Investigation of the Factors Regulating Ecdysteroid Release by Ovaries from Adult Female *Rhodnius prolixus* During Egg Development

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GENERAL ABSTRACT

Ecdysteroids are released in rhythmic fashion from ovaries during egg development in *Rhodnius prolixus*. Transfer of ovaries from arrhythmic (LL) animals to continuous darkness *in vitro* did not stimulate ecdysteroid release. PERIOD (PER) protein was observed in follicle cells of the ovary only at specific stages of oocyte development in both entrained (LD) and arrhythmic (LL) animals arguing against the presence of an endogenous ovarian clock. I showed that whole and fractionated brain extracts stimulated ecdysteroid release by ovaries from both LD and LL animals. The low molecular weight fraction (containing insulin-like proteins, ILPs) was tenfold more potent than the high molecular weight fraction (containing prothoracicotropic hormone, PTTH). None of recombinant PTTH, vertebrate insulins or corazonin had any significant stimulatory effect on ovarian ecdysteroid release. The circadian rhythm of ecdysteroid release by ovaries appears to be driven exogenously, probably by rhythmic release of unidentified hormones.

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Abbreviations

20E	20-hydroxyecdysone
ACTH	Adrenocorticotropic hormone
BIGFLP	*Bombyx* IGF-like peptide
CA	Corpus allatum
CC	Corpus cardiacum
CNS	Central nervous system
CRY	Cryptochrome, clock protein
CYC	Cycle, clock protein
DBT	Double-time, kinase of PER protein
dCLK	*Drosophila* clock, clock protein
DD	Continuous dark environment
DNs	Dorsal clock neurons
EcR	Ecdysteroid receptor
EDNH	Egg development neurosecretory hormone
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GCs	Granulosa cells
HPA	Hypothalamus-pituitary axis
IGFs	Insulin-like growth factors
ILP	Insulin-like peptide
JH	Juvenile hormone
LH	Luteinizing hormone
LL	Continuous light environment
LNs	Lateral clock neurons
MNCs	Medial neurosecretory cells
MO	Molecular oscillator
OEH	Ovary ecdysteroidogenic hormone
PAS domain	Per-ARNT-Sim domain
PDF	Pigment-dispersing factor
PER	PERIOD, clock protein
PGs	Prothoracic glands
PP2A	Protein phosphatase-2-alpha
PTTH	Prothoracicotropic hormone
RIA	Radioimmunoassay
SCN	Suprachiasmatic nucleus
StAR	Steroidogenic acute regulatory protein
TCs	Thecal cells
TIM	TIMELESS, clock protein
TRITC	Tetramethylrhodamine
TTFL	Transcription-translation feedback loop
TTX	Tetrodotoxin

I. GENERAL INTRODUCTION.

1.1 Physiology of the Ovary and Egg Development in Insects

Three main types of ovaries exist in insects, the panoistic, polytrophic, and telotrophic ovaries (Snodgrass, 1935; Bonhag, 1958). Panoistic ovaries are comprised of only follicle cells surrounding oocytes, whereas polytrophic and telotrophic ovaries (collectively known as meroistic) are comprised of oocytes, nurse cells, and follicle cells (Huebner and Anderson, 1972a). The telotrophic ovary is characteristic of many hemipterans, with *Rhodnius prolixus* providing an example of the cellular organization of this complex ovary type (King and Akai, 1984). The polytrophic ovary type has been well studied in many insects, while fewer studies have focused on the ultrastructural organization and development of the far more complex telotrophic ovary (King and Akai, 1984). Insect ovaries are made up of compartmentalized subunits referred to as ovarioles, and the number of ovarioles in telotrophic ovaries ranges from a few to thousands. Many hemipterans, including *R. prolixus*, have seven ovarioles per ovary, which hangs from a terminal filament in the body cavity or haemacoel (King and Akai, 1984). The telotrophic ovary consists of nurse cells, also known as trophocytes, confined to a trophic chamber in the anterior end of the ovariole, with trophic chords connecting the chamber to individual oocytes for the transport of nutrients and other material to the developing oocyte (Fig. 1.1) (Huebner and Anderson, 1972a,b,c). Posterior to this trophic chamber is the vitellarium, consisting of the oocytes at various stages of development and their surrounding follicle cells.

In *R. prolixus*, the development of oocytes into fully formed eggs is initiated by a blood meal. Development of oocytes occurs in two main stages, which are classified as previtellogenesis and vitellogenesis. During previtellogenesis, follicle cells begin as prefollicular tissue (Huebner and Anderson, 1972a). Small oocytes are also embedded within this tissue. Once stimulated, the prefollicular tissue undergoes drastic morphological changes in tandem with oocyte growth, forming a thin layer of follicle cells around developing oocytes (Huebner and Anderson, 1972a; Bonhag, 1955). During previtellogenesis, the trophocytes in the tropharium form a syncytial core (Huebner and Anderson, 1972b), which extends to the developing oocytes and transfers nutrients, such as ribosomes and mRNA (Huebner, 1981). This intercellular transport of nutrients is achieved by an electrical gradient that drives negatively charged nurse cell products (most soluble proteins and organelles in cytoplasm) into the oocyte (Telfer *et al.*, 1981). At the onset of vitellogenesis of the developing oocyte, the trophic cord reduces in size and then degenerates completely by mid-vitellogenesis (Huebner, 1981). Vitellogenesis is a period of rapid growth and development of the oocyte during which yolk is deposited (Hagedorn and Kunkel, 1979), and occurs between Days 2 and 9 following a blood meal in adult female *R. prolixus* (Patchin and Davey, 1968). During vitellogenesis, lipids and proteins such as the yolk precursor protein vitellogenin are transferred across the follicular epithelium, and these yolk precursors are incorporated into the oocyte where they form the major egg yolk protein, vitellin (Huebner and Anderson, 1972c). During a cycle of egg development in *R. prolixus*, each ovariole only has one vitellogenic oocyte at a time (referred to as the terminal oocyte). The preceding oocyte (known as the penultimate oocyte) remains in the pre-vitellogenic stage until the terminal oocyte has completed vitellogenesis (Huebner, 1981). Following vitellogenesis, the mature oocyte is

covered in a shell, known as the chorion, and the completed egg is released from the vitellarium (Beament, 1946). Therefore, within a single ovariole many eggs are produced in succession during a single reproductive cycle.

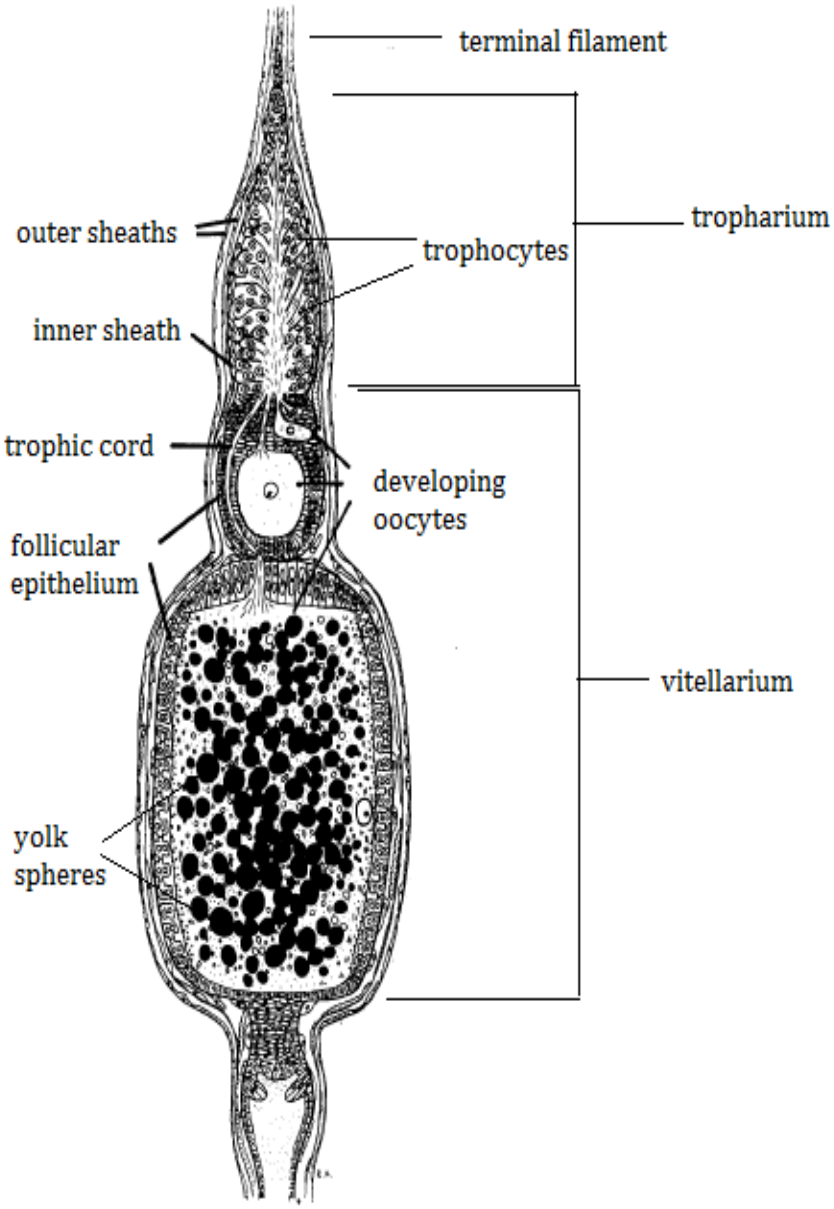

Figure 1.1 A line drawing of a single ovariole from the telotrophic ovary of *R. prolixus* (adapted from Huebner and Anderson, 1972b). The ovariole is divided into two separate compartments, the tropharium and the vitellarium. Developing oocytes at various stages, and their surrounding follicle cells are located in the vitellarium.

1.1.1 The Physiology and Function of Ovarian Follicle Cells

The follicle cells have a variety of functions that are critical to oogenesis and embryonic growth and development. The single layer of binucleate follicle cells is responsible for the uptake of yolk proteins (Huebner and Anderson, 1972a), the formation of the chorion (Beament, 1946), and vitellogenin synthesis (Melo *et al.*, 2000). Furthermore, in some insects the follicular epithelium is the primary site of synthesis of ovarian ecdysteroids (Goltzene *et al*., 1978).

The follicular epithelium is derived through mitosis from prefollicular cells during pre-vitellogenesis (Huebner and Anderson, 1972a). These cells are organized into a columnar epithelium, and are close in proximity to the previtellogenic oocyte (Huebner and Anderson, 1972a). Follicle cells become binucleate during the later stages of previtellogenesis (Huebner and Anderson, 1972a). Throughout vitellogenesis, follicle cells on the anterior surface of the developing oocyte remain columnar and tightly organized (80-90 µ), while the lateral cells become squamous (25-30 µ, each nucleus 10-12 µ long) and significantly altered to create extracellular spaces, a phenomenon known as patency (Huebner and Anderson, 1972a). Despite these differences, the follicle cells are in close association with each other and the oocytes by gap junctions throughout vitellogenesis (Huebner and Anderson, 1972a). Patency is important for the initiation of vitellogenesis, mainly for the passage of yolk precursors into the oocyte through this previously impermeable barrier (Huebner and Anderson, 1972a). Vitellogenin in *R. prolixus* is synthesized in the fat body, and patency of the follicular epithelium appears and increases gradually throughout vitellogenesis, correlating with the rate of vitellogenin uptake into the oocyte from the hemolymph (Huebner and Injeyan, 1980). It

was shown that in addition to incorporating vitellogenin produced by the fat body into the developing oocyte, the follicle cells produce vitellogenin, which is taken up by the oocyte towards the end of vitellogenesis after patency has diminished (Melo *et al*., 2000). Subsequent to vitellogenesis, it is the single layer of binucleate follicle cells that synthesizes and deposits the chorion (Beament, 1946).

