Review of PhD Thesis

Circadian genes and regulation of Diapause in insects (by Adam Bajgar)

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The PhD thesis focuses on the role of circadian clock genes and the endocrinal regulation of the photoperiodic response of the insect *Pyrrhocoris apterus*, which is manifested as a developmental arrest of ovaries in females (i.e. reproductive diapause) induced by short days (presumably allowing the females to survive winter in temperate regions). The thesis consists of an Introduction, and five result chapters that represent distinct manuscripts, two of which have already been published.

The Introduction is quite extensive and provides a literature review on the circadian and the photoperiodic clocks. The review is based on recent references and is likely to provide an updated reflection of the field (although fig2. for example, is rather outdated and misses important clock genes). It would have been useful to include some information regarding the endocrine control of development and particularly the role of Juvenile hormone (JH), given the major part that this hormone takes in this dissertation. However, the main problem of the Introduction is the language, which would benefit from an extensive editing and word-proofing. There are many mistakes and style problems that at some points make the text to become unclear. There are also many errors in the references list that should be amended, and in some cases I was not sure that the correct citations were used.

In Chapter One, the authors establish that the expression level of clock genes *cry2* and *Pdp1* in the gut, is photoperiodic dependent, and is regulated by JH receptor *Met* and clock genes *cyc* and *Clk*. Interestingly, the two genes *cry2* and *Pdp1* seems to be reciprocally regulated. This work had made use of various techniques including qPCR, dsRNAi knockdown, and immunoblotting. The work provides a first and important insight into the downstream molecular processes that follow the photoperiodc induction to the diapause response. Appropriately, the work has been published in PNAS and the student is the first author. There are however, a few details which are not clear, particularly methodical details which are not extensively explained (further details below). It is also not clear what is the evidence for *cry2* (mammalian-like) to be regarded as a circadian clock gene, and what is the actual impact of expression changes in the gut on diapause (in the ovaries). The photoperiodic expression changes in the gut might not be related to diapause, in which case it would have been interesting to test whether these expression changes are mirrored in the males.

Chapter Two addresses the photoperiodic at the cellular level, specifically the role of the neuoroendocrine glands, the *corpus allatum* (CA) and *pars intercerebralis* (PI). Taking advantage of the fact that chirurgical removal of these glands in their model is possible, the authors study the impact of JH on the expression of *cry2*/*Pdp1* in the gut. The results are summarised in a nice model (Fig. 5), which interestingly suggest a novel factor(s) secreted from the PI that promote expression of *cry2*. Again, this paper (unpublished) would benefit from an extensive revision of the English. There is also a significant lack of technical details, for example description of the statistics that has been used (e.g. Fig.1), or what were the time-points (Zt) when the samples were taken (Fig. 3). The description of the error bars in the

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figures (here and elsewhere) is vague ("error bars show measurement divergeneces"), and the sample sizes are not provided. There are many problems with the format of the references (e.g. not uniform). The Material and Methods (here, and in other chapters) are brief and could not allow the reader to replicate the experiment. Obviously, journal publications are space limited, but a PhD dissertation is not, and should include all the required details. For example, the primers for the qPCR are provided, but not the PCR programme that has been used (for qPCR, the PCR efficiencies should also be stated). The actual qPCR method is not explained. The primers for the dsRNAi are provided, but not the size of the actual fragment, nor the coordinates of the fragment in the gene. It is also not clear how the fragments were selected and exactly how they were tested for off-targets matches (which are one of the major problems of this technique).

Chapter Three analyse the expression of various genes in the ovaries and aimed at comparing the signalling pathways of JH in different tissues in context of different phenotypes (epidermis>> larval metamorphosis, fat body >> vitellogenesis, gut >> metabolic function). This study provides more data on the gene *Met*, which as the sole JH receptor, is expectedly involved in all JH functions. However, *met* binds various bHLA-PAS transcription factor in different tissues, for different functions. Overall, this is an interesting story, although some details are not clear or not provided, as described above. This paper (as well as others) would benefit from better explaining in the Introduction, the choice of genes to be analysed (e.g. the gene *Kr-h1*).

