



**Circadian control of the daily rhythm of adult emergence by regulation of the timing of
ecdysone action in *Drosophila melanogaster***

Tesis entregada a

LA UNIVERSIDAD DE VALPARAÍSO

en Cumplimiento Parcial de los requisitos para optar al grado de Doctor en Ciencias con
Mención en Neurociencia

Facultad De Ciencias

Por

Liliana Andrea Bustos González

Marzo, 2022

Dirigida por

Dr. John Ewer Lothian

TABLE OF CONTENTS

SUMMARY	1
CHAPTER 1: INTRODUCTION.....	3
<i>Molecular organization of the circadian clock in Drosophila melanogaster</i>	<i>4</i>
<i>Circadian and endocrine regulation of adult emergence in Drosophila melanogaster</i>	<i>5</i>
<i>Role of the Ecdysone Receptor in the circadian regulation of the daily rhythm of adult emergence.....</i>	<i>8</i>
<i>Role of Ecdysone Responsive Genes in the circadian regulation of the daily rhythm of adult emergence.....</i>	<i>10</i>
REFERENCES.....	12
CHAPTER 2: THE CIRCADIAN CLOCK GATES EMERGENCE IN DROSOPHILA BY CONTROLLING ECDYSONE ACTION.....	17
ABSTRACT	18
INTRODUCTION.....	19
MATERIALS AND METHODS	21
<i>Fly rearing and fly stocks</i>	<i>21</i>
<i>White prepupae collection</i>	<i>21</i>
<i>Filming setup</i>	<i>22</i>
<i>Image analyses.....</i>	<i>23</i>
<i>20HE injections.....</i>	<i>24</i>
<i>ETH injections.....</i>	<i>24</i>
<i>Population assay for eclosion.....</i>	<i>25</i>
<i>Locomotor activity assay</i>	<i>25</i>

<i>Statistical analyses</i>	26
RESULTS	26
<i>Time course of molting fluid resorption</i>	26
<i>Role of ecdysone signaling in the circadian control of the timing of emergence</i>	30
<i>The circadian clock controls the time when the last steps of metamorphosis are initiated</i> ...	39
DISCUSSION	41
<i>New insights into the emergence assay: Expanding and improving its utility</i>	41
<i>Use of the TARGET system: the effect of temperature on emergence and periodicity analysis</i>	42
<i>Homology to the circadian control of glucocorticoid action in mammals</i>	43
<i>A proposed model for the circadian regulation of ecdysone action</i>	44
CONCLUSIONS	48
REFERENCES	49

**CHAPTER 3: THE CIRCADIAN CLOCK SET THE TIMING OF ADULT EMERGENCE BY
REGULATING ECDYSONE RECEPTOR FUNCTION55**

ABSTRACT	56
INTRODUCTION	58
MATERIALS AND METHODS	61
<i>Fly rearing and stocks</i>	61
<i>UAS-EcR and UAS-EcR¹lys generation</i>	61
<i>Immunohistochemistry</i>	62
<i>Drosophila brain and ring gland complex preparation for FISH</i>	63
<i>Whole-mount FISH</i>	63
<i>Image analysis</i>	64
<i>Population assay for eclosion</i>	65
<i>Locomotor activity assay</i>	65
<i>Statistical analyses</i>	65

RESULTS.....	66
<i>Ecdysone receptor immuno-localization varies during the course of the day.....</i>	66
<i>EcR transcription presents daily variations in PG cells.....</i>	68
<i>The pattern of transcriptional activity varies during the course of the day in PG cells.....</i>	71
<i>The expression in the PG of an EcR with mutated putative nuclear localization signal alters the pattern of daily emergence.</i>	77
DISCUSSION	82
<i>Circadian control of the intracellular localization of EcR in PG cells.....</i>	82
<i>The requirement of photic input for the circadian rhythmicity of EcR expression.....</i>	83
<i>Circadian regulation of transcriptional activity and chromatin remodeling.....</i>	84
<i>Impact of Dlys substitution in EcR on the circadian control of emergence.</i>	86
CONCLUSIONS	87
REFERENCES.....	88

CHAPTER 4: THE CIRCADIAN CLOCK CONTROLS THE RHYTHM OF EMERGENCE IN

***DROSOPHILA* BY REGULATING OTHER ELEMENTS OF THE ECDYSONE PATHWAY 94**

ABSTRACT	95
INTRODUCTION.....	96
MATERIALS AND METHODS	98
<i>Fly rearing and fly stocks.....</i>	98
<i>Identification of E-box candidate sequence.....</i>	99
<i>Population assay for eclosion.....</i>	99
<i>Locomotor activity assay</i>	99
<i>Drosophila brain and ring gland complex preparation for fluorescent in situ hybridization (FISH)</i>	100
<i>Whole-mount FISH</i>	100
<i>Image analysis.....</i>	100