1.1.2 Hormonal Regulation of Patency, Vitellogenesis, and Ecdysteroids in Rhodnius prolixus

The release of regulatory factors that occurs during the entire period of egg development in insects has been the focus of extensive research. Classical studies showed that normal egg development is under control of the corpus allatum (CA) (see Section 1.6.3) (Wigglesworth, 1936). Juvenile hormones (JH) (Wigglesworth, 1940) are acyclic sesquiterpenoids produced in the CA, a pair of endocrine glands in the retrocerebral complex close to the brain. JH is crucial for many aspects of insect physiology, including the development of patency during vitellogenesis (Ilenchuk and Davey, 1987). JH also regulates previtellogenic growth of the follicle in some insect species (Wyatt and Davey, 1996). In *R. prolixus*, exposure of prefollicular tissue to JH is required during larval development in order to acquire competence of the follicle cells to respond to JH in the vitellogenic follicle in the adult (Abu-Hakima and Davey, 1975). While the identity of the *R. prolixus* JH is not known, surgical removal of the CA in mated females results in delayed vitellogenesis and the inhibition of a patent follicular epithelium (Pratt and Davey, 1972). Through binding assays on isolated follicle cells it was shown that JHI directly binds to the plasma membrane

(Ilenchuk and Davey, 1985), and causes an increase in specific JH-sensitive membrane bound Na/K-ATPase activity. Additionally, JH induced follicle cell shrinkage is inhibited in the presence of ouabain, an Na/K-ATPase inhibitor (Abu-Hakima and Davey, 1979). Therefore, JH acts directly on the follicle cells to induce patency, permitting vitellogenin uptake into the oocyte (Pratt and Davey, 1972; Wyatt and Davey, 1996).

In most insects, synthesis of vitellogenins in the fat body is dependent on stimulation by JH (Wyatt and Davey, 1996). Studies involving the removal and replacement of the CA demonstrated the requirement of JH for normal vitellogenin production (Coles, 1965). In *R. prolixus*, adult females lacking the CA produce low levels of vitellogenin and fewer amounts of eggs, and these processes can be restored to normal levels with administration of JH (Wang and Davey, 1993). Additionally, the function of the accessory sex glands in the reproductive system of some insects is dependent on the CA (Scharrer, 1946). The accessory glands produce mainly protein, and it has been demonstrated that JH stimulates protein synthesis in these glands (Wyatt and Davey, 1996). In *Rhodnius* (Barker and Davey, 1982), *Locusta* (Braun and Wyatt, 1995), and *Melanoplus* (Gillot and Friedel, 1976) removal of the CA causes a reduction in protein synthesis by accessory glands and application of JH (or JH analogues) can restore normal rates of protein synthesis. Additionally, an antigonadotropin that inhibits the response of follicle cells to JH was identified (Huebner and Davey, 1973). Four pairs of adbominal neurosecretory organs were found to be the source of this antigonadotropin (Davey and Kuster, 1981), which inhibits the action of JH in causing patency.

In addition to JH, ecdysteroids play an important role in the regulation of vitellogenesis in insects (Brown *et al*., 2009). In Diptera, ovarian ecdysteroids released into

the hemolymph stimulate the fat body to synthesize and release vitellogenin, which is then incorporated into the developing oocyte (Van de Velde *et al.*, 2008). In many adult insects, the follicle cells are the source of ecdysteroids, which are taken up by the oocyte and/or released into the hemolymph for circulation. While the exact location and function of ovarian ecdysteroidogenesis in *R. prolixus* has not been elucidated, studies in a cockroach demonstrated ecdysteroid secretion by ovarian follicle cells (Zhu *et al*., 1983). Ovarian ecdysteroids have numerous roles in adult insects, in addition to initiation of vitellogenesis. Ecdysteroids are converted to various conjugates, which are incorporated into the developing oocyte where they may function in activating and regulating early embryogenesis (Lagueux *et al.*, 1979). In *R. prolixus*, ecdysone (E) and 20-hydroxyecdysone (20E) were the only free ecdysteroids found in the hemolymph (Cardinal-Aucoin *et al.*, 2013).

Because the telotrophic ovaries of *R. prolixus* are suspended in the hemocoel, hormonal regulation of various processes during oogenesis via the hemolymph has been a primary area of study (Pratt and Davey, 1972). Earlier studies involving surgical lesions and cauterization excluded the involvement of direct innervation in the regulation of egg development (discussed in Büning, 1994). In addition to the regulatory factors discussed, many other molecules involved in the regulation of the egg development have been identified in other insects (see Section 4.1.4).

1.2 Importance and Development of Circadian Rhythms

Organisms undergo rhythmic changes in behavioural, physiological, and biochemical processes in the course of a day. Many of these changes occur in response to environmental

stimuli, such as light:dark (LD) cycles (Pittendrigh and Minis, 1964). Rhythms in organisms that occur with a periodicity matching that of the earth's rotation, but persist in the absence of environmental cues, are termed circadian. Circadian rhythms are biological rhythms generated by circadian clocks (Pittendrigh, 1960). Circadian rhythms have roughly 24h periodicity, and convey critical timing information to diverse cells and tissues. Circadian rhythms are essential for coordinating and synchronizing cellular and physiological activities into a temporal sequence in organisms, from bacteria to humans (reviewed by Harmer *et al*., 2001). These endogenously controlled biological rhythms are synchronized to external, environmental, and internal hormonal controls to regulate the timing of cellular, tissue, and systemic events (Panda *et al*., 2002). The circadian clock permits anticipation of changes in the environment and thereby prepares the organism for these changes (discussed in Saunders, 1977).

Not only do circadian rhythms persist in the absence of external cues, they are also temperature compensated (Pittendrigh, 1960). Therefore, over a wide range of temperatures the phase and period length of a circadian rhythm does not change. Additionally, the phase and period length can be adjusted so that the endogenous timing of organisms matches that of the surrounding environment; this process is known as entrainment (Pittendrigh and Minis, 1964). Zeitgebers (Beling, 1929), or "time-givers", are signals such as light, hormones, nutrients, and temperature, which govern and synchronize the timing of biochemical and physiological processes (Panda *et al*., 2002). Therefore, endogenously controlled biological rhythms are synchronized to external environmental timing signals as well as internal hormonal controls by Zeitgeibers.

1.3 The Molecular Oscillator

Circadian clocks are found throughout all eukaryotes and bacteria; from cyanobacteria (Kondo *et al*., 1993), which are photosynthetic prokaryotes, to mammals. Circadian oscillations in physiological and biochemical functions are generated by specialized cell types in multicellular organisms. The traditional model of the circadian clock has three functional components: (1) a core molecular oscillator that generates endogenous rhythms with approximately a 24 hour period; (2) input pathways that entrain the molecular oscillator (via environmental signals which are converted to sensory information) and (3) output pathways that transmit circadian information to various biochemical, physiological and behavioural processes (Dunlap *et al*., 2004; Bloch *et al*., 2013). Clock genes, initially cloned from *D. melanogaster*, have the primary role of participating in molecular oscillations that generate circadian rhythms (Vafopoulou and Steel, 2005). Only a limited number of groups of cells possess these molecular oscillators, in which the clock genes are transcribed with circadian periodicity resulting in circadian changes in levels of clock gene mRNA and protein (Vafopoulou and Steel, 2005). Furthermore, cytoplasmic clock proteins move into the nucleus with circadian periodicity, where they act as transcription regulators (Vafopoulou and Steel, 2005), resulting in transcription-translation feedback loops (TTFL). To be a clock cell, circadian cycling of clock gene mRNA and/or protein levels, as well as rhythmic protein migration into the nucleus, must free-run in continuous darkness (DD) (Vafopoulou and Steel, 2005). In certain cells that exhibit daily cycling of clock gene expression, mRNA levels, and clock protein abundance, transfer to DD results in the failure of rhythmicity to free run. Hence, these cellular oscillators are not clock cells, and the rhythmicity in these cells must be

driven by true clock cells via hormones or nerves (Vafopoulou and Steel, 2005). Additionally, true clock cells are almost always found in groups, and are coupled together by gap junctions and/or hormonal or neural inputs to generate circadian rhythmicity. True circadian clocks, as well as driven oscillators, have been identified in the brain and peripheral tissues (discussed in Vafopoulou and Steel, 2005).

In many animals, a circadian clock in the brain coordinates and synchronizes circadian rhythms in peripheral tissues and organs (reviewed by Helfrich-Forster, 2004). Furthermore, circadian clocks or oscillators have been identified in virtually all other tissues, such as the eyes, liver, kidneys (reviewed by Herzog and Tosini, 2001), ovaries (reviewed by Sellix, 2015) and many other tissues in vertebrates, and the prothoracic glands (reviewed by Vafopoulou X and Steel, 2006), Malphigian tubules, fat body, rectum, and reproductive tissues in insects (reviewed by Giebultowicz, 2001).

1.3.1 Molecular Basis for Circadian Rhythmicity

In most clock cells examined to date, the TTFL is central to the circadian oscillator (Yu and Hardin, 2006). The first molecular model of the circadian oscillator consisted of a single autoregulatory feedback loop in gene expression (Hall, 2003), and the discovery of additional clock genes in *D. melanogaster* added to its complexity. The current model of the *D. melanogaster* circadian oscillator is composed of two interlocked feedback loops (Hardin, 2004; Hardin, 2005; Stanewsky, 2003). These feedback loops are regulated by transcription factors that induce the expression of clock genes, that then indirectly negatively regulate their own transcription, thus creating oscillatory patterns of gene, mRNA, and protein expression

(described in Yu and Hardin, 2006). Additionally, posttranscriptional modifications, such as the coordinated effects of kinases and phosphatases, are important in the regulation of the TTFL (Yu and Hardin, 2006). The TTFL drives rhythmic expression of target downstream genes, called clock-controlled genes (*ccgs*), thus regulating specific outputs in a circadian manner (Wijnen *et al*., 2006). As in *D. melanogaster*, the mammalian molecular oscillator is composed of interlocked transcriptional feedback loops, and many orthologs or functional equivalents between both models have been identified (discussed in Yu and Hardin, 2006).

1.3.2 The Molecular Oscillator in Insects

The molecular and genetic studies of circadian rhythms began in *Drosophila melanogaster*. Originally, three fly mutants with different eclosion periods were identified, and all three strains were found to have a mutation in the same gene locus, *period (per^{long}, per^{short}, per^{01})* (Konopka and Benzer, 1971). The *dPer* gene, and its protein PERIOD (PER), was the first clock component identified. *Timeless (dTim)* was the second clock gene to be cloned in *D. melanogaster*, as dTIM was found to bind to and stabilize dPER via a PAS domain (Gekakis *et al*., 1995).

The current model of the circadian clock in *D. melanogaster* shows that two genes *clock* (*Clk)* and *cycle (Cyc),* encode transcription factors containing basic helix-loop-helix (bHLH) DNA-binding domains and PAS domains (Darlington *et al*., 1998; Allada *et al*., 1998; Rutila *et al*., 1998). Starting from mid-day, dCLK and CYC heterodimerize in the nucleus and bind to E-Box sequences, which are found in the promoter region of many circadian regulated genes, such as *per* and *tim (*Figure 1.2, indicated by "+") (Hardin, 2005).

At the same time, *per* and *tim* mRNA levels begin to accumulate, but PER and TIM protein levels do not peak until the late evening (Williams and Sehgal, 2001). PER and TIM levels remain low during the day as a result of the blue-light photoreceptor cryptochrome (CRY), which undergoes a light-induced conformational change that promotes the formation of CRY-TIM complexes (Ashmore *et al*., 2003). As a result, TIM is degraded by the ubiquitin/proteasome pathway (Ashmore and Sehgal, 2003), and PER is targeted for phosphorylation and degradation. Without TIM, PER is subject to phosphorylation by Double-Time (DBT) kinase (Price *et al*., 1998), and then targeted for ubiquitination and subsequent degradation by *supernumerary limbs (slmb)* protein, SLMB (Grima *et al*., 2002). PER levels are also regulated by protein phosphatase 2A (PP2A) in the cytoplasm, where PP2A rhythmically dephosphorylates PER, thereby regulating PER stability (not shown in Figure 1.2)(Sathyanarayanan *et al*., 2004). Interestingly, rhythmic dephosphorylation of PER is achieved by the rhythmic expression of PP2A regulatory subunits, *twins (tws)* and *widerborst (wdb)* in the cytoplasm (Sathyanarayanan *et al*., 2004). This demonstrates the importance of posttranslational modification (phosphorylation being the most studied) of PER and TIM in the cytoplasm, which plays crucial roles in the generation of a 24 h rhythm by regulating the stability of PER and the timing of nuclear entry of PER-TIM complexes (discussed in Harms *et al*., 2004).

