneira autônoma, independentemente do controle dos s-$LN_v$. Isto está caracterizado nos tubos de Malpighi (órgão de excreção), nas antenas (órgão sensorial) e nas glândulas assessórias (pertencentes ao aparelho reprodutor masculino) dentre outros (Plautz et al, 1997; Giebultowicz & Hege, 1997; Krishnan et al, 1999; Beaver et al, 2002). Por exemplo, experimentos em que tubos de Malpighi foram isolados *in vitro* mostraram que a expressão cíclica de um gene repórter era mantida pelo seu relógio. Outro experimento em que tubos de um doador foram transplantados para um receptor em fases luminosas opostas mostraram que o órgão do doador continuava a se comportar com sua antiga periodicidade, não entrando em fase com o relógio do receptor. Estes resultados indicaram claramente que o relógio biológico deste tecido funciona independentemente do controle dos s-$LN_v$ (Giebultowicz et al, 2000).

Sendo assim, enquanto em mamíferos o relógio central reside no NSQ e este controla todos os outros relógios do corpo (Reppert & Weaver, 2002), nas drosófilas tal controle está disperso, ou seja, o relógio cerebral é responsável pelo controle de alguns eventos enquanto que outros órgãos periféricos controlam seus produtos através de um oscilador próprio (revisado em Glossop & Hardin, 2002). Possivelmente, isto acontece porque *D. melanogaster*, sendo um animal de pequeno

porte e "translúcido", pôde evoluir de forma que seus órgãos continuassem respondendo à luz, permanecendo, de certa forma, independentes de um controle central.

## 1.8 Diferentes mecanismos para diferentes tecidos

O modelo proposto de funcionamento do marcapasso central em *D. melanogaster* foi fundamentado em uma série de estudos, sendo que a grande maioria deles utilizou cérebros (cabeças) destas moscas como fonte de RNAm e de proteína. Isto porque é nesta região onde se localizam os s-$LN_v$, neurônios controladores da atividade locomotora circadiana e da emergência (Helfrich-Förster, 1995; Helfrich-Förster, 1998). Estudos com "extratos de cabeças" deveriam então revelar o funcionamento do marcapasso central. Entretanto a análise de diferentes tecidos mostrou que relógios periféricos podiam funcionar independentemente dos s-$LN_v$ (Jackson et al, 2001; Glossop & Hardin, 2002). Inclusive na própria cabeça existe um tecido com relógio periférico, a antena, que regula a resposta olfativa de *D. melanogaster* (Krishnan et al, 1999). Isso levantou a hipótese de que o modelo proposto poderia conter informações de diferentes tecidos, não dando uma noção exata do que realmente acontece. Estudos posteriores do relógio periférico da antena revelaram que CRY, além de participar no mecanismo de INPUT, participa na geração do ritmo *per se* (Krishnan et al, 2001; Collins et al, 2006). Este tipo de regulação se assemelha muito com o observado em camundongos, onde o parceiro de mPER

(mousePER) responsável por sua estabilização e localização nuclear é mCRY e não mTIM (Reppert & Weaver, 2002).

Recentemente foi descrito algo semelhante com os próprios s-$LN_v$. Em um experimento realizado por imunocitoquímica foi observado que nestes neurônios é detectada a presença de PER no núcleo aproximadamente 3hs antes de TIM, sugerindo que a formação do dímero não é necessária para a localização nuclear de PER (Shafer et al, 2002). Isto vai essencialmente contra tudo que havia sido descrito anteriormente, porém tais trabalhos utilizaram ou extratos de cabeças ou sistemas heterólogos como as células Schneider 2 (S2) de *D. melanogaster* (Price et al, 1995; Saez & Young, 1996).

Outra forma variante do modelo proposto foi encontrada nos ovários. A expressão de *per* neste tecido é constitutiva e sua proteína é encontrada no citoplasma durante todo o dia. Apesar disto o tecido mostra claramente eventos com periodicidade circadiana como a vitelogênese e a oviposição (revisado em Giebultowicz, 2000). Se estes ritmos são controlados pelo próprio ovário ou pelos s-$LN_v$ ainda está por ser desvendado.

Quase todos os órgãos de *D. melanogaster* apresentam expressão de genes de relógio e eventos com periodicidade circadiana (Giebultowicz, 2000). Embora existam particularidades do relógio biológico para cada órgão, o modelo proposto ainda pode ser utilizado com certa abrangência. Por exemplo, em machos não foi notada diferença no padrão de expressão de *per* entre cabeça e abdômen, sendo o resultado observado com o corpo inteiro muito semelhante ao

resultado obtido com as partes separadas. Já em fêmeas o fato de *per* ser constitutivo nos ovários implicou em uma diferença significativa na expressão de *per* entre cabeça e abdômen (Hardin, 1994).

## 1.9 O controle molecular do relógio biológico de mamíferos

O relógio circadiano de diferentes organismos apresenta muitas similaridades ao longo da escala evolutiva. *D. melanogaster* e camundongos compartilham quase todos os genes envolvidos na geração dos ritmos circadianos estudados, sendo a diferença mais marcante o número de cópias de cada gene envolvido e o padrão de expressão de alguns deles (ver Tabela 1; Dunlap, 1999; Bell-Pedersen et al, 2005; Ko & Takahashi, 2006; Gallego & Virshup, 2007). Além disso, em mamíferos o marcapasso molecular também é controlado por uma interação entre duas alças regulatórias conectadas pelos mesmos fatores de transcrição (ver adiante).

Os genes que compõem o marcapasso central no NSQ em camundongos são: *per* (*mper1*, *mper2* e *mper3*); *cry* (*mcry1* e *mcry2*); *mClk*; *bmal1* (que é homólogo ao *cyc*); *Rev-erbα* e *Rorα*. De todos estes apenas *Rev-erbα* e *Rorα* não têm papel descrito no relógio de *Drosophila*, apesar de desempenharem função análoga. Adicionalmente, as enzimas que controlam estabilidade e localização celular (quinases e fosfatases) em camundongos são exatamente as homológas das descritas no relógio de *D. melanogaster* (Tabela 1).

Tabela 1: Genes de relógio descritos em *D. melanogaster*, seu padrão de expressão, modificações pós-traducionais e homólogos em camundongo.

| **Gene em *Drosophila*** | **Padrão de expressão gênica** | **Padrão de expressão protéica** | **Regulação pós-traducional** | **Homólogo em mamífero** |
|---|---|---|---|---|
| CLK | Cíclico | Constitutivo | Fosforilação rítmica | mCLK |
| CYC | Constitutivo | Constitutivo | Não | BMAL1 |
| PER | Cíclico | Cíclico | Fosforilação progressiva | mPER1, mPER2,mPER3 |
| TIM | Cíclico | Cíclico | Fosforilação progressiva | mTIM |
| PDP1ε | Cíclico | Cíclico | Não | D-element binding protein (DBP) |
| VRI | Cíclico | Cíclico | Fosforilação | E4BP4 |
| CRY | Cíclico | Cíclico, degradado pela luz | – | mCRY1, mCRY2 |
| DBT | Constitutivo | Constitutivo | Não | CK1ε, CK1δ |
| CK2 | Constitutivo | Constitutivo | – | CK2 |
| SGG | Constitutivo | – | – | Glycogen synthase kinase 3(GSK3) |
| PP2a | Cíclico (uma das subunidades) | – | – | PP2a |
| – | – | – | – | Rev-Erbα |
| – | – | – | – | RORα |

*O hífen (-) simboliza indisponibilidade de dados.*

Na primeira alça regulatória, no começo do dia os fatores de transcrição mCLK e BMAL1 se ligam às regiões E-box em promotores de genes alvo e ativam sua expressão. Estes genes alvo incluem *mper1*, *mper2*, *mper3*, *mcry1* e *mcry2*. Em mamíferos o dímero que se forma e reprime mCLK:BMAL1 é mPER:mCRY, e não PER:TIM como em *Drosophila*. Assim sendo, uma alça regulatória semelhante à observada em moscas existe em mamíferos, com exceção da substituição de TIM

por mCRY (Gekakis et al, 1998; Kume et al, 1999; Vitaterna et al, 1999; Bunger et al, 2000).

A segunda alça regulatória apresenta diferenças interessantes entre essas duas espécies, apesar do mecanismo ser muito parecido. Em mamíferos *mClk* é expresso constitutivamente, enquanto *bmal1* cicla tanto ao nível transcricional quanto traducional. Dessa forma não é surpresa imaginar que o mecanismo de retro-alimentação negativa gira em torno de BMAL1, e não de CLK como em *Drosophila*. Além de ativar *mper1*, *mper2*, *mper3*, *mcry1* e *mcry2*; o heterodímero mCLK:BMAL1 também ativa *Rev-erbα* e *Rorα* no início do dia. Analogamente ao acúmulo diferencial de VRI e PDP1ϵ, REV-ERBα e RORα acumulam em tempos distintos. Além disso, ambos competem pelo mesmo sítio RORE (retinoic acid–related orphan receptor response element) na região promotora de *bmal1*. Enquanto REV-ERBα inibe a transcrição de *bmal1* em um primeiro momento, RORα ativa logo após seus níveis sobrepujarem os níveis de REV-ERBα. Esse espaçamento entre inibição e ativação de *bmal1* gera uma oscilação em sua transcrição com um período de aproximadamente 24hs (Preitner et al, 2002; Sato et al, 2004; Triqueneaux et al, 2004; Akashi & Takumi, 2005; Guillaumond et al, 2005). Dessa forma, apesar da segunda alça em mamíferos utilizar moléculas diferentes, o fato do mecanismo ser extremamente parecido constitui um belo exemplo de evolução convergente.

A conservação dos mecanismos geradores de ritmo circadiano em espécies tão distantes na escala evolutiva sugere que organismos mais

próximos apresentariam relógios biológicos extremamente parecidos. Mas não é isso que se observa no grupo dos insetos.

### 1.10 Diferentes mecanismos para diferentes insetos

Apesar das óbvias semelhanças entre os marcapassos centrais de *Drosophila* e mamíferos, comparações entre as seqüências de diferentes espécies de *Drosophila* e outros insetos indicam a rápida evolução dos genes de relógio neste grupo (Tauber et al, 2004). Além da diferenciação estrutural, esta evolução mais rápida em insetos parece estar em alguns casos associada à diferenças na regulação destes genes.

Por exemplo, em *Antheraea pernyi* (mariposa) o gene *per* cicla tanto na sua expressão quanto na sua localização celular (Sauman & Reppert, 1996). O mecanismo de regulação encontrado nas células neuro-secretórias do cérebro é bastante peculiar quando comparado ao observado em *D. melanogaster*. Neste local existe a expressão cíclica de uma seqüência de RNAm que é complementar a *per* (*per* anti-senso), em fase oposta à dele. Isso faz com que a expressão de *per* seja cíclica, mas não devido a um controle pré-transcricional como ocorre em *D. melanogaster* (Sauman & Reppert, 1996).

A possibilidade dos mesmos genes estarem sendo regulados de forma diversa em diferentes organismos implica que cada espécie de interesse deva ser avaliada quanto à evolução e peculiaridades na regulação dos seus genes do relógio. Isto, naturalmente, também vale

para insetos vetores de doenças tropicais como, por exemplo, flebotomíneos.

## 1.11 *Lutzomyia longipalpis*

Flebotomíneos (Fig 1.8) são dípteros hematófagos da família Psychodidae, subfamília Phlebotominae e incluem o gênero *Lutzomyia*, que possui diversas espécies já incriminadas como vetores de *Leishmania* no Novo Mundo (Young & Duncan, 1994; Tesh & Guzman, 1996). *L. longipalpis* é o principal vetor da leishmaniose visceral na América Latina, estando distribuído por quase todo o Brasil (Young & Duncan, 1994).

Fig 1.8: Fêmea de *Lutzomyia longipalpis* se alimentando de sangue através da pele humana (Fonte: WHO/TDR/Stammers).

Machos e fêmeas de flebotomíneos se alimentam de fontes de carboidratos (sucos vegetais) (Souza et al, 1995), sendo a hematofagia praticada apenas pelas fêmeas. As formas adultas se abrigam durante o dia predominantemente em locais úmidos e com pouca luminosidade, normalmente em fendas entre rochas e grutas (Forattini, 1973).

Com o avanço das populações humanas para áreas florestais, *L. longipalpis* acaba se instalando em abrigos artificiais como, por exemplo, em galinheiros, currais e canis. Dessa forma, esta espécie encontra locais com condições favoráveis para sua sobrevivência, além de estar próxima ao animal no qual poderá realizar um repasto sanguíneo. Como *L. longipalpis* apresenta tendência para sugar animais de sangue quente, passa a picar também o homem (Forattini, 1973; Feliciangeli, 2004). Entretanto, apesar de transmitir uma doença em franco crescimento no Brasil graças a sua chegada aos centros urbanos (FUNASA, 2002), *L. longipalpis* ainda é pouco estudada sob muitos aspectos, sobretudo do ponto de vista molecular. Este é o caso dos ritmos circadianos desta espécie.

O comportamento de insetos vetores tem fundamental importância na dinâmica das doenças por eles transmitidas (Klowden, 1996). Por exemplo, o padrão de atividade de hematofagia de mosquitos está certamente sob controle de um relógio biológico (Clements, 1999), que por sua vez está sob controle genético (Dunlap, 1999). Apesar disso, o estudo molecular nesta área recebeu pouca atenção da comunidade científica até o momento, apesar da razoável quantidade de dados de atividade circadiana, seja no campo ou no laboratório (Clements, 1999).

Dados coletados em uma área de Leishmaniose endêmica na Colômbia demonstram que a atividade de *L. longipalpis* neste local é crepuscular/noturna (Morrison et al, 1995). O mesmo é observado na população brasileira da Gruta da Lapinha, Minas Gerais (Nataly de Souza, comunicação pessoal).

Nosso laboratório está estudando a genética molecular de genes do relógio circadiano em insetos vetores. Neste trabalho, nós isolamos e seqüenciamos o gene *cyc* em *L. longipalpis*. Além da clonagem de *cyc*, nós estudamos o padrão da expressão circadiana de *per*, *tim*, *Clock* e *cyc*, e a expressão destes genes após o repasto sanguíneo em *L. longipalpis*. Além do seu papel nos ritmos circadianos, foi atribuída a *cyc* função na resposta ao estresse oxidativo em *D. melanogaster* (Shaw et al, 2002), o que o torna particularmente interessante no estudo de insetos vetores, já que a alimentação com sangue está associada a este tipo de estresse (Oliveira et al, 1999).

Os resultados obtidos em flebotomíneos nos levaram a um interesse sobre aspectos evolutivos dos relógios biológicos. Assim, estudamos o funcionamento de um domínio de CYC encontrado em flebotomíneos e mosquitos, mas ausente em *D. melanogaster*. Analisamos a função desta região a fusionando com CYC de *D. melanogaster* e utilizando este quimérico em ensaios com células Scheider2 (S2) e gerando *D. melanogaster* transgênicas. Esperamos com nossos resultados contribuir para um melhor entendimento da evolução dos relógios biológicos em insetos.

**Capítulo 2 – Objetivos**

- Iniciar o estudo das bases moleculares dos ritmos circadianos em *Lutzomyia longipalpis*, pela clonagem do gene *cyc* e análise da expressão circadiana dos genes que controlam a primeira alça regulatória. Além disso, analisar o impacto da alimentação sanguínea no comportamento e na expressão dos genes do relógio desta espécie.

- Analisar a evolução do mecanismo molecular do relógio biológico de *Drosophila melanogaster*, com ênfase no papel do domínio C-Terminal de CYC de insetos vetores (em especial *Aedes aegypti*) e sua interação com dCRY de *D. melanogaster*.

**Capítulo 3**

# The biological clock of an hematophagous insect: Locomotor activity rhythms, circadian expression and downregulation after a blood meal

Antonio C.A. Meireles-Filho, Gustavo B. da S. Rivas, João S.M. Gesto, Ricardo C. Machado, Constança Britto, Nataly A. de Souza, Alexandre A. Peixoto

Neste artigo publicado em 2006 na FEBS Letters analisamos a atividade locomotora e a expressão circadiana dos genes *period*, *timeless* e *Clock*, componentes da primeira alça regulatória do relógio de *Lutzomyia longipalpis*. Examinamos também o impacto da alimentação sanguínea na expressão destes genes e na atividade locomotora deste vetor.

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# The biological clock of an hematophagous insect: Locomotor activity rhythms, circadian expression and downregulation after a blood meal

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**Abstract** **Despite the importance of circadian rhythms in vector-borne disease transmission, very little is known about its molecular control in hematophagous insect vectors. In *Drosophila melanogaster*, a negative feedback loop of gene expression has been shown to contribute to the clock mechanism. Here, we describe some features of the circadian clock of the sandfly *Lutzomyia longipalpis*, a vector of visceral leishmaniasis. Compared to *D. melanogaster*, sandfly *period* and *timeless*, two negative elements of the feedback loop, show similar peaks of mRNA abundance. On the other hand, the expression of *Clock* (a positive transcription factor) differs between the two species, raising the possibility that the different phases of *Clock* expression could be associated with the observed differences in circadian activity rhythms. In addition, we show a reduction in locomotor activity after a blood meal, which is correlated with downregulation of *period* and *timeless* expression levels. Our results suggest that the circadian pacemaker and its control over the activity rhythms in this hematophagous insect are modulated by blood intake.**
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*Keywords:* *Period*; *Timeless*; *Clock*; Circadian rhythms; Blood-feeding; Insect vector; *Luzomyia longipalpis*

## 1. Introduction

A diversity of organisms has circadian variations of behavior and physiology that are indeed controlled by an internal biological clock. In *Drosophila melanogaster* a number of genes controlling this mechanism are known [1]. The core clock mechanism is based on two interacting molecular feedback loops in which the transcription factors encoded by the genes *Clock* (*Clk*) and *cycle* (*cyc*) form a heterodimer that binds to upstream E-boxes sequences (CACGTG) and activate transcription of *period* (*per*), *timeless* (*tim*), *vrille* (*vri*) and *PAR domain protein 1* (*Pdp1*) [1–4]. Whereas the heterodimer PER/TIM interacts with CLK/CYC, inhibiting its function, VRI and PDP1ε compete for the same site in the *Clk* promoter, regulating its transcription.

These two interlocked feedback loops create circadian rhythms of gene expression of its components, except for *cyc*, which is constitutively expressed [1,5]. The repressor genes *per* and *tim*, for example, have their peaks of mRNA expression around Zeitgeber Time (ZT) 13 [6,7], while the transcriptional activator *Clk* reaches its maximum abundance in the late night/early morning, around ZT22-2 [8]. These cycles in the expression of negative and positive elements of the clock are important for the generation of circadian rhythms. Besides its central role in the link between the two feedback loops, the CLK/CYC heterodimer is believed to bind to regulatory sequences of downstream genes, controlling different aspects of physiology and behavior, as suggested by microarray experiments with the loss-of-function $Clk^{Jrk}$ strain (e.g. [9]).

In contrast to the abundance of information about the molecular control and circadian expression of clock genes in *D. melanogaster*, very little is known about it in hematophagous insect disease vectors. Nevertheless, for some mosquito species there is a large amount of data on circadian activity rhythms (reviewed in [10]).

Blood-feeding, which is critical to disease transmission, is tightly adjusted to a specific time of day and certainly controlled by the circadian pacemaker [10]. In addition, there is circumstantial evidence that it might have an effect on the clock. In the malaria vector *Anopheles stephensi*, for example, blood-feeding is followed by a marked reduction of circadian flight activity for 2–3 days [11]. Based on these findings and on molecular data from *D. melanogaster*, we hypothesized that the inhibitory effect of blood on circadian locomotor activity could involve regulation of clock gene expression levels.

For the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae), despite its medical importance as the main vector of American visceral leishmaniasis [12–14], neither molecular nor behavioral data on circadian locomotor activity rhythms in controlled laboratory conditions are available.

To initiate the molecular study of circadian rhythms in insect vectors, we analyzed the circadian expression of *per*, *tim* and *Clk* in *L. longipalpis*. We also examine the effect of blood-feeding on the expression of these three genes and recorded the circadian locomotor activity of this species in controlled laboratory conditions.

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## 2. Materials and methods

### 2.1. Insects

*Lutzomyia longipalpis* sandflies from a Lapinha (Minas Gerais State, Brazil) laboratory colony were reared as previously described [15]. *D. melanogaster* from the Canton S strain was raised on oatmeal medium at room temperature.

### 2.2. Analysis of locomotor activity rhythms

Newly emerged males and females of *L. longipalpis* were kept together in an incubator at 25 °C and LD 12:12 (cycles of 12 h of light and 12 h of darkness). Two days before recording, 3–5 days old sandflies were transferred to the Drosophila Activity Monitoring System (Trikinetics Inc., Waltham, MA, USA). *Drosophila* males used in the activity experiments for comparison were treated in a similar way.

### 2.3. Cloning and sequence analysis of the gene fragments used in the expression experiments

Fragments homologous to the *Drosophila per*, *tim*, *Clk* and *rp49* genes used in the circadian gene expression experiments were initially amplified from *L. longipalpis* genomic DNA using the degenerate primer PCR technique. The amplified fragments were subcloned and sequenced at Instituto Oswaldo Cruz on an ABI 377XL using BigDye Terminator v3.0 (Applied Biosystems). Sequence analysis was conducted using the GCG software and the NCBI website (www.ncbi.nlm.nih.gov/BLAST). The sequences of the fragments used in the expression experiments were submitted to the GenBank (Accession No. DQ207732; DQ207733; DQ207734; DQ207735; DQ207736; DQ207737; DQ207738). The full sequences of the three *L. longipalpis* clock genes will be published elsewhere.

### 2.4. Sample preparation for quantitative RT-PCR analysis of circadian gene expression

Sandflies were entrained for 3 days in LD12:12. On the fourth day, we collected 3 independent replicates samples with circa 40 sandflies for each time point at ZTs 1, 5, 9, 13, 17 and 21. Because *per* mRNA levels are differentially expressed between head and body in females of *D. melanogaster* (but not in males) [16], female sandfly heads were manually dissected from bodies on a frozen dish in dry ice. Heads and bodies of each ZT were stored at −80 °C until mRNA extraction. Males were not dissected. mRNA was extracted from whole males, female heads and bodies with the QuickPreptrade Micro mRNA Purification kit (Amersham Biosciences) according to manufacturer instructions. Approximately 10 ng of each mRNA sample were reverse-transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems) using the oligo-dT primer.

### 2.5. Quantitative RT-PCR

Levels of *per*, *tim* and *Clk* RNA relative to non-cycling levels of *rp49* were assayed by quantitative real time PCR using an ABI PRISM® 7000 (Applied Biosystems). For all genes analyzed two different sets of primers were used, except for the *rp49* constitutive control (primer sequences are available upon request). At least one of each primer in the pair spanned an exon/intron boundary to prevent amplification from any genomic DNA contamination. Indeed, all PCR reactions generated only the expected amplicons as shown by the melting-temperature profiles of the final products and by gel electrophoresis (data not shown). Standard curves were used to confirm that primers pairs had similar reaction efficiencies. Reactions were carried out in quadruplicates in a final reaction volume of 30 μl using 2X SYBR® Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 500 nM. Amplifications were carried out for 50 cycles as follows: (i) 95 °C, 10 s; (ii) 60 °C, 60 s; (iii) 78 °C, 25 s (florescence recorded); (iv) repeat. Raw data were exported to EXCEL (Microsoft) for analysis.

### 2.6. Experiments testing the effect of blood-feeding in locomotor activity and gene expression

Two- to three-day-old females were blood-fed on an anaesthetized hamster during 10 min at the light–dark transition (the hamster was placed in the cage at ZT11:55 and removed at ZT12:05). This procedure allowed about half of the females in the cage to blood-feed. Afterwards blood-fed and unfed controls (from the same cage) were separated and kept in different cages in an incubator at 25 °C and LD12:12 until frozen (for the gene expression analysis) or directly placed in the activity monitor for behavioral analysis (with recording starting one day later). Since blood-fed and unfed controls had to be visually separated after the feeding period, they were subjected to a phase-delay of 2 h, that is, placed in a different incubator with lights turning on and off 2 h later than the previous one where they were entrained. In fact, this procedure did not alter sandfly circadian locomotor behavior after one-day entrainment (see Section 3). The sandflies used in the expression experiment were collected and frozen at ZT13 in the following day (27 h after the blood meal – the same 2 h needed to separate blood-fed and unfed controls plus 25 h to reach the ZT13 in the next day).

## 3. Results

### 3.1. Locomotor activity rhythms

Fig. 1 shows the locomotor activity of the sandfly *L. longipalpis* compared to that of *D. melanogaster*. The graphs show the mean normalized activity of males of each species during two consecutive days, one in LD12:12 followed by one day at constant darkness (DD). We can see that *L. longipalpis* has a nearly unimodal pattern with a small startle response to lights-on. The activity peak is centered around the light-to-dark transition, showing clearly the characteristic anticipation to lights-off and higher level of activity during the dark phase than during the day (Fig. 1). Sandfly females show a very similar pattern (see below). The first day of DD shows a peak about 23 h later than that observed in LD and the activity is nearly unimodal. In the same conditions *D. melanogaster* shows a bimodal activity pattern in LD, which dampens in the first day in DD (Fig. 1). This is in agreement with several reports showing that after some days in DD the morning activity peak tends to disappear while the evening peak is sustained (e.g. [17]). *D. melanogaster* also shows far more activity in the subjective day than *L. longipalpis*. That is consistent with the fact that the latter is a crepuscular/nocturnal insect in the wild [13,14]. Indeed the difference in the proportion of diurnal over total activity between *D. melanogaster* and *L. longipalpis* is highly significant in both LD ($t = -8.713$; d.f. 108; $P < 0.001$) and DD ($t = -4.034$; d.f. 99; $P < 0.001$).