<i>Statistical analyses</i>	101
RESULTS	101
<i>Identification of other elements of the 20E pathway that may be involved in the circadian control of emergence</i>	101
<i>Knockdown of “early” genes in the PG disrupts the circadian rhythm of emergence</i>	103
<i>The knockdown of selected “early genes” in the PG affects the molecular clock in <i>Drosophila melanogaster</i></i>	107
DISCUSSION	111
<i>Role of E75 in the circadian control of emergence behavior</i>	111
<i>A role for Juvenile Hormone pathway in the control of emergence behavior?</i>	112
<i>hr4 as a possible candidate of direct circadian regulation of 20E action</i>	113
CONCLUSIONS	116
REFERENCES	117
CONCLUSIONS	123

INDEX OF TABLES

CHAPTER 3:

Table 3.1 Statistical dependence of acetyl-lysine-IR vs. nuclear area in PG cells.....	76
---	----

CHAPTER 4:

Table 4.1. List of “early genes” searched for the presence of E-box motifs in their regulatory region.	102
--	-----

Table 4.2. List of early genes containing E-box motifs in their putative regulatory region.....	103
--	-----

Table 4.3. Daily emergence rhythmicity phenotypes following knockdown of selected “early” genes in the PG.	106
---	-----

INDEX OF FIGURES

CHAPTER 1:

FIGURE 1.1	The core molecular clock in <i>Drosophila melanogaster</i>	5
FIGURE 1.2.	Gated and non-gated events during metamorphosis of <i>Drosophila victoria</i>	6
FIGURE 2.1.	Schematic of the workflow and the custom-built setup used to record the progression through metamorphosis.	23
FIGURE 2.2.	Time course of head roughening.....	29
FIGURE 2.3.	Role of ecdysone in the circadian control of emergence.....	32
FIGURE 2.4.	Interfering with ecdysone signaling in the PG during the final steps of metamorphosis renders arrhythmic the pattern of emergence.	34
FIGURE 2.5.	Effects of interfering with ecdysone signaling in brain clock neurons.	36
FIGURE 2.6.	Expression of EcR DN alters the anatomy of PDF-expressing neurons.	38
FIGURE 2.7.	Effects of injections of ETH on adult emergence.	40
FIGURE 2.8.	Proposed model for clock control of the timing of emergence.	47
FIGURE 3.1	Daily pattern of EcR-IR in the <i>Drosophila</i> PG.	67
FIGURE 3.2	Characterization of EcR-transcriptional rhythms in the PG during the day.	70
FIGURE 3.3	Nuclear sizes in the PG vary during the course of the day.	72
FIGURE 3.4	Daily pattern of transcriptional activity in the <i>Drosophila</i> PG.....	75
FIGURE 3.5	Identification of the putative nuclear localization signal (NLD) within EcR.	78
FIGURE 3.6	Effects on the rhythms of emergence and locomotor activity caused by expressing EcRDlys in the PG.....	79

FIGURE 3.7 RREKK residues at the hinge region of EcR are important for the maintenance of the gating of emergence under DD.....	80
FIGURE 3.8 Effects on the pattern of emergence of co-expressing EcRDlys or EcR together with EcR DN in the PG.....	81
FIGURE 3.9. PTH innervation of PG cells.....	85
FIGURE 4.1 Knockdown in the PG of selected “early genes” that rendered arrhythmic the rhythm of eclosion.....	105
FIGURE 4.2 Effects of knockdown of selected “early genes” on the molecular clock.	110
FIGURE 4.3 Stopping the clock in the CA does not affect the circadian rhythm of adult emergence.....	111
FIGURE 4.4 A proposed model for the role of E75 and DHR4 in the circadian control of emergence.....	115