At night when it is dark, PER and TIM accumulate and form a complex, because TIM is no longer destabilized by light-induced degradation (Fig. 1.2, indicated by "-"). PER-TIM complexes enter the nucleus (described in Harms *et al*., 2004), and the complex binds to CLK/CYC dimers resulting in hyperphosphorylation of CLK, preventing CLK/CYC complexes from binding to DNA (Hardin, 2005). As a result, transcription of *per* and *tim* is

inhibited (Hardin, 2005). Therefore, dPER and dTIM mediate negative feedback and dCLK and CYC regulate the positive elements of the TTFL (Yu and Hardin, 2006). The transcription of *Clk* is regulated by a second feedback loop that interlocks with the *tim/per* loop (Hardin, 2004; Yu and Hardin, 2006), which will not be discussed here.

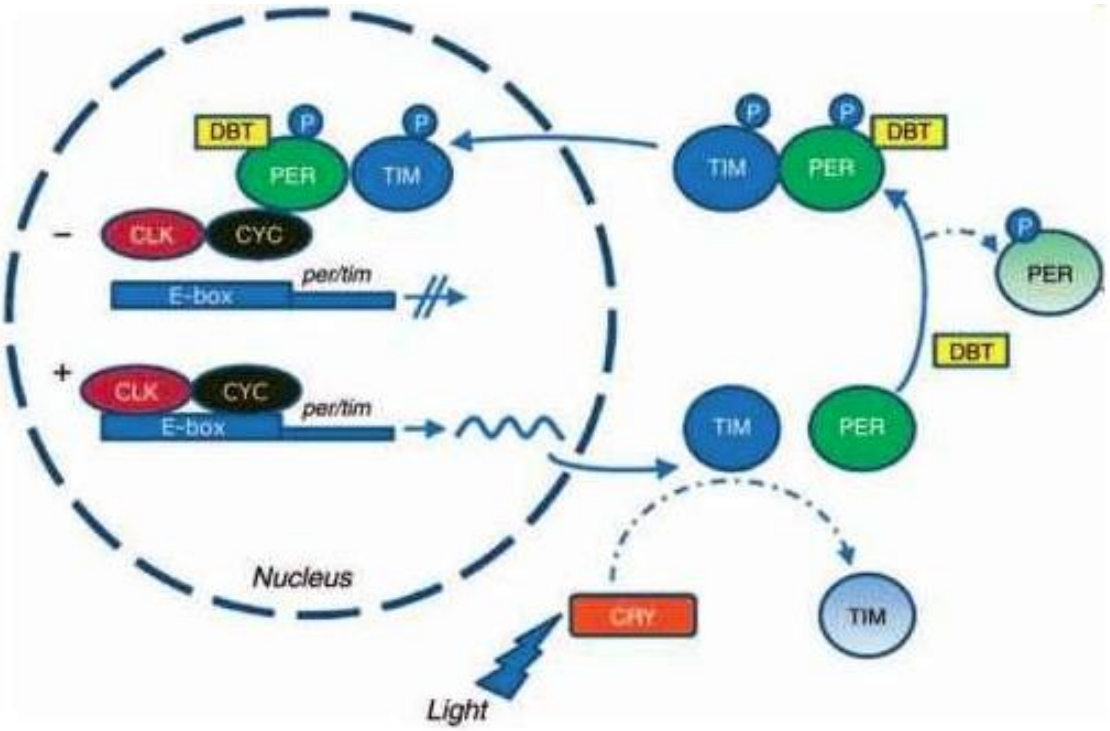

Figure 1.2 A diagram illustrating the core molecular machinery comprising the transcription-translation feedback loop (TTFL) of the *D. melanogaster* circadian clock (adapted from Hardin, 2005). Only the PER/TIM negative feedback loop is shown. See text for details.

1.3.3 The Molecular Oscillator in Mammals

In mammals, the TTFL is comprised of genes homologous to the *D. melanogaster* clock genes (Harmer *et al*., 2001). Three *per* homologs have been identified in mammals, *Per1*, *Per2*, and *Per3* (Sun *et al*., 1997; Zylka *et al*., 1998). Similar to the insect clock, the bHLH/PAS transcription factors CLK and BMAL1 (the mouse homolog of CYCLE) comprise the positive elements of the mammalian clock (Gekakis *et al*., 1998; Vitaterna *et al*., 1994). The negative feedback loop in the mammalian model is more complex, and has been reviewed in detail (Harmer *et al*., 2001). In short, all three PER proteins are involved in the negative feedback inhibition of CLK/BMAL1-mediated gene activation, and the action of PER is aided by two cryptochromes, CRY1 and CRY2, independently of TIM (Griffin *et al*., 1999; Kume *et al*., 1999).

1.4 Multioscillator Organization of Circadian Systems

Circadian clocks and oscillators are present in many organs and tissues in animals. Because true circadian clocks generate the rhythmic inputs that drive rhythmicity in cellular oscillators (both in the CNS and in peripheral tissues), this leads to the notion that the circadian system is comprised of a hierarchal organization of oscillators (Vafopoulou and Steel, 2005). This hierarchal arrangement of the circadian system consists of master clocks driving rhythmicity in other oscillators (via nerves and/or hormones), as well as the integrated roles of semi-autonomous clocks.

1.4.1 Consequences of Circadian Disruption

The implications of desynchronized circadian systems and the resulting disruption of internal temporal order as a result of exposure to abnormal circadian light cycles is becoming readily apparent (Aschoff, 1969; Pittendrigh and Minis, 1972). With the widespread use of electrical lighting, as well as the increasing number of shift workers, exposure to light at night is resulting in repeated and long term desynchronized circadian systems (Navara and Nelson, 2007). Not only can light pollution affect many behavioural and physiological processes in animals that rely on seasonal changes in day length, such as in birds (Brigham and Barclay, 1992) and rodents (Kotler, 1984), human exposure to a low-level incandescent bulb at night can suppress levels of the pineal gland hormone melatonin to 50% in 39 minutes (Lewy *et al*., 1985). Melatonin exhibits diurnal variations in circulating levels which peak at night, and this rhythm is critical for conveying photoperiodic information to entrain the circadian system in mammals, birds, and insects (Redman *et al*., 1983; Lewy *et al*., 1985; Vivien-Roels and Pevet, 1993). Additionally, shift work and light exposure at night has been associated with insulin resistance, hypertension, and heart disease, cancer, and many other diseases in humans (reviewed in Haus and Smolensky, 2006). Many reproductive processes rely on variation in light, and disruptive effects of unnatural, long-term light exposure on reproductive behavior and physiology have been documented (reviewed in Reiter, 1980).

1.4.2 The Circadian Clock in the Insect Brain

While clock gene expression has been identified in virtually all tissues examined to date, the center of circadian timekeeping is located in the brain of insects. Circadian master clocks in the insect brain were identified by lesioning, transplantation, and *in vitro* experiments, which demonstrated candidate structures that were critical for circadian rhythmicity. Two distinct areas in the brain possessing circadian clocks have been identified; the lateral and dorsal protocerebrum (detailed in Vafopoulou and Steel, 2005). However, the interconnectivity of anatomically separate groups of clock cells in the brain has been demonstrated in many insect species, indicating that the protocerebral and optic lobe clocks are not distinct structures but are components of a complex clock network in the brain (discussed in Vafopoulou and Steel, 2005).

Early transplantation studies in the cockroach, *Leucophaea maderae*, demonstrated that the optic lobe contains a circadian clock (Page, 1982). Implantation of an optic lobe into a lobeless recipient animal was sufficient to restore activity rhythms, and the period of the locomotor rhythm was that of the optic lobe donor, rather than the recipient. Futhermore, isolated optic lobes *in vitro* showed a circadian rhythm in neural activity (Colwell and Page, 1990).

The central oscillator of *L. maderae* was localized between the medulla and the lobula (Fig. 1.3) (reviewed by Helfrich-Forster *et al*., 1998)). Pigment-dispersing hormones (PDHs) identified in several crustacean species (Rao and Riehm, 1993), and similar peptides in insects called pigment-dispersing factors (PDFs) (Rao *et al*., 1987), showed dense immunoreactive

staining in cell bodies of neurons localized to the optic lobe. These pigment-dispersing factor-immunoreactive neurons (PDFMe) that innervate the optic lobe region fulfilled the anatomical criteria proposed for circadian clock neurons (Homberg *et al*., 1991; Page, 1984). In crickets, grasshoppers, and flies, the optic lobe was also identified as the location of the master clock, with PDH-immunoreactive (PDH-ir) neurons connecting the lamina, medulla, and different targets in the central brain (reviewed by Helfrich-Forster *et al*., 1998).

In *D. melanogaster*, PER was localized to a group of lateral neurons (LNs) in the optic lobe, which was sufficient to sustain behavioural rhythms in flies with a null mutation in *per* in all other cells (Frisch *et al*., 1994). In contrast to the centrally organized master clock in cockroaches and crickets, several clock gene expressing dorsal neurons of the protocerebrum in *D. melanogaster* have been identified (reviewed by Helfrich-Forster, 2003). The dorsal neurons (DNs), however, require functioning LNs to control activity rhythms under constant conditions (Helfrich-Forster, 2004). In some insects, the dorsal brain clock neurons seem just as important as those in the optic lobe clock (Helfrich-Forster, 2004; reviewed in Vafopoulou and Steel, 2006). While the role of the optic lobe in circadian timekeeping has been frequently described in some insect groups (Helfrich-Forster *et al*., 1998), its function in other insects such as *R. prolixus* is questionable as an area for the integration of timing information (Vafopoulou and Steel, 2005). Interestingly, rhythmic clock gene (PER/TIM) expression in PDF-immunoreactive LNs has only been demonstrated in *D. melanogaster* and *R. prolixus* (see Section 1.6.3) (Helfrich-Forster, 2004; Vafopoulou and Steel, 2006).

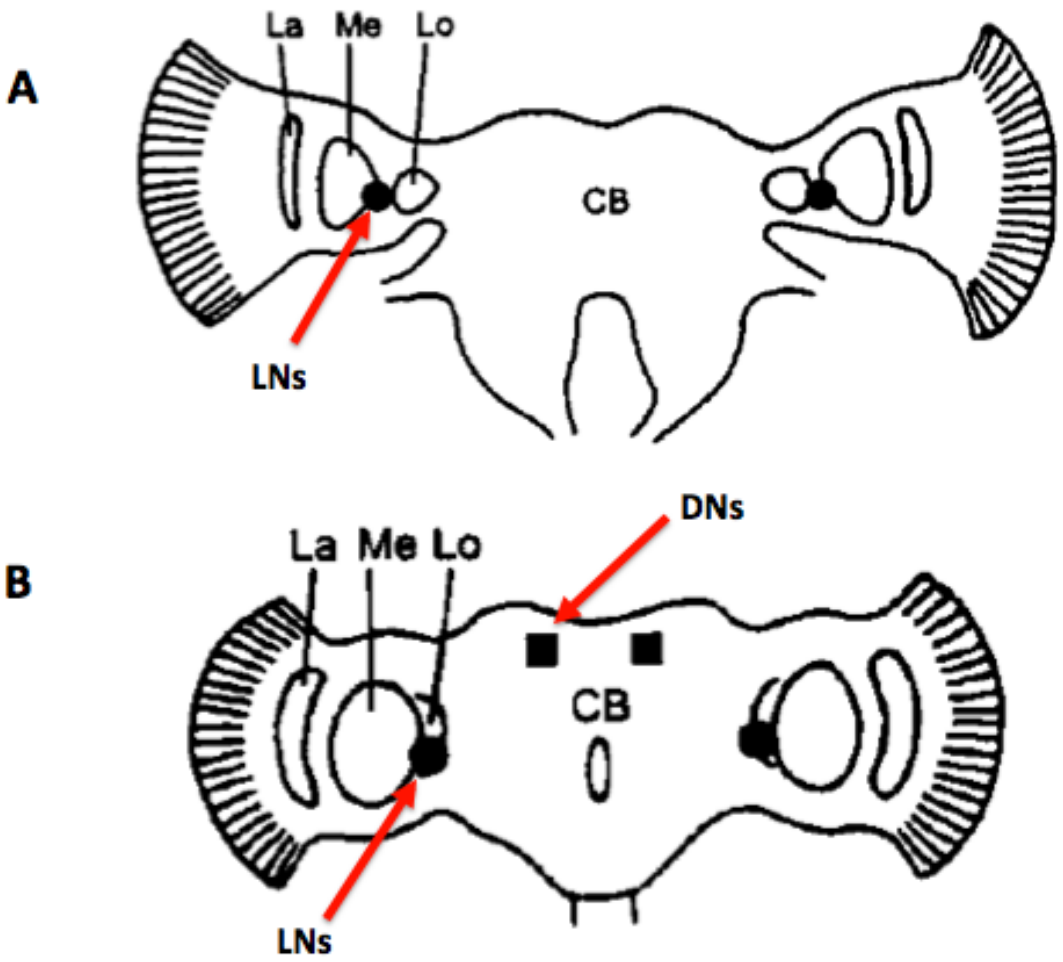

Figure 1.3 Schematic drawings of the brains of the (a) cockroach, *Leucophaea maderae*, and (b) the fruit fly, *Drosophila melanogaster* (adapted from Helfrich-Forster, 1998). The lamina (La), medulla (Me), and lobula (Lo) in the optic lobe are indicated. The general locations of circadian pacemaker centers are indicated (filled circles and squares). The lateral neurons (LNs) are localized between the lobula and medulla in the optic lobe. In *D. melanogaster*, additional clock neurons, dorsal neurons (DNs), are located in the region of the central brain.