### 3.2. Temporal clock gene expression patterns in males and female heads

Quantitative real time RT-PCR analysis of males and female heads have not revealed significant differences, therefore, the results were combined. Analysis of mRNA abundance indicates low amplitude circadian oscillation of *per*, *tim* and *Clk* in *L. longipalpis* relative to the *rp49* constitutive control. *per* and *tim* mRNAs oscillate with a peak at ZT13 (Fig. 2A and B) presenting a pattern somewhat similar to that observed in *D. melanogaster* [6,7,18]. A posteriori (LSD – least significant difference) comparisons indicate significant differences in *per* mRNA levels between ZT13 and ZTs 1 and 9 ($P < 0.05$). Although the difference in *tim* mRNA levels between peak (ZT13) and through (ZT1) fail to reach significance in the LSD analysis ($0.05 < P < 0.10$), *per* and *tim* show similar patterns with higher mRNA levels during the dark phase. Note that the amplitude of *per* and *tim* mRNA cycling in sandflies (1.5–2-fold difference between peak and through) is not as high as reported for *Drosophila*. However, similar results were found in other insects (e.g. [19]). Analysis of *Clk* mRNA revealed an expression pattern (Fig. 2C) that is very different

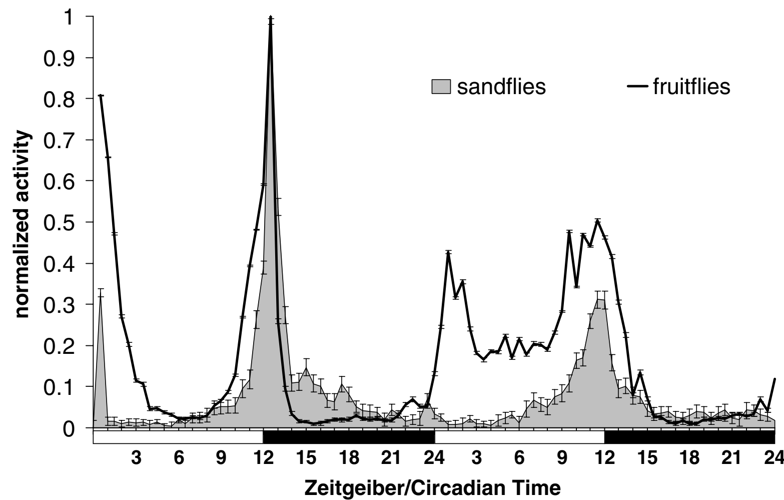


Fig. 1. Locomotor activity profile of *L. longipalpis* ($n$ = 68) compared to *D. melanogaster* ($n$ = 68). The graph shows the mean normalized activity during one day at LD12:12 followed by one day at DD. Bars represent the standard error of the mean (S.E.M.).

from *Drosophila*. The sandfly *Clk* expression begins to increase a couple of hours before *per* and *tim* and although it also reaches its maximum around ZT13, it is strongly repressed soon after, at ZT17, when *Drosophila Clk* is increasing and it is almost at its peak [8]. A posteriori LSD comparisons indicate significant differences in *Clock* mRNA levels between ZT13 and ZTs 1, 17 and 21 ($P < 0.05$ in all cases).

### *3.3. Temporal clock gene expression patterns in female bodies*

As reported for *D. melanogaster* [16], sandfly *per* mRNA abundance remained fairly constant throughout the day in female bodies (Fig. 3A) and no significant differences between ZTs were observed. On the other hand, analysis of sandfly *tim* indicates a circadian oscillation of its mRNA with a fairly high amplitude (2.34-fold) in opposite phase compared to heads (Fig. 3B) and in fact LSD comparisons indicate significant differences in *tim* mRNA levels between ZT13 and ZTs 1, 17 and 21 ($P < 0.05$ in all cases). In addition, sandfly *Clk* expression is also rhythmic, peaking at the same time (ZT13) as in heads (Fig. 3C). LSD comparisons also indicate significant differences in *Clock* mRNA levels between ZT13 and ZTs 1, 17 and 21 ($P < 0.01$ in all cases).

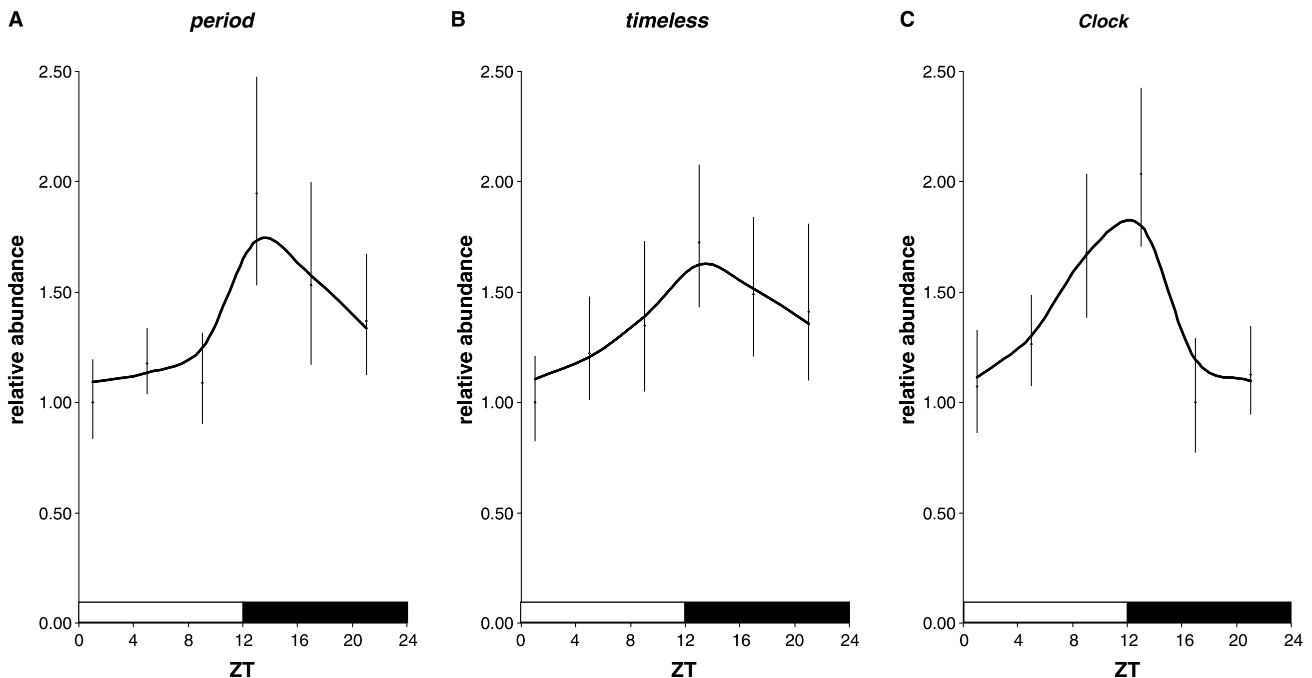


Fig. 2. Circadian expression of *L. longipalpis period* (A), *timeless* (B) and *Clock* (C) (pooled data for males and female heads). The lines are smoothed by calculating the mean between the relative abundance at a certain ZT and the interpolated value based on the previous and following time points. Bars depict the mean relative abundance and the range based on the S.E.M. Note that the amplitude of sandfly clock gene oscillations are not as high as reported for *D. melanogaster*, but still similar to other insects [18].

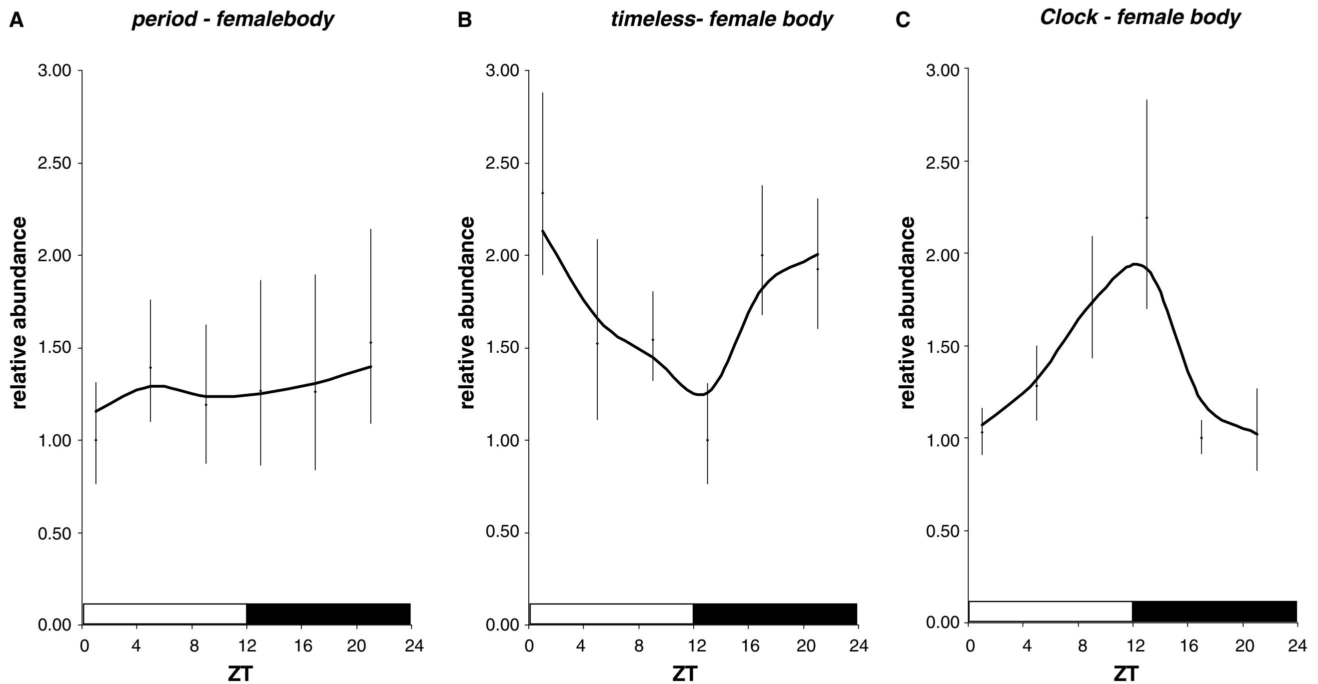


Fig. 3. Circadian expression of *period* (A), *timeless* (B) and *Clock* (C) in female bodies of *L. longipalpis*. As in Fig. 2, the lines are smoothed by calculating the mean between the relative abundance at a certain ZT and the interpolated value based on the previous and following time points. Bars depict the mean relative abundance and the range based on the S.E.M.

### 3.4. Locomotor activity rhythm of blood-fed females

To address if there is a reduction of locomotor activity after blood-feeding in *L. longipalpis* as reported for mosquitoes [10] we assayed locomotor activity rhythms of unfed and fully engorged female sandflies. Circadian activity of unfed females in this experiment (Fig. 4) was very similar to the male data presented in Fig. 1, showing that after one-day entrainment the phase shift and the presence of the host one day before recording did not affect sandfly locomotor activity pattern (compare Fig. 1 with Fig. 4). Nevertheless, Fig. 4 clearly shows that blood-feeding caused a ∼40% reduction in the activity peak. A two-way analysis of variance shows a highly significant difference between blood-fed and unfed ($F_{1,170} = 16.776$; $P < 0.001$) and non-significant

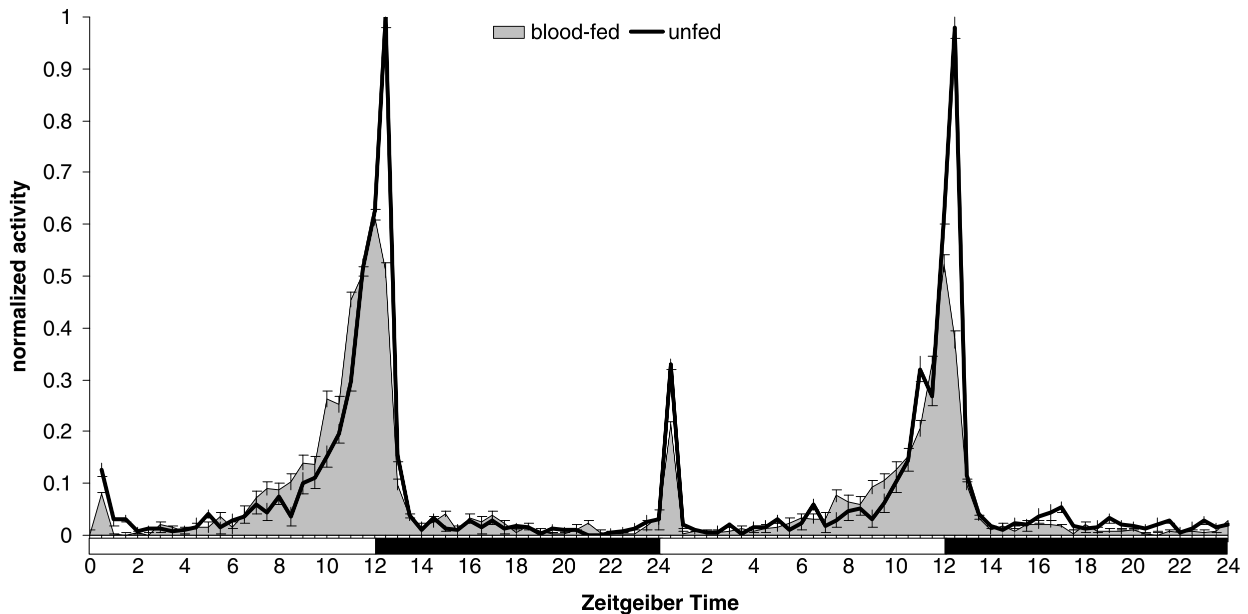


Fig. 4. Locomotor activity profile of *L. longipalpis* unfed ($n = 31$) and blood-fed ($n = 56$) females. The graph shows the mean normalized activity during two days at LD12:12. Bars represent the S.E.M.

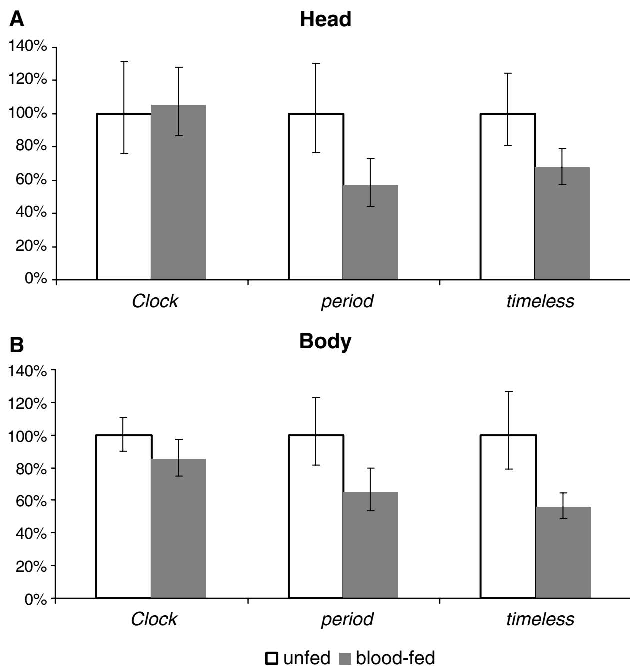


Fig. 5. Relative expression of *period*, *timeless* and *Clock* in unfed and blood-fed *L. longipalpis* female heads (A) and bodies (B). Columns represent the normalized relative abundance and bars represent the range based on the S.E.M. Levels of *Clk*, *per* and *tim* mRNA relative to *rp49* levels were assayed by quantitative real time PCR.

difference between the two days or interaction between day and feeding status.

*3.5. Clock gene expression in blood-fed females*

To further address if the molecular clock machinery might be involved in the reduction of activity after blood intake, we assayed *per*, *tim* and *Clk* expression levels in female sandflies 27 h after a full blood meal (corresponding to ZT13). A significant downregulation of *per* and *tim* is observed in heads and bodies after females had fed on blood (Fig. 5). Interestingly, in contrast to *per* and *tim*, *Clk* expression levels were not significantly affected in ingurgitated females compared to unfed controls (Fig. 5). A two-way analysis of variance confirmed these results. It shows a highly significant effect of body part for all three genes ($P < 0.001$ in all cases) as the expression in the head is about 100 times higher than in the body. However, only *per* ($F_{1,16} = 4.557$; $P < 0.05$) and *tim* ($F_{1,16} = 6.334$; $P < 0.05$) show a significant effect of feeding on their expression level.

## 4. Discussion

Blood-sucking insects spread a number of diseases that affect millions of people worldwide. Since vector-borne disease transmission is tightly associated to the insect behavior [20], understanding circadian locomotor activity and its modulation by blood-feeding may add important insights to the study of pathogen transmission dynamics.

In this study, we were able to correlate different circadian activity with differential expression pattern between *L. longipalpis* and *D. melanogaster*. Similar to *D. melanogaster* and other insects, female heads and males of *L. longipalpis* showed a clear rhythm of *per* and *tim* mRNA levels, peaking at ZT13 [1,6,7,18,19]. The main difference observed between sandflies and fruitflies concerned *Clk* expression: while in the former the higher mRNA levels concentrate between ZT9-13, in the latter the peak is around ZT22-2, giving a 12 h (or half a circadian cycle) delay compared to *Lutzomyia* [8]. Although in *D. melanogaster*, *per* and *tim* are clearly in anti-phase to *Clk*, our results are also consistent with a negative feedback loop model: indeed, *per* and *tim* levels start to rise just after the increase of *Clk* expression at ZT9. As CLK protein and mRNA in *Drosophila* cycle with similar temporal profiles, this suggests that CLK expression begins to drive *per* and *tim* transcription almost immediately in sandflies. Moreover, after ZT13 (when *per* and *tim* reach their peaks) *Clk* is strongly repressed, reaching its lowest levels at ZT17, when it is 2-fold reduced compared to its peak at ZT13. In this scheme, *per* and *tim* transcripts would probably have a longer half-life, giving rise to proteins that repress CLK activity through the night. In accordance, *per* and *tim* expression is still present in ZT21 (compared to ZT1), at the time where *Clk* mRNA levels are low (Fig. 2). In addition, perhaps PER and TIM in sandflies are themselves more stable than in *Drosophila*, reflecting different levels of interactions with their kinases, DBT, CK2, GSK3, so that PER/TIM proteins initiate CLK repression rather sooner that in the fruitfly. Our results in sandflies illustrate a new scenario in insect circadian clocks, one that is perhaps more easily understood and less complex than in fruitflies. As previously noted, in *Drosophila per* and *tim* start to accumulate when CLK levels are decreasing, which cannot be satisfactorily explained only by a simple feedback loop model [1].

Manipulations of *Clk* expression in *D. melanogaster* have shown that its altered timing and/or levels did not change the mRNA profile of *per, tim* and *cyc* [21]. In addition, when ectopically expressed, *Clk* induces normal-phase *tim* expression in different brain regions, independent of the promoter activating sequences used [22]. However, although these treatments did not cause phase changes in other clock components, they did alter circadian locomotor activity to a lower [21] or a higher [22] extent. Therefore, as changes in *Clk* expression patterns affect locomotor behavior, our data might suggest that late-day *Clk* expression in *L. longipalpis* could be responsible for its crepuscular activity behavior, without necessarily shifting *per* and *tim* expression phases in relation to *Drosophila*. Further analyses of *vri* and *Pdp1* homologues in sandflies might provide a better understanding of *Clk* particular expression pattern in this species [3,4].

Our data on clock gene expression in female *L. longipalpis* bodies are in agreement with the idea that the same clock molecules can play different roles and can have different expression patterns in different *Drosophila* body tissues [23,24]. While *per* is constitutive in female bodies, *tim* and *Clk* are highly rhythmic (Fig. 3). The difference in *per* expression between head and body suggests that PER might be involved in non-circadian aspects of *L. longipalpis* physiology. Indeed, in *D. melanogaster* PER is found with this same pattern in the ovarian follicle cells [23]. In contrast, while *Drosophila* TIM is also constitutively expressed in ovaries [23], in *L. longipalpis* female bodies *tim* mRNA cycles in anti-phase compared to heads (Figs. 2B and 3B), suggesting that *per* and *tim* might be differently regulated throughout the body. Since *Clk* expression in the female body

was also rhythmic (peaking at ZT13), the same molecules may play different roles in the periphery, as observed for example in *Drosophila* antennae [24].

Analysis of blood-feeding effect on *L. longipalpis* behavior revealed a dampening of locomotor activity (markedly at dusk) (Fig. 4), in accordance with data from several mosquito species [10,11]. As the blood meal weighs around 2–3 times the mean weight of the unfed female, this could interfere with sandfly mobility. However, we do not believe that is the case because partially engorged females (which show ingestion of small amounts of blood without significant enlargement in volume) showed similar reduction on locomotor activity as fully engorged ones (data not shown). Moreover, we found good evidence that this reduction of activity could involve the central pacemaker because, although *Clk* expression was not altered, *per* and *tim* levels were reduced in heads and bodies (Fig. 5).

In mammals, CLK and NPAS2 (a CLK paralogue) activity are sensitive to cellular redox status, namely the ratio of $NAD(P)H/NAD(P)^+$ [25]. This balance is largely affected by energy metabolism, which is able to entrain the molecular clock in the liver [26]. In hematophagous insects, blood-feeding also alters this balance, causing metabolic burst and oxidative stress [27,28]. It is possible that the change in cellular redox potential caused by blood-feeding might impair CLK's ability to bind DNA, as shown for its mammalian orthologues [25]. This would in turn downregulate *per* and *tim* levels as we have observed (Fig. 5). Interestingly, fluctuation of intracellular redox potential has also been found to entrain molecular rhythms in the mammalian forebrain [25], although this is still somewhat controversial [29]. Moreover heme, a byproduct of blood digestion in hematophagous insects, has been shown to have a role in the mammalian clock. It downregulates *mPer2* but not *Clk* expression [30], just as we have observed.

The circadian clock is involved in the coordination and proper functioning of developmental processes, detoxification, olfaction, neuropeptide signaling, nutritional state, and immunity in *Drosophila* [9]. Therefore, the clock modulation observed in sandflies after blood-feeding might control a number of physiological aspects previously thought to be an effect of the blood digestion itself [31,32]. Further studies on downstream (output) genes will give a better idea about the extent to which the blood meal affects hematophagous insect behavior. In addition, the complete characterization of *L. longipalpis per*, *tim* and *Clk* genes will allow future interspecific transformation experiments in loss-of-function *D. melanogaster* mutants (e.g. [33]), that can further illuminate the role of sandfly clock genes on crepuscular behavior, and their regulation by specific components present in the blood meal.

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## References

[1] Hardin, P.E. (2005) The circadian timekeeping system of *Drosophila*. Curr. Biol. 15, R714–R722.

[2] Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D., Weitz, C.J., Takahashi, J.S. and Kay, S.A. (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science 280, 1599–1603.

[3] Glossop, N.R., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M. and Hardin, P.E. (2003) VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator. Neuron 37, 249–261.

[4] Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R., Hardin, P.E., Young, M.W., Storti, R.V. and Blau, J. (2003) *vrille*, *Pdp1*, *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112, 329–341.

[5] Bae, K., Lee, C., Hardin, P.E. and Edery, I. (2000) dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. J. Neurosci. 20, 1746–1753.

[6] Hardin, P.E., Hall, J.C. and Rosbash, M. (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature 343, 536–540.

[7] Sehgal, A., Rothenflush-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P. and Young, M.W. (1995) Rhythmic expression of *timeless*, a basis for promoting circadian cycles in *period* gene autoregulation. Science 270, 808–810.

[8] Bae, K., Lee, C., Sidote, D., Chuang, K.Y. and Edery, I. (1998) Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators. Mol. Cell. Biol. 18, 6142–6151.

[9] McDonald, M.J. and Rosbash, M. (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell 107, 567–578.

[10] Clements, A.N. (1999) The biology of mosquitoesSensory Reception and Behaviour, vol. 2, CABI Publishing, New York.

[11] Rowland, M. (1989) Changes in the circadian flight activity of the mosquito *Anopheles stephensi* associated with insemination, blood-feeding, oviposition and nocturnal light-intensity. Physiol. Entomol. 14, 77–84.

[12] Soares, R.P.P. and Turco, S.J. (2003) *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae): a review. An. Acad. Bras. Ciênc. 75, 301–330.

[13] Deane, L.M. and Deane, M.P. (1962) Visceral leishmaniasis in Brazil: geographical distribution and transmission. Rev. Inst. Med. Trop. São Paulo 4, 198–212.

[14] Morrison, A.C., Ferro, C., Pardo, R., Torres, M., Wilson, M.L. and Tesh, R.B. (1995) Nocturnal activity patterns of *Lutzomyia longipalpis* (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. J. Med. Entomol. 32, 605–617.

[15] Souza, N.A., Andrade-Coelho, C.A., Barbosa, A.F., Vilela, M.L., Rangel, E.F. and Deane, M.P. (1995) The influence of sugars and aminoacids on the blood-feeding behaviour, oviposition and longevity of laboratory colony of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae, Phlebotominae). Mem. Inst. Oswaldo Cruz 90, 751–757.

[16] Hardin, P.E. (1994) Analysis of *period* mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. Mol. Cell. Biol. 14, 7211–7218.

[17] Helfrich-Forster, C. (2000) Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster* – sex-specific differences suggest a different quality of activity. J. Biol. Rhythm 15, 135–154.

[18] So, W.V. and Rosbash, M. (1997) Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J. 16, 7146–7155.

[19] Goto, S.G. and Denlinger, D.L. (2002) Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. J. Insect Physiol. 48, 803–816.

[20] Klowden, M. (1996) Vector behavior in: The Biology of Disease Vectors (Beaty, B.J. and Marquardt, W.C., Eds.), pp. 34–50, University Press of Colorado, Colorado.

[21] Kim, E.Y., Bae, K., Ng, F.S., Glossop, N.R., Hardin, P.E. and Edery, I. (2002) *Drosophila* CLOCK protein is under posttranscriptional control and influences light-induced activity. Neuron 34, 69–81.

