AN INTEGRATED APPROACH TO THE ANALYSIS OF THE CIRCADIAN CLOCK OF THE BLOW FLY *Lucilia cuprina*

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ABSTRACT

The Australian sheep blow fly *Lucilia cuprina* is an economically important dipteran pest whose circadian behavioural rhythms have been the subject of considerable scrutiny. The underlying biochemical nature of these rhythms however, has remained a mystery. The primary objective of this thesis was therefore to investigate the molecular control of circadian rhythms in *L. cuprina* using an integrative approach. To these ends, a dynamic molecular simulation model for *L. cuprina* was formulated using existing biochemical data on insect circadian clocks. The validity of this simulation model was subsequently tested at both molecular and behavioural levels.

The basic molecular assumptions of the simulation model were tested by cloning a full length *L. cuprina per* cDNA and analysing its mRNA and protein expression levels. Isolation of the 4 Kb *L. cuprina per* cDNA revealed the conservation of three functional domains known to be important for circadian clock function; namely the PAS dimerisation motif (with 92% identity to *D. melanogaster* at the amino acid level), and the cytoplasmic and nuclear localisation domains (with 85% and 80% identity respectively). A fourth domain, the threonine-glycine (TG) repeat region, was also found to be conserved, but severely truncated in *L. cuprina*. No length variation was found in the TG repeat of flies collected from several different latitudinal zones, and no correlation was detected between sequences flanking the repeat and latitude of collection of flies. Thus, the contention that the TG repeat region plays a role in temperature compensation of the circadian clock is cast in doubt. Expression analyses (using quantitative RT-PCR) showed *per* mRNA levels to undergo diel oscillations with a period (24 h) and peak phase (Zt 12) consistent with the *Drosophila* data. PER-immunoreactive protein oscillations were also demonstrated, with peak immunoreactivity lagging approximately 3 h behind peak mRNA levels.

The behavioural predictions of the model were tested by recording adult locomotor activity under different light regimes. The simulation model successfully predicted free-run, entrainment, the effect of short light pulses, and the effects of constant

lighting on behavioural rhythms. Disparities between the simulated and real phase response curves for *L. cuprina* are hypothesised to be indicative of an earlier nuclear entry time of the PER-TIM dimer in *L. cuprina* compared with *D. melanogaster*.

The three different approaches of simulation modelling, molecular analysis and behavioural investigation are integrated in the discussion in order to help provide a comprehensive explanation of circadian function in *L. cuprina*. The benefits of an integrated approach to the analysis of circadian function are discussed, as is the relevance of the present findings to the development of a clock-based control strategy for this economically important pest species.

Nothing puzzles me more than time and space; and yet nothing troubles me less, as I never think about them (Charles Lamb, Letter to Southy, 9 Aug. 1815).

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GLOSSARY OF CHRONOBIOLOGY TERMS

The terminology used here is based on that of Saunders (1982) with modifications.

Active phase (α) — The time during the sleep-wake cycle during which an animal is active.

Aschoff's Rule — The period of the free-running oscillation (τ) lengthens on transfer from DD to LL in dark active animals, and shortens for light active animals.

Circadian (rhythm) — An endogenous oscillation with a natural period close to, but seldom equal to 24 h.

Circadian time (Ct) — Time scale covering one full period of an oscillation. Ct 0 is defined arbitrarily (in the present study Ct 6 is defined as the midpoint of the active phase and therefore Ct 0 occurs 6 hours prior to this point).

Desynchronisation — Loss of synchrony between two populations of endogenous oscillators involved in the generation of a rhythm.

Diel rhythm — A 24 h rhythm that has been measured only in natural or artificial day-night cycles, and not yet known to persist in constant conditions.

Diurnal — Active during the day (photophase)

Endogenous rhythm — A self-sustained rhythm which continues in the absence of external entraining factors (zeitgeber).

Endogenous oscillator	A self-sustained and temperature compensated oscillator responsible for endogenous rhythmicity.
Entrainment	The synchronisation of an endogenous oscillation to the period of a zeitgeber.
Free-running	A rhythm in its unentrained state (isolated from zeitgeber).
Free-running period (τ)	The period of a free-running rhythm.
Oscillator	The unseen 'driving' organ (the biological clock) whose influence from within the organism causes the measurable changes seen as the overt rhythm.
Period	The length of time between the same phase point on two consecutive oscillations.
Phase ($\varnothing$)	The instantaneous state of an oscillation within a period. eg. onset of activity.
Phase advance ($+\Delta\varnothing$)	The shortening of the period of the rhythm in response to a light or temperature perturbation.
Phase angle (ψ).	The relationship between two phase points on the same or different oscillations (phase relationship).
Phase delay ($+\Delta\varnothing$)	The lengthening of the period of the rhythm in response to a light or temperature perturbation.

Phase response curve A plot of phase shift (Δ∅) caused by a single perturbation at different phases.

Phase shift (Δ∅) A single displacement of an oscillation along the time axis.

Photoperiod The daylength (period of light in the daily cycle).

Photophase The light portion of the day-night cycle.

Rhythm A periodically occurring event.

Scotophase The dark portion of the day-night cycle.

Shattering Loss of a single cohesive active phase into many shorter active phases (usually with no observable rhythm).