1.4.3 The Circadian Clock in the Mammalian Brain

In mammals, the master clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus in the brain (Klein *et al*., 1991). The SCN is composed of between 5,000-30,000 neurons depending on the species, and is organized into several neuronal subpopulations based on different neuroactive substances (described in Helfrich-Forster, 2004). Only a few SCN cells are considered true clock cells, and individual SCN cells *in vitro* have highly variable period lengths in electrical firing patterns and membrane potential (Hamada *et al*., 2001). Therefore, the coupling and integration of populations of SCN cells is required to fulfill the properties of a circadian clock, as has been demonstrated in the insect brain. Transplantation studies using the *tau* mutant hamster, with a behavioral rhythm period of about 20 h (in comparison to 24 h rhythm in wild type hamsters), demonstrated that the SCN is essential for determining the period of this rhythm (Ralph *et al*., 1990). Similar to the accessory medulla in insects, the SCN is linked with many parts of the brain, modulating neuroendocrine functions in a rhythmic manner (Helfrich-Forster, 2004).

1.4.4 Peripheral Oscillators and Clocks in Insects

The idea that one central clock in the brain controls internal temporal order (Pittendrigh, 1960) has been questioned as a result of the study of spatial patterns of clock-gene expression in *D. melanogaster* (Plautz *et al*., 1997), suggesting the presence of a multi-oscillatory circadian organization. Clock genes cycle outside of the CNS, in many tissues and organs involved in development, reproduction, metabolism, and excretion (reviewed in

Giebultowicz, 2001). Peripheral oscillators are not necessarily circadian clocks because the requirements of a true clock have not been met, and rhythmicity is often driven by external factors (see Section 1.2) (Vafopoulou and Steel, 2005). For example, in *D. melanogaster* rhythmic clock gene expression in the Malpighian tubules, which could be reset by light, was demonstrated using a luciferase reporter for the promoter of *per* (Hege *et al*., 1997). However, when tubules were transplanted into another insect, light sensitivity was lost and the tubules were unable to respond to humoral signals from the host insect (Giebultowicz *et al*., 2000). While it is not known what the functional significance of the tubule rhythm is, it is clear that entrainment of this rhythm occurs via intact connections between the Malpighian tubules and the whole animal (Steel and Vafopoulou, 2005).

The most fully characterized peripheral circadian clock in insects is found in the paired prothoracic gland (PG) of larval *Rhodnius prolixus* (reviewed in Vafopoulou and Steel, 2006). The photosensitive clock in this tissue generates rhythmic synthesis and release of the ecdysteroid molting hormones, which persists in aperiodic conditions (Vafopoulou and Steel, 1991). This strong rhythm in titer had a free-running period length of approximately 24 h, and was temperature compensated (Vafopoulou and Steel, 1991). It is now well understood that the circadian orchestration of developmental hormones in *R. prolixus* is achieved by a multioscillator system consisting of four photosensitive circadian clocks, one in each of the paired PGs and two in the brain (see Section 1.5) (reviewed in Vafopoulou and Steel, 2006).

1.4.5 Peripheral Oscillators and Clocks in Mammals

The hierarchical organization of the mammalian circadian system is demonstrated by the coordination of the central clock with peripheral clocks to mediate behavioural and physiological outputs (reviewed by Bass and Takahashi, 2010). Peripheral clocks are regulated through direct neural and humoral signals from the brain clock, as well as by regulation from local, endogenous cellular oscillators that function independently of the brain clock. Peripheral clocks have been characterized in many tissues and organs in mammals, including the liver, muscle, fat, pancreas, and the cardiovascular system (reviewed by Bass and Takahashi, 2010). Bile acid synthesis, lipogenesis, cardiovascular function, inflammation, and glucose homeostasis are just a few biochemical and physiological processes regulated by peripheral oscillators (reviewed by Bass and Takahashi, 2010).

Ovarian clocks have been detected in mammals, and a clear rhythm in circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary is tightly regulated by light:dark (LD) signals transmitted from the SCN (Boden and Kennaway, 2006). The mammalian ovarian follicle is comprised of a heterogeneous population of cells, namely theca cells (TCs), granulosa cells (GCs), and androgen- and estrogen-producing cells surrounding the outside of the follicle (Sellix, 2015). The TCs and GCs undergo specific changes in levels of steroidogenic enzymes (Espey and Richards, 2006), and this process is dependent upon the appropriate timing of a surge in circulating LH (Karsch *et al*., 1997; Sellix and Menaker, 2010). Furthermore, rhythmic clock gene expression has been reported in mature GCs (Chu *et al*., 2011, Chu *et al*., 2012), as well as isolated GCs from rats providing evidence of circadian clock function in these cells (Chen *et al*., 2013a; Chen *et al*., 2013b).

Circadian clocks in the mammalian ovary also play a critical role in steroid hormone synthesis and secretion, which show clear rhythms in rodents (Sellix, 2015). In *bmal1-/-* mice, which are infertile, reduced expression of steroidogenic acute regulatory protein (StAR) and low levels of circulating progesterone were observed (Ratajczak *et al*., 2009). It was suggested that clock disruption alone, which also abolishes rhythmic StAR expression in isolated *bmal1-/-* mature GCs from rats (Chen *et al*., 2013a; Chen *et al*., 2013b), might lead to altered patterns of ovarian steroid hormone synthesis and secretion (Sellix, 2015). These findings demonstrate that rhythmic events such as the timing and amplitude of steroid hormone secretion, which are essential to fertility in the mammalian ovary, are reliant on the coordination of multiple peripheral oscillators in endocrine tissues and the primary circadian clock in the SCN (reviewed by Sellix, 2015).

1.5 Entrainment of Circadian Clocks and Oscillators

Every eukaryote and prokaryote examined to date has some mechanism for circadian timekeeping. While circadian rhythms are self-sustained and persist with a free-running period in the absence of external cues, circadian clocks are entrained by periodic environmental signals, which allow organisms to anticipate environmental changes (Pittendrigh, 1960). Light, temperature, social cues (Levine *et al*., 2002), and even magnetism (Yoshii *et al*., 2009) can act as Zeitgebers (Peschel and Helfrich-Forster, 2011). Circadian clocks also convey timing information to peripheral clocks and oscillators through the use of internal Zeitgebers, where hormones are the most common entraining signals to such clocks and oscillators (Vafopoulou and Steel, 2005).

1.5.1 Entrainment of Clocks by Light

The predominant Zeitgeber for entrainment is light (or dark) (reviewed in Peschel and Helfrich-Forster, 2011). Furthermore, the circadian clock is sensitive to the timing of light exposure. In *D. melanogaster*, a brief exposure to light at night causes a delayed or advanced onset of activity on the next day, depending on the timing of the light pulse given (Peschel and Forster, 2011). Furthermore, in constant light (LL) a fly's behaviour becomes arrhythmic (Konopka *et al*., 1989). It was suggested that light has a strong influence by either degrading a component of the TTFL mechanism that typically declines during photophase, or that a component that usually increases during photophase is being induced by light (Emery *et al*., 1998). The stability of TIM protein is light-sensitive, and dTIM is rapidly degraded in response to light (Lee *et al*., 1996).

Three different photoreceptive organs containing different photopigments for light entrainment of clock neurons have been described in *D. melanogaster* (Peschel and Helfrich-Forster, 2011). The compound eyes, the ocelli, and the Hofbauer-Buchner eyelets are photosensitive organs involved in light entrainment pathways of clock neurons in the brain (Peschel and Helfrich-Forster, 2011). In some insects, it has been suggested that the only photoreceptors for entrainment by light are the compound eyes (Nishiitsutsuji-Uwo and Pittendrigh, 1986b). Severing of the optic nerve, removal of both compound eyes, or even partial removal of the compound eye of crickets (Tomioka *et al*., 1990), results in weakened or complete loss of entrainment in both aperiodic and LD conditions. In cockroaches, lesion studies demonstrated that photoreceptors in the compound eye are necessary for light

entrainment (Nishiitsutsuji-Uwo and Pittendrigh, 1968a). The sensitivity of the compound eye is also controlled by a circadian clock (Tomioka and Chiba, 1982). After severing the optic tracts in the cricket *Gryllus bimaculatus*, thereby isolating the optic lobe (OL)-compound eye (CE) system from the CNS, a circadian electroretinogram (ERG) rhythm persisted in DD and constant temperature conditions. From this, the possibility of a self-sustaining oscillator in the OL has been raised (Tomioka and Chiba, 1982).

Non-ocular entrainment by light has been well studied, with the use of genetically eyeless or blind *D. melanogaster* that can be entrained to LD cycles (Helfrich-Forster, 1997). Cryptochromes, which are blue-light photoreceptors expressed in specific subsets of clock neurons and compound eyes (Yoshii *et al*., 2008), were found to transmit light information to the circadian clock. In *D. melanogaster*, dCRY interacts with dTIM in response to light, and this interaction is correlated with dTIM degradation (Ceriani *et al*., 1999). dCRY-mediated degradation of TIM prevents PER-TIM dimer formation when there is light, confining PER-TIM dimerization to darkness (Emery *et al*., 1998). Therefore, in LL conditions dCRY-mediated dTIM degradation prevents increased levels of dTIM and stabilization of dPER, which affects the TTFL mechanism and causes arrhythmic behaviour (Harmer *et al*., 2001). Interestingly, mutant cry^b flies which have a null mutation encoding the blue light photoreceptor do not become arrhythmic under constant light conditions (Stanewsky *et al*., 1998), in comparison to wild type flies, demonstrating a unique role of dCRY in extraocular circadian photoreception (Emery *et al*., 2000).

In vertebrates, the primary site for photoreception and entrainment is the retina, which contains a separate anatomical and functional system that mediates light information to the clock in the brain (Hannibal and Fahrenkrug, 2002). The retinohypothalamic tract (RHT) is a

projection of retinal ganglion cells that transmits light signals from the retina to the SCN directly, but transmission to the SCN can also be achieved indirectly through projections to the lateral geniculate nucleus (LGN) (reviewed by King and Takahashi 2000). Melanopsin is a photopigment expressed exclusively in certain retinal ganglion cells, and melanopsin containing ganglion cells are intrinsically photosensitive (Hannibal and Fahrenkrug, 2002; Morin *et al*., 2003; Gompf *et al*., 2014; Lucas *et al*., 2014). This specific subset of ganglion cells can detect light when isolated from the rest of the retina, and specific ablation of these cells abolishes non-visual responses such as photic input to the circadian system (Lucas *et al*., 2003; reviewed in Lucas *et al*., 2014).