[22] Zhao, J., Kilman, V.L., Keegan, K.P., Peng, Y., Emery, P., Rosbash, M. and Allada, R. (2003) *Drosophila* clock can generate ectopic circadian clocks. Cell 113, 755–766.
[23] Beaver, L.M., Rush, B.L., Gvakharia, B.O. and Giebultowicz, J.M. (2003) Noncircadian regulation and function of clock genes *period* and *timeless* in oogenesis of *Drosophila melanogaster*. J. Biol. Rhythm 18, 463–472.
[24] Tanoue, S., Krishnan, P., Krishnan, B., Dryer, S.E. and Hardin, P.E. (2004) Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. Curr. Biol. 14, 638–649.
[25] Rutter, J., Reick, M., Wu, L.C. and McKnight, S.L. (2001) Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science 293, 510–514.
[26] Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M. (2001) Entrainment of the circadian clock in the liver by feeding. Science 291, 490–493.
[27] Dansa-Petretski, M., Ribeiro, J.M., Atella, G.C., Masuda, H. and Oliveira, P.L. (1995) Antioxidant role of *Rhodnius prolixus* heme-binding protein. Protection against heme-induced lipid peroxidation. J. Biol. Chem. 270, 10893–10896.
[28] Kumar, S., Christophides, G.K., Cantera, R., Charles, B., Han, Y.S., Meister, S., Dimopoulos, G., Kafatos, F.C. and Barillas-Mury, C. (2003) The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. Proc. Natl. Acad. Sci. USA 100, 14139–14144.
[29] Zhang, Q., Piston, D.W. and Goodman, R.H. (2002) Regulation of co-repressor function by nuclear NADH. Science 295, 1895–1897.
[30] Kaasik, K. and Lee, C.C. (2004) Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature 430, 467–471.
[31] Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M., Wides, R., Salzberg, S.L., Loftus, B., Yandell, M., Majoros, W.H., Rusch, D.B., Lai, Z., Kraft, C.L., Abril, J.F., Anthouard, V., Arensburger, P., Atkinson, P.W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chaturverdi, K., Christophides, G.K., Chrystal, M.A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C.A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M.E., Hladun, S.L., Hogan, J.R., Hong, Y.S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J.J., Lobo, N.F., Lopez, J.R., Malek, J.A., McIntosh, T.C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Broder, S., Gardner, M.J., Fraser, C.M., Birney, E., Bork, P., Brey, P.T., Venter, J.C., Weissenbach, J., Kafatos, F.C., Collins, F.H. and Hoffman, S.L. (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298, 129–149.
[32] Ribeiro, J.M. (2003) A catalogue of *Anopheles gambiae* transcripts significantly more or less expressed following a blood meal. Insect Biochem. Mol. Biol. 33, 865–882.
[33] Tauber, E., Roe, H., Costa, R., Hennessy, J.M. and Kyriacou, C.P. (2003) Temporal mating isolation driven by a behavioral gene in *Drosophila*. Curr. Biol. 13, 140–145.

**Capítulo 4**

**Rhythmic expression of the *cycle* gene in a hematophagous insect vector**

Antonio C.A. Meireles-Filho, Paulo R. Amoretty, Nataly A. Souza, Charalambos P. Kyriacou, Alexandre A. Peixoto

Neste artigo publicado em 2006 na BMC Molecular Biology clonamos e caracterizamos a estrutura do gene *cycle* de *Lutzomyia longipalpis* e examinamos sua expressão circadiana. Investigamos também o efeito da alimentação sanguínea na expressão deste gene.

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# Rhythmic expression of the *cycle* gene in a hematophagous insect vector

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## Abstract

**Background:** A large number of organisms have internal circadian clocks that enable them to adapt to the cyclic changes of the external environment. In the model organism *Drosophila melanogaster*, feedback loops of transcription and translation are believed to be crucial for the maintenance of the central pacemaker. In this mechanism the *cycle* (or *bmal1*) gene, which is constitutively expressed, plays a critical role activating the expression of genes that will later inhibit their own activity, thereby closing the loop. Unlike *Drosophila*, the molecular clock of insect vectors is poorly understood, despite the importance of circadian behavior in the dynamic of disease transmission.

**Results:** Here we describe the sequence, genomic organization and circadian expression of *cycle* in the crepuscular/nocturnal hematophagous sandfly *Lutzomyia longipalpis*, the main vector of visceral leishmaniasis in the Americas. Deduced amino acid sequence revealed that sandfly *cycle* has a C-terminal transactivation domain highly conserved among eukaryotes but absent in *D. melanogaster*. Moreover, an alternative form of the transcript was also identified. Interestingly, while *cycle* expression in *Drosophila* and other Diptera is constitutive, in sandflies it is rhythmic in males and female heads but constitutive in the female body. Blood-feeding, which causes down-regulation of *period* and *timeless* in this species, does not affect *cycle* expression.

**Conclusion:** Sequence and expression analysis of *cycle* in *L. longipalpis* show interesting differences compared to *Drosophila* suggesting that hematophagous vector species might present interesting new models to study the molecular control of insect circadian clocks.

## Background

A diversity of organisms, ranging from bacteria to humans, shows circadian rhythms in physiology and behavior that are controlled by endogenous oscillators. In mammals and flies, the core clocks are generated by two negative feedback loops that are interconnected to the same two positive basic helix-loop-helix (bHLH)/PAS-containing transcription factors CLOCK (CLK) and CYCLE (CYC) (also called BMAL1)(reviewed in [1,2]).

In *D. melanogaster*, CLK and CYC form a heterodimer that binds to upstream E-box sequences (*CACGTG*) in *period* (*per*) and *timeless* (*tim*), which in turn control their own expression by negatively regulating CLK/CYC mediated activation [1,2]. In the second loop, the products of *vrille* (*vri*) and *PAR domain protein 1 epsilon* (*Pdp1ε*), which are also activated by CLK/CYC, regulate *Clk* transcription by competing for the same site in its promoter. Whereas VRI represses *Clk* production just after lights off, PDP1ε activates it in the middle of the night, separating the phases of *Clk* transcription and repression [3,4]. These oscillations of gene expression and posttranslational regulation are necessary for the robustness and accuracy of overt physiological and behavioral rhythms.

Although the core clock molecules are relatively conserved between mammals and *D. melanogaster*, there are some interesting differences in, for example, the transcriptional control of *Clk* and *cyc* expression. In the suprachiasmatic nuclei (SCN) of mammals (where the central pacemaker is located), *Clk* is constitutively expressed [5] and *Bmal1* is rhythmic, reaching its maximum abundance at dawn [6,7]. In contrast, *cyc* is constitutively expressed in *D. melanogaster* heads [8,9], while *Clk* shows rhythmic expression on the mRNA level, peaking during the night-day transition (ZT 22-2) [10,11]. Although the *Drosophila* CLK protein has also been reported to cycle with the same phase of its mRNA [10,11], recent data indicates that was a result of a methodological artifact [12,13]. Its ability to bind E-boxes and activate transcription in a cyclic manner in fact resides in its phosphorylation pattern, with only the late day/early night hypophosphorylated forms being capable of promoting *per* and *tim* expression [12,13].

The molecular study of circadian rhythms in insect vectors is still in its infancy. In sandflies the circadian expression profiles of *per*, *tim* and *Clk* has been studied in *Lutzomyia longipalpis*, the main vector of visceral leishmaniasis in the Americas [14]. While *per* and *tim* cycle as in other insects, peaking around ZT 13 [15-17], *Clk* expression peaks around ZT 9–13, about half a day later than in *D. melanogaster* [10,11,14]. This difference in *Clk* expression is correlated with differences in locomotor activity. *Drosophila* shows a bimodal/diurnal pattern, whereas *Lutzomyia* is predominately unimodal/nocturnal [14]. In addition, blood feeding causes a reduction in sandfly locomotor activity that is accompanied by a reduction in *per* and *tim*, but not *Clk* levels [14]. Thus, as the *Clk* profile of *L. longipalpis* is different from that of *D. melanogaster*, we wondered if the same would occur for its partner *cyc*.

We therefore cloned the *L. longipalpis cyc* gene and report here its genomic structure and the putative amino acid sequence. The presence of an alternative transcript is also identified. In addition we have analyzed the daily expression of *cyc* in males and females, as well as its expression after a blood meal.

## Results

### *Cloning and sequence analysis*

The sequence of the *Lutzomyia longipalpis cycle* gene (GenBank accession number DQ841151) was obtained using a PCR gene walking approach. An initial fragment was obtained using degenerate primers. Based on this first sequence new specific primers were designed and used in new reactions to obtain further sequences. The 5' and 3' ends were obtained using RACE techniques (see Methods for details). Introns were mapped by comparing PCR fragments generated using cDNA and genomic DNA as templates. Fig 1 shows a schematic representation of the *L. longipalpis cyc* gene with its seven introns. The figure also shows the regions coding for the different domains of the protein and the position of an alternative splice form (see below).

*L. longipalpis cycle* codes for a putative protein of 622 amino acids homologous to CYC from other species (Fig 2). Alignment of the sandfly CYC sequence with insect orthologues (*Anopheles gambiae*, *D. melanogaster*, *Bombyx mori* and *Antheraea pernyi*) and one mammal (*Mus musculus*) reveals high conservation in some particular regions such as the bHLH DNA binding domain and the protein dimerization PAS A and PAS B regions, especially when compared to *A. gambiae* and *D. melanogaster* (see Table 1). In addition, we were able to find in sandfly CYC the BMAL1 C-terminal region ("BCTR"), which was characterized as an activation of the CLK/BMAL1 heterodimer in a mammalian cell culture [18] (Fig 2). This region in *L. longipalpis* CYC is 96.87% identical to the mosquito predicted CYC sequence, 87.5% identical to the moth *A. pernyi* and 93.75% identical to the mouse BMAL1 sequences (Table 1). Interestingly, this motif is not present at all in *Drosophila* (Fig 2; see Discussion). Fig 3 shows a Neighbor-joining tree using the alignment of the protein sequences shown in Fig 2. As expected *L. longipalpis* CYC clustered with the *A. gambiae* sequence.

The approximate positions of the seven introns of the L. longipalpis cycle gene are also marked in fig 2 by inverted triangles. Inspection of cDNA and genomic sequences available for Anopheles gambiae cyc revealed that only three out of the seven intron positions of L. longipalpis cyc (2, 3 and 7) are conserved between the two species (data not shown). Comparison of different cDNA sequences also revealed the existence of a rare alternative splice transcript missing only one Arginine codon (Fig 1 and 2). Nevertheless this single difference potentially alters the ability of the putative protein to be phosphorylated (see below). This minor transcript corresponds to about 20% of all sequenced cDNA fragments (7/35).

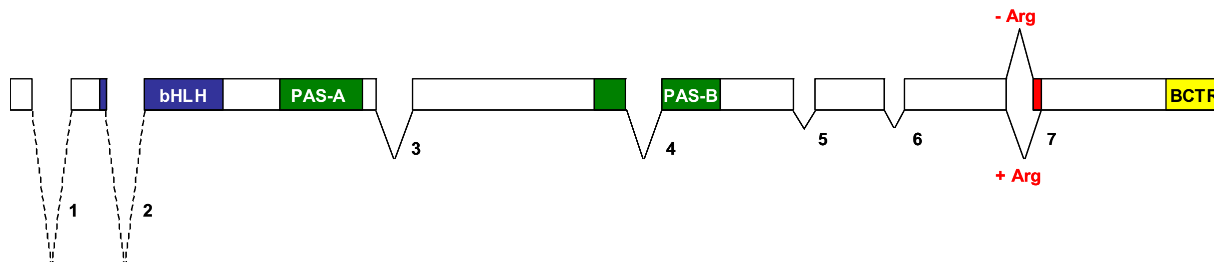


**Figure 1**
**Genomic structure *L. longipalpis cycle* gene**. Schematic representation of the genomic structure *L. longipalpis cycle* gene. The introns are numbered and were drawn to scale. The first and the second introns are depicted by a broken line to indicate that their complete sequence has not been determined. The coding region of the *L. longipalpis cycle* gene consists of 8 exons and 7 introns and the conserved motifs are shown in blue (bHLH), green (PAS A and B respectively) and yellow (BCTR). In red is marked the approximate position of the alternatively spliced Arg (see text).

We also searched in sandfly CYC sequence possible conserved residues for pos-translational modification (Fig 4a,b). We were able to find a lysine at the position Lys-225 in a conserved sumo consensus site (ΨKXE/D, where Ψ is a hydrophobic residue and X may be any amino acid) in the PAS link region, at an approximate position where its mammalian homologue is sumoylated *in vivo* (Lys-259) [19] (Fig 4a). In addition we were able to find Ser-502 and a Thr-510 at conserved positions, corresponding to the mammalian Ser-527 and Thr-534, which are phosphorylated *in vitro* [20]. This region was further examined for potential kinase substrate sites using Scansite 2.0 (stringency levels = high) [21] and the Ser-502 was identified as potential casein kinase-1 phosphorylation site (Score: 0.3474; Percentile: 0.081%) (Fig 4b). Interestingly, the rare alternative transcript identified as missing a single Arg did not reach significance in the phosphorylation prediction at high stringency, suggesting another level of sandfly CYC regulation.

### *Temporal cyc expression analysis*
As *per* mRNA levels are differentially expressed between the head and body in females of *D. melanogaster*, female sandfly heads and bodies were analyzed separately. Sandfly males were not dissected since no differences are observed in *per* expression between heads and bodies in *Drosophila* [22].

Analysis of *L. longipalpis cyc* expression in males and female heads under LD12:12 relative to the *rp49* constitutive control indicates clear cycling in mRNA abundance. As for sandfly *per*, *tim* and *Clk* [14] no significant differences between males and female heads were observed, and therefore these results were pooled. ANOVA indicates that *cyc* mRNA levels are significantly different among ZT groups ($F_{5,72} = 4.481$; $P < 0.001$) with a peak around ZT 5–9, with levels over two-fold higher compared to the levels at ZT 17 ($P < 0.001$, LSD – Least significant difference analysis) (Fig 5a). This is similar to the observed *Bmal1* cycling in mammals [6,7] but different compared to most insects analyzed to date, where its expression is constitutive [8,9,16,23]. On the other hand, in female bodies, *cyc* expression was constitutive ($F_{5,34} = 0.086$; $P = 0.994$) (Fig 5b), in contrast to *Bmal1* expression in all mammalian tissues analyzed so far [24,25].

**Table 1: Percentage identities between the *Lutzomyia longipalpis* CYCLE and its orthologues in some other organisms in the whole protein (excluding regions with gaps) and in some particular domains**

| | Total | bHLH | PAS A | PAS B | BCRT |
|---|---|---|---|---|---|
| *Anopheles gambiae* | 84.97 | 94.34 | 92.73 | 92.31 | 96.87 |
| *Drosophila melanogaster* | 67.36 | 84.91 | 80.00 | 69.23 | - |
| *Bombyx mori* | 60.36 | 83.02 | 76.36 | 65.38 | 87.50 |
| *Antheraea pernyi* | 60.62 | 84.91 | 72.73 | 63.46 | 87.50 |
| *Mus musculus* | 62.18 | 73.58 | 76.36 | 69.23 | 93.75 |

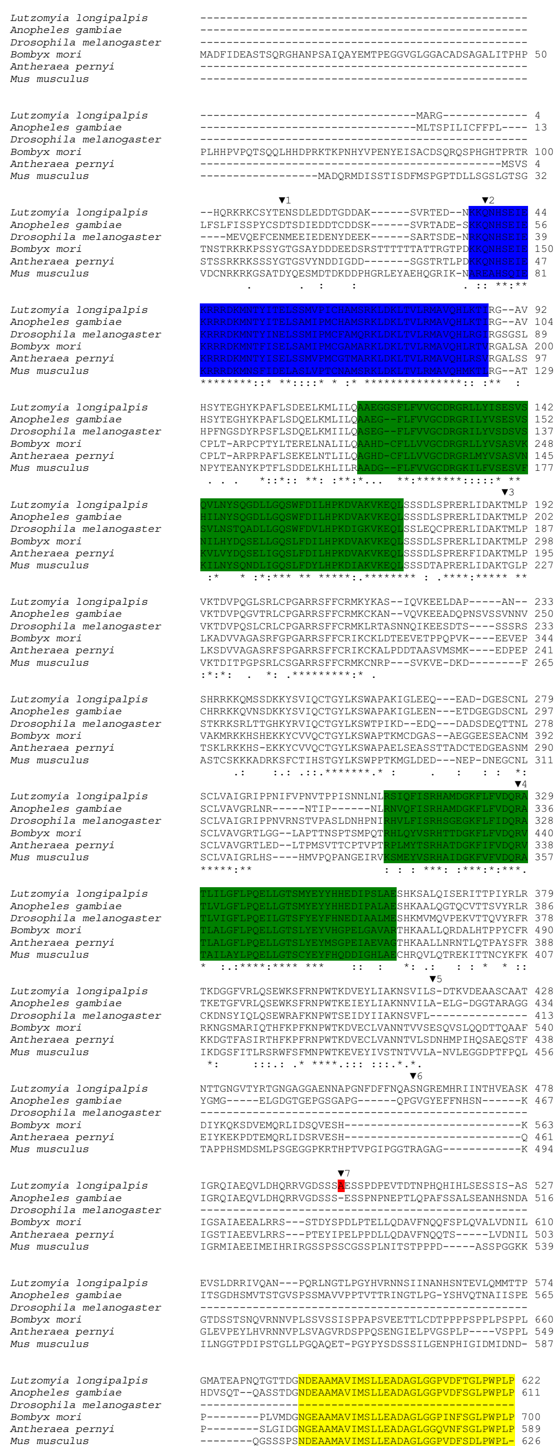

**Figure 2**
**Alignment of CYCLE protein sequences**. Protein alignment of the *L. longipalpis* CYCLE sequence with orthologues from *Anopheles gambiae* (Ensembl: ENSANGP00000027387), *Drosophila melanogaster* (GenBank™: AAC39124), *Bombyx mori* (GenBank™: BAB91178), *Antheraea pernyi* (GenBank™: AAR14937) and *Mus musculus* (GenBank™: AAH25973). The marked motifs use the same colors as in Fig 1.

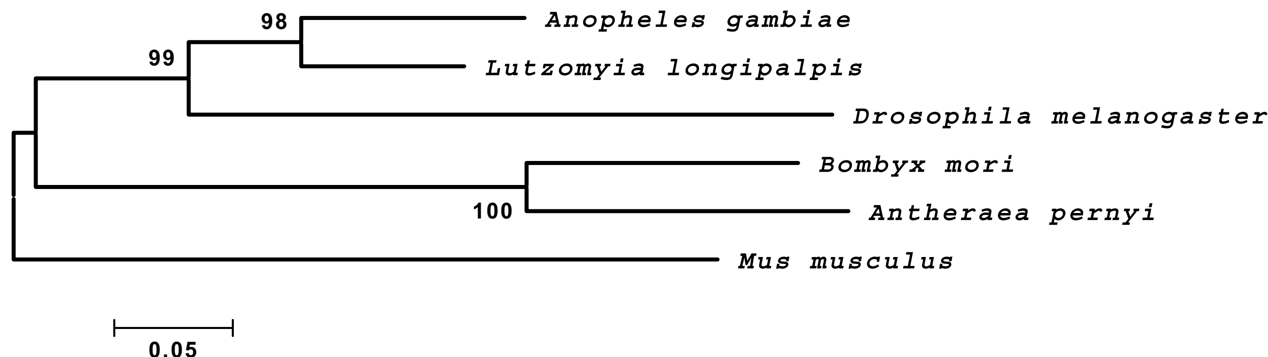


**Figure 3**
**Neighbor-joining tree of CYCLE protein sequences**. Neighbor-joining tree using the alignment of the protein sequences shown in Fig 2, complete deletion and Poisson corrected distances. *Mus musculus* was used as the outgroup.

#### cyc *expression analysis in blood-fed females*

In order to know if blood feeding has an effect on *cyc* expression as it does for *per* and *tim* [14], we assayed its mRNA levels 27 hs after a full blood meal. But, as for *Clk* [14], *cyc* levels were not significantly altered in heads and bodies of ingurgitated females compared to unfed controls (Fig 6).

## Discussion

In this study we characterized the sequence, genomic structure and expression of the *cycle* gene in the hematophagous sandfly *L. longipalpis*. Analysis of predicted protein sequence revealed its homology with *cyc* from others species (Fig 2). Interestingly, the BMAL1 C-terminal region ("BCTR"), which was characterized as responsible for the activation of the CLK/BMAL1 heterodimer in a mammalian cell culture [18], was also found in sandfly CYC. The conservation of this region in all animals analyzed so far (except *Drosophila*) suggests that sandfly CYC may also possess a C-terminal transactivation domain [8,23,26,27]. Chang et al [26] studying moth clock genes have suggested that the BCTR is very ancient, being lost in *Drosophila* CYC probably because it became redundant after the fruitfly CLK had acquired a new transactivation domain, a large poly-Q region. This latter domain is not found in the moth CLK orthologue and we are currently cloning the sandfly *Clk* to determine if the same is true for this vector species.

An important feature of mammalian CYC regulation is the phosphorylation and sumoylation of its serine/threonine and lysine residues respectively [19,20,28]. Aligning CYC homologues from different species we were able to find a lysine in the PAS link region of sandfly CYC at an approximately similar position where its homologue in mammals is sumoylated (Fig 4a). In addition, prediction phosphorylation site analysis identified Ser-502 as a potential target for posttranslational modification, but only in the more abundant form. In the alternative transcript identified, the missing Arg alters the ability of the Ser to be phosphorylated. This difference is noteworthy since in mammals only the hypophosphorylated form is able to bind to E-boxes *in vitro*, showing that phosphorylation of BMAL1 might play an important role in pacemaker regulation [20,28]. Taken together, these results suggest that sandfly CYC might be regulated at different levels (transcriptional and posttranslational), which may be important for its role in the sandfly pacemaker.

Our results on daily gene expression in males and female heads, unexpectedly, resemble data from mammals where *cyc* expression is also rhythmic (Fig 5a) [6,7,24]. Unlike most insects analyzed so far (where no oscillation of *cyc* mRNA was detected [9,16,23], but see Rubin et al [27]) sandfly *cyc* cycled robustly, beginning to rise at the end of the night (ZT 21) and peaking in the middle of the day ZT 5–9 (Fig 5a).