Chapter 4 provide a more detailed look at the expression of *cry2*/*Pdp1* in the gut. It expands the expression analysis of Chapter 1, by recoding the expression diurnal profiles and in both gut and fat bodies tissues. I found the Introduction for this paper redundant and not written very well. The same problems mentioned earlier (e.g. lack of technical details) apply here as well. Most of the data are clear, but it would be appropriate to back the statements/conclusions with statistics (for example, for deciding if expression of a gene shows daily cycle). The gene expression profile is different in different tissues, which brings again the question how these findings aid in understanding the role in diapause.

Chapter Five is a digression to a study of diapause mutant of the Chymomyza drosophild. This chapter (published) describe a functional analysis of the promoter of the *timeless* gene which seems to be the locus underlying the diapause mutation of this strain. The expression of other clock genes is also compare between the diapause and the non-diapause strains. In general, this study provides one of the few examples of the involvement of the circadian clock in photoperiodic timing, a hugely debated question in chronobiology.

Overall, this thesis provides interesting and novel insights to this rather uncharted area photoperiodism, and the amount of data that is summarised here is rather impressive and convincing. The main problem is with the presentation, which should be revised. Given that most of the work presented here is a collaborative efforts (the student is first author on four papers, but share first authorship in one of them, and is a second author of the fifth paper), the main goal of the oral viva would be to verify the contribution of the student to these studies, and to ascertain the scholarship of the student.

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Report on "Circadian Genes and Regulation of Diapause in Insect" by Adam Bajgar
Reviewed by Terry L. Page

Overall:
Bajgar (with some contributions from colleagues) presents an outstanding body of work examining the mechanisms of gene regulation in the gut of *Pyrrhocoris apterus* by photoperiodically controlled signals that arise in the central nervous and associated endocrine systems (corpus allata). Overall, with the possible exception of the Introduction, the chapters are very well written, the data are clearly presented, and the conclusions are fully justified by the results. Comments and some questions regarding specific chapters follow.

Introduction
The introduction is a bit disjointed, difficult to follow, and sometimes superficial. There seems to be a competing emphasis on the variability between species while at the same time promoting a unity of mechanism (e.g., on page 18 the sentence "The *Drosophila* clock mechanisms differ significantly from other species and regulatory pathways directing peripheral oscillators are practically missing" is preceded by "Their highly conserved aminoacid sequence and similarity of the clock mechanism in phylogenetically distant species enable us to generalize result even from research of insect model organisms to mammals.") The facts seem to suggest to me that generalizing, even among the insects, is fraught with difficulty at both the level of physiology and at the level of molecular mechanism. In fact, I would suggest that a central message from this thesis is that molecular mechanisms can differ significantly even between tissues within the same individual. One of the general problems is that discussion of individual differences (sometimes, for example, between insects mammals) is often intermixed with statements of generalization without clear delineation of the how far the generalization extends – is it generally true of all animals, all insects, all diptera, or only all *Drosophila*?).

I think many of the problems I have with the introduction could be alleviated by a focus specifically on circadian clocks and photoperiodism in insects coupled with an effort to carefully delineate which species have been used for which experiments. When generalizations are made, how many and which species are they based on? What are the exceptions?

I was also surprised that there wasn't a more extensive review of the physiological role of JH in development and diapause and of the control of JH synthesis and release since it plays such an important role in the investigations presented. In this regard, it would be very helpful to the reader to have a section of the introduction devoted to a review what is already known about seasonal and circadian rhythms specifically in the linden bug.

Finally, I found some of the referencing in the introduction a bit curious. In several instances much of the early literature that established many of the fundamental properties of circadian rhythms and photoperiodism are omitted. For example, the notion that temperature cycles can entrain (pg. 3) is attributed to Glaser and Stanewsky, 2005 and Schadova et al., 2009 when temperature entrainment of ectotherms was well established at least three decades prior to those publications. Similarly (same page) the discovery of the first circadian clock genes was attributed to Bargielo et al., 1984 and Sehgal et al., 1994 although Konopka and Benzer, 1971

(who discovered the *per* locus) and Feldman and Hoyle, 1973(*frq* in *Neurospora*) are generally given credit for initiating the approach.