1.5.2 Entrainment of Clock Cells by Temperature

In comparison to light, which can be perceived by the compound eyes and extra-ocular pathways and entrains the circadian clock, temperature changes can act directly on the TTFL mechanism (Rensing and Ruoff, 2002). Under constant darkness (DD) or constant light (LL), the activity rhythm of wild type and arrhythmic mutant *per^{o} D. melanogaster* could be entrained to periodic temperatures (12:12 h) oscillating with a ±5 C° difference (Tomioka *et al*., 1998). In *Drosophila pseudoobscura*, the circadian rhythm of eclosion can be entrained by temperature cycles of 8 C° to 10 C° amplitude in the absence of light cues (Zimmerman *et al*., 1968). Temperature cycles also have an indirect effect on signaling cascades, such as cAMP, cGMP, and protein kinases A and C, which may influence phosphorylation of clock components in the TTFL mechanism (reviewed by Rensing and Ruoff, 2002). In *D. melanogaster*, entrainment of locomotor activity rhythms by a temperature cycle of ±3 C° was

observed in blind flies (Wheeler *et al*., 1993), and many wild type individuals are entrainable by temperature cycles that vary by only ±2 C° (Helfrich-Forster, 1998). Similar findings were demonstrated in crickets and cockroaches with their optic lobes removed, in which locomotor rhythms persisted in response to temperature cycles (Page, 1985b).

1.5.3 Entrainment of Peripheral Tissue Clocks and Oscillators

In multi-oscillatory systems, behavioural and physiological experiments have demonstrated that the clock in the brain often regulates the circadian rhythm of physiological functions in peripheral tissues with endogenous circadian oscillators (Pittendrigh, 1974). Through neural and hormonal inputs, the master clock can regulate and set the phase of peripheral circadian oscillators, while some of these peripheral oscillators can independently be entrained by light cycles *in vitro* (reviewed by Tomioka and Abdelsalam, 2004).

In the hemimetabolous insect, *R. prolixus*, the peripheral clocks in the paired PGs control the synthesis and release ecdysteroids with a circadian rhythm. The circadian clock in PG cells is photosensitive in culture, but hormonal entrainment via the photosensitive clock in the brain acts to set the phase of rhythmic ecdysteroidogenesis and release (Pelc and Steel, 1997). The brain neuropeptide, prothoracicotropic hormone (PTTH), is released from the brain with a circadian rhythm, and synchronizes left and right PGs to regulate the output of ecdysteroids into the hemolymph (Vafopoulou and Steel, 1996). Interestingly, the photosensitive clock in the brain has also been shown to be responsive to internal Zeitgebers. Expression of the ecdysteroid receptor (EcR) is induced by ecdysteroids in many target tissues

(epidermal cells, brain neurons, fat body, rectal epithelium and oenocytes [Vafopoulou and Steel, 2006]), and the level of EcR expression closely parallels the hemolymph ecdysteroid titer during development (Vafopoulou *et al*., 2005). Furthermore, ecdysteroids were found to exert rhythmic feedback on the lateral clock neurons in the brain of *R. prolixus*, which exhibit circadian cycling of EcR (Vafopoulou and Steel, 2006). In this mechanism of feedback, ecdysteroids exert a rhythmic influence on the clock cells that generates rhythmicity (Vafopoulou and Steel, 2006).

In mammals most cells and tissues contain endogenous circadian oscillators, but cyclic clock gene expression is more readily dampened in the absence of inputs from the central clock. Therefore, Zeitgebers such as SCN signals (neural and hormonal), feeding-fasting cycles, and cycles in body temperature are essential for synchronizing peripheral oscillators (reviewed in Son *et al*., 2011).

The hypothalamus-pituitary-adrenal (HPA) axis is of interest because it constitutes the neuroendocrine regulation of the stress response in mammals. Additionally, adrenal glucocorticoids (GCs) are adrenal steroid hormones that are released with a robust circadian rhythm and act on many tissues and the brain (reviewed in Son *et al*., 2011). Each adrenal gland harbors an endogenous circadian clock that mediates the circadian GC rhythm by gating the sensitivity of the adrenals to ACTH (Oster *et al*., 2006). This is remarkably similar to the previously well-described circadian system in larval *R. prolixus*, which regulates the productions of ecdysteroids. The action of ACTH parallels that of PTTH in insects, acting on endocrine glands that harbor a photosensitive circadian clock (the adrenal glands and PGs, respectively).

Neurosecretory neurons in the hypothalamus are stimulated to release corticotrophin-releasing hormone, which in turn induces the synthesis and release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH targets and activates adrenal synthesis and secretion of GCs. Circulating GCs will interact with specific receptors in target tissues to enable the organism to cope with the stress (reviewed by Chrousos and Kino, 2007), and will also inhibit further HPA neuroendocrine activity by its upstream regulators (Sapolsky *et al*., 2000).

Interestingly, the adrenal peripheral clock is not only entrained by the action of the SCN central clock, but also by food entrainable oscillators in the dorsomedial hypothalamus (Damiola *et al*., 2000; Fuller *et al*., 2008). This finding was demonstrated with the use of nocturnal rodents that were restricted to daytime feeding, in which the daily GC rhythm showed two peaks (Holmes *et al*., 1997). The significance of this system is the integrated activity of multiple regulatory mechanisms in the brain and peripheral tissue to regulate the rhythm of circulating GCs, which has also been implicated in synchronizing circadian timing in peripheral clocks (reviewed in Son *et al*., 2011).

1.6 Circadian Orchestration of Development in the Model Organism, Rhodnius prolixus

***1.6.1 Rhodnius prolixus* as a Model Organism**

R. prolixus is a hemimetabolous insect belonging to the order Hemiptera, and the family Reduviidae, more commonly known as the assassin bug family (Schofield and Galvão, 2009). *R. prolixus* is a triatomine bug that has adapted to feed on vertebrate blood, and has a

typical hemimetabolous life cycle. Using humans as hosts, it is the predominant vector of *Trypanosoma cruzi*, or Chagas disease (Dias, 1953). Each of the five larval stages, or instars, requires a single blood meal to initiate a series of developmental events leading to moulting, culminating in metamorphosis of fifth instar larvae (Buxton, 1930). As adults, a single blood meal will initiate a cascade of reproductive processes, resulting in egg production and ovulation in females (Buxton, 1930). Between blood meals, *R. prolixus* remain in a state of suppressed growth, metabolism, and reproduction that is analogous to developmental diapause (Wigglesworth, 1964). Development, metamorphosis, and reproduction resumes only when the insect receives a sufficient blood meal (Wigglesworth, 1972).

R. prolixus was pioneered as a principal model organism for the study of insect physiology and endocrinology (Wigglesworth, 1934). Additionally, *R. prolixus* is an ideal model organism for circadian studies because individuals in a population are developmentally and reproductively synchronized with each blood meal following hatching, allowing precise experimental control (Vafopoulou *et al*., 2010). The most substantial body of literature documenting the endocrinology and hierarchal organization of the circadian system in insects was established using *R. prolixus* as a model organism (Vafopoulou and Steel, 2006).

1.6.2 Regulation of Ecdysteroid Synthesis and Release by a Photosensitive Clock in the PGs

Ecdysteroids are essential developmental hormones of larval stages, regulating molting and metamorphosis. Ecdysone from the PGs is converted to 20-hydroxyecdysone (20E), the active form, by peripheral tissues and targets the epidermal cells to initiate the molting process (Gilbert *et al*., 1980; Riddiford, 1980). When the titer of a second hormone, juvenile hormone

(JH), declines following development, ecdysteroids initiate metamorphosis into the adult (Riddiford, 1980). Ecdysteroids are released with a circadian rhythm, and virtually all cells respond to changes in titer of hemolymph ecdysteroids during development (reviewed in Steel and Vafopoulou, 2006). The PGs are known to be the primary site of ecdysteroid synthesis and secretion in larvae, and the PG synthetic activity and hemolymph ecdysteroid titer undergo gradual changes of increase and decrease during a developmental cycle in *R. prolixus* following a blood meal (Steel *et al*., 1982). When hemolymph samples were taken multiple times a day, a significant daily oscillation in hemolymph ecdysteroid titer was observed, with peak levels during scotophase, and deep troughs during each photophase (Ampleford and Steel, 1985). These findings led to the notion of an endogenous circadian clock driving the daily oscillations in ecdysteroid titer, which was investigated using aperiodic conditions (Vafopoulou and Steel, 1991).

In continuous light (LL) environments, ecdysteroid rhythms generated by circadian clocks dampen after a few cycles, resulting in arrhythmic animals (Vafopoulou and Steel, 1993). This has been demonstrated in *R. prolixus;* in animals exposed to long-term LL conditions there is a loss in rhythmic ecdysteroid synthesis and release by the PGs. Transfer to continuous darkness, DD, serves as a lights-off cue, reinitiating ecdysteroid rhythms (Vafopoulou and Steel, 2001). When animals were transferred to either LL or DD, circadian rhythms in ecdysteroid titer persisted. Furthermore, in DD this free running rhythm persisted with a phase similar to the entrained state, a period of approximately 24 hours, and the free running period length was temperature compensated (Vafopoulou and Steel, 1991). Similar results were found when PGs were explanted and ecdysteroid titer was measured *in vitro* following transfer to DD. However, transfer to LL resulted in a phase reversal of ecdysteroid

synthesis and hemolymph titer indicating a second oscillator that regulates ecdysteroidogenesis in the absence of dark cues (Vafopoulou and Steel, 1991).

The finding that an endogenous circadian clock was located in the PGs was achieved by removing PGs from LL animals, incubating them in vitro in LL for several hours, followed by an abrupt transfer to DD. Ecdysteroid synthesis was stimulated by this "lights off" signal, and this was initiated and maintained by the PGs (in DD) in the absence of neural and hormonal input. It was concluded that the PGs possess a photosensitive clock that regulates ecdysteroid synthesis.

1.6.3 Regulation of Ecdysteroid Synthesis and Release from the PGs by Prothoracicotropic Hormone (PTTH)

PTTH is the central cerebral neurohormone that regulates growth and development in insects (Vafopoulou and Steel, 2012). PTTH has classically been considered a larval hormone, with the PGs being the only known target. In *R. prolixus* it was found that PTTH is released rhythmically on almost every day throughout development, rather than being confined to a brief period early in a developmental cycle (Vafopoulou and Steel, 1996). PTTH hemolymph titer peaked during scotophase when the brain would release one third of its PTTH content, which was shown to be replenished during the following photophase (Vafopoulou and Steel, 1996).

The PTTH peptides of *R. prolixus* are composed of 17kDa subunits, and have a high homology to PTTH of the silkworm, *Bombyx mori* (Vafopoulou and Steel, 2002). An *in vitro* PTTH assay was employed to quantify PTTH release, and this entailed incubating fifth instar

larval brains for several hours and then quantifying the content based on the ability of the medium to augment ecdysteroid synthesis by the PGs (Vafopoulou and Steel, 1996). With the use of anti-PTTH from *B. mori*, it was demonstrated that PTTH from *Rhodnius* is immunologically similar to *Bombyx* PTTH, as all biological activity from the PTTH assay medium was removed (Vafopoulou and Steel, 2002).

PTTH daily rhythms are also under circadian control. This was demonstrated by the findings that the free running period of PTTH release persisted for several cycles in DD and LL, and that transfer of larval animals raised in LL (which lack PTTH release, Vafopoulou and Steel, 2001) to DD initiated the rhythmic release of PTTH, which free ran in DD (Vafopoulou and Steel, 2001). In the 5^{th} instar of *R. prolixus*, two neurosecretory cells in the dorsolateral region of the protocerebrum (in each brain hemisphere) showed strong immunoreactivity to *Bombyx* anti-PTTH (Fig. 1.4), and a clear daily rhythm in the content of PTTH in the cell bodies was demonstrated by quantification of the intensity of staining by anti-PTTH (Vafopoulou and Steel, 2006; Vafopoulou *et al*., 2007). This rhythm corresponded with the rhythm of PTTH content of brains measured with the PTTH *in vitro* bioassay (Vafopoulou and Steel, 2001). Additionaly, a cluster of lateral clock neurons in close proximity to the optic lobe were identified using anti-PER, anti-TIM, and anti-PDF (see Section 1.4.2) (Vafopoulou *et al*., 2010). The axons of LNs are tripolar, with one branch transversing the protocerebrum in extremely close proximity to the two PTTH cell axons, indicating a neuroanatomical pathway for the circadian regulation of PTTH by the lateral clock neurons (reviewed in Steel and Vafopoulou, 2006).