In *Drosophila* posttranslational mechanisms are necessary to provide optimal levels and subcellular localization of clock proteins. Earlier data have indicated that *per* and *tim* start to accumulate when CLK levels are decreasing [10,11], and this cannot be satisfactorily explained by a simple feedback loop model [1,2]. This contradiction was recently clarified by two papers that show that CLK levels in fact do not cycle [12,13]. Nevertheless, CLK transcriptional activity is rhythmic, via its phosphorylation levels. While hyperphosphorylated CLK predominates during times of transcriptional repression (late night/early morning), hypophosphorylated CLK is more abundant during times of transcriptional activation (late day/early night) [12,13]. The authors of these studies suggest that hypo-

**4a**

**Sumo modification**

```
                                                   ΨKXE/D

Mus musculus              RLCSGARRSFFCRMKCNRP---SVKVEDKDFAST
Lutzomyia longipalpis     RLCPGARRSFFCRMKYKAS--IQVKEELDAPTNS
Anopheles gambiae         RLCPGARRSFFCRMKCKAN--VQVKEEADQPNSV
Drosophila melanogaster   RLCPGARRSFFCRMKLRTASNNQIKEESDTSSSS
                          ***.***********  .    .:* *  .   .
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**4b**

**Phosphorylation**

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Lutzomyia longipalpis     KIGRQIAEQVLDHQRRVGDSSSAESSPDPEVTDTNPH
Mus musculus              KIGRMIAEEIMEIHRIRGSSPSSCGSSPLNITSTPPP
Anopheles gambiae         KIGRQIAEQVLDHQRRVGDSSS-ESSPNPNPEPTLQPA
                          **** ***:::: :*  *.*.*  .*.  : .    *
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**Figure 4**

**Potential Sumo modification and Phosphorylation sites in the *L. longipalpis* CYCLE sequence**. Potential Sumo modification and Phosphorylation sites in the *L. longipalpis* CYCLE sequence. (A) Alignment of the *M. musculus*, *A. gambiae*, *D. melanogaster* and *L. longipalpis* PAS linker region. The three insect species listed contains a potential SUMO consensus motif and the correspondent Lys that is sumoylated in mammals. (B) A Ser-rich region (Ser underlined) is shown for *M. musculus*, *A. gambiae* and *L. longipalpis* CYC. Ser and Thr residues are phosphorylated in mammals and their potential cognates in *L. longipalpis* and *A. gambiae* CYC are highlighted in yellow. In red is marked the position of the alternatively spliced Arg (see text).

phosphorylated CLK forms complexes with CYC at midday, bind to E-boxes and initiate *per* and *tim* transcription. Once the TIM/PER/DBT complex enters the nucleus it represses transcription by inhibiting CLK/CYC E-box binding and promoting CLK hyperphosphorylation and degradation [12,13]. On the other hand our previous report on *per*, *tim* and *Clk* expression in sandflies satisfied a simple feedback loop model, since *per* and *tim* levels rise at the time when *Clk* levels reaches its peak [14]. Given that in head oscillators *cyc* expression is earlier than *Clk* and that we identified at least one strong putative motif for phosphorylation, we propose that sandfly CYC might be subject to posttranslational modification, which would provide the necessary time delay for its accumulation at the appropriate time of day (ZT 13, when it can dimerize with the product of *Clk* and drive *per* and *tim* transcription [9,14]).

In contrast to heads, *cyc* expression in female bodies was shown to be constitutive (Fig 5b). In *Drosophila per* was shown to be constitutively expressed in ovaries [22] causing a strong damping in *per* cycling in female bodies. In fact, sandfly *per* is also constitutive in female bodies [14]. The differential regulation of *cyc* through the sandfly body suggests that, as in *Drosophila* and mammals [29], clock genes in *L. longipalpis* may also play different roles in different tissues, reflecting particular interactions with different molecules, what would finally lead to the coordination of other aspects of sandfly physiology. Interestingly the mammalian orthologue BMAL1 was shown to interact with non-circadian transcription factors, which in turn could respond to different kinds of stimuli [30].

Finally, data on blood-fed females shows that, although *per* and *tim* expression are downregulated, *Clk* and *cyc* are

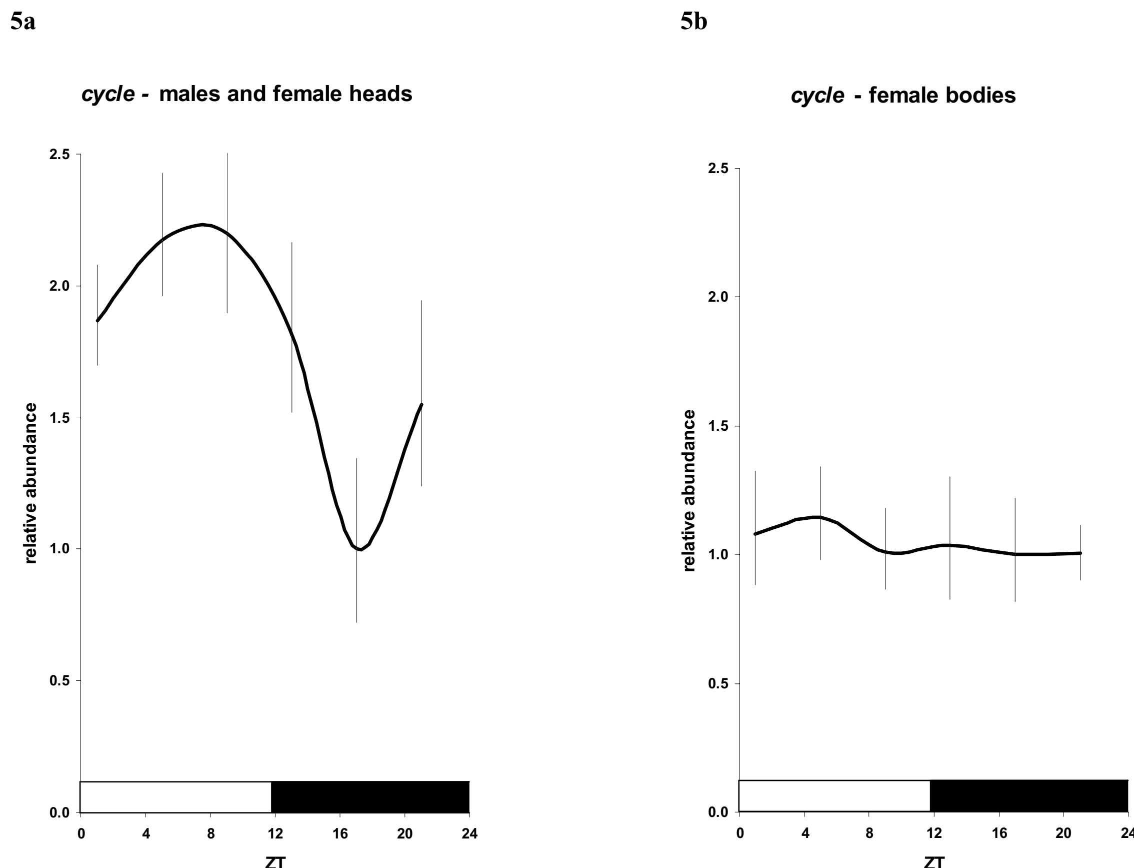


**Figure 5**
**Daily expression of *L. longipalpis cycle***. Daily expression of *L. longipalpis cycle*. A. Pooled data for males and female heads. B. Female bodies. Bars represent the mean relative abundance +/- the range based on the SEM (Standard Error of the Mean).

not [[14] and this report]. Since the latter two are activators of the formers, we believe that blood-feeding might regulate negatively CLK and CYC function at the posttranscriptional level, leading to diminished *per* and *tim* activation. This could be mediated by changes in NAD(P)H/NAD(P)+ levels, which can be altered by blood-feeding in other insect species [31,32]. Furthermore, changes in redox state have been observed to alter mammalian CLK activity *in vitro* [33]. This latter observation is consistent with the observations that feeding and fasting, which would be expected to change the redox profile, can entrain mammalian peripheral clocks independently of the LD cycles [34,35]. However, restricted-feeding regimes in *Drosophila* do not appear to influence circadian behavior or molecular rhythms of *per* and *tim* [36].

## Conclusion

The present results, together with our previous data, show that the molecular clock of *L. longipalpis* shows interesting differences compared to *Drosophila*, suggesting that blood-sucking insect vector species might present very interesting comparative models to study circadian rhythms and its molecular control. In addition, since the circadian clock drives activity and feeding behavior in insect vectors, understanding the molecular machinery of the clock may add important information in the dynamics of vector-borne disease transmission.

## Methods

### *Insects*

*L. longipalpis* sandflies from a Lapinha (Minas Gerais State, Brazil) laboratory colony were reared as previously described [14,37]. Briefly, for the temporal gene expression experiments three independent replicate samples with circa 40 sandflies were collected on the fourth day of entrainment at ZTs 1, 5, 9, 13, 17 and 21. Only females were dissected due to their differential pattern of expression between heads and body tissues [14]. For blood-feed-

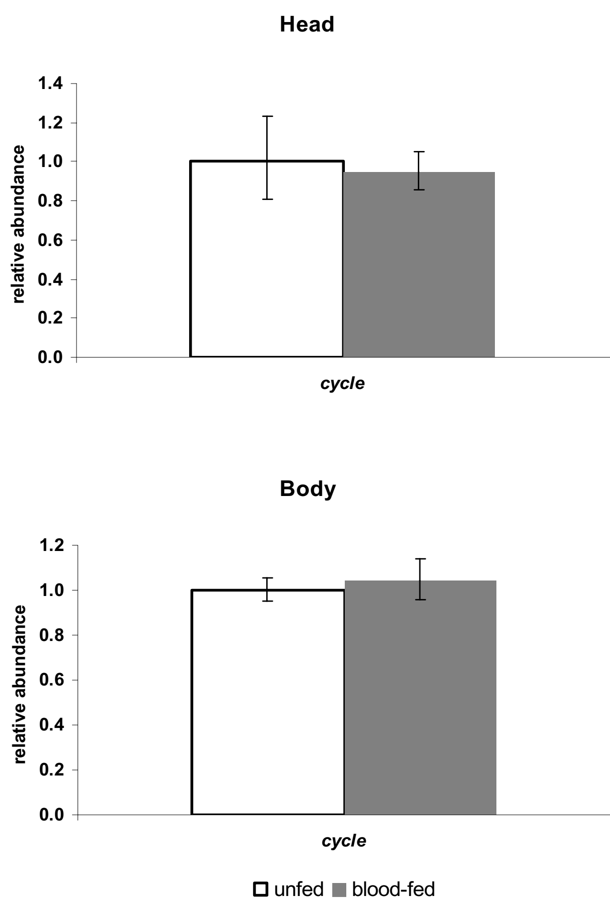


**Figure 6**
***cycle* expression in unfed and blood-fed *L. longipalpis* females**. Relative expression of *cycle* in unfed and blood-fed *L. longipalpis* female heads (top) and bodies (bottom). Columns (+/- the range based on the SEM) represent the normalized relative abundance.

ing experiments two to three-day-old females were blood-fed on an anaesthetized hamster during 10 min at the light-dark transition. Afterwards blood-fed and unfed controls (from the same cage) were separated and kept in different cages in an incubator at 25°C and LD12:12. Since blood-fed and unfed controls had to be visually separated after the feeding period, they were subjected to a phase-delay of 2 h, that is, placed in a different incubator with lights turning on and off 2 h later than the previous one where they were entrained. They were collected and frozen at ZT 13 in the following day (27 h after the blood meal – 2 h needed to separate blood-fed and unfed controls plus 25 h to reach the ZT 13 in the next day). This procedure was shown not to affect sandfly behavior nor *per*, *tim* and *Clk* expression [14].

### *Cloning of sandfly* cyc

Genomic sandfly DNA from circa 20 individuals was extracted with the GenomicPrep™ (Amersham Biosciences) kit according to manufacturer instructions. A fragment homologous to the *Drosophila cyc* was first amplified from *L. longipalpis* genomic DNA using the degenerate primer PCR technique. The primer sequences were as follows: 5'CYCdeg1, 5' *A(A, G)(A, C)GN(A, C)GN(A, C)GNGA(T, C)AA(A, G)ATGAA* 3' & 3'CYCdeg1, 5' *AC(C, T)TTNCC(A, G, T)AT(A, G)TC(C, T)TTNGG(A, G)TG* 3'. Sequential reactions were carried out to reach the 3' and 5' end of the gene as follows. For the missing 5' of the gene we used the "5' Race System for Rapid Amplification of cDNA Ends" kit (Gibco BRL). Primer used in the 1st strand synthesis 3'llCYCexp1: 5' *TTATGGAAGTGGCCATGGGAGTCC* 3'. Then the first PCR reaction was done with the primers 5'RACE AAP: 5' *GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG* 3' & 3'llCYC8: 5' *CTCCTTGACCTTAGCCACATC* 3'. Reamplification of this material was done with the nested AUAP 5'*GGCCACGCGTCGACTAGTAC* 3' & 3' llCYC7 5' *TGGGAGTAATTGAGGACCTGC* 3' primers according to manufacturer instructions. For the 3' region a preliminary reaction with specific and degenerate primers was done before the 3'RACE: initial reaction with primers 5'llCYC2 5' *GGTCCTCAATTACTCCCAAG* 3' & 3'CYCdeg2 5' *TTCATNC(G, T)(A, G)CA(A, G)AA(A, G)AA* 3' and later with the primers 5'llCYC3 5' *CAATGCTTCCGGTGAAGACG* 3' & 3' CYCdeg3 5' *(G, C)(A, T)NGTNCCNA(A, G)(A, G, T)AT(C, T)TC(C, T)TG* 3'. The 3' extreme end of the gene was obtained with the following primers: 5'llCYC7 5' *CAGTTCATCTCTCGTCATGCC* 3' & oligo dT and later a nested reaction: 5'llCYC6 5' *CGTTGATTCTGGGCTTCCTAC* 3' & oligo dT. Gene fragments were cloned in a pMOS vector (Amersham Biosciences) and sequenced at the Department of Biochemistry and Molecular Biology, Instituto Oswaldo Cruz – FIOCRUZ on an ABI 377XL DNA analyzer using BigDye Terminator v3.0 (Applied Biosystems). Sequence analysis was performed with the GCG software and the NCBI website [38]. Potential phosphorylation sites were detected using Scansite 2.0, with high stringency levels [21]. The sandfly *cyc* sequence was submitted to the GenBank under the accession number DQ841151.

### *Quantitative RT-PCR*

Firstly, mRNA was extracted with the QuickPrep™ Micro mRNA Purification kit (Amersham Biosciences) and reverse-transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems) using the oligo-dT primer according to manufacturer instructions. Levels of *cyc* mRNA relative to non-cycling levels of *rp49* were assayed by quantitative Real Time PCR using an ABI PRISM® 7000 (Applied Biosystems) as previously described [14]. We used 3 different sets of primers for *cyc*

and one for *rp49*. *cyc* primer pairs: 5' *TGCCAAAACAATGCTTCCGG* 3' & 5' *ACGTTGCCCTTTGATCGACA* 3'; 5' *AATTGATGCCAAAACAATGC* 3' & 5' *AGAATCAACGTTGCCCTTTG* 3'; 5' *GATGCCAAAACAATGCTTCC* 3' & 5' *GTGCCCAGGACTTGAGGTAG* 3'. *rp49* primer pair: 5' *CGATATGCCAAGCTAAAGCA* 3' & 5' *GGGCGATCTCAGCACAGTAT* 3'. At least one of each primer in the pair spanned an exon/intron boundary to prevent amplification from any genomic DNA contamination. Indeed, melting-temperature curves showed a single amplified product and the absence of primer-dimer formation, which was confirmed by gel electrophoresis (data not shown). Non-template controls were included for each primer pair to check for any significant levels of contaminants. Standard curves were used to confirm that primers pairs had similar reaction efficiencies. Reactions were carried out in quadruplicates in a final reaction volume of 30 μl using 2× SYBR® Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 500 nM. Amplifications were carried out for 50 cycles as follows: (i) 95°C, 10 sec; (ii) 60°C, 60 sec; (iii) 78°C, 30 sec (florescence recorded); (iv) repeat. Raw data were exported to EXCEL (Microsoft) for analysis.

## Abbreviations

Bmal1, Brain and muscle Arnt-like protein-1; SCN, suprachiasmatic nuclei; qRT-PCR, quantitative Reverse Transcription – Polymerase Chain Reaction; ZT, zeitgeber time; LD, light-dark; bHLH-PAS, basic helix-loop-helix-Per-Arnt-Sim.

## Authors' contributions

ACAMF carried out most of experiments and drafted the manuscript. PRA did part of the cloning steps and sequencing. NAS helped in the acquisition of sandfly samples and to design the blood-feeding experiment. CPK participated in the coordination, helped to write the manuscript and supervised ACAMF during his stay in Leicester. AAP is the principal investigator, participated in its design and coordination, and helped to write the manuscript. All authors read and approved the final manuscript.

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## References


1. Hardin PE: **The Circadian Timekeeping System of *Drosophila*.** *Curr Biol* 2005, **15:**R714-R722.
2. Stanewsky R: **Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals.** *J Neurobiol* 2003, **54:**111-47.
3. Glossop NR, Houl JH, Zheng H, Ng FS, Dudek SM, Hardin PE: **VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator.** *Neuron* 2003, **37:**249-61.
4. Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NR, Hardin PE, Young MW, Storti RV, Blau J: ***vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock.** *Cell* 2003, **112:**329-41.
5. Shearman LP, Zylka MJ, Reppert SM, Weaver DR: **Expression of basic helix-loop-helix/PAS genes in the mouse suprachiasmatic nucleus.** *Neuroscience* 1999, **89:**387-97.
6. Honma S, Ikeda M, Abe H, Tanahashi Y, Namihira M, Honma K, Nomura M: **Circadian oscillation of BMAL1, a partner of a mammalian clock gene *Clock*, in rat suprachiasmatic nucleus.** *Biochem Biophys Res Commun* 1998, **250:**83-7.
7. Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N: **Antiphase circadian expression between *BMAL1* and *period* homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats.** *Biochem Biophys Res Commun* 1998, **253:**199-203.
8. Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC: **CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila *period* and *timeless*.** *Cell* 1998, **93:**805-14.
9. Bae K, Lee C, Hardin PE, Edery I: **dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex.** *J Neurosci* 2000, **20:**1746-53.
10. Bae K, Lee C, Sidote D, Chuang KY, Edery I: **Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators.** *Mol Cell Biol* 1998, **18:**6142-51.
11. Lee C, Bae K, Edery I: **The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex.** *Neuron* 1998, **21:**857-67.
12. Houl JH, Yu W, Dudek SM, Hardin PE: ***Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells.** *J Biol Rhythms* 2006, **2:**93-103.
13. Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE: **PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription.** *Genes Dev* 2006, **20:**723-33.
14. Meireles-Filho AC, da S Rivas GB, Gesto JS, Machado RC, Britto C, de Souza NA, Peixoto AA: **The biological clock of an hematophagous insect: locomotor activity rhythms, circadian expression and downregulation after a blood meal.** *FEBS Letters* 2006, **580:**2-8.
15. So WV, Rosbash M: **Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling.** *EMBO Journal* 1997, **16:**7146-55.
16. Goto SG, Denlinger DL: **Short-day and long-day expressionpatterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*.** *J Insect Physiol* 2002, **48:**803-16.
17. Hall JC: **Genetics and molecular biology of rhythms in *Drosophila* and other insects.** *Adv Genet* 2003, **48:**1-280.
18. Takahata S, Ozaki T, Mimura J, Kikuchi Y, Sogawa K, Fujii-Kuriyama Y: **Transactivation mechanisms of mouse clock transcriptionfactors, *mClock* and *mArnt3*.** *Genes Cells* 2000, **5:**739-47.
19. Cardone L, Hirayama J, Giordano F, Tamaru T, Palvimo JJ, Sassone-Corsi P: **Circadian clock control by SUMOylation of BMAL1.** *Science* 2005, **309:**1390-4.
20. Sanada K, Okano T, Fukada Y: **Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1.** *J Biol Chem* 2002, **277:**267-71.
21. Obenauer JC, Cantley LC, Yaffe MB: **Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs.** *Nucleic Acids Res* 2003, **31:**3635-41.
22. Hardin PE: **Analysis of *period* mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators.** *Mol Cell Biol* 1994, **11:**7211-8.
23. Markova EP, Ueda H, Sakamoto K, Oishi K, Shimada T, Takeda M: **Cloning of *Cyc* (*Bmal1*) homolog in *Bombyx mori*: structural analysis and tissue specific distributions.** *Comp Biochem Physiol B: Biochem Mol Biol* 2003, **134:**535-42.
24. Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, Takumi T: **Transcriptional oscillation of canonical clock genes in mouse peripheral tissues.** *BMC Mol Biol* 2004, **5:**18.

25. Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N: **Antiphase circadian expression between BMAL1 and *period* homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats.** *Biochem Biophys Res Commun* 1998, **253:**199-203.
26. Chang DC, McWatters HG, Williams JA, Gotter AL, Levine JD, Reppert SM: **Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*.** *J Biol Chem* 2003, **278:**38149-158.
27. Rubin E, Shemesh Y, Cohen M, Elgavish S, Robertson HM, Bloch G: **Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock.** *Genome Res* in press.
28. Eide EJ, Vielhaber EL, Hinz WA, Virshup DM: **The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon.** *J Biol Chem* 2002, **277:**17248-54.
29. Glossop NR, Hardin PE: **Central and peripheral circadian oscillator mechanisms in flies and mammals.** *J Cell Sci* 2002, **115:**3369-77.
30. Hogenesch JB, Gu YZ, Jain S, Bradfield CA: **The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors.** *Proc Natl Acad Sci USA* 1998, **95:**5474-9.
31. Dansa-Petretski M, Ribeiro JM, Atella GC, Masuda H, Oliveira PL: **Antioxidant role of *Rhodnius prolixus* hemebinding protein. Protection against heme-induced lipid peroxidation.** *J Biol Chem* 1995, **270:**10893-6.
32. Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C: **The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*.** *Proc Natl Acad Sci USA* 2003, **100:**14139-44.
33. Rutter J, Reick M, Wu LC, McKnight SL: **Regulation of CLOCK and NPAS2 DNA binding by the redox state of NAD cofactors.** *Science* 2001, **293:**510-4.
34. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M: **Entrainment of the circadian clock in the liver by feeding.** *Science* 2001, **291:**490-93.
35. Kobayashi H, Oishi K, Hanai S, Ishida N: **Effect of feeding on peripheral circadian rhythms and behaviour in mammals.** *Genes Cells* 2004, **9:**857-64.
36. Oishi K, Shiota M, Sakamoto K, Kasamatsu M, Ishida N: **Feeding is not a more potent Zeitgeber than the light-dark cycle in *Drosophila*.** *Neuroreport* 2004, **15:**739-43.
37. Souza NA, Andrade-Coelho CA, Barbosa AF, Vilela ML, Rangel EF, Deane MP: **The influence of sugars and aminoacids on the blood-feeding behaviour, oviposition and longevity of laboratory colony of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae, Phlebotominae).** *Mem Inst Oswaldo Cruz* 1995, **90:**751-7.
38. **NCBI** [http://www.ncbi.nlm.nih.gov/BLAST/]



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## Capítulo 5

## A chimeric *cyc* gene reveals ancestral features of the *Drosophila* circadian clock

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Neste artigo, ainda em fase de preparação, analisamos a evolução do mecanismo molecular do relógio biológico de *Drosophila melanogaster*. Estudamos principalmente o papel do domínio C-Terminal de CYCLE de *Aedes aegypti*, que está presente em todos os insetos analisados até o momento com exceção de *Drosophila*, e sua interação com CRY de *D. melanogaster*.