Skeleton photoperiod A light regime using two shorter periods of light to simulate dawn and dusk effects of a longer, complete photoperiod.

Singularity (T*S*) A pulse of critical duration, intensity and timing, which results in the damping of a rhythm (ie. stops the clock). First proposed by Winfree (1970).

Subjective day The first half of the circadian cycle (Ct 0 to Ct 12).

Subjective night The second half of the circadian cycle (Ct 12 to Ct 24).

Transients One of the more temporary oscillatory states between two steady states caused, for instance, by light or

temperature perturbations.

Ultradian (rhythm) An endogenous oscillation with a period many times shorter than the solar day and unrelated to any geophysical cycle.

Zeitgeber The forcing geophysical oscillation which entrains a biological oscillation.

Zeitgeber time (Zt) Time (in hours) relative to the zeitgeber. (ie. in LD 12:12 Zt 0 is defined as the L-D transition and Zt 12 as the D-L transition).

GLOSSARY OF MOLECULAR TERMS

Base pair (bp) — A single pair of complementary nucleotides from opposite strands of the DNA double helix. The number of base pairs is used as a measure of a length of double stranded DNA.

cDNA clone — A DNA clone derived from a complementary DNA (cDNA) transcript of a mRNA.

cDNA library — A collection of phage containing complementary DNA (cDNA) clones of all of the mRNA species represented in a particular tissue at a particular developmental stage.

Cloning — The isolation and multiplication of a particular gene by incorporating it into specifically modified phage or plasmid and introducing it into a bacterial cell where the DNA of interest is replicated along with the phage or plasmid DNA and can subsequently be recovered from bacterial culture in large amounts.

Dimer — A protein made up of two subunits.

DNAse — Deoxyribonuclease: An enzyme which degrades DNA.

DNA polymerase — Any of several enzymes which catalyse DNA synthesis by addition of deoxyribonucleotide units to a DNA chain using DNA or (in the case of retroviruses) RNA as a template.

Electrophoresis — A technique for separating molecules such as proteins or nucleic acid fragments on the basis of their net charge and mass, by their differential migration through a paper, polyacrylamide or agarose gel in an electric field.

Kilobase (Kb) — Unit of length used for nucleic acids and polynucleotides corresponding to 1000 base bairs or bases.

Kilodalton (kD) — Unit of mass equal to 1000 daltons. One dalton is the unit of mass almost equal to the weight of a hydrogen atom and is used interchangeably with molecular weight.

Northern Blotting — A technique in which RNAs (usually separated by electrophoresis) are transferred to a suitable medium for subsequent hybridisation with radioactive probes for the identification and isolation of RNAs of interest.

Phage (bacteriophage) — A virus infecting bacteria, such as lambda (which infects *E. coli*).

Plasmid — Small self-replicating circular DNA independent of the chromosome in bacteria and unicellular eucaryotes such as yeast, which is maintained at a characteristic stable number from generation to generation. Plasmids typically carry genes for antibiotic resistance and are widely used in genetic engineering as vectors into which foreign genes are inserted for subsequent cloning or expression in bacterial cells.

Poly (A) tail — A stretch of polyadenylic acid residues found at the 3' ends of many eucaryotic messenger RNAs which is added in the nucleus by the enzyme poly (A) polymerase after transcription.

Reverse transcriptase — A DNA polymerase found in retroviruses which synthesises DNA on an RNA template.

Reverse transcription — The synthesis of DNA on an RNA template, catalysed by the enzyme reverse transcriptase.

RNAse — Ribonuclease: an enzyme which degrades RNA or cleaves it into shorter oligonucleotides.

RNA polymerase — Any of several enzymes which catalyse the synthesis of RNA from a DNA template by the process of transcription

Southern Blotting — A technique in which DNA fragments separated by gel electrophoresis in an agarose gel are transferred by blotting to a nylon or nitrocellulose filter for subsequent hybridisation with radioactively labelled nucleic acid probes for the identification and isolation of sequences of interest.

Transcription — Copying of a DNA strand to an RNA strand by an RNA polymerase.

Translation — Process by which RNA directs the synthesis of specific proteins.

Vector — Specifically modified plasmid or phage into which

foreign genes can be inserted for introduction into bacterial or other cells for multiplication or studies of gene expression.

Western Blotting A technique used to transfer the pattern of proteins separated by electrophoresis to a medium in which they can be further analysed.

LIST OF ABBREVIATIONS

α	alpha (active phase)
bp	base pairs
Ct	circadian time
°C	degrees Celsius
DD	constant dark
h	hour
Kb	kilobase
kD	kilodalton
L	litre
LL	constant light
LD	light-dark cycle (numbers following indicate hours occupied by each)
µg	microgram
µL	microlitre
µM	micromolar
M	molar
mg	milligram
min	minute
mM	millimolar
ng	nanogram
g	gram
per	period gene (italicised)
PER	period protein (uppercase, plain face)
pers. comm.	personal communication
pg	picogram
PCR	polymerase chain reaction
PRC	phase response curve
RT	reverse transcription
sec	second
T	period of the zeitgeber
tim	timeless gene (italicised)
TIM	timeless protein (uppercase, plain face)
τ	tau (free-running period)
Zt	zeitgeber time
∅	phase