Chapter 1

I have little to say about chapter 1. It has been published in one of the top scientific journal in the world and has already been vetted by expert reviewers. This chapter clearly shows that the photoperiodically controlled regulation of transcription in the gut of *Pyrrhocoris apterus* is regulated (at least in part) by JH. Interestingly the regulation of "downstream" genes (whose products play a role in seasonally regulated metabolic activities of the gut) involves the transcription factors CRY2 and PDP1. At least in some insects, the genes for these two factors are rhythmically transcribed and play a role in the generation of circadian oscillations. However in the gut the transcripts do not oscillate with a circadian rhtyhm, but do show very robust, reciprocal photoperiodically controlled changes in abundance which in turn regulates the levels of transcripts of downstream genes. The data indicate that the expression of these genes is under the regulation of the JH receptor in conjunction with the clock proteins CLOCK and CYCLE. In addition, there is clear evidence that the molecular components involved in seasonal regulation of the ovaries differs from those involved in regulation of the gut.

Some general questions I think would be interesting for further discussion that arose as I read this Chapter are:

1. Why don't the clock genes in the gut show circadian oscillations – what is missing? What is known about the molecular mechanism underlying the generation of central and peripheral circadian oscillations in this species. In this context it is interesting to note that there is at least some evidence that JH levels exhibit a circadian rhythm (cricket). What is the temporal expression of JH like in the linden bug?

2. Could one speculate on the effects of the RNAi injections (particularly for CRY2 and PDP1) on the circadian/photoperiodic clocks in the brain? Do the data provide any new information on the mechanism of photoperiodic time measurement?

Chapter 2

This chapter (manuscript in progress) focusses on the role of the *pars intercerebralis* (PI) and corpora allata (CA) on in the regulation of photoperiod dependent gene transcription (*cry2* and *pdp1* in particular) in the gut. The data clearly indicate that the PI plays a major role through the regulation of the CA and JH release basically extending earlier observations of Hodkova on the signaling pathways involved in photoperiodic regulation.

Questions that arose during my reading of this chapter (that may arise because of my lack of familiarity with the anatomy):

1. I wonder about the absence of any controls involving "sham" operations. Is there any chance that the surgery might have damaged the photoperiodic clock or interrupted photoreceptive function necessary for the photoperiodic response? If so, what impact would this have on the

interpretation of the results? Is anything known about the anatomical location of components of the circadian/photoperiodic clock systems beyond the role of the PI and CA as output pathways? For example, the implication from Fig. 5 is that the eyes contain the photoreceptors for the photoperiodic response – is there any evidence to support this notion?

2. How was the success/extent of the surgery evaluated? What other structures might have been damaged?

Chapter 3
The focus of the third chapter is on identification of the binding partners of the JH receptor in promotion of larval and ovarian development using RNAi approaches to knock down levels of candidate transcription factors. The results are straight-forward and are nicely summarized in Fig. 6 of this chapter and show that the signal transduction pathways by which JH and the JH receptor regulate various aspects of physiology employ distinct JH receptor binding partners which in turn activate distinct downstream transcription factors. One interesting question that arises is why. Is there is a selective advantage to having distinct signal transduction pathways downstream of JH for the different functions is regulates?

Chapter 4
This chapter presents interesting data on the differences in the expression and functional roles of clock genes in the gut and in the fat body. Differences in the regulation of these genes by JH and by photoperiod are documented. It would be interesting to know more about the downstream consequences of the differences in order to have a better understanding of the functional relevance of the differences. It would also be quite useful to better understanding the underlying reasons for the tissue specific differences (e.g., why do the levels of *cry2* exhibit a daily rhythm in fat body but not in gut?).

Chapter 5
This chapter departs from the general theme of the rest of the dissertation. It is a published chapter that details an analysis of the *tim* promoter with a focus on the *npd* mutation in the *tim* promoter region of the fly *Chymomyza* which renders the fly incapable of normal photoperiodic responses. The findings are rather straight forward showing that the deletion in the promoter of the *npd* gene abolishes transcriptional activity and results in a loss of circadian variation in a variety of other normally rhythmic genes in the fly's head. This would appear to be a very useful system for exploring the relationship between circadian oscillations and photoperiodic time measurement.

Final Conclusions
This is a well-written, succinct summary of the work is presented here along with a useful schematic model of the findings.

[Note: I have made several suggestions for improving English usage directly on the dissertation]