# A chimeric *cyc* gene reveals ancestral features of the *Drosophila* circadian clock

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Abstract

Most organisms present molecular timekeepers known as circadian clocks, which determine their daily biological rhythms in behavior and physiology. Although many aspects of these molecular pacemakers are conserved in animals, some differences among insects suggest that their circadian clocks have diversified significantly during the course of evolution. For example, the very conserved C-Terminal activation domain of the CYCLE (CYC) transcriptional factor, which is important to the maintenance of circadian rhythmicity in a number of species, is absent in *Drosophila melanogaster*. In this species, the activation domain is found in its partner, CLOCK (CLK). Besides, while the *Drosophila* CRYPTOCHROME (CRY) works as a photoreceptor in the central brain, its paralogues in other species are transcriptional repressors, a role that CRY also plays in peripheral clocks of *Drosophila*. To shed light into the functional evolution of the *Drosophila* circadian clock, we tested the hypothesis that CLK and CYC have swapped the role of transcriptional activator during the course of evolution in flies. Our studies revealed that *Drosophila* can sustain a mammalian-like pacemaker and that CRY, besides its well described role in photoreception, might have had an ancient role in the fruit fly clockwork mechanism.

Keywords: *Drosophila*, circadian clock, *bmal1*, *cryptochrome*

## Introduction

Circadian rhythms are genetically controlled biological cycles with a period close to, but not exactly 24 hours. These rhythms enable organisms (ranging from prokaryotic and eukaryotic microbes to plants and animals) to adapt themselves to cyclic changes of the external environment (reviewed in Bell-Pedersen et al 2005; Hardin, 2005; Gallego and Virshup, 2007). In *Drosophila melanogaster*, endogenous oscillators can be found in a variety of cellular types and arise from interlocked transcriptional negative feedback loops, which are connected by rhythmic activity of the bHLH/PAS transcription factors dCLOCK (dCLK) and dCYCLE (dCYC) (Hardin, 2005).

In the main feedback loop dCLK and dCYC heterodimerize, bind to upstream E-box sequences (CACGTG) of the genes *period* (*per*) and *timeless* (*tim*) and activate their expression (Allada et al1998; Bae et al 1998; Rutila et al 1998; Darlington et al 1998). A glutamine-rich (poly-Q) transactivation domain in the dCLK C-terminal region is indispensable for the heterodimer transcriptional activity. Indeed, mutant flies that lack this region ($Clk^{Jrk}$) are arrhythmic and have constitutive low levels of *per* and *tim* (Allada et al 1998). After PER accumulation, dimerization and consequent stabilization by TIM, the complexes and individual proteins enter into the nucleus were PER binds to dCLK and represses dCLK/dCYC activity, inhibiting its own expression and consequently closing the loop (Bae et al 2000; Yu et al 2006). Besides its central role in the generation and maintenance of the

circadian expression of clock genes, the dCLK/dCYC heterodimer is believed to bind to regulatory sequences of downstream genes that will control periodic physiological and behavioral rhythms. In this system, photic entrainment is achieved by the dCRYPTOCHROME (dCRY) protein, which promotes the degradation of TIM in a light-dependent manner and resets the clock early morning in the main pacemaker (Emery et al 1998; Stanewsky et al 1998; Ceriani et al 1999; Lin et al 2001). Despite its role in photoreception, dCRY is believed to work as a repressor of the dCLK/dCYC heterodimer in peripheral tissues, but the precise molecular mechanism is yet unclear (Krishnan et al 2001; Ivanchenko et al 2001; Collins et al 2006).

Although the presence of many common players suggests that both insects and mammals inherited their clocks from a common ancestor, some differences in the pacemaker mechanism suggest that they have strongly diverged during the course of evolution. In mammals, the positive transcription factors are also mCLK and BMAL1 (BMAL1 is the homologue of CYC), and the formation of the heterodimer also activates the transcription of circadian genes, including three *per* genes (*mPer1* – *mPer3*) and two *cry* genes (*mCry1* and *mCry2*). The resultant mPER and mCRY proteins are translocated into the nucleus where the mCRY proteins, in contrast to dCRY's photoreceptive function in the fly main pacemaker, act as negative regulators by directly interacting with the C-Terminal region of BMAL1 to inhibit transcription, closing the negative feedback loop (Gekakis et al 1998; Kume et al 1999; Vitaterna et al 1999; Bunger et al 2000). In the

mammalian clockwork, circadian photoreception is taken by retinal photopigments, which entrain the suprachiasmatic nucleus (SCN, the main pacemaker in the brain). In fact, the differential role of CRY is not only one important distinction between fly tissues and between flies and mammals' main clocks, but also among other species. Interestingly, some insects contain two CRYs, one working as a photoreceptor while the other acts as a repressor of CLK/CYC (Zhu et al 2005; Yuan et al 2007).

Another interesting difference between fly and mammal pacemakers is the localization of the region responsible for the transcriptional activation of CLK and CYC. While in flies the transactivation domain of the heterodimer resides in dCLK (the poly-Q region), in mammals and all other species analyzed so far it is in BMAL1 (Takahata et al 2000; Chang et al 2003; Markova et al 2003; Meireles-Filho et al 2006a; Rubin et al 2006). Therefore, it seems that during the course of evolution a functional transfer of the transactivation domain from dCYC to dCLK occurred in the *Drosophila* lineage.

Thus, although a general clock mechanism exists, the same molecules seem to carry slightly modified tasks between these two species. Interestingly, analyses of clock gene structure and expression in other insects have shown that they somehow are more similar to the mammalian than to the fly clock (Chang et al 2003; Meireles-Filho et al 2006a; Meireles-Filho et al 2006b; Rubin et al 2006). This suggests that, after the insect-vertebrate split, *Drosophila* diverged faster than other insect species from a common ancestral pacemaker (Tauber et al

2004). The degree of species-specific variation in the clockwork of insects, in addition to the high degree of divergence of the *D. melanogaster* clock raised the question of how fruit flies have evolved from a common insect clock ancestor.

To shed light onto the functional evolution of the *Drosophila* circadian clock we tested the hypothesis that dCLK and dCYC have swapped the transactivation domain during the course of evolution. Specifically, we tested if the "lost" transactivation domain of dCYC would retain functionality in *Drosophila* by making a chimeric protein carrying the full sequence of the *Drosophila cyc* gene fused to a fragment of the mosquito *Aedes aegypti cyc* encoding the C-Terminal region (dCYC-aaCYCct), which is homologous to the activation domain of BMAL1. The idea is to observe if this construct is able to restore transcriptional activity to the defective $dCLK^{Jrk}$ (which lacks the poly-Q activating region), mimicking the mammalian mCLK/BMAL1 heterodimer structure and function. Our results add new insights in clock evolution, and the implications of that are discussed.

## Material and Methods

### Fly stocks and transgenic flies.

*w* ($w^{1118}$) flies used in *P*-element transformation have been described previously (Hazelrigg et al 1984).

To construct the *P*-element vector for injection, we first amplified by PCR a 4.2 kb genomic fragment from the *D. melanogaster cyc* promoter extending from –4210 to –47, with reference to the transcription start of the *cyc* gene. We substituted the transcription start site of the *cyc* promoter with that of *hsp70* gene to obtain a stronger expression of the construct (see below). We carried out three separate reactions using whole fly genomic DNA. For the downstream part of the promoter sequence we used the primers 5'-AGCTCGGTGGAGGAGTCAA-3' and 5'-ACTAGTTTCGGAAGAATACCAAGGGA-3', SpeI site underlined. The 5' primer was designed to bind just after a natural EcoRV site. These restriction sites were later used to clone this fragment into pBluescriptKS+ digested with the same enzymes. The inner *cyc* promoter sequence, which comprehends sequences between natural restrictions sites SalI and EcoRV, was amplified with the primers 5'-TGTCGTGGCTATTGTTGCTC-3' and 5'-CAGATAGCGAGCACGAATCA-3', which overlapped the more downstream fragment and the most upstream one, that was amplified with the primers 5'-AGGCCTAAGAAGCCCTTGCTGAGTAA-3' and 5'-AACCGGCACTACCTTCAATG-3' (StuI restriction site is underlined). These other two fragments were assembled together with the first one in pBluescript KS+, sequenced to confirm the integrity of *cyc* promoter region, excised from it as a StuI–SpeI fragment and cloned into the StuI and SpeI sites of pCaSpeR4 transformation vector (Thummel et al

1988), where an 800bp EcoRI fragment containing the SV40 polyadenylation site had been previously incorporated.

To achieve strong expression of the cloned construct and to provide proper stability to the transcribed construct, we amplified the *hsp70* TATA box and its 5' UTR (from -45 to +203) from the pUAST vector (Brand and Perrimon 1993) with the primers 5'-TTACTAGTAGAGCGCCGGAGTATAAATA-3' and 5'-TTGCGGCCGCATTCAGAGTTCTCTTCTTGTAT-3' (SpeI and NotI sites underlined respectively) and cloned them into the SpeI and NotI sites of *dcyc*-pCaSpeR4 to form *dcyc-hsp70*-SV40-pCaSpeR4.

To construct a dCYC protein fused to the C-Terminal region of the *A. aegypti* CYC (aaCYCct), designated dCYC-aaCYCct, we first amplified the *dcyc* open reading frame by RT-PCR from adult head RNA with the primers 5'-TTGCGGCCGCATGGAAGTTCAGGAGTTCTG-3' and 5'-TTCTCGAGTAAGAACACGGAATTCTTGG-3' (NotI and XhoI sites underlined respectively) substituting the original *dcyc* stop codon with a XhoI site. After that we amplified from reverse transcribed adult mosquito head RNA the aaCYCct region with the primers 5'-TTCTCGAGGTGGACCTGGCCGAAGGAAC-3' and 5'-TTGGTACCTCATGGTAACGGCCACGGC-3'; XhoI and KpnI sites underlined respectively. These two fragments were cloned sequentially into pBluescriptKS+, excised with NotI and KpnI and cloned into the NotI and KpnI sites of *dcyc-hsp70*-SV40-pCaSpeR4 to form *dcyc-hsp70*-dCYC-aaCYCct-SV40-pCaSpeR4 (Fig1). This full vector was re-sequenced to verify integrity, and then used for microinjection.

*Drosophila* transformation was carried out essentially as described by Spradling (1986) and transformed flies were balanced using w; CyO/Sco; D/TM6B flies.

Plasmid constructs for cell experiments

Plasmids pAc-dCLK (pAc5.1/V5-HisA - dCLK), pRLcopia (for Renilla luciferase (R-LUC) control) and pGL3-(4-*per*-E-box)hs:luc (for expression of firefly luciferase (FF-LUC) from a minimal heat shock promoter with four *per*-E-box elements) were kindly provided by Frank Weber (University of Heidelberg, Germany). The pAc-dCLK$^{Jrk}$ construct was generated by using pAc-dCLK as a template for a PCR with the primers 5'-TTTGAATTCGGGATGGACGACGAGAGCGACGA-3' and 5'-TTTTCTAGACTAGTGCTGCGTGTGCAGAT-3' (EcoRI and XbaI restriction sites underlined, respectively) and cloning the resulting PCR product into pAc5.1/V5-HisB EcoRI/XbaI digested vector. This recombinant protein is the same truncated protein predicted to be produced by *Clk*$^{Jrk}$ flies (Allada et al 1998). The chimeric dCYC-aaCYCct construct was excised from *dcyc-hsp70*-dCYC-aaCYCct- SV40-pCaSpeR4 with NotI and KpnI and cloned into a modified pAc5.1/V5-HisB vector (pAcMOD) digested with the same enzymes. The pAcMOD vector was generated by excising its multiple cloning site (MCS) with SacII and KpnI and substituting it by the MCS from pBluescriptKS+ digested with the same enzymes. mCRY1 was a kind gift from Takeshi Todo (Osaka University, Japan). mCRY1 was excised from pcDNA3.1/HisC-mCRY1 vector with

EcoRI and XbaI and cloned into pAc5.1/V5-HisB digested with the same enzymes. dCRY was kindly provided by Nicolai Peschel and its cloning will be published elsewhere.

Cell culture and transfection

S2 cells were maintained in InsectExpress medium (Cambrex) supplemented with 10% fetal bovine serum (Invitrogen) at 25°C. In 12-well plates, 0.8 mL of cells (density of $1 \times 10^6$ cells/ml) were allowed to seed for 24 h. After seeding, cells were transfected with 0,5μL of JetPei (Polyplus Transfection) and a total DNA amount of 250ηg (corrected with pAc empty vector). All wells contained 25 ηg pRLcopia, 10 ηg pGL3-(4-per-E-box)hs::luc+ and 5ηg of dCLK or $dCLK^{Jrk}$ or dCYC-aaCYCct or combinations of them as indicated. The amounts of pAc-PER, pAc-dCRY, pAc-mCRY1 are indicated in the respective figures. Cells were harvested 48 h after transfection, and enzyme activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The plates containing cells transfected with dCRY were wrapped with two layers of aluminum foil and kept in the same chamber. LUC activity was calculated from ratios of FF-LUC/R-LUC of a lysate sample to control for transfection efficiency and lysate concentration (Darlington et al 1998; Weber and Kay 2003). The averages of at least three experiments are shown as a percentage of the control value.

## Analysis of behavioral rhythms

3 to 5 days old adult male and virgin female *w* and *w*;+; dCYC-aaCYCct flies were entrained in LD cycles at 25°C for 5 days and transferred into DD for at least 10 days. Locomotor activity behavior was monitored using Drosophila activity monitors (Trikinetics, Waltham, MA), and activity was calculated as the number of activity events occurring every 30 min. Data collected were subjected to periodogram analysis using Matlab software and a custom signal processing toolbox (Levine et al 2002).

# Results

## dCLK$^{Jrk}$/dCYC-aaCYCct heterodimer form a mammalian-like clock in S2 cells

To investigate how the particular domains of the main transcription factors operate, we performed transient transfection assays in *Drosophila* Schneider (S2) cells. Untransfected S2 cells express endogenous constitutive levels of CYC, and addition of dCLK is sufficient to induce expression of a reporter gene controlled by the *per* promoter or by a synthetic promoter with 4 copies of the *per* E-box (4-*per*-E-box; Darlington et al 1998, Weber and Kay, 2003). On the other hand, a dCLK protein lacking part of its poly-Q activating region is unable to induce E-box transcription in S2 cells (Darlington et al 1998).

We then cotransfected the reporter 4-*per*-E-box and dCLK or $dCLK^{Jrk}$ (which lacks all poly-Q repeats) under the control of the *actin* promoter. In agreement with previous results (Darlington et al 1998, Weber and Kay 2003) the addition of dCLK promotes E-box dependent transcription. As expected, $dCLK^{Jrk}$ alone is insufficient to activate reporter expression (Fig2). However, when $dCLK^{Jrk}$ is transfected in concert with dCYC-aaCYCct, E-box dependent expression was rescued to levels comparable to the wild type dCLK (Fig2). This activation seems to be a result of the heterodimerization of $dCLK^{Jrk}$ and dCYC-aaCYCct, since dCYC-aaCYCct itself (or in an eventual combination with endogenous CYC) does not induce luciferase expression (Fig2). In addition, although exogenous dCYC-aaCYCct is necessary for high-level $dCLK^{Jrk}$-mediated transactivation, its combination with dCLK did not cause a further increase in transcriptional activity (Fig2). Based on these observations, our data show that $dCLK^{Jrk}$/dCYC-aaCYCct heterodimers can support E-box dependent expression in S2 cells.

To assay if PER is able to repress $dCLK^{Jrk}$/dCYC-aaCYCct activity as it does for dCLK/dCYC, we cotransfected these transcription factors with increasing amounts of PER, also under the control of the *actin* promoter. As expected from previous studies (Darlington et al 1998; Chang and Reppert 2003; Weber and Kay 2003), PER suppressed dCLK/dCYC transcription in a concentration-dependent manner (Fig3). Moreover, PER also significantly inhibited $dCLK^{Jrk}$/dCYC-aaCYCct expression compared with control levels. At 10 ng of PER, LUC expression was reduced to 70% of control, while at 100 ng LUC levels

were almost completely eliminated (Fig3). These results show that the heterodimer dCLK$^{Jrk}$/dCYC-aaCYCct is efficiently inhibited by *Drosophila* PER as the wild type dCLK/dCYC heterodimer.

mCRY1 is a core component of the molecular oscillator in vertebrates, as it has been shown that it represses mCLK-BMAL1-mediated transcription by binding to the C-Terminal region of BMAL1. This process is essential for rhythmicity, both at the molecular and behavioral levels (van der Horst et al 1999; Kiyohara et al 2006; Kwon et al 2006; Sato et al 2006). Due to the presence of a similar transactivation domain in dCYC-aaCYCct we tested if, in addition to its activation function, this region could also be repressed by mCRY1. Indeed, consistent with the previously published results for mammals and some insects (Zhu et al 2005; Kiyohara et al 2006; Kwon et al 2006; Sato et al 2006; Yuan et al 2007), increasing amounts of transfected mCRY1 led to dose-dependent dCLK$^{Jrk}$/dCYC-aaCYCct inhibition, while the same was not observed with dCLK/dCYC (Fig4). These results show that the transactivation domain added to dCYC is fully functional in S2 cells, given that it has not only activation function but also can be inhibited by a known repressor.

Previous studies showed that dCRY, in addition to its function as a photoreceptor, also works as a repressor in *Drosophila* peripheral clocks. This difference in dCRY role is probably the reason why some of these studies failed to shown dCRY repression in S2 cells; these cells simply might not support dCRY repressor function. Since we were able to repress dCLK$^{Jrk}$/dCYC-aaCYCct with mCRY1, we analyzed if the

addition of the mosquito activation domain in dCYC would elicit repressor function to dCRY. Fig 5 shows that increasing amounts of dCRY did not repress dCLK/dCYC activation as expected. However unexpectedly, increasing amounts of dCRY leaded to a small but significant increase in activation function of $dCLK^{Jrk}$/dCYC-aaCYCct (Fig5). This suggests that the addition of the mosquito CYC C-Terminal region to dCYC make it inducible by dCRY. PER and dCRY were shown to interact physically in yeast two-hybrid assays and in S2 cells, but the meaning of this interaction is still not known (Rosato et al 2001). We then asked if the augment in E-box mediated signal was due to a sequestration (i.e., de-repression) of PER by dCRY, as one would expect if $dCLK^{Jrk}$/dCYC-aaCYCct have produced low levels of endogenous PER that would in turn repress them. Therefore we next cotransfected PER and dCRY in the presence of $dCLK^{Jrk}$/dCYC-aaCYCct. As shown in Fig 5, the addition of PER caused the expected reduction in E-box mediated activation by $dCLK^{Jrk}$/dCYC-aaCYCct. Increasing amounts of dCRY in the presence of PER leaded to a dose-dependent variation in the increase of transcription levels that was similar to the variation of transcription without exogenous PER. This suggest that the activation of transcription observed was not due to a derepression of PER by dCRY. It is interesting to note that dCRY also increased E-box mediated expression of dpCLK and dpCYC in S2 cells (from the monarch butterfly *Danaus plexippus*, in which the latter also has the conserved C-Terminal activating region) (Zhu et al 2005).