ABBREVIATIONS

20E: 20- hydroxyecdysone

BPE: Before predicted eclosion

CA: *Corpora allata*

clk: clock

CLK: CLOCK

CNS: Central nervous system

cry: cryptochrome

CYC: CYCLE

D:D: Constant darkness

E: Ecdysone

EcR: Ecdysone receptor

EcR DN: Ecdysone receptor dominant negative

EH: Eclosion hormone

ETH: Ecdysone Triggering Hormone

FISH: Fluorescent *in situ* hybridization

GC: Glucocorticoid

GR: Glucocorticoid receptor

h: Hour

IHC: Immunohistochemistry

L:D: Light:Dark cycle

mM: millimolar

mRNA: Messenger ribonucleic acid

nl: Nanoliters

NPF: Neuropeptide F

NRs: Nuclear receptors

ORF: Open reading frame

p: Period

per. period

PER: PERIOD

Pdp1: PAR Domain Protein 1

pdf: pigment dispersing factor

PDF: Pigment dispersing factor neuropeptide

pmol: Picomol

PTMs: Post-translational modifications

PTTH: Prothoracicotropic hormone

RI: Rhythmicity Index

sLNvs: Small lateral ventral neurons

SNC: Suprachiasmatic nucleus

sNPF: small Neuropeptide F

tb: time of bristle pigmentation

te: time of eclosion

tim: timeless

th: time of head eversion

TIM: TIMELESS

tp: Time of prepupal formation

TTFL: Transcription-translation feedback loops

ty: time of yellow eye pigmentation

UAS: Upstream activating sequences

USP: Ultraspiracle

vri: vrille

WP: white-prepupae

ZT: Zeitgeber

In *Drosophila melanogaster*, the circadian clock imposes a daily rhythm to the pattern of adult emergence (eclosion) by a process that has been described as “gating”. My research is aimed at identifying the mechanism by which the daily “gating” of the time of emergence occurs.

In this insect, the circadian clock sets the time of emergence through the coupling between the central clock located in the brain and a peripheral clock contained in the Prothoracic Gland (PG), an endocrine gland whose only known function is the production of the molting hormone, ecdysone (E). The levels of E increase to cause the larval molts and metamorphosis, and then drop to signal the end of each molt. Previous work in the insect, *Rhodnius prolixus*, suggests that ecdysone is involved in the gating of eclosion because injections of increasing doses of E delay the time of eclosion to later times *in a continuous fashion*, suggesting that the titer of E itself is central to the gating mechanism. Moreover, in *Rhodnius*, molting is accompanied by circadian oscillations in ecdysone titers, produced as a consequence of its rhythmic release. Nevertheless, this rhythm of E does not seem to be a general feature among insects. In *Drosophila*, in particular, no circadian oscillations in E have been detected during metamorphosis. However, whether the clock regulates ecdysone signaling through a different mechanism, remains to be explored. Thus, the main goal of my Doctoral thesis is **to use *Drosophila* to explore the role of E in the circadian gating of eclosion** by asking whether **there are elements of the E pathway that are under circadian control and what are the molecular mechanisms used by the clock to impose a daily rhythmicity to adult emergence**. To address this question, in **Chapter 2** I first asked whether the clock regulates the time when flies commit to end metamorphosis and whether the clock can set the time of adult emergence by regulating ecdysone levels. Since my results support a mechanism where the clock acts downstream of E, I also disrupted E signaling in the PG via its receptor, EcR, and evaluated the consequences of these manipulations on the rhythm of eclosion and locomotor activity. Next, in **Chapter 3** I evaluated the role of EcR in the circadian regulation of the rhythm of emergence. For this purpose, I used immunohistochemistry and fluorescent *in situ* hybridization to determine whether the clock regulates the expression of EcR during the course of the day. Moreover, I analyzed whether the clock acts by synchronizing the cellular size within the PG (as a proxy for cellular activity) and, in addition, I determined which are the possible molecular mechanisms used by the clock to regulate EcR action. Finally, in **Chapter 4** I asked whether there are other elements of the E transduction pathway that may be under circadian control. For this I assessed the effects on the rhythm of eclosion of downregulating in the PG candidate genes involved in transducing 20E actions.

Results: I found that the time when flies initiate the final steps of metamorphosis is correlated with the time of emergence, which suggests that the circadian clock gates emergence by controlling the time when the animal commits to complete metamorphosis, and not by simply preventing the emergence of animals that completed metamorphosis before the gate opens (work done in collaboration with Brandon Mark, Guadalupe Cascallares, and Felipe Conejera). Moreover, I found that injecting increasing doses of E prior to eclosion produced a dose-dependent delay in the time of adult emergence but did not disrupt its circadian rhythmicity, which suggests that the clock acts downstream of E. Consistent with this hypothesis, I found that interfering with E signaling in the PG renders arrhythmic the pattern of emergence. In addition, my findings reveal that the circadian clock controls EcR function by regulating its intracellular location as well as its level of expression. Finally, I found that downregulating genes directly induced by ecdysone also eliminated the rhythm of eclosion.

Conclusions: My results suggest that the circadian clock imposes a daily rhythm to the pattern of emergence by regulating the process of E signal transduction in the PG. The study of the pathways linking development and the circadian system will help elucidate how the clock regulates the timing of emergence, in addition to helping in the understanding of how daily steroid hormone rhythms are generated in animals.