While the autonomous PG clock continues to drive rhythmic synthesis and release of ecdysteroids *in vitro*, removal of PTTH signaling through decapitation or injection of a

sublethal dose of tetrodotoxin (TTX) in whole animals was found to cause a phase reversal of the rhythm of ecdysteroid release (Pelc and Steel, 1997). This parallels later findings, which demonstrated phase reversals in ecdysteroidogenesis when explanted PGs were maintained in LL (see Section 1.6.2) (Vafopoulou and Steel, 2001). The PGs are highly responsive to PTTH around the onset of scotophase, but not during photophase (Vafopoulou and Steel, 1999). Therefore, light and dark cues are capable of initiating rhythms in both PTTH release and ecdysteroid release independently in the brain and PG clock. However, PTTH is the dominant mechanism for entrainment of the PG clock, and acts to set the phase of ecdysteroidogenesis during development (reviewed in Steel and Vafopoulou, 2006).

The activation, or stimulation, of the PGs by PTTH to produce ecdysteroids occurs through intracellular signaling pathways. In the PG cells of *Manduca sexta*, PTTH signaling occurs through a G-protein coupled receptor (GPCR), triggering an influx of cytosolic Ca^{2+}, increased cAMP synthesis resulting in protein kinase A (PKA) activation, and other mechanisms leading to ecdysteroid synthesis (Rybczynski *et al*., 2001; Rybczynski and Gilbert, 2003; Fellner *et al*., 2005).

1.6.4 Regulation of Ecdysteroid Synthesis and Release from the PGs by Insulin and Insulin-like peptides (ILPs)

Insulin-like peptides (ILPs) regulate a multitude of biological and physiological events in insects including growth, development, metabolic processes, and reproduction. The first invertebrate ILP to be characterized, bombyxin II, was identified in the silkmoth, *B. mori* (Nagasawa *et al*., 1984). Bombyxin is a small neurosecretory hormone [a molecular weight

(MW) of 4400] that was found to be highly stimulatory on PGs in some insects, but not in *B. mori*, indicating that it was not the true PTTH of *B. mori* (reviewed in detail in Mizoguchi and Okamoto, 2013). To date, a total of 5 bombyxin species have been isolated from *B. mori* brains (Mizoguchi and Okamoto, 2013). In *D. melanogaster*, at least seven ILP genes have been identified (*dilp1-7*) (Brogiolo *et al*., 2001). In the brain of *B. mori*, bombyxin-producing neurosecretory cells have been localized to medial neurosecretory cells (MNCs) with axon terminals around the periphery of the corpus allatum (CA) (Mizoguchi *et al*., 1987).

In the brains of larval *R. prolixus*, binding of anti-bombyxin allowed for the localization of eleven ILP neurosecretory cells in a group of MNCs, as well as an additional four cells located laterally outside of the MNC area (Vafopoulou and Steel, 2012).

Upon intake of a blood meal, ILP fluorescence with anti-bombyxin in the neurosecretory cells decreased within four hours, and later increased in intensity and persisted throughout development and after ecdysis, indicating synthesis of ILPs (Vafopoulou and Steel, 2012). The presence of ILPs in the hemolymph of *R. prolixus* using dot blots and anti-bombyxin confirmed its release from the brain following a blood meal (Vafopoulou and Steel, 2012). Furthermore, the release of ILPs from the brain following a blood meal occurs with a clear daily rhythm. ILP fluorescence intensity peaks during scotophase, but decreases during mid-photophase, indicating a depletion of fluorescent material during mid- to late-scotophase (Vafopoulou and Steel, 2012).

Bombyxin II had a stimulatory effect on ecdysteroid synthesis by *R. prolixus* PGs *in vitro* in a dose dependent manner, but with much higher (40 fold) concentrations in comparison to *Bombyx* PTTH (Vafopoulou and Steel, 1997). In *B. mori*, high concentrations of bovine insulin stimulated ecdysteroidogenesis by the PGs during a long-term (24 h)

incubation period, in a dose dependent manner *in vitro* and *in vivo* (Gu *et al*., 2009). Stimulation of the PGs was blocked by an inhibitor of the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Gu *et al*., 2009). Interestingly, injection of anti-bovine insulin into fifth instar *R. prolixus* larvae immediately after feeding, and on specific days post feeding, prevented molting (Sevala *et al*., 1992). Extensive studies of the functional roles of insulin and ILPs, mediated in part through the insulin/insulin-like growth factor signaling pathway (IIS), in many insect species has illustrated functional similarities to the IIS in vertebrates (reviewed by Vafopoulou and Steel, 2015).

1.7 Circadian Rhythmicity of PTTH, ILPs, and Ecdysteroid Production in Adults

1.7.1 Regulation of PTTH and ILPs by the Adult Brain

Extensive research has shown that ecdysteroid biosynthesis and the factors regulating it are not restricted to larvae (Gilbert *et al*., 1980). In adult insects, which lack PGs following metamorphosis, ecdysteroids are still present and alternative sites of production have been described. Additionally, production (Vafopoulou *et al*., 2007) and release (Vafopoulou *et al*., 2012) of PTTH and ILPs (Vafopoulou and Steel, 2012) in adult *R. prolixus* brains has been demonstrated.

During the metamorphic molt cycle, the number of PTTH neurons in the lateral neurosecretory cell group increased from two (in unfed 5^{th} instar larvae) to five with axons

that project into the retrocerebral complex, which is comprised of the corpus cardiacum (CC) and corpus allatum (CA) (Fig. 1.4) (Vafopoulou *et al*., 2007). In comparison to two PTTH cells in each brain hemisphere of unfed larval brains and five PTTH cells in each brain hemisphere through larval-adult development, the brains of unfed adults contain four PTTH neurons in each brain hemisphere (Fig. 1.4) (Vafopoulou *et al*., 2007). Additionally, PTTH-immunofluorescence in the axons of PTTH cells appears rapidly following a blood meal, as in larvae, indicating transport of PTTH to be released (Vafopoulou *et al*., 2007). Circadian cycling in relative PTTH-immunofluorescence was found, with peak levels during the onset of scotophase and low levels in both axons and somata between late scotophase and early photophase, suggesting release of PTTH into the hemolymph (Vafopoulou *et al*., 2007). These findings imply an important role of PTTH in the circadian organization of the adult, as it does in larvae (Vafopoulou and Steel, 2007).

The close association of larval PTTH neurons with PDF immunoreactive axons is maintained in adults (see Section 1.6.3) (Vafopoulou *et al*., 2007), indicating that circadian control of PTTH persists in adults (Vafopoulou *et al*., 2007). More recently, it was shown that PTTH is released from the adult brain, and the adult PTTH is closely similar to that of larvae in molecular weight (multiples of 17 kDa subunits), immunoreactivity, and biological activity on the PGs (Vafopoulou *et al*., 2012).

In the brains of *R. prolixus*, the number of ILP cells, identified using anti-Bombyxin II (see Section 1.6.4) increased from 15 (3 groups) in larvae to 24 (4 groups) in adults in each brain hemisphere (Vafopoulou and Steel, 2012). In addition to 19 cells located in the MNC, five small ILP-immunoreactive neurons in the nervus corpus cardiacum (NCC) develop in the adult (Vafopoulou and Steel, 2012). ILP-immunofluorescent axons terminate in the CC and

CA, with terminal varicosities in these complexes suggesting that ILPs are released from this complex. ILP immunoreactive neurons are located in close proximity with the LNs, and rhythmic changes in ILP fluorescence intensity within cell bodies were shown, with depletion of immunoreactive material occurring during late scotophase or early photophase after feeding and continuing throughout reproduction (Vafopoulou and Steel, 2012). Therefore, the production and release of PTTH and ILPs are tightly coupled to the central clock in the brain, and rhythms in these circulating brain neurohormones may orchestrate the timing of cellular responses in diverse tissues in adults, as in larvae.

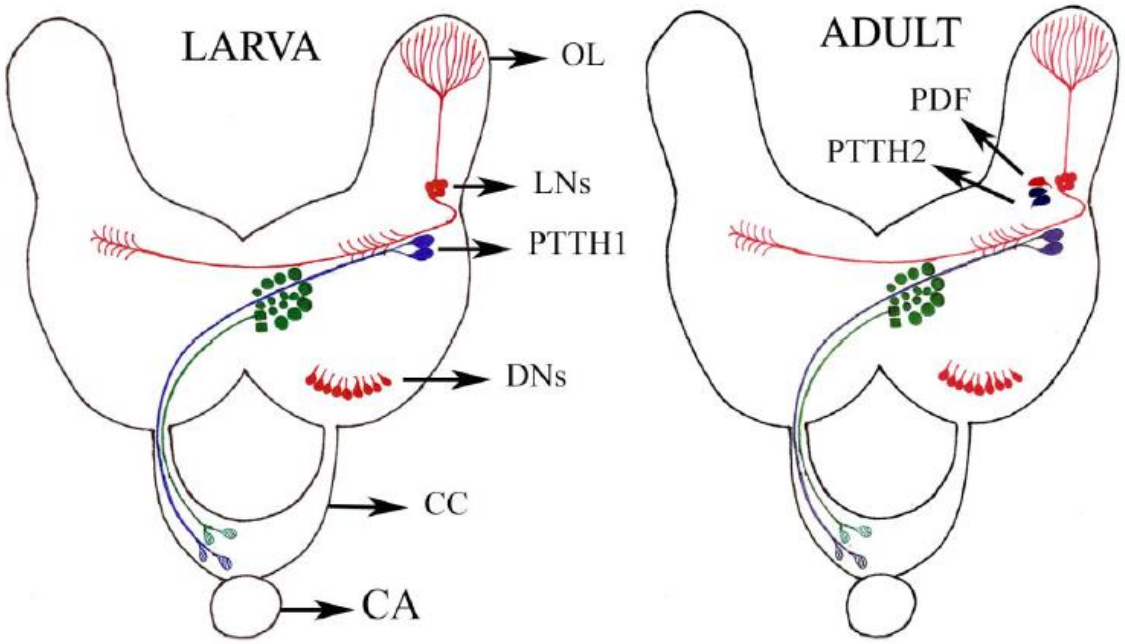

Figure 1.4 Schematic illustration of the timekeeping system in the brains of larval and adult *R. prolixus* (from Vafopoulou et al., 2007). The regions of the optic lobe (OL), corpius cardiacum (CC), and corpus allatum (CA) are indicated. The CC and CA comprise the retrocerebral complex, which is the site of release of many neurosecretory hormones from neuroendocrine cells (shown in green). In larva, the lateral clock neurons (LNs) (shown in red) are found in close proximity with a single pair of PTTH neurons (indicated by PTTH1). The location of dorsal clock neurons (DNs) is indicated, but the details of projections are not known. In the adult brain, an additional pair of PTTH neurons (indicated by PTTH2) is seen. Additional PDF-immunoreactive neurons (used to trace axons of clock neurons) also appear (indicated by PDF). ILP positive neurons were immunolocalized in the dorsal protocerebrum (not shown) (Vafopoulou and Steel, 2012).

1.7.2 Circadian Regulation of Ovarian Ecdysteroids

Ecdysteroids in adult insects are regarded as possible insect sex steroid hormones; they regulate cell growth in egg follicles, and promote cell division in spermatocytes (De Loof *et al*., 2001). The ovary of *R. prolixus* is the main source of hemolymph ecdysteroids in adult females during egg development (Cardinal-Aucoin *et al.,* 2013). While the exact location and function of ovarian ecdysteroidogenesis in *R. prolixus* has not been elucidated, initial studies in a few lepidopteran species demonstrated ecdysteroid secretion by ovarian follicle cells (Zhu *et al*., 1983).

Ovarian ecdysteroids have numerous roles in adult insects, in addition to initiation of vitellogenesis (Brown *et al*., 2009). Ecdysteroids are converted to various conjugates, which are incorporated into the developing oocyte where they are deconjugated and may function in activating and regulating early embryogenesis (Lagueux *et al.*, 1979). In *R. prolixus*, ecdysone (E) and 20-hydroxyecdysone (20E) were the only free ecdysteroids found in the hemolymph (Cardinal-Aucoin *et al.,* 2013). The concentration of these immunoreactive ecdysteroids was 7-8 fold higher within the ovary compared to levels in the hemolymph, but it is unknown whether this represents *de novo* synthesis within the ovary, or a deconjugation of stored ecdysteroids (Cardinal-Aucoin *et al*., 2013).

In adult females, a blood meal initiates a cycle of egg development (Buxton, 1933). A daily rhythm in hemolymph ecdysteroid titer, as well as release of ecdysteroids by ovaries *in vitro* occurs within hours after a blood meal and continues throughout a 10-day sampling period over the course of egg development (Cardinal-Aucoin *et al*., 2013). These rhythms appear to be under circadian control, persisting for three 24-hour cycles following transfer of

the insects to LL or DD. The ovaries appear to be the major contributor to hemolymph ecdysteroid titer in adult *R. prolixus*, as the ovarian ecdysteroid content and their *in vitro* release of ecdysteroids were enough to account for the levels seen in the hemolymph (Fig. 1.5) (Cardinal-Aucoin *et al*., 2013). The presence of the major developmental hormones, including PTTH, insulin-like peptides (ILPs), and ecdysteroids persisting in the adult insect and released with a daily circadian rhythm, invites comparison to the larval circadian control system. As previously mentioned, the adult and larval brains of *R. prolixus* contain the same machinery for circadian regulation of PTTH and ILPs, which are produced and released in high quantities throughout egg production (Vafopoulou and Steel, 2012; Vafopoulou *et al*., 2012).

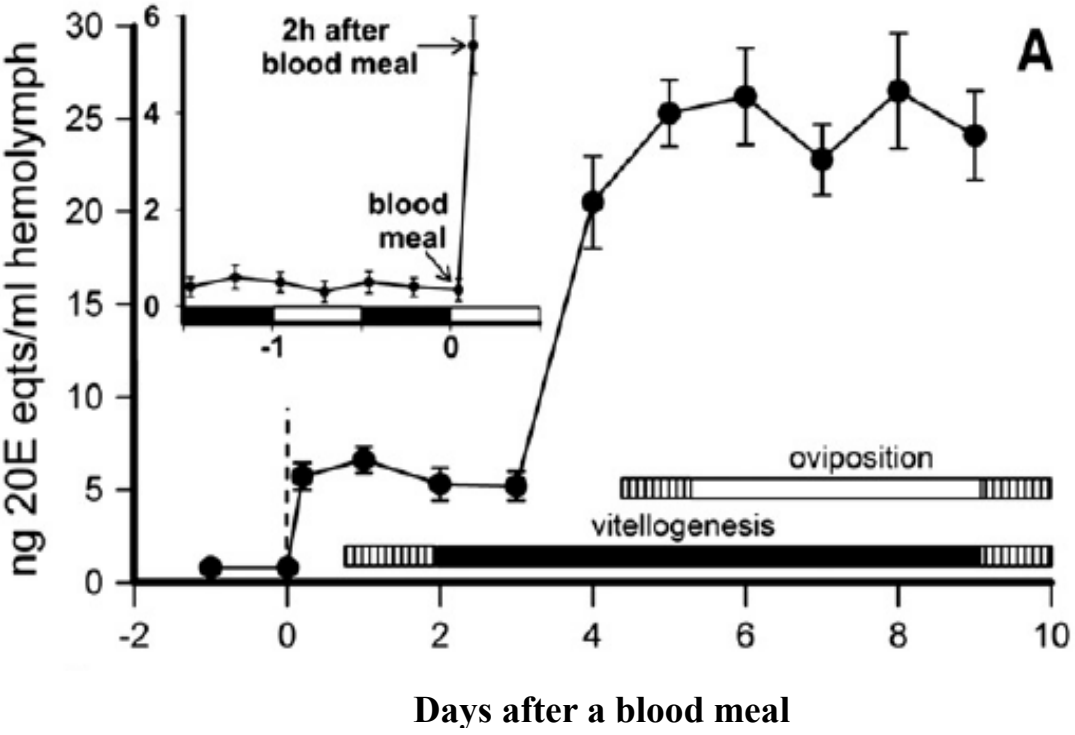

Figure 1.5 Circulating ecdysteroid levels in hemolymph during egg development following a blood meal, termed Day 0 (dashed line) (from Cardinal-Aucoin et al., 2013). The x-axis indicates days before and after a blood meal was given. Period of vitellogenesis and oviposition is indicated by the solid bar (Huebner and Anderson, 1972a,b,c). Ecdysteroid levels increase within 2 h following a blood meal, and much higher levels of ecdysteroids occur around Day 4 following a blood meal. Vitellogenesis and oviposition continue for 10 days after a blood meal.

1.8 PER in Ovarian Follicle Cells in Insects

In adult female *D. melanogaster*, the follicular cells surrounding previtellogenic ovarian egg chambers contain high levels of cytoplasmic PER and TIM (Kotwica *et al*., 2009). In follicle cells surrounding oocytes at the onset of vitellogenesis, PER localization in the nucleus was observed and later disappeared in more advanced vitellogenic follicles. Similarly, DBT was present in the cytoplasm of follicle cells at the onset of vitellogenesis, coinciding with nuclear localization of PER protein in those cells (Kotwica *et al*., 2009). These findings demonstrated a non-circadian, developmental pattern of PER in ovarian follicle cells of *D. melanogaster*.

More recently, the presence of PER in follicle cells of the *R. prolixus* ovary has been investigated (Hajia Yakubu, unpublished results). The first seven days of a reproductive cycle were examined, and the localization of PER in the nucleus of follicle cells was observed only on the fifth day (during scotophase), with decreased levels of PER in the nucleus on the sixth day. At this point, terminal oocytes had ended vitellogenesis (Huebner and Anderson, 1972a,b,c). These findings led to the notion that PER is developmentally regulated in ovarian follicle cells in *R. prolixus*, and its presence is not a manifestation of a circadian rhythm.

1.9 Objectives

The purpose of this study was to elucidate the regulatory mechanisms that control ovarian release of ecdysteroids during egg production in *R. prolixus*. A parallel axis of neurohormonal rhythms and ecdysteroid rhythms in larvae and adults leads to the possibility of neuroendocrine control (PTTH, ILPs) of ovarian ecdysteroid production and release in *R. prolixus*. Furthermore, circadian rhythmicity of ovarian ecdysteroids could be driven by rhythmic inputs originating outside of the ovary, or controlled by an ovarian clock. Recent work from our lab detected non-cyclical PER in ovarian follicle cells during early vitellogenesis, suggesting a non-circadian function of this protein in the ovary. It was hypothesized that (1) PER will appear at a specific developmental stage in each mature oocyte, and this phenomenon will not be abolished in LL animals. Further, it was predicted that (2) ecdysteroid release by ovaries *in vitro* is not photosensitive, and (3) the rhythm of ecdysteroid release is controlled by neurohormonal factors. Therefore, the objective of this study was to investigate the effects of neurohormonal treatment on ecdysteroid release by ovaries, as well as the presence of nuclear PER in both arrhythmic (LL) and entrained (LD) animals at specific developmental stages during late vitellogenesis (days 8-10).

II. MATERIALS AND METHODS

2.1 Animals and tissue collection:

Adult female *R. prolixus* were reared in a 12 h light:12 h dark (LD) cycle at 28 ± 0.5 °C. Animals that are fed a single blood meal initiate a synchronous cycle of egg development within a population. The day of feeding is referred to as day 0. Females that had undergone one cycle of egg production were used throughout.

For circadian studies, adult females were reared in continuous light (LL) at 28 ± 0.5 °C. Insects were transferred from LL to DD, with half of the population remaining in LL, on Day 5 after feeding, and ovaries were excised one hour after transfer and then incubated for 4 hours in *R. prolixus* saline (Lane *et al*., 1975). Incubation of photophase and LL ovaries was performed in light and incubation (including removal/replacement of media) of scotophase and DD ovaries was performed in darkness. Following incubation, media were removed and stored in methanol at -20 °C until assayed by RIA. For circadian studies, a minimum of 5 insects was sampled per treatment.

Ovaries used for assays of different neurohormonal treatments were excised under saline on specific days after feeding, as specified. One ovary from each animal was incubated with test medium for 4 hr (herein referred to as Experimental), and the contralateral ovary was incubated in saline as a control (unless stated otherwise). For neurohormonal treatments, ovaries from 3 to 5 animals (as specified) were pooled together in groups to obtain measurable amounts of ecdysteroids. A minimum of 5 groups of ovaries was sampled per treatment.

2.2 Immunohistochemistry and Antibodies:

Ovaries were prepared following an established protocol (Vafopoulou and Steel, 2007). Ovaries were excised in *R. prolixus* saline and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.5; Tsang and Orchard, 1991) for 2 h in darkness, then washed in three 15 min changes of PBS. Ovaries were then incubated for one hour in blocking solution, consisting of PBS, 4.0% normal serum, and 4% Triton X-100 to permeabilize the tissues and minimize non-specific antibody binding. Tissues were washed in three 15 min changes of PBS and then incubated with primary antibody against PER and EcR (see below) at 4°C for 24 h. Tissues were washed in three 15 min changes of PBS, and then incubated with secondary antibody (see Antibodies) for 2 h. Following washes in three 15 min changes of PBS, tissues were mounted in mounting medium, composed of 90% glycerol in PBS with 1% 1,4-diazabicyclo[2,2,2,]-octane (DABCO) (Sigma-Aldrich). In technical controls, ovaries were incubated in non-immune serum in primary buffer, with no other changes. No immunostaining was observed and autofluorescence was not detected.

Digital optical sections at 1.5μM intervals were visualized using an Olympus FV300 confocal laser scanning microscope. Microscope parameters (offset, gain, PMT) were kept constant. Images were processed using ImageJ 1.41 (NIH) and Adobe Photoshop 7.0.

A custom made (Genscript, NJ) purified guinea pig polyclonal antibody raised against a synthetic peptide corresponding to the conserved (protein-protein dimerization motif) of the PAS region of PER (residues 605-618; KSSTETPPSYNQLN; known as PER-S) was used for PER immunolabelling. This antibody recognizes the native PER of *Rhodnius* (Vafopoulou *et al.*, 2010), and was used at a dilution of 1:200 in primary buffer (PBS, 0.4% Triton X-100, 4.0% normal serum, and 0.1% NaN_3) for all experiments.

EcR 9B9 mouse monoclonal antibody against the *Manduca sexta* EcR purchased from the Developmental Studies Hybridoma Bank (University of Iowa), was raised against the common region of the molecule (ecdysteroid receptor) (described in Vafopoulou, 2009). The present work employs this antiserum only as a tool to outline plasma and nuclear membranes, which is not distinguishable with anti-PER staining alone. EcR 9B9 was used at a dilution of 1:1000 in primary buffer (PBS, 0.4% Triton X-100, 4.0% normal serum, and 0.1% NaN_3) for all experiments. The secondary antibodies goat anti-guinea pig IgGs conjugated to the green fluorophore fluorescein isothiocyanate (FITC) and goat anti-mouse IgGs conjugated to the bright orange flurophore tetramethylrhodamine (TRITC) (both purchased from Sigma-Aldrich) were used at a 1:200 dilution in secondary buffer (PBS, 10% normal serum, and 0.1% NaN_3) in all experiments.

2.3 In vitro Assay for Brain Extract:

Brain-retrocerebral complexes from fifth instar larvae were excised and washed thoroughly in *R. prolixus* saline (Lane *et al.,* 1975) on Day 14 after a blood meal. Brains from fifth instar larvae were used because the neuroarchitecture of the timing system has been more fully characterized in comparison to adult brains. Furthermore, there are technical difficulties associated with excision of adult brains. Brain complexes were excised from donors in late-photophase (2 hr before lights off) and early-scotophase (1-2 hr after lights on) and were pooled in groups of 10 and homogenized in 150µl saline on ice. The homogenate was immediately heat-treated at 100°C for 3 min, and centrifuged at 10,000 x *g* for 10 min. The supernatant was removed and stored at -80°C until use. The activity of an extract was assayed by its ability to stimulate ecdysteroid release by *Rhodnius* adult ovaries *in vitro.* The

contralateral control ovaries were incubated in saline. Samples of brain extract were also assayed to ensure that no ecdysteroids were present (data not shown). All data on ecdysteroid stimulating activity are expressed as the effect of one brain equivalent.

2.4 Fractionation of Brain Extract:

Fractionation of whole brain extract was achieved by ultrafiltration (Microcon 10 YM membrane, Amicon; 10 kDa cut off). Ultrafiltration at 15000 x g for 15 min was performed after samples of whole brain extract were homogenized on ice, boiled at 100°C and then centrifuged at 10,000 *g* for 10 min (see above). Retenates (>10 kDa fractions) were reconstituted to the starting volume of 150µl using saline, and filtrates (<10 kDa fractions) were reconstituted to the starting volume of 150µl using ddH_2O. "High" and "low" molecular weight fractions were tested for their ability to stimulate ecdysteroid release by ovaries from adult female *R. prolixus in vitro*. All data on ecdysteroid stimulating activity are expressed as the effect of one brain equivalent.

2.5 Extraction of ecdysteroids from ovaries (ovarian content):

Ovaries were homogenized in 25 µl water in a 1.5 ml graduated microtube. An additional 200 µl water, 525 µl methanol, and 750 µl hexane was added, and the mixture was vortexed and then centrifuged at 10 000 g for 5 min. The upper (lipid) fraction was removed and the aqueous fraction, containing the ecdysteroids, was stored at -20 °C until assayed by RIA. The procedure was standardized to account for any loss when removing the lipid phase, by removing a constant volume of the remaining aqueous phase (0.25 ml from 0.75 ml of the aqueous phase) from each sample. Therefore, each sample contained exactly 0.5 ml of the

aqueous phase, which was assayed by RIA. This standardized procedure has been shown to result in a mean loss of ecdysteroids of 25% (meaning an ecdysteroid extraction efficiency of approximately 75%) (described in Cardinal-Aucoin *et al*., 2013). The data presented (ovarian ecdysteroid content) have not been corrected for this mean loss of ecdysteroids of 25%.

2.6 Peptides:

Recombinant *Bombyx* PTTH (rPTTH) (expressed in *E. Coli*) (gift from A. Mizoguchi; Nagoya University, Nagoya, Japan) was used at a 6×10^{-10} M dilution in all experiments. This peptide at the concentration stated above exhibits steroidogenic activity on the PGs of *Rhodnius* (Vafopoulou and Steel, 1997). Controls were incubated in saline with 0.04% BSA. Bovine insulin in 0.1% acetic acid (pH=2.8) in *R. prolixus* saline was used at dilutions of 2.8×10^{-7} M and 1.7×10^{-6} M, where specified. The working concentrations of bovine insulin were determined based on a dose response curve of Bombyxin-II on *R. prolixus* PGs, which showed significant stimulation elicitied by concentrations of 80 ng/ml to 320 ng/ml, and an increased response was seen using concentrations up to 1.7×10^{-6} M (Vafopoulou and Steel, 1997). Controls were incubated in 0.1% acetic acid (pH=2.8) in *R. prolixus* saline. Human recombinant insulin expressed in *E. coli* (Humulin-R, a kind gift from Dr. Gary Sweeney; York University, Toronto, Canada) was diluted in *R. prolixus* saline at a working concentration of 2.8×10^{-7} M. Corazonin (a kind gift from Dr. Ian Orchard, University of Toronto Mississauga, Mississauga, Ontario) was used at a working concentration of 10^{-6} M.

2.7 Ecdysteroid quantification

Ecdysteroids were quantified by RIA, as previously described (Steel *et al*., 1982). H-21B antiserum which recognizes ecdysone and 20E, was used to quantify ecdysteroids. The antiserum was a kind gift from Dr. Ernest F. Chang (University of California, Davis, CA, USA). The labeled ligand was α-[23,24-3H(N)]ecdysone (sp. act. 88.2 Ci/mmol; 1Ci=37 GBq) (PerkinElmer, Billerica, MA, USA). Results are expressed as ng 20E equivalents, since 20-hydroxyecdysone (20E) (Sigma) was used as the standard.

2.8 Statistical Analyses

Stimulation of ecdysteroid release by neurohormones was measured as the difference in the amount of ecdysteroid release between control and experimental members of ovaries. Results are expressed as the mean difference (± SEM) (in ng 20E equivalents) between control and experimental groups of ovaries. Differences were analyzed using the Mann-Whitney U Test (Rank Sum Test). *: indicates significance ($p < 0.05$), versus control.

2.8 Trypan Blue Exclusion Assay

In this exclusion assay, live cells will exclude the dye, whereas dead cells will take up the dye and will appear blue (Strober, 2001). This assay has been used in previous studies examining vitellogenin uptake by ovaries from *Aedes aegypti* following a blood meal, which showed that trypan blue surrounds but does not penetrate living follicle cells of ovaries *in vitro* (Yonge and Hagedorn, 1977). This assay was employed to ensure that follicle cells in incubation media were viable for the length of the incubation period used. Pairs of ovaries were excised and incubated in either *R. prolixus saline*, 0.1% acetic saline, or 0.4% BSA in

saline for up to 24 hours. At 1, 4, 8, and 24-hour intervals, ovaries were carefully submerged in 4% Trypan blue for 30 seconds. Ovaries were then rinsed and viewed under a light microscope. Follicle cells were viable (for up to 12 hours) in each incubation medium (see Appendix).

III. RESULTS

3.1 Is a Light Cue Detected by Ovaries and Does this Effect Ecdysteroid Release?

Transfer of ovaries from LL animals to DD *in vitro* was used as a test protocol to investigate the effects on ovarian ecdysteroid release by a "lights-off" signal. *Rhodnius* PGs synthesize and secrete high levels of ecdysteroids in response to a signal of darkness (Vafopoulou and Steel, 1992). In the present study, ovaries from arrhythmic LL animals were excised and incubated *in vitro*. One member of the ovary pair was immediately placed in DD and the other member of the ovary pair was incubated in LL. Removal of incubation medium and replacement of fresh saline (using far-red lighting for ovaries in DD) occurred at 1 and 7 hours of incubation. After one hour of incubation, the level of ecdysteroid release of ovaries placed in DD (grey bar) was relatively the same as the LL controls (black bar) (Fig. 2.1). After 7 hours in incubation, the level of ecdysteroid release by ovaries in DD was not significantly different than the release of LL controls (Fig. 2.1). Sampling at later time points would be unreliable, as the tissue *in vitro* begins to degenerate after 12 hours (See Section 2.7).

3.2 Nuclear PER Localization During Egg Development in Entrained (LD) and Arrhythmic (LL) Animals

The presence of nuclear PER in follicle cells of terminal oocytes from two different cycles (each occurring around Day 5 and Day 8 following a blood meal) of vitellogenesis within ovarioles during egg development was examined. Around Day 5, many terminal

oocytes within each ovariole have just completed vitellogenesis and are beginning secondary coat formation. Around Day 8, a second (or even third) set of terminal oocytes undergoes vitellogenesis and secondary coat formation. Terminal oocytes in ovaries from LD animals examined on Day 5 (Fig. 2.2A), and Day 8 (Fig. 2.2B), showed nuclear localization of PER (for detailed microscopic anatomy of follicle cells see Huebner and Anderson, 1972a). Widespread PER was observed in many terminal oocytes during photophase on both days, but not at other time points examined (images not shown). Interestingly, ovarian follicle cells of terminal oocytes from LL animals, examined on Day 5 post blood meal, exhibited the same pattern of nuclear PER presence as was observed in LD animals (Fig. 2.3A). EcR immunostaining was localized to the cytoplasm of follicle cells on all days examined. No autofluorescence and immunostaining of secondary antibodies was observed in negative controls (Fig. 2.3B).

3.3 Ecdysteroid Release by Ovaries from Entrained (LD) and Arrhythmic (LL) Animals and Ovarian Ecdysteroid Content from Unfed LD Animals

Initial experiments aimed to verify that the endogenous variation in ecdysteroid release between contralateral ovary pairs was insignificant. Left and right ovaries from LD animals on Day 8 following a blood meal released very similar amounts of ecdysteroids *in vitro* (Fig 2.4A). This was also true for ovaries from LL animals on Day 2 following a blood meal (Fig 2.4B), however more variability in levels of ecdysteroid release between animals was observed, in comparison to LD animals. Based on the findings that contralateral ovaries release the same amount of ecdysteroids *in vitro*, assays investigating the effects of

neurohormonal treatment employed this method using one ovary as a control for the contralateral experimental ovary. Additionally, the ovarian content of ecdysteroids from unfed LD animals was almost undetectable (Fig 2.4C), with levels of ecdysteroids similar to the *in vitro* release of ovaries from unfed animals incubated in saline as controls.

3.4 Effect of Whole Brain Extract on Ecdysteroid Release by Ovaries from Arrhythmic (LL) and Entrained (LD) Animals in vitro

Ovaries from arrhythmic (LL) animals were initially used to examine the effect of brain extract on ecdysteroid release because the ecdysteroid rhythm is abolished in these animals (See Section 1.6.2). Furthermore, the release of PTTH from the brain ceases in LL (Vafopoulou and Steel, 2001). The expectation was that neurohormones have an action equivalent to darkness (not of light), which has been demonstrated in larval *R. prolixus.* PGs from LL animals transferred to DD *in vitro* abruptly synthesized high levels of ecdysteroids (Vafopoulou and Steel, 2002). PTTH from the brains of fifth instar larvae *Rhodnius* had the same effect on PGs from LL animals, stimulating ecdysteroid release *in vitro* (Vafopoulou and Steel, 1996).

Ovaries from LL animals on Day 2 following a blood meal were examined, because around this day ovaries (from LD animals) are releasing relatively low levels of ecdysteroids in comparison to later days in egg development (Fig. 1.5). Therefore, the amount of ecdysteroid secretion by the ovaries into the hemolymph on Day 2 is not yet at a maximum and may be responsive to whole brain extract to further secrete ecdysteroids. Additionally,

ecdysteroid release by ovaries from LL animals on Day 4 was examined as a comparison because at this point, ecdysteroid secretion into the hemolymph is at a maximum (Fig. 1.5). The present data shows that ovaries from LL animals on Day 2 post blood meal treated with whole brain extract exhibited a significant release of ecdysteroids *in vitro* ($p < 0.05$) (Fig. 2.5A). The effect of whole brain extract on Day 4 ovaries from LL animals was not significant (Fig. 2.5B).

Surprisingly, a high degree of variability in the levels of ecdysteroid release between groups of ovaries from LL animals was observed (Fig. 2.5), similar to what was observed in control experiments (Fig. 2.4). To test whether this variability was a consequence of LL, experiments were repeated using ovaries from LD animals to see if the variability could be reduced. Furthermore, ovaries from unfed and newly fed animals were examined, limiting the amount of external and internal variables involved during egg development following a blood meal.

In ovaries from unfed and newly (2 hr post blood meal) fed LD animals, whole brain extract had a highly significant stimulatory effect ($p = 0.002$ and $p < 0.001$, respectively; Fig. 2.6). Levels of stimulation of ecdysteroid release by ovaries from newly fed animals were similar to that of ovaries from unfed animals (compare Fig. 2.6A with 2.6B, whole brain extract). Additionally, the levels of ecdysteroid release were much less variable compared to those from LL animals (Fig. 2.5). Interestingly, the content of ecdysteroids in ovaries from unfed animals is extremely low (Fig 2.4C), and is not enough to account for the high amounts of ecdysteroids being released upon incubation with whole brain extract (see Discussion). Results are shown as the activity of whole brain extract from one brain complex per experimental ovary.

3.5 Effect of Fractionated Brain Extract on Ecdysteroid Release of Ovaries from Entrained (LD) Animals in vitro

Fractionated brain extracts corresponding to ILP-like and PTTH-like activity also exhibited a stimulatory effect on ecdysteroid release of ovaries from newly (2 hr) fed animals ($p = 0.008$ and $p<0.001$, respectively; Fig. 2.7). The <10 kDa fraction (ILP-like activity) was much more stimulatory (12x fold) than the >10 kDa fraction (PTTH-like activity) (compare Fig. 2.7A with Fig. 2.7B). Levels of ecdysteroid release by ovaries from newly fed animals in response to each brain fraction were much higher in comparison to the content of ecdysteroids in unfed ovaries, as was seen with treatment of whole brain extract. Results are shown as the activity of fractionated brain extract from one brain complex per experimental ovary.

3.6 Effect of Bovine Insulin and Bombyx rPTTH on Ecdysteroid Release by Ovaries from Arrhythmic (LL) Animals in vitro

Because fractionated brain extracts corresponding to ILP-like and PTTH-like activity stimulated ecdysteroid release in LD ovaries (Fig. 2.7), the effects of hormones related to insulin-like peptides and PTTH (either structurally, functionally, or both) on ecdysteroid release was examined. Bovine insulin at concentrations of 2.8×10^{-7} M (Fig. 2.8A) and 1.7×10^{-6} M (Fig. 2.8