Altogether, we favor the notion that *Drosophila* S2 cells can support a non-Drosophilid clockwork and that dCRY might have had a yet unappreciated function in the ancestral *Drosophila* pacemaker.

Increased Morning Activity during Light-Dark Cycles in female, but not male, dCYC-aaCYCct flies.

Due to the fact that we were able to induce expression of a reporter gene in S2 cells based on the transactivation C-Terminal domain of dCYC-aaCYCct, and that this chimeric protein showed increased activity when interacting with dCRY, we hypothesized that the same mechanism could be reproduced *in vivo* and that this interaction would induce changes at the behavioral level. To that we generated transgenic flies whereby the expression of dCYC-aaCYCct construct is driven by *cyc* regulatory sequences (see Fig1 and Material and Methods). We obtained 3 independent transgenic fly lines, which were used in the next experiments. We measured locomotor activity of these flies along with control flies under DD conditions after 5 days of entrainment in LD cycles. Under DD conditions dCYC-aaCYCct flies manifested activity rhythms with normal period lengths close to 24 hr, indistinguishable from wild type controls (data not shown). This is somehow expected since the dCYC-aaCYCct protein did not show an obvious effect on E-box mediated expression when coexpressed with wild type dCLK in S2 cells (see Fig 2). Under LD conditions, dCYC-aaCYCct flies showed normal bimodal activity pattern with the

characteristic morning and evening peak (Fig 6). But interestingly, a distinct sex-specific behavior was observed in all 3 independent lines generated in LD: female dCYC-aaCYCct flies exhibited a significantly higher portion of their daily activity during the light phase, while males did not show any obvious difference compared to wild type flies (Fig 6). Thus, this data suggest that the dCYC-aaCYCct alters light-driven locomotor activity in female flies.

## Discussion

Fruit flies and mice have contributed to a great extent with experimental data and insights into the molecular dissection of the circadian clock in the animal kingdom. However the large evolutionary distance between them did not allow proper dissecting of the molecular mechanisms involved in *Drosophila* clock evolution. To that, other insects might provide better tools to study the molecular nature of interspecific divergence in clock regulatory function.

We described here that the addition of a highly conserved C-Terminal region of mosquito CYC to fruit fly CYC makes the *Drosophila* circadian clock run in a mammalian fashion in S2 cells. Some pieces of evidence support this affirmation: (i) the dCLK$^{Jrk}$/dCYC-aaCYCct heterodimer, which is similar in structure to mCLK/BMAL1, induces E-box-mediated expression; (ii) PER is able to repress it; (iii) as well as mCRY1 (Fig 2,3,4). The reconstruction of a feedback loop in S2 cells

supports the idea that *Drosophila* and mammal clocks evolved from a common ancestral pacemaker.

The description of an activation domain in an insect CYC C-Terminal region is not unprecedented. Chang et al showed that the circadian clock of the silkmoth *Antheraea pernyi* can operate in S2 cells in a similar way as we reported here: in this species the apCYC has the same indispensable activation domain in its C-Terminus, while apCLK does not present any glutamine repeats (Chang et al 2003). These authors concluded that the C-Terminus present in almost all CYC analyzed is very ancient, being lost in *Drosophila* probably because it became redundant after the fruitfly CLK had acquired the new large poly-Q transactivation domain. We then provide further support of this hypothesis, showing that substituting the poly-Q region of dCLK by adding the C-Terminal region of mosquito CYC to dCYC restitutes a mammalian-like functional clock in a *Drosophila* cell line.

In addition, our unexpected results showing that dCRY can activate the heterodimer formed by $dCLK^{Jrk}$/dCYC-aaCYCct indicate that dCRY and the insect CYC C-Terminal region might have had a peculiar role in the ancestral *Drosophila* circadian clock.

Gehring and Rosbash proposed that clocks may have evolved originally to sequester processes that are particularly sensitive to UV-light damage to the dark phase of the daily cycle (Gehring and Rosbash 2003). In this way, the (6-4) photolyases, which use blue light to repair UV induced DNA damage, would be intimately linked to clock evolution. Indeed, it is believed that cryptochromes evolved from a photolyase-like

ancestral gene (Cashmore et al 1999; Green 2004). Moreover, it has been suggested that the ability of cryptochromes to repress CLK/CYC-mediated transcription and to work as photoreceptor have evolved independently several times, an example of "repeated evolution", as vertebrate and invertebrate CRYs with repression function are more similar to vertebrate and invertebrate (6-4) photolyases (which have no circadian function describe so far) than they are to *Drosophila* CRY (Cashmore et al 1999; Green 2004). Nevertheless, according to Rubin et al (2006) the presence of the repressor CRY in the basal insect *Tribolium castaneum* and in the basal deuterostome sea urchin suggests a more ancient origin of the repressor-type CRY, which implies that circadian photoreception was not necessarily primordial (Rubin et al 2006).

However, our data support a third hypothesis. We observed that dCRY is able to interact with the C-Terminal region of aaCYC and induce a small but significant increase in its transcriptional activity. Interestingly, a similar transcriptional activation was observed in the Monarch butterfly with dCRY (Zhu et al 2005) and with the dp (6-4) photolyase (Yuan et al 2007), suggesting that perhaps the ancestral photolyase-like protein of insects and vertebrates possessed not only light sensitivity but also the ability to interact with the C-terminal domain of non-Drosophila CYC (and BMAL1). Therefore, in this hypothesis the repression activity observed in repressor CRYs is in fact a derived character that perhaps evolved from an ancestral ability to activate transcription.

Interestingly, the addition of the mCRY1 tail region (aminoacids from 371 to 606) to *Arabidopsis thaliana* (6-4) photolyase conferred to this chimeric protein mCLK/BMAL1 transcription-inhibitory capacity, suggesting that the variable C-Terminus region of mCRY1 defines its role in the mammalian clock mechanism (Cashmore et al 1999; Green 2004; Chaves et al 2006). In addition, the fact that mCRY1 tail alone did not inhibit mCLK/BMAL1 suggests that *A. thaliana* (6-4) photolyase was able to bind to BMAL1 (Chaves et al 2006). Together with our results, this suggests that repressor CRYs, dCRY and (6-4) photolyases share an ancient common ancestor that was probably capable of affecting transcription and photoreception as mentioned before.

In addition, we propose that the ability of *Drosophila* CRY to work mainly as a photoreceptor in the central clock structures was accompanied by the loss of the C-Terminus of CYC and the acquisition of the poly-Q by CLK. Precisely, CLK must have acquired the poly-Q region first, making the CYC C-Terminus redundant and consequently dispensable for activation (Fig 2 – compare activation by dCLK/dCYC-aaCYCct with by dCLK$^{Jrk}$/dCYC-aaCYCct). In addition, as PER was still able to repress CLK (Fig 3), this might have allowed dCRY to work exclusively as a photoreceptor in the central brain pacemaker. This scenario in *Drosophila* is indeed reasonable, since all insects analyzed so far (that have the C-terminus of CYC) have two *cry* (one repressor and the other photoreceptor) or only the repressor one (Yuan et al 2007). Then, dCYC and dCRY might have coevolved in *Drosophila* so that the lost of dCYC C-terminus might have been necessary to permit

dCRY to work exclusively as a photoreceptor in the central brain pacemaker. Otherwise dCRY might still work as an activator. In addition, since butterflies and other Diptera such as mosquitoes possess both types of CRYs (Zhu et al 2005; Yuan et al 2007), the loss of the C-terminus in CYC might have also allowed the loss of repressor-like CRY in the *Drosophila* lineage. One intriguing possibility is that the light sensitive CRY might still have a role as transcriptional activator in insects that possess both types of CRYs, contributing to the activation of CLK/CYC complex in early morning.

We can not explain with the available information how dCRY induces activation of dCLKJrk/dCYC-aaCYCct. It is possible that the aaCYCct co-opted dCRY to stimulate expression, although we need formal proof to that. By now we also can not rule out the possibility that dCYC-aaCYCct might have a direct role in the light input pathway or in regulating clock responsiveness to light through its interaction with dCRY. But one evidence that this activation might reflect an important ancestral role is the impact of dCYC-aaCYCct expression in the locomotor behavior of adult flies. Despite normal activity in DD, activity of transformed female flies in LD was largely increased compared to wild type females, while no difference was observed between males. Similar results were observed in flies over-expressing CLK from the *per* promoter, what could lead us to suggest that the mechanism driving light dependent activity in our flies is the same, since CYC the partner of dCLK in clock mechanism (Kim et al 2002). However, Kim and colleagues analyzed and reported an increase in

light-driven activity only for males, suggesting that our results are not exactly the same of them. Indeed, in that work they argued that the levels of dCLK were important in modulating the duration of light-induced bouts of activity in dCLK overexpressing flies. In a different way, the data presented here suggest that the dCYC-aaCYCct chimeric protein is involved in sex-specific light-driven activity and, taken together with the results from S2 cells, that this might involve dCRY mediated activation. Besides, although dCLK and dCYC dimerize to activate transcription of clock genes, in mammals the dCYC homologue BMAL1 interacts with other partners than mCLK, and this might allow BMAL1 to affect locomotor behavior in different ways (Hogenesch et al 1998). Additionally, besides its role in the molecular clockwork mechanism, *dcyc* is involved in different non-circadian aspects of fly physiology, ranging from sleep and longevity to stress response (Shaw et al 2002; Hendricks et al 2003). And some of these aspects are believed to be sex-specifc. For example, the compensatory rebound of activity after rest deprivation in *dcyc*$^{01}$ mutant flies was reduced or absent in males while exaggerated in females (Hendricks et al 2003). The nature of this disparity was not described, but it is interesting that our chimeric *cyc* construct affects differently male and female fly behavior as it was shown for other aspects of fly physiology.

Furthermore, several wild type strains of *D. melanogaster* showed sex-specific variations in locomotor activity rhythms, but the reasons underlying these differences are yet unknown (Helfrich-Förster 2000). We further speculate that wild type *cyc* might somehow be involved in

controlling natural *D. melanogaster* sex-specific locomotor activity since dCYC-aaCYCct affects locomotor behavior in this way.

The study of interspecific chimeric genes offers opportunities to examine the evolution of circadian gene regulation and can contribute to a better understanding of the evolution of single clock molecules. Our studies revealed that *Drosophila* can sustain a mammalian-like pacemaker and that dCRY, besides its well described role in photoreception in the central pacemaker, might have had an ancient role in the fruit fly clockwork mechanism. These findings reveal novel aspects of *Drosophila* molecular clock evolution.

References

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 93:791-804.

Bae K, Lee C, Hardin PE,Edery I (2000) dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. J Neurosci 20:1746-1753.

Bae K, Lee C, Sidote D, Chuang KY,Edery I (1998) Circadian regulation of a Drosophila homolog of the mammalian Clock gene: PER and TIM function as positive regulators. Mol Cell Biol 18:6142-6151.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL,Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544-556.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118:401-415.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS,Bradfield CA (2000) Mop3 is

an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

Cashmore AR, Jarillo JA, Wu YJ, Liu D (1999) Cryptochromes: blue light receptors for plants and animals. Science.284:760-5.

Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ,Kay SA (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285:553-556.

Chang DC, McWatters HG, Williams JA, Gotter AL Levine JD,Reppert SM (2003) Constructing a feedback loop with circadian clock molecules from the silkmoth, Antheraea pernyi. J Biol Chem 278:38149-38158.

Chang DC, Reppert SM (2003) A Novel C-Terminal Domain of Drosophila PERIOD Inhibits dCLOCK:CYCLE-Mediated Transcription. Curr Biol 13:758-62.

Chaves I, Yagita K, Barnhoorn S, Okamura H, van der Horst GT, Tamanini F (2006) Functional evolution of the photolyase/cryptochrome protein family: importance of the C terminus of mammalian CRY1 for circadian core oscillator performance. Mol Cell Biol. 26:1743-53.

Collins B, Mazzoni EO, Stanewsky R,Blau J (2006) Drosophila CRYPTOCHROME is a circadian transcriptional repressor. Curr Biol 16:441-449.

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TD, Weitz CJ, Takahashi JS,Kay SA (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. Science 280:1599-1603.

Emery P, So WV, Kaneko M, Hall JC,Rosbash M (1998) CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669-679.

Gallego M, Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 8:139-148.

Gehring W, Rosbash M (2003) The coevolution of blue-light photoreception and circadian rhythms. J Mol Evol. 57:S286-9.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS,Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science 280:1564-1569.

Green CB (2004) Cryptochromes: tail-ored for distinct functions. Curr Biol. 2004 14:R847-9.

Hardin PE (2005) The circadian timekeeping system of Drosophila. Curr Biol 15:R714-22.

Hazelrigg T, Levis R, RubinGM (1984) Transformation of white locus DNA in Drosophila: dosage compensation, zeste interaction, and position effects. Cell 36:469–81.

Helfrich-Förster, C (1995) The Period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. Proc. Natl Acad. Sci. USA 92, 612–6.

Helfrich-Förster C (2000) Differential control of morning and evening components in the activity rhythm of Drosophila melanogaster-sex-specific differences suggest a different quality of activity. J Biol Rhythms 15: 135-54.

Hendricks JC, Lu S, Kume K, Yin JC, Yang Z, Sehgal A (2003) Gender dimorphism in the role of cycle (BMAL1) in rest, rest regulation, and longevity in Drosophila melanogaster. J Biol Rhythms. 18(1):12-25.

Hogenesch JB, Gu YZ, Jain S, Bradfield CA (1998) The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc Natl Acad Sci USA 95: 5474-9.

Ivanchenko M, Stanewsky R, Giebultowicz JM (2001) Circadian photoreception in Drosophila: functions of cryptochrome in peripheral and central clocks. J Biol Rhythms. 16:205-15.

Kaneko M, Helfrich-Förster C, Hall JC (1997) Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of Drosophila: newly identified pacemaker candidates and novel features of clock gene product cycling. J Neurosci 17: 6745-60.

Kim EY, Bae K, Ng FS, Glossop NR, Hardin PE, Edery I (2002) Drosophila CLOCK protein is under posttranscriptional control and influences light-induced activity. Neuron 34: 69-81.

Kiyohara YB, Tagao S, Tamanini F, Morita A, Sugisawa Y, Yasuda M, Yamanaka I, Ueda HR, van der Horst GT, Kondo T, Yagita K (2006) The BMAL1 C terminus regulates the circadian transcription feedback loop. Proc Natl Acad Sci USA 103:10074-9.

Krishnan B, Levine JD, Lynch MK, Dowse HB, Funes P, Hall JC, Hardin PE,Dryer SE (2001) A new role for cryptochrome in a Drosophila circadian oscillator. Nature 411:313-317.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH,Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98:193-205.

Kwon I, Lee J, Chang SH, Jung NC, Lee BJ, Son GH, Kim K, Lee KH (2006) BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. Mol Cell Biol. 26: 7318-30.

Levine JD, Funes P, Dowse HB, Hall JC (2002) Signal analysis of behavioral and molecular cycles. BMC Neurosci. 3:1.

Lin FJ, Song W, Meyer-Bernstein E, Naidoo N, Sehgal A (2001) Photic signaling by cryptochrome in the Drosophila circadian system. Mol Cell Biol. 21:7287-94.

Markova EP, Ueda H, Sakamoto K, Oishi K, Shimada T,Takeda M (2003) Cloning of Cyc (Bmal1) homolog in Bombyx mori: structural analysis and tissue specific distributions. Comp Biochem Physiol B Biochem Mol Biol 134:535-542.

Meireles-Filho AC, Amoretty PR, Souza NA, Kyriacou CP,Peixoto AA (2006) Rhythmic expression of the cycle gene in a hematophagous insect vector. BMC Mol Biol 7:38.

Meireles-Filho AC, da S Rivas GB, Gesto JS, Machado RC, Britto C, de Souza NA,Peixoto AA (2006) The biological clock of an hematophagous insect: locomotor activity rhythms, circadian expression and downregulation after a blood meal. FEBS Lett 580:2-8.

Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, Hall JC (2000) Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc Natl Acad Sci USA 97: 3608-13.

Rosato E, Codd V, Mazzotta G, Piccin A, Zordan M, Costa R, Kyriacou CP (2001) Light-dependent interaction between Drosophila CRY and the clock protein PER mediated by the carboxy terminus of CRY. Curr Biol. 11:909-17.

Rubin EB, Shemesh Y, Cohen M, Elgavish S, Robertson HM ,Bloch G (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (Apis mellifera) and shed new light on the molecular evolution of the circadian clock. Genome Res 16:1352-1365.

Rutila JE, Suri V, Le M, So WV, Rosbash M ,Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. Cell 93:805-814.

Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB (2006) Feedback repression is required for mammalian circadian clock function. Nat. Genet. 38: 312-9.

Shaw PJ, Tononi G, Greenspan RJ, Robinson DF (2002) Stress response genes protect against lethal effects of sleep deprivation in Drosophila. Nature. 417:287-91.

Spradling AC (1986) P element mediated transformation. In Drosophila: A Practical Approach, D.B. Roberts, ed. (Oxford: IRL Press) 175-96.

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M,Hall JC (1998) The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. Cell 95:681-692.

Takahata S, Ozaki T, Mimura J, Kikuchi Y, Sogawa K,Fujii-Kuriyama Y (2000) Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. Genes Cells 5:739-747.

Tauber E, Last KS, Olive PJ,Kyriacou CP (2004) Clock gene evolution and functional divergence. J Biol Rhythms 19:445-458.

Thummel CS, Boulet AM, Lipshitz,HD (1988) Vectors for P element-mediated transformation and tissue culture transfection. Gene 74, 445-56.

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-30.

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS,Sancar A (1999) Differential regulation of mammalian period genes and circadian

rhythmicity by cryptochromes 1 and 2. Proc Natl Acad Sci U S A 96:12114-12119.

Yu W, Zheng H, Houl JH, Dauwalder B,Hardin PE (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. Genes Dev 20:723-733.

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 24:948-955.

Weber F, Kay SA (2003) A PERIOD inhibitor buffer introduces a delay mechanism for CLK/CYC-activated transcription. FEBS Lett. 555: 341-5.

Zhu H, Yuan Q, Briscoe AD, Froy O, Casselman A, Reppert SM (2005) The two CRYs of the butterfly. Curr Biol. 15: R953-4.

Legends:

Figure 1: *dcyc-hsp70*-dCYC-aaCYCct construct is shown along with the *cyc* genomic region for reference. Closed bars indicate untranslated exons and open bars indicate translated portions of exons. For details see Material and Methods.

Figure 2: dCLK$^{Jrk}$/dCYC-aaCYCct activates E-box dependent expression. The presence (+) or absence (-) of expression plasmids (5 ng dCLK; 5 ng dCLK$^{Jrk}$; 5 ng dCYC-aaCYCct) is depicted. Luciferase activity was determined in cotransfection assays as described in Material and Methods. CYC is expressed endogenously in S2 cells (Darlington et al 1998). Each value is the mean ± SEM of three replicates as percent of control, which is luciferase activity in a transfection with pAc-CLK alone.

Figure 3: PER inhibits dCLK$^{Jrk}$/dCYC-aaCYCct and dCLK/CYC luciferase reporter gene expression in a dose dependent manner. The amounts of PER (in ng) are indicated. As in Fig. 2, each value is the mean ± SEM of three replicates as percent of control, which is luciferase activity in a transfection with pAc-CLK alone.

Figure 4: mCRY1 inhibits dCLK$^{Jrk}$/dCYC-aaCYCct, but not dCLK/CYC luciferase reporter gene expression in a dose dependent manner. The amounts of mCRY1 (in ng) are indicated. As in Fig. 2 and 3, each value

is the mean ± SEM of three replicates as percent of control, which is luciferase activity in a transfection with pAc-CLK alone.

Figure 5: Increasing amounts of dCRY lead an induction of luciferase reporter gene expression in a dose dependent manner driven by $dCLK^{Jrk}$/dCYC-aaCYCct but not dCLK/CYC. The amounts of dCRY (in ng) are indicated. As in Fig. 2, 3 and 4, each value is the mean ± SEM of three replicates as percent of control, which is luciferase activity in a transfection with pAc-CLK alone.

Figure 6: Rest-activity patterns of transformant and control male and female flies under Light:Dark cycles. Vertical bars represent activity recorded in 30 min bins; white bars in indicate lights-on and black bars indicate lights off. The three independent transformed fly lines are described after the genotype (M1, M2 and M3). M1 flies are shown in the heterozygous configuration because the homozygous is lethal.

Figure 1

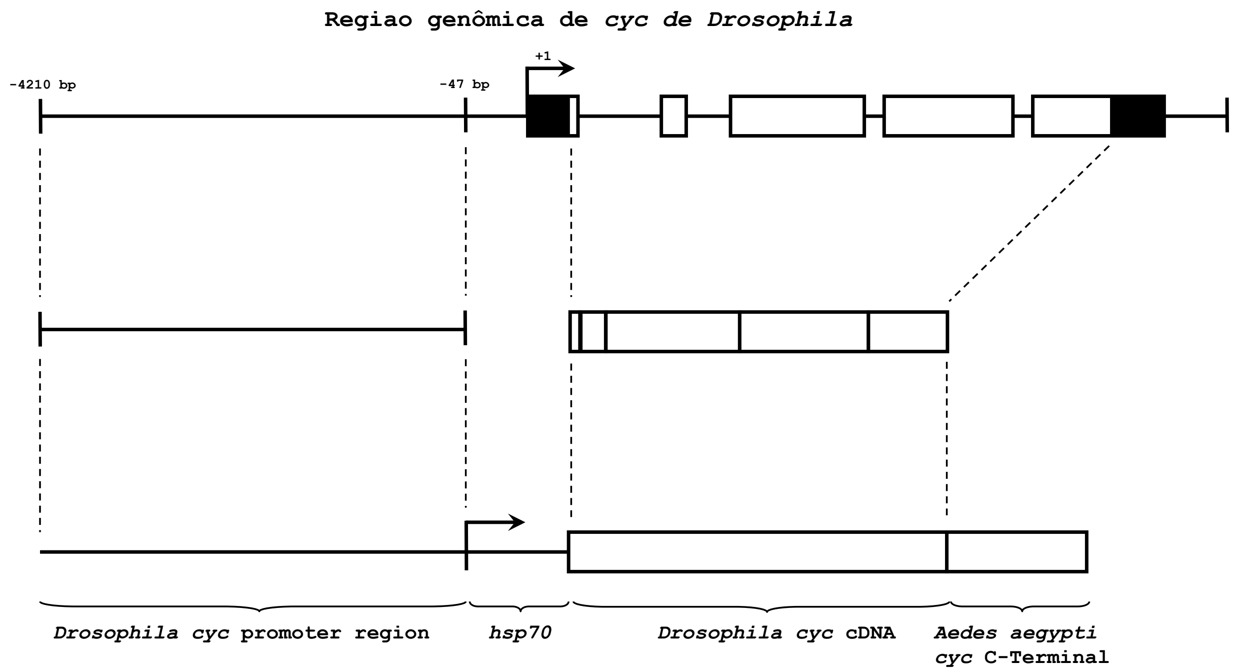


Figure 2

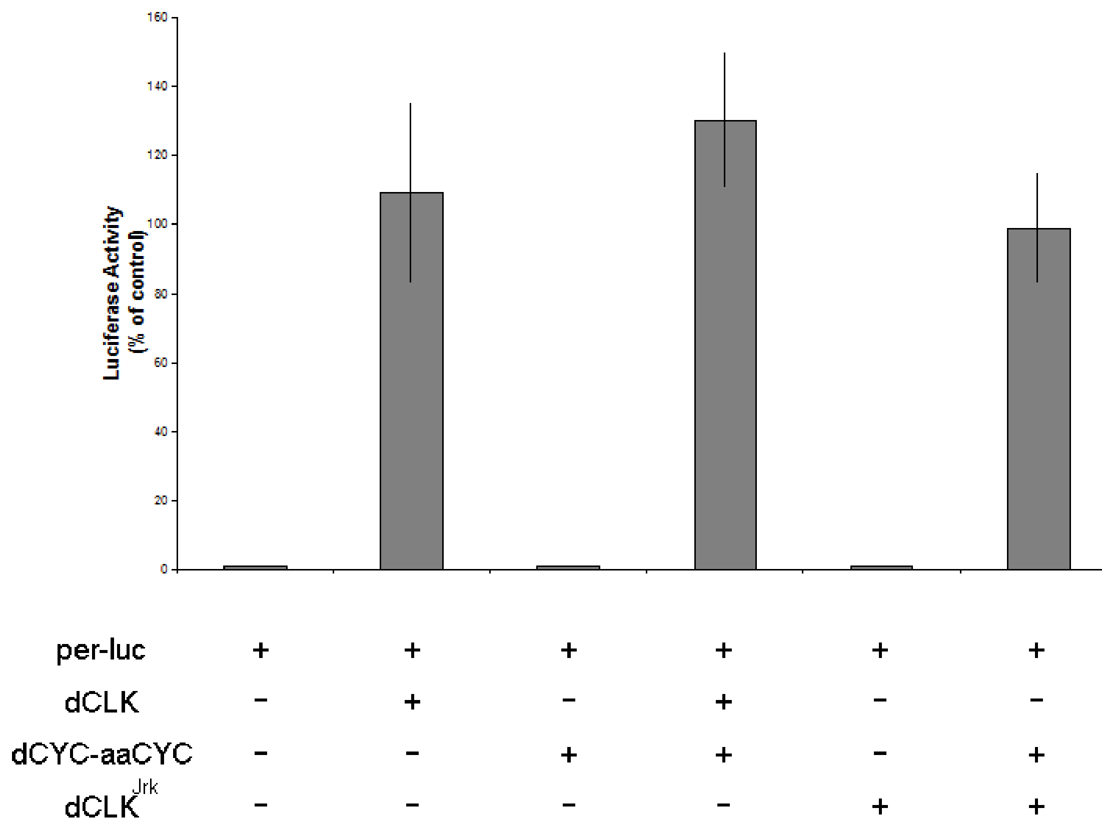


Figure 3

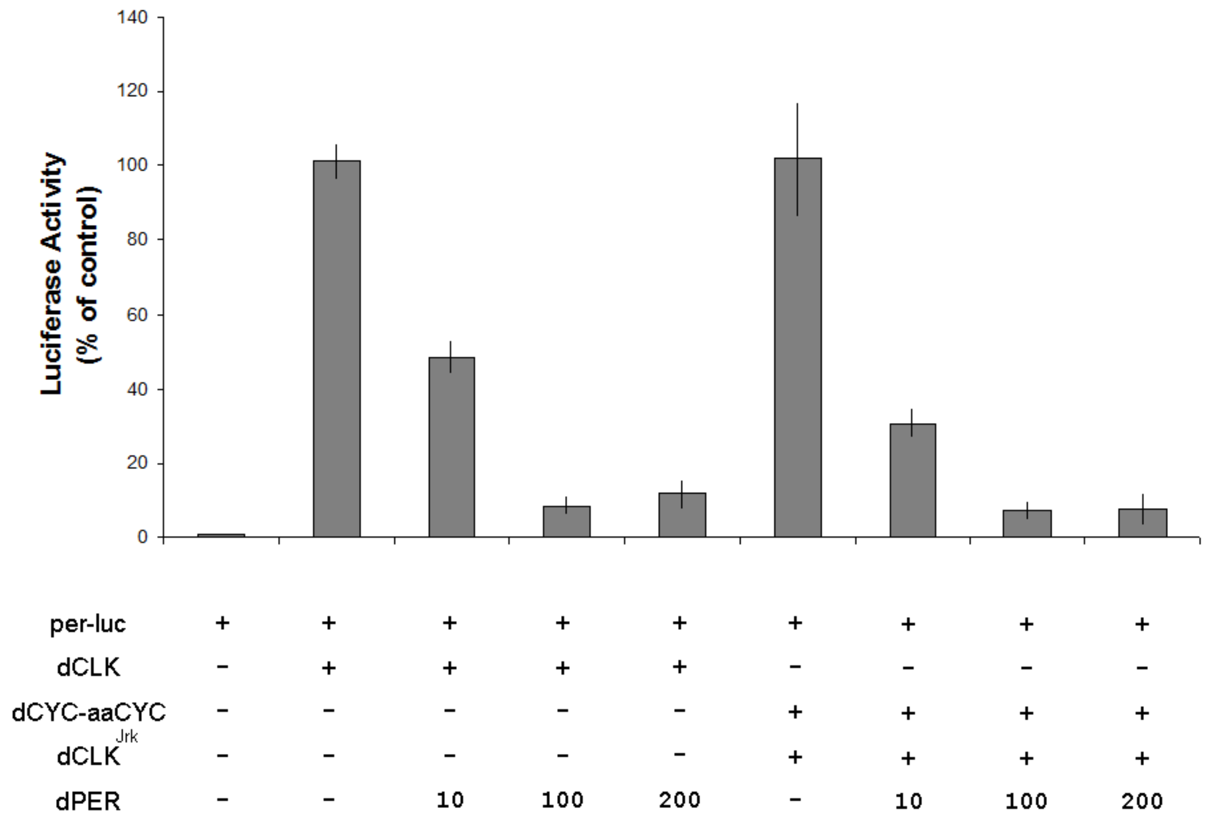
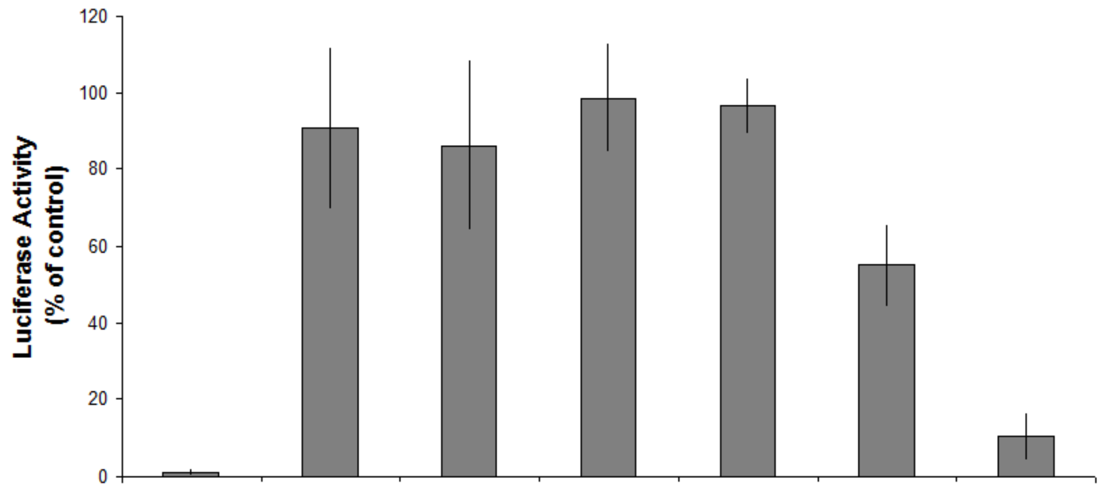


Figure 4




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| per-luc | + | + | + | + | + | + | + |
| dCLK | - | + | + | + | - | - | - |
| dCYC-aaCYC | - | - | - | - | + | + | + |
| $dCLK^{Jrk}$ | - | - | - | - | + | + | + |
| mCRY1 | - | - | 10 | 100 | - | 10 | 100 |

Figure 5

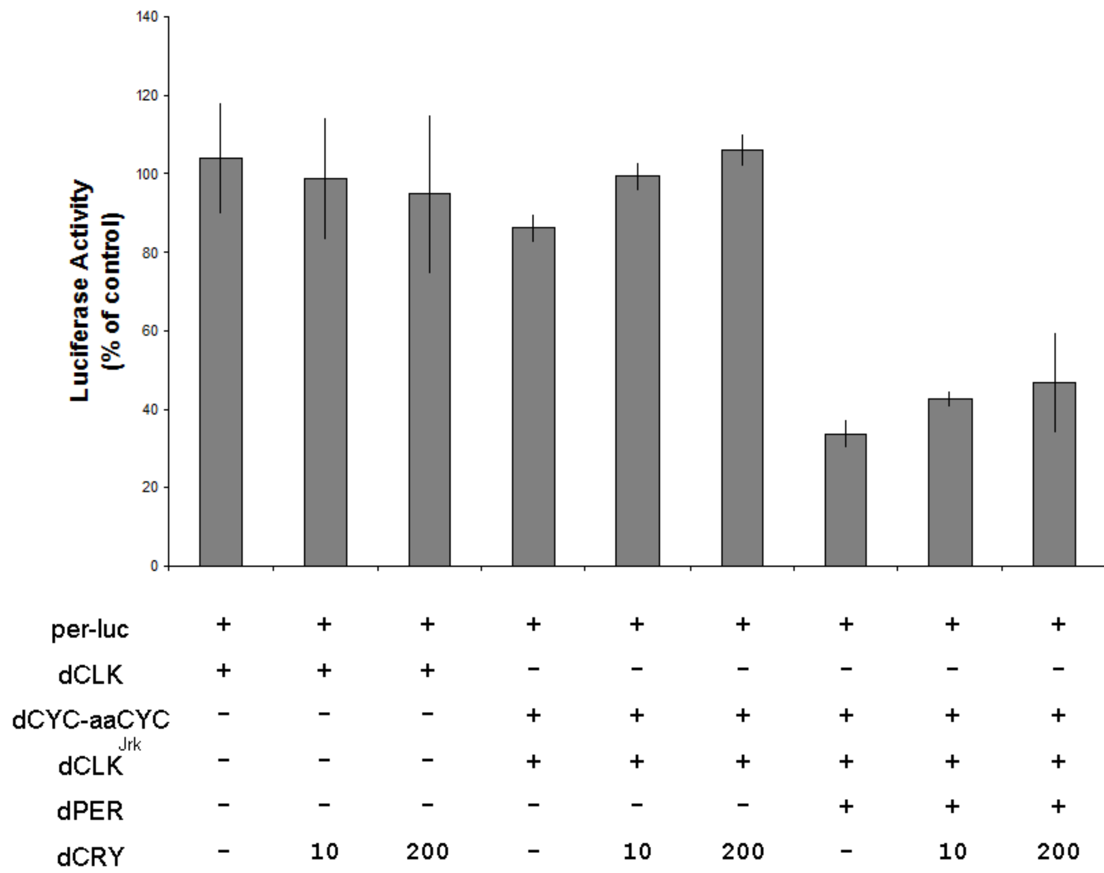
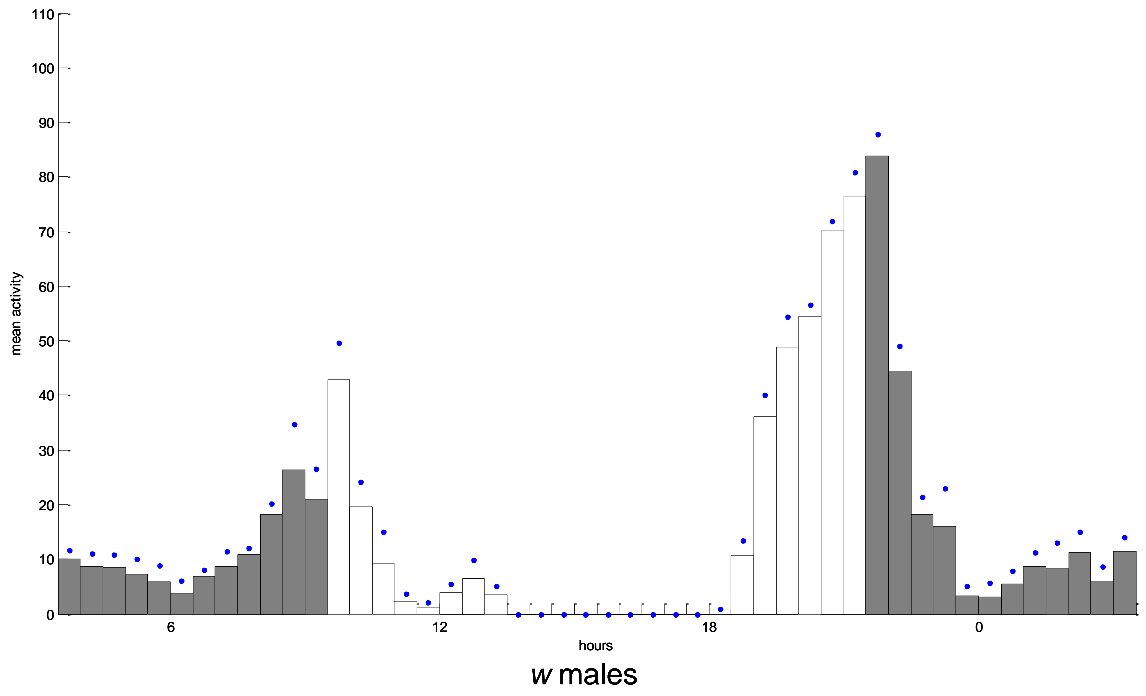
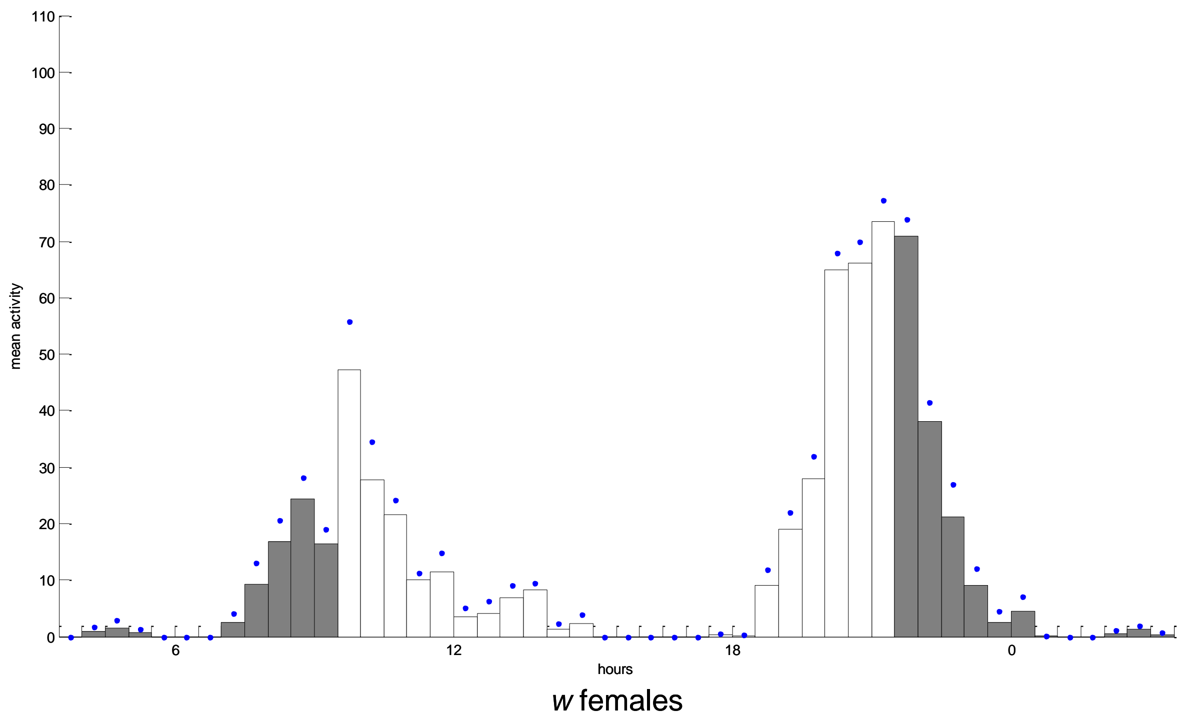


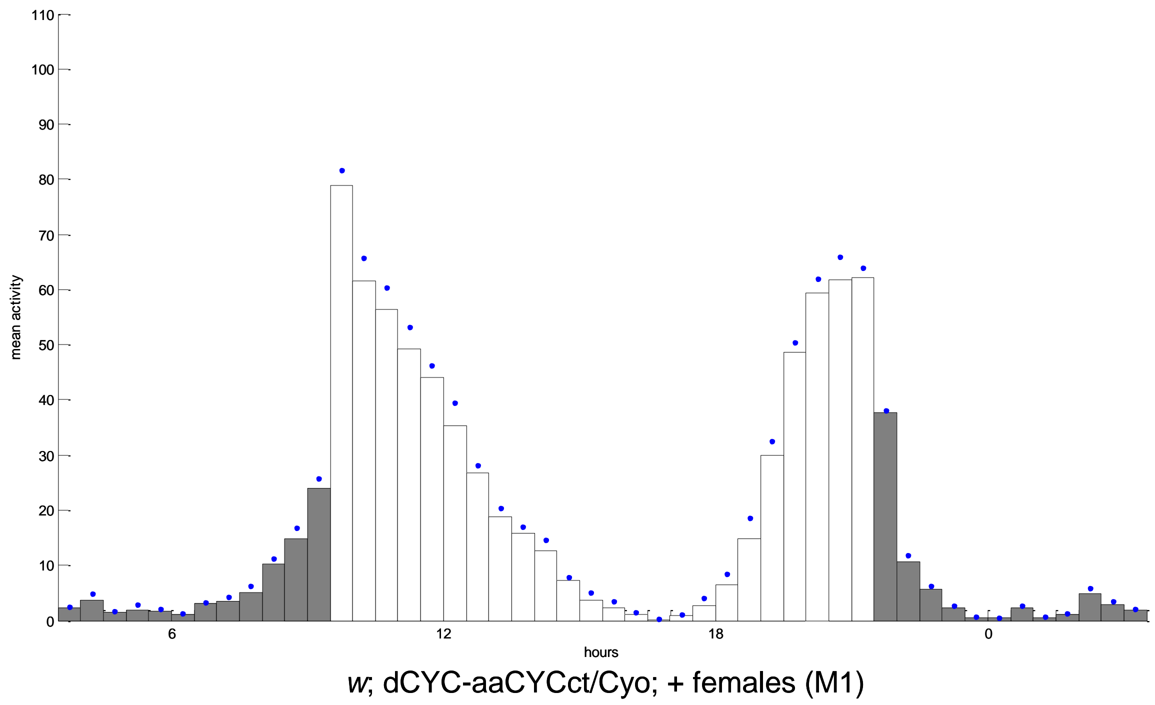
Figure 6



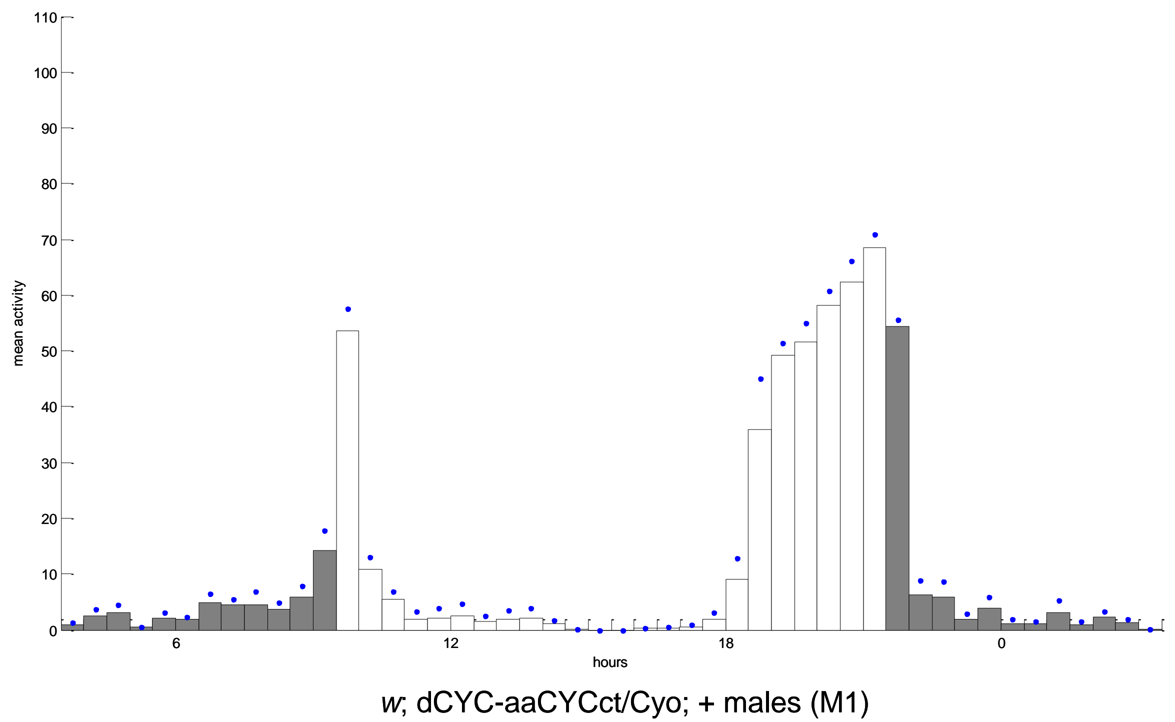
*w* males



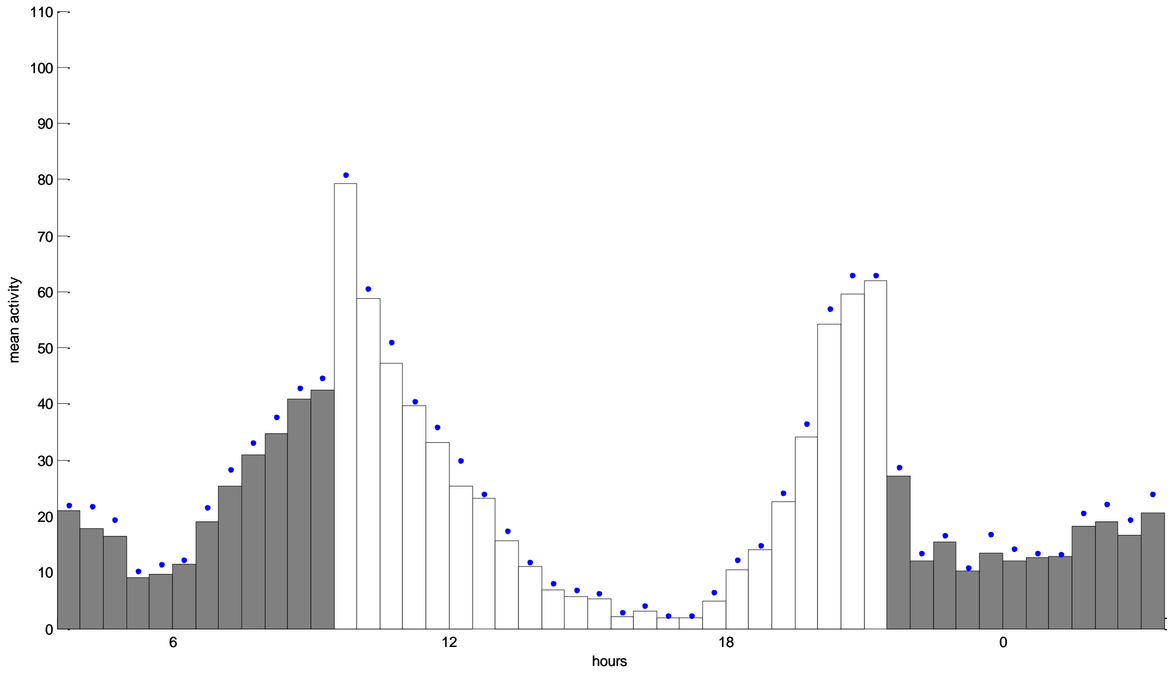
*w* females



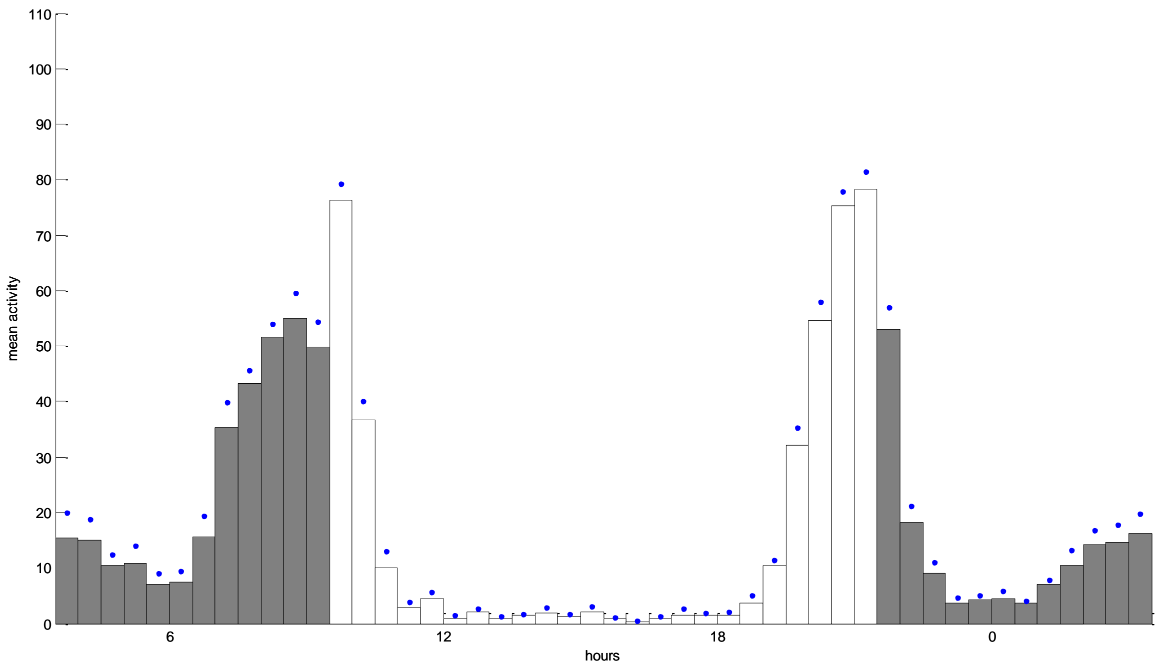

*w*; dCYC-aaCYCct/Cyo; + females (M1)



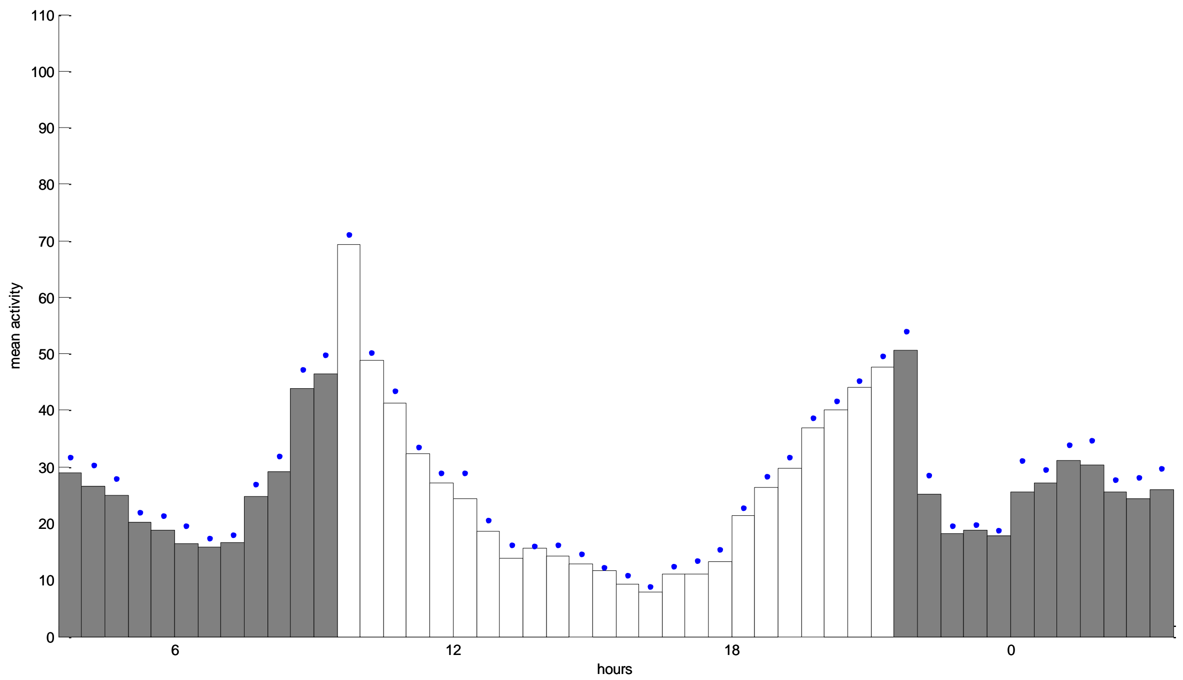

*w*; dCYC-aaCYCct/Cyo; + males (M1)



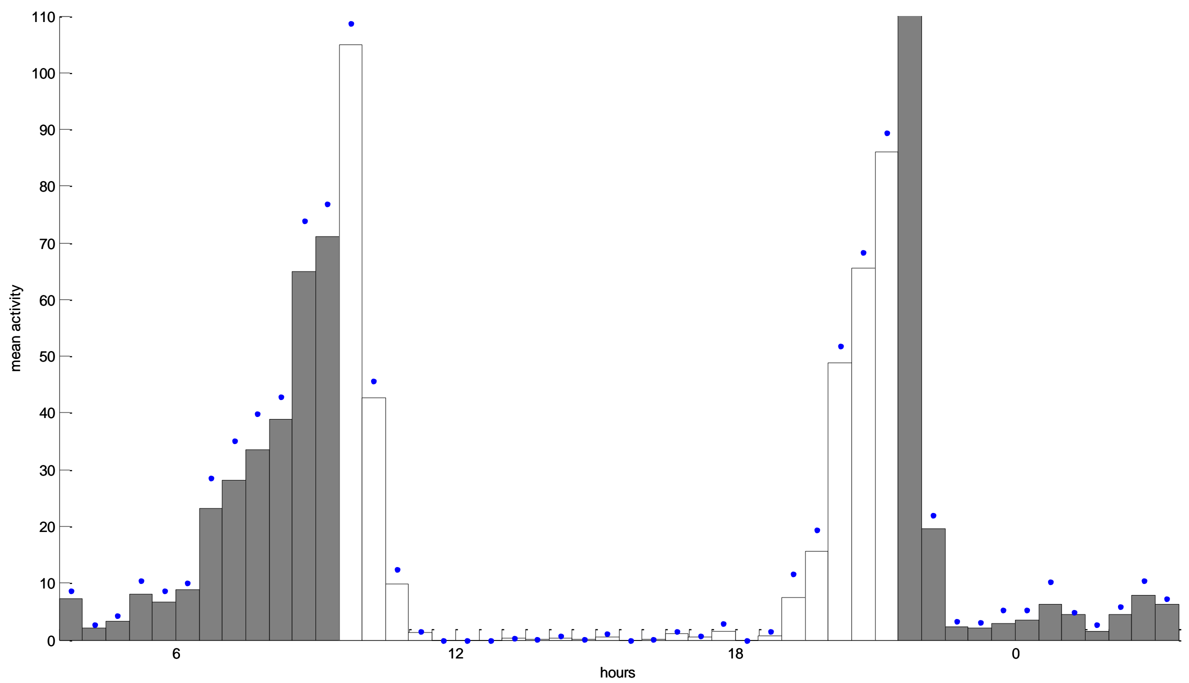

*w*; +; dCYC-aaCYCct  females (M2)




*w*; +; dCYC-aaCYCct  males (M2)




*w*; dCYC-aaCYCct; + females (M3)




*w*; dCYC-aaCYCct; + males (M3)

## Capítulo 6 - Discussão

Atualmente grande parte dos estudos sobre Cronobiologia foca na elucidação das bases moleculares do relógio biológico em diferentes espécies. A comparação entre organismos distintos revelou uma surpreendente conservação estrutural e funcional, sugerindo que o mecanismo de marcação temporal tenha sido adquirido pelos seres vivos a bastante tempo na escala evolutiva (Tauber et al, 2004). Entretanto as diferenças observadas entre espécies relativamente próximas sugerem que cada espécie deve ser individualmente analisada no intuito de se entender como seu relógio biológico controla seu comportamento e sua fisiologia. Além disso, o estudo comparativo entre espécies próximas ajuda no entendimento da evolução molecular dos relógios biológicos.

Este trabalho é basicamente dividido em duas partes. Na primeira analisamos a atividade locomotora de *L. longipalpis*, a expressão gênica dos componentes da primeira alça regulatória, o impacto da alimentação sanguínea neste controle e clonamos o gene *cyc* desta espécie (Capítulos 3 e 4). Na segunda parte, devido a questões evolutivas levantadas por nossos primeiros resultados, analisamos potenciais mudanças ao longo da evolução do mecanismo molecular do relógio biológico de *D. melanogaster*, com ênfase no papel do domínio C-Terminal de CYC de insetos vetores (em especial *Aedes aegypti*) que parece ter sido perdido em *Drosophila* e sua interação com dCRY de *D. melanogaster* (Capítulo 5).

## 6.1 – O relógio biológico de *L. longipalpis*

O comportamento de insetos vetores está diretamente relacionado com a epidemiologia das doenças por eles transmitidas (Klowden, 1996). Por exemplo, os ritmos de atividade e a hematofagia tem fundamental importância na dinâmica de transmissão dos parasitos de que são vetores, e seu controle certamente se dá por meio do relógio circadiano (Klowden, 1996; Clements, 1999; Dunlap, 1999).

A clonagem do gene *cyc* e a análise expressão gênica de *per*, *tim Clk* e *cyc* em *L. longipalpis* foi o primeiro passo na tentativa de se compreender as bases moleculares dos ritmos circadianos em insetos vetores. Concentramos nossas análises nos genes da primeira alça regulatória porque ela é a principal na manutenção dos ritmos de atividade locomotora (Hardin, 2005) e porque ela era a melhor descrita em *D. melanogaster* no início de nossos estudos. Acredita-se que a segunda alça exista para prover estabilidade no funcionamento da primeira, visto que mudanças no padrão de expressão de genes que participam desta alça alteram o comportamento das moscas de maneira modesta (Hardin, 2005; Benito et al, 2007).

A análise da atividade locomotora e da expressão dos genes da primeira alça em *L. longipalpis* mostrou que o comportamento e os padrões de oscilação de *Clk* e *cyc* são bastante diferentes dos observados em *D. melanogaster* (Capítulos 3 e 4). Enquanto *D. melanogaster* é essencialmente diurna, apresenta *cyc* expresso de maneira constitutiva e *Clk* com pico de RNAm em ZT22-2; *L. longipalpis*

apresenta comportamento crepuscular-noturno e expressão rítmica de *cyc* e *Clk* com picos em ZT5-9 e ZT13 respectivamente (Capítulos 3 e 4; Bae et al, 1998; Lee et al, 1998; Rutila et al, 1998; Houl et al, 2006; Yu et al, 2006). Como a atividade destes genes é de fundamental importância no controle da atividade locomotora em *D. melanogaster* (Kim et al, 2002), propomos uma correlação entre as diferenças no padrão de expressão de *Clk* e *cyc* e a atividade locomotora em *L. longipalpis*. Para confirmar tal hipótese, o padrão de expressão de *Clk* e *cyc* poderia ser manipulado em flebotomíneos, aumentando, diminuindo ou alterando suas fases de transcrição e observando como isso afetaria o ritmo de atividade locomotora em adultos. Entretanto, além das dificuldades relacionadas à sua manutenção em cativeiro (Munstermann, 2004), em *L. longipalpis* ainda não existem ferramentas que permitam este tipo de manipulação e apenas recentemente esta espécie teve seu projeto genoma proposto (http://www.vectorbase.org/). Desta forma, nosso laboratório hoje foca no estudo e elucidação dos mecanismos moleculares de outros insetos vetores como *Aedes aegypti*, que além de apresentarem uma gama de aspectos de sua fisiologia e comportamento sabidamente controlados pelo relógio biológico como em *L. longipalpis*, tem seu genoma já seqüenciado e técnicas para o desenvolvimento de transgênicos já desenvolvidas (Coates et al, 1998; Jasinskiene et al, 1998; Adelman et al, 2007; Nene et al, 2007).

Outra hipótese levantada nestes trabalhos foi a de que a alimentação sanguínea causa uma diminuição da atividade locomotora mediada pelo relógio biológico (Capítulo 3). Esta suposição foi baseada

em observações do padrão de atividade de flebotomíneos no campo. Por volta do crepúsculo, flebotomíneos machos e fêmeas começam a chegar ao local onde se acasalam e as fêmeas fazem o repasto sanguíneo. Esta atividade é certamente controlada pelo relógio circadiano, visto que apresenta grande precisão (dia após dia se repete aproximadamente no mesmo horário) e varia pouco com a época do ano, apesar da variação de luminosidade (Nataly de Souza, comunicação pessoal; Morrison et al, 1995). Após o repasto as fêmeas se afastam do hospedeiro e repousam, provavelmente para que o sangue seja digerido. Esta diminuição da atividade sempre foi creditada ao aumento do peso do inseto, que em flebotomíneos pode chegar a duas vezes seu próprio peso. Entretanto nossos dados sugerem fortemente que isso envolva um controle interno pelo marcapasso central, visto que a expressão dos genes *per* e *tim* é inibida na cabeça (ou seja, onde o controle neural da atividade locomotora se localiza) após a alimentação sanguínea (Capítulo 3).

Nossa hipótese é que o repasto sanguíneo altera o nível redox celular e que isto inibiria a expressão de *per* e *tim* indiretamente pela repressão de CLK:CYC. Isto pode ser testado em sistemas celulares heterólogos como os usados nos experimentos descritos no Capítulo 3 e disponíveis para células de *L. longipalpis* (Saraiva et al, 2000). Para isso, a clonagem dos genes envolvidos na primeira alça regulatória de flebotomíneos vem sendo conduzida em nosso laboratório, sendo que alguns deles já completados como o gene *cyc* (Capítulo 4).

Além disso, a transformação de *D. melanogaster* com genes de *L. longipalpis* pode ser realizada para superar a falta de ferramentas

genéticas específicas em flebotomíneos, permitindo a análise do funcionamento de genes desta espécie em um "organismo hospedeiro". Entretanto, sua aplicação é limitada pelo fato de que as outras moléculas do organismo seriam da própria *Drosophila*, permitindo apenas uma extrapolação relativa do que possa ocorrer em flebotomíneos. Apesar disso, transgênicos inter-específicos podem proporcionar resultados extremante interessantes tanto no estudo das bases moleculares do funcionamento do relógio circadiano como de sua evolução (e.g. Petersen et al, 1988; Peixoto et al, 1998; Capítulo 4).

Finalmente, o fato de *Clk* e *cyc* controlarem vários genes de OUTPUT envolvidos em processos diversos como oviposição, detoxificação e imunidade (McDonald & Rosbash, 2001; Ceriani et al, 2002; Lee & Edery, 2008) sugere que muitas das diferenças observadas no comportamento e na fisiologia entre *D. melanogaster* e flebotomíneos possam ser devidas à ação destes genes.

## 6.2 – A evolução do relógio biológico de *D. melanogaster*

*D. melanogaster* tem sido um dos organismos modelo mais bem estudados em Biologia. E muito do que se sabe dos relógios biológicos, especialmente do ponto de vista molecular, se deve a estudos realizados com esta espécie. Assim sendo, não é surpreendente que muitos dos trabalhos envolvendo o estudo da evolução dos marcapassos circadianos tenha sido dedicada à comparação dos resultados obtidos com *Drosophila* e outros insetos (revisado em Peixoto, 2002; Hall, 2003).

A análise evolutiva do gene *per* em *D. melanogaster* e outros Diptera revelou que ele é um dos genes que evoluem com maior rapidez em insetos, o que o torna particularmente interessante em estudos de genética de populações e evolução (Peixoto, 2002). Já os outros componentes do relógio não despertaram muito interesse da comunidade científica, visto que o grau de conservação entre eles e seus homólogos em outras espécies é bem maior que o observado para *per* (Dunlap, 1999; Peixoto, 2002).

Entretanto, apesar do apreciável grau de identidade existente ao nível de seqüência, existem diferenças consideráveis com relação à regulação gênica. Além disso, proteínas homólogas têm sido descritas interagindo com moléculas diferentes no relógio de diferentes espécies. Isso mostra que a análise pura da seqüência pode encobrir diferenças importantes nos padrões de expressão temporal e espacial dos genes de relógio. Estas diferenças podem fazer com que as mesmas moléculas desempenhem funções bem diferentes, como de fato o fazem, do que o imaginado (Peixoto, 2002; Bell-Pedersen et al, 2005).

Um bom exemplo disso é o gene *cyc*. Inicialmente clonado em *D. melanogaster*, ele despertou pouca atenção dos cronobiologistas por se tratar de um gene expresso constitutivamente, enquanto todos os outros descritos até então para o mecanismo do relógio biológico apresentavam expressão cíclica (Rutila et al, 1998; Hardin, 2005). Seu homólogo em mamíferos foi incriminado como participante do marcapasso central mais tarde, apesar da seqüência já ser conhecida desde 1997 (Hogenesch et al, 1997; Bunger et al, 2000). Hoje em dia ele

tem ganhado muita atenção visto que está ligado não apenas ao marcapasso circadiano, mas também a uma série de outros aspectos da fisiologia, inclusive a certas doenças em mamíferos. O mutante nulo de *bmal1* em camundongos apresenta perda de peso, problemas de sono, susceptibilidade a hipertensão e a diabete tipo 2, dentre outros (Rudic et al, 2004; Shimba et al, 2005; Curtis et al 2007; Woon et al, 2007).

Os resultados da clonagem de *cyc* em *L. longipalpis* (descritos no Capítulo 4) levantaram questões acerca da origem evolutiva deste gene. Além de apresentar homologia geral apenas pouco maior a moscas (67,36%) que a camundongos (62,18%), a presença em flebotomíneos de uma importante cauda de ativação ausente em *D. melanogaster* e com 93,75% de homologia com *Mus musculus*, somado ao padrão de expressão rítmico mais similar a camundongos colocava este gene como possivelmente importante no processo evolutivo do relógio em *D. melanogaster* (Capítulo 4).

Deste modo resolvemos estudar o efeito que esta cauda de ativação, que está presente em todas as espécies analisadas até o momento com exceção dos drosofilídeos, teria no relógio de *D. melanogaster* (Capítulo 5). Para isto utilizamos tanto a cauda de CYC de *L. longipalpis* (resultados preliminares não apresentados nesta tese) como a de *Aedes aegypti* (Capítulo 5). Os resultados obtidos desta análise, além de sugerirem como funcionaria o relógio ancestral de *Drosophila*, ajudam também a entender o papel que CRY desempenha atualmente.

Está descrito em *Mus musculus* e *Xenopus laevis* que CRY interage com a cauda de BMAL1, e isto é fundamental para a repressão do complexo ativador CLK:BMAL1 e conseqüente manutenção dos ritmos circadianos (Kiyohara et al 2006; Kwon et al 2006; Sato et al 2006; van der Schalie et al, 2007). Isso pode ser destacado como uma das principais diferenças entre os relógios de vertebrados e *Drosophila*, já que nesta espécie CRY funciona primordialmente como fotoreceptor no mecanismo de INPUT, se ligando a TIM e o degradando em uma reação dependente de luz (Emery et al, 1998; Stanewsky et al, 1998; Ceriani et al, 1999; Lin et al 2001).

Apesar disso, CRY parece ter função repressora no marcapasso central em órgãos periféricos como antena, túbulos de Malpighi e nos olhos (Krishnan et al, 2001; Ivanchenko et al, 2001; Collins et al, 2006). Contudo os mecanismos moleculares envolvidos na repressão em órgãos periféricos ainda não são conhecidos, e certamente não são similares aos de vertebrados devida à ausência da região C-Terminal de CYC em *D. melanogaster*.

Deste modo, a evolução de CRY é peça chave não só para o entendimento da evolução do relógio biológico em *Drosophila* como um todo, mas também na elucidação do mecanismo no qual ele está envolvido nos relógios periféricos nesta espécie.

Assim sendo nossos estudos revelaram que o relógio circadiano de *D. melanogaster* pode funcionar de uma maneira semelhante ao de vertebrados, confirmando que a homologia entre espécies é significativa. Além disso, nossos resultados sugerem que a perda da porção C-

Terminal de CYC foi decisiva para que CRY desempenhasse uma nova função no relógio central, e que estes eventos foram de fundamental importância na evolução do relógio biológico de *Drosophila*.

No futuro pretendemos estender nossas análises para dar mais embasamento a nossas hipóteses. Por exemplo, apesar dos experimentos em células S2 indicarem fortemente que dCRY está interagindo com a cauda aaCYCct na medida em que ativa o complexo, enquanto o controle sem a cauda não reponde a dCRY (Capítulo 5, Fig 5), pretendemos realizar experimentos de co-imunoprecipitação para confirmar a interação entre dCRY e dCYC-aaCYCct.

Outro experimento para se confirmar essa interação seria utilizando as células S2. A idéia é cotransfectar os fatores de transcrição ($dCLK^{Jrk}$:dCYC-aaCYCct paralelo ao controle dCLK:dCYC) com combinações crescentes e combinadas de mCRY1 e dCRY. Como mCRY1 sabidamente reprime $dCLK^{Jrk}$:dCYC-aaCYCct (Capítulo 5, Fig 4), quantidades crescentes de dCRY deveriam competir e desreprimir a ativação de $dCLK^{Jrk}$:dCYC-aaCYCct caso realmente aja a interação entre aaCYCct e dCRY por nós proposta. De maneira inversa, quantidades crescentes de mCRY1 deveriam suprimir a ativação de $dCLK^{Jrk}$:dCYC-aaCYCct causada por dCRY.

Além disso, para confirmarmos nossos dados *in vivo*, pretendemos transformar em *D. melanogaster* o gene *dcyc* sob controle de seu promotor. A única diferença entre este transformante e o por nós usado seria a cauda de *cyc* mosquito. Com isso afastaríamos a hipótese de que a alteração no comportamento das fêmeas em LD seria devida única e

exclusivamente a uma superexpressão de CYC, independente da cauda aaCYCct.

Um outro experimento programado é cruzar nossas moscas expressando dCYC-aaCYCct com moscas mutantes *cry*$^{b}$ (Stanewsky et al, 1998). *cry*$^{b}$ é um mutante que apresenta dificuldades em ser sincronizado pela luz, o que o caracterizou como importante molécula no mecanismo de fotorecepção em *D. melanogaster*. Como não trabalha no relógio central dos neurônios que controlam o comportamento, o ritmo de atividade locomotora destas moscas é normal, apesar de dCRY quase não ser detectado no cérebro nestes mutantes (Stanewsky et al, 1998; Emery et al, 2000). Portanto, nossa idéia é usar este mutante para testar se o aumento da atividade dependente de luz observado em fêmeas expressando dCYC-aaCYCct é dependente de dCRY. Moscas expressando dCYC-aaCYCct seriam cruzadas com mutantes *cry*$^{b}$ e sua atividade locomotora seria avaliada. Caso o aumento da atividade em fêmeas fosse de fato devido a ativação de dCYC-aaCYCct por dCRY, deveríamos observar uma indiferença no padrão de atividade locomotora entre estas moscas e moscas selvagens.

Outra hipótese que queremos testar e que está em andamento, é a de que, assim como nas células S2, dCYC-aaCYCct também recupere a atividade de dCLK$^{Jrk}$ *in vivo.* Para tal, cruzamos moscas expressando dCYC-aaCYCct com moscas duplo-mutante *Clk*$^{Jrk}$;*cyc*$^{01}$ (Allada et al, 1998; Rutila et al, 1998). A idéia é que dCYC-aaCYCct, além de recuperar o fenótipo de *cyc*$^{01}$ também recupere o fenótipo de *Clk*$^{Jrk}$, já que é isso que acontece em células S2. Entretanto, dados preliminares

sugerem que não há recuperação de ritmo na atividade locomotora. A mutação *Clk*$^{Jrk}$ afeta a anatomia cerebral, principalmente eliminando os s-$LN_v$ e suas projeções, que são indispensáveis para ritmicidade circadiana (Park et al, 2000). Desta forma comparamos cérebros por análises imunocitoquímicas (usando um anticorpo contra PDF) de moscas selvagens, *Clk*$^{Jrk}$ e dCYC-aaCYCct/*Clk*$^{Jrk}$ e constatamos que não havia diferença entre o mutante e o mutante com a construção. Foi descrito que a severidade da mutação *Clk*$^{Jrk}$ é difícil de ser recuperada, visto que a proteína dCLK$^{Jrk}$ apresenta fenótipo semi-dominante (Allada et al, 1998; Allada et al, 2003). Tentativas foram feitas expressando dCLK sob controle do promotor de *pdf* e *cry*, mas os resultados não foram satisfatórios (0% e 18% de recuperação de atividade locomotora respectivamente; Allada et al, 2003). Portanto, existem evidências de que a mutação não tem seu fenótipo recuperado por uma incapacidade de se restaurar os neurônios importantes para o controle do relógio biológico.

Concluindo, esperamos com estes resultados contribuir para um melhor entendimento da genética molecular dos ritmos circadianos em insetos vetores e *D. melanogaster*. Esperamos também com a análise comparativa destas espécies, colaborar para um melhor entendimento da evolução destes mecanismos. Isto é importante não só no estudo da Cronobiologia, mas da Biologia de maneira geral visto que os mecanismos aqui descritos estão envolvidos em vários aspectos do comportamento e da fisiologia de quase todos os organismos presentes no planeta.

**Capítulo 7 – Referências Bibliográficas**

Adelman ZN, Jasinskiene N, Onal S, Juhn J, Ashikyan A, Salampessy M, MacCauley T, James AA (2007) nanos gene control DNA mediates developmentally regulated transposition in the yellow fever mosquito Aedes aegypti. Proc Natl Acad Sci U S A 104:9970-9975.

Akashi M, Takumi T (2005) The orphan nuclear receptor RORa regulates circadian transcription of the mammalian core-clock Bmal1. Nat Struct Mol Biol 12:441-448.

Allada R, Kadener S, Nandakumar N, Rosbash M (2003) A recessive mutant of Drosophila Clock reveals a role in circadian rhythm amplitude. EMBO J 22:3367-3375.

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 93:791-804.

Bae K, Lee C, Hardin PE, Edery I (2000) dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. J Neurosci 20:1746-1753.

Bae K, Lee C, Sidote D, Chuang KY, Edery I (1998) Circadian regulation of a Drosophila homolog of the mammalian Clock gene: PER and TIM function as positive regulators. Mol Cell Biol 18:6142-6151.

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioural rhythms by gene transfer in Drosophila. Nature 312:752-754.

Beaver LM, Gvakharia BO, Vollintine TS, Hege DM, Stanewsky R, Giebultowicz JM (2002) Loss of circadian clock function decreases reproductive fitness in males of Drosophila melanogaster. Proc Natl Acad Sci U S A 99:2134-2139.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6:544-556.

Benito J, Zheng H, Hardin PE (2007) PDP1epsilon functions downstream of the circadian oscillator to mediate behavioral rhythms. J Neurosci 27:2539-2547.

Blau J, Young MW (1999) Cycling vrille expression is required for a functional Drosophila clock. Cell 99:661-671.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ, Kay SA (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285:553-556.

Ceriani MF, Hogenesch JB, Yanovsky M, Panda S, Straume M, Kay SA (2002) Genome-wide expression analysis in Drosophila reveals genes controlling circadian behavior. J Neurosci 22:9305-9319.

Chang DC, Reppert SM (2003) A novel C-terminal domain of drosophila PERIOD inhibits dCLOCK:CYCLE-mediated transcription. Curr Biol 13:758-762.

Coates CJ, Jasinskiene N, Miyashiro L, James AA (1998) Mariner transposition and transformation of the yellow fever mosquito, Aedes aegypti. Proc Natl Acad Sci U S A 95:3748-3751.

Collins B, Mazzoni EO, Stanewsky R, Blau J (2006) Drosophila CRYPTOCHROME is a circadian transcriptional repressor. Curr Biol 16:441-449.

Curtis AM, Cheng Y, Kapoor S, Reilly D, Price TS, Fitzgerald GA (2007) Circadian variation of blood pressure and the vascular response to asynchronous stress. Proc Natl Acad Sci U S A 104:3450-3455.

Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NR, Hardin PE, Young MW, Storti RV, Blau J (2003) vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell 112:329-341.

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TD, Weitz CJ, Takahashi JS, Kay SA (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. Science 280:1599-1603.

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271-290.

Dunlap JC, Loros JJ (2006) How fungi keep time: circadian system in Neurospora and other fungi. Curr Opin Microbiol 9:579-587.

Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669-679.

Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, Rosbash M (2000) Drosophila CRY is a deep brain circadian photoreceptor. Neuron 26:493-504.

Forattini, OP (1973) Entomologia Médica. IV Psychodidae, Phlebotominae, Edgar Blucher, São Paulo.

Gallego M, Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 8:139-148.

Gardner MJ, Hubbard KE, Hotta CT, Dodd AN, Webb AA (2006) How plants tell the time. Biochem J 397:15-24.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS,Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science 280:1564-1569.

Giebultowicz JM (2000) Molecular mechanism and cellular distribution of insect circadian clocks. Annu Rev Entomol 45:769-793.

Giebultowicz JM, Hege DM (1997) Circadian clock in Malpighian tubules. Nature 386:664.

Giebultowicz JM, Stanewsky R, Hall JC, Hege DM (2000) Transplanted Drosophila excretory tubules maintain circadian clock cycling out of phase with the host. Curr Biol 10:107-110.

Glossop NR, Hardin PE (2002) Central and peripheral circadian oscillator mechanisms in flies and mammals. J Cell Sci 115:3369-3377.

Glossop NR, Houl JH, Zheng H, Ng FS, Dudek SM, Hardin PE (2003) VRILLE feeds back to control circadian transcription of Clock in the Drosophila circadian oscillator. Neuron 37:249-261.

Glossop NR, Lyons LC, Hardin PE (1999) Interlocked feedback loops within the Drosophila circadian oscillator. Science 286:766-768.

Greenspan RJ (2008) The origins of behavioral genetics. Curr Biol. 18:R192-8.

Guillaumond F, Dardente H, Giguere V, Cermakian N (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms 20:391-403.

Hall JC (2003) Genetics and molecular biology of rhythms in Drosophila and other insects. Adv Genet 48:1-280.

Hao H, Allen DL, Hardin PE (1997) A circadian enhancer mediates PER-dependent mRNA cycling in Drosophila melanogaster. Mol Cell Biol 17:3687-3693.

Hardin PE (2005) The circadian timekeeping system of Drosophila. Curr Biol 15:R714-22.

Hardin PE (1994) Analysis of period mRNA cycling in Drosophila head and body tissues indicates that body oscillators behave differently from head oscillators. Mol Cell Biol 14:7211-7218.

Hardin PE, Hall JC, Rosbash M (1990) Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature 343:536-540.

Helfrich-Forster C (1998) Robust circadian rhythmicity of Drosophila melanogaster requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. J Comp Physiol [A] 182:435-453.

Helfrich-Forster C (1995) The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. Proc Natl Acad Sci U S A 92:612-616.

Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu YZ, Pray-Grant M, Perdew GH, Bradfield CA (1997) Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. J Biol Chem 272:8581-8593.

Houl JH, Yu W, Dudek SM, Hardin PE (2006) Drosophila CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. J Biol Rhythms 21:93-103.

Huang ZJ, Edery I, Rosbash M (1993) PAS is a dimerization domain common to Drosophila period and several transcription factors. Nature 364:259-262.

Ivanchenko M, Stanewsky R, Giebultowicz JM (2001) Circadian photoreception in Drosophila: functions of cryptochrome in peripheral and central clocks. J Biol Rhythms 16:205-215.

Iwasaki H, Kondo T (2004) Circadian timing mechanism in the prokaryotic clock system of cyanobacteria. J Biol Rhythms 19:436-444.

Jackson FR, Schroeder AJ, Roberts MA, McNeil GP, Kume K, Akten B (2001) Cellular and molecular mechanisms of circadian control in insects. J Insect Physiol 47:833-842.

Jasinskiene N, Coates CJ, Benedict MQ, Cornel AJ, Rafferty CS, James AA,Collins FH (1998) Stable transformation of the yellow fever mosquito, Aedes aegypti, with the Hermes element from the housefly. Proc Natl Acad Sci U S A 95:3743-3747.

Kim EY, Bae K, Ng FS, Glossop NR, Hardin PE, Edery I (2002) Drosophila CLOCK protein is under posttranscriptional control and influences light-induced activity. Neuron 34:69-81.

Kiyohara YB, Tagao S, Tamanini F, Morita A, Sugisawa Y, Yasuda M, Yamanaka I, Ueda HR, van der Horst GT, Kondo T, Yagita K (2006) The BMAL1 C terminus regulates the circadian transcription feedback loop. Proc Natl Acad Sci U S A 103:10074-10079.

Kloss B, Price JL, Saez L, Blau J, Rothenfluh A, Wesley CS, Young MW (1998) The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. Cell 94:97-107.

Klowden M (1996) Vector Behavior. pp: 34-50 In: The biology of disease vectors. BJ Beaty & WC Marquardt, eds. University Press of Colorado, Colorado.

Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Hum Mol Genet 15 Spec No 2:R271-7.

Konopka RJ, Benzer S (1971) Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A 68:2112-2116.

Krishnan B, Dryer SE, Hardin PE (1999) Circadian rhythms in olfactory responses of Drosophila melanogaster. Nature 400:375-378.

Krishnan B, Levine JD, Lynch MK, Dowse HB, Funes P, Hall JC, Hardin PE, Dryer SE (2001) A new role for cryptochrome in a Drosophila circadian oscillator. Nature 411:313-317.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH,Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98:193-205.

Kwon I, Lee J, Chang SH, Jung NC, Lee BJ, Son GH, Kim K, Lee KH (2006) BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. Mol Cell Biol 26:7318-7330.

Lee C, Bae K, Edery I (1998) The Drosophila CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. Neuron 21:857-867.

Lee JE, Edery I (2008) Circadian Regulation in the Ability of Drosophila to Combat Pathogenic Infections. Curr Biol 18:195-199.

Lin FJ, Song W, Meyer-Bernstein E, Naidoo N, Sehgal A (2001) Photic signaling by cryptochrome in the Drosophila circadian system. Mol Cell Biol 21:7287-7294.

Lin JM, Schroeder A, Allada R (2005) In vivo circadian function of casein kinase 2 phosphorylation sites in Drosophila PERIOD. J Neurosci 25:11175-11183.

Martinek S, Inonog S, Manoukian AS, Young MW (2001) A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. Cell 105:769-779.

McDonald MJ, Rosbash M (2001) Microarray analysis and organization of circadian gene expression in Drosophila. Cell 107:567-578.

Morrison AC, Ferro C, Pardo R, Torres M, Wilson ML, Tesh RB (1995) Nocturnal activity patterns of Lutzomyia longipalpis (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. J Med Entomol 32:605-617.

Moore-Ede MC, Sulzman FM, Fuller CA (1982) The clock that time us - Physiology of the circadian timing system, Harvard University Press.

Munstermann L (2004) Care, Maintenance, and Experimental Infection of Phlebotomine Sand Flies. In Biology of Disease Vectors 2nd Edition. ed. Marquardt WC et al. Elsevier Academic Press. Amsterdam.

Myers EM, Yu J, Sehgal A (2003) Circadian control of eclosion: interaction between a central and peripheral clock in Drosophila melanogaster. Curr Biol 13:526-533.

Myers MP, Wager-Smith K, Wesley CS, Young MW, Sehgal A (1995) Positional cloning and sequence analysis of the Drosophila clock gene, timeless. Science 270:805-808.

Naidoo N, Song W, Hunter-Ensor M, Sehgal A (1999) A role for the proteasome in the light response of the timeless clock protein. Science 285:1737-1741.

Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, Debruyn B, Decaprio D, Eiglmeier K, Eisenstadt E, El-Dorry H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, Jaffe D, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CF, Miller JR, Montgomery

P, Mori A, Nascimento AL, Naveira HF, Nusbaum C, O'leary S, Orvis J, Pertea M, Quesneville H, Reidenbach KR, Rogers YH, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JM, Vanzee JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW (2007) Genome sequence of Aedes aegypti, a major arbovirus vector. Science 316:1718-1723.

Oliveira MF, Silva JR, Dansa-Petretski M, de Souza W, Lins U, Braga CM, Masuda H, Oliveira PL (1999) Haem detoxification by an insect. Nature 400:517-8.

Park JH, Helfrich-Forster C, Lee G, Liu L, Rosbash M, Hall JC (2000) Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc Natl Acad Sci U S A 97:3608-3613.

Peixoto AA (2002) Evolutionary behavioral genetics in Drosophila. Adv Genet 47:117-150.

Peixoto AA, Hennessy JM, Townson I, Hasan G, Rosbash M, Costa R, Kyriacou CP (1998) Molecular coevolution within a Drosophila clock gene. Proc Natl Acad Sci U S A 95:4475-4480.

Petersen G, Hall JC, Rosbash M (1988) The period gene of Drosophila carries species-specific behavioral instructions. EMBO J 7:3939-3947.

Pittendrigh CS (1967) Circadian systems. I. The driving oscillation and its assay in Drosophila pseudoobscura. Proc Natl Acad Sci U S A 58:1762-1767.

Pittendrigh CS (1954) On Temperature Independence in the Clock System Controlling Emergence Time in Drosophila. Proc Natl Acad Sci U S A 40:1018-1029.

Plautz JD, Straume M, Stanewsky R, Jamison CF, Brandes C, Dowse HB, Hall JC, Kay SA (1997) Quantitative analysis of Drosophila period gene transcription in living animals. J Biol Rhythms 12:204-217.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U (2002) The orphan nuclear receptor REV-ERBa controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110: 251-260.

Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW (1998) double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. Cell 94:83-95.

Price JL, Dembinska ME, Young MW, Rosbash M (1995) Suppression of PERIOD protein abundance and circadian cycling by the Drosophila clock mutation timeless. EMBO J 14:4044-4049.

Reddy P, Zehring WA, Wheeler DA, Pirrotta V, Hadfield C, Hall JC, Rosbash M (1984) Molecular analysis of the period locus in Drosophila melanogaster and identification of a transcript involved in biological rhythms. Cell 38:701-710.

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell 99:791-802.

Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418:935-941.

Rothenfluh A, Young MW, Saez L (2000) A TIMELESS-independent function for PERIOD proteins in the Drosophila clock. Neuron 26:505-514.

Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, Fitzgerald GA (2004) BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol 2:e377.

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. Cell 93:805-814.

Saez L, Young MW (1996) Regulation of nuclear entry of the Drosophila clock proteins period and timeless. Neuron 17:911-920.

Saraiva E, Fampa P, Cedeno V, Bergoin M, Mialhe E, Miller LH (2000) Expression of heterologous promoters in Lutzomyia longipalpis and Phlebotomus papatasi (Diptera: Psychodidae) cell lines. J Med Entomol 37:802-806.

Sathyanarayanan S, Zheng X, Xiao R, Sehgal A (2004) Posttranslational regulation of Drosophila PERIOD protein by protein phosphatase 2A. Cell 116:603-615.

Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43:527-537.

Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB (2006) Feedback repression is required for mammalian circadian clock function. Nat Genet 38:312-319.

Sauman I, Reppert SM (1996) Circadian clock neurons in the silkmoth Antheraea pernyi: novel mechanisms of Period protein regulation. Neuron 17:889-900.

Sehgal A, Price JL, Man B, Young MW (1994) Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. Science 263:1603-1606.

Sehgal A, Rothenfluh-Hilfiker A, Hunter-Ensor M, Chen Y, Myers MP, Young MW (1995) Rhythmic expression of timeless: a basis for promoting circadian cycles in period gene autoregulation. Science 270:808-810.

Shafer OT, Rosbash M, Truman JW (2002) Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of Drosophila melanogaster. J Neurosci 22:5946-5954.

Shaw PJ, Tononi G, Greenspan RJ, Robinson DF (2002) Stress response genes protect against lethal effects of sleep deprivation in Drosophila. Nature 417:287-291.

Shimba S, Ishii N, Ohta Y, Ohno T, Watabe Y, Hayashi M, Wada T, Aoyagi T, Tezuka M (2005) Brain and muscle Arnt-like protein-1

(BMAL1), a component of the molecular clock, regulates adipogenesis. Proc Natl Acad Sci U S A 102:12071-12076.

Souza NA, Andrade-Coelho CA, Barbosa AF, Vilela ML, Rangel EF, Deane MP (1995) The influence of sugars and amino acids on the blood-feeding behaviour, oviposition and longevity of laboratory colony of Lutzomyia longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae, Phlebotominae). Mem Inst Oswaldo Cruz 90:751-757.

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC (1998) The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. Cell 95:681-692.

Tauber E, Last KS, Olive PJ, Kyriacou CP (2004) Clock gene evolution and functional divergence. J Biol Rhythms 19:445-458.

Tesh RB, Guzmann H (1996) Sand Flies and the agents they transmit. In: The biology of disease vectors. BJ Beaty & WC Marquardt, eds. University Press of Colorado, Colorado. 117-127.

Triqueneaux G, Thenot S, Kakizawa T, Antoch MP, Safi R, Takahashi JS, Delaunay F, Laudet V (2004) The orphan receptor Rev-erba gene is a target of the circadian clock pacemaker. J Mol Endocrinol 33:585-608.

van der Schalie EA, Conte FE, Marz KE, Green CB (2007) Structure/function analysis of Xenopus cryptochromes 1 and 2 reveals differential nuclear localization mechanisms and functional domains important for interaction with and repression of CLOCK-BMAL1. Mol Cell Biol 27:2120-2129.

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS, Sancar A (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. Proc Natl Acad Sci U S A 96:12114-12119.

Woon PY, Kaisaki PJ, Braganca J, Bihoreau MT, Levy JC, Farrall M, Gauguier D (2007) Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. Proc Natl Acad Sci U S A 104:14412-14417.

Young DG, Duncan MA (1994) Guide to the identification and geographic distribution of Lutzomyia sand flies in Mexico, the West Indies, Central and South America (Diptera:Psychodidae). Mem. Amer. Ent. Inst. 54:1-881.

Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. Genes Dev 20:723-733.

Zeng H, Qian Z, Myers MP, Rosbash M (1996) A light-entrainment mechanism for the Drosophila circadian clock. Nature 380:129-135.

Zerr DM, Hall JC, Rosbash M, Siwicki KK (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. J Neurosci 10:2749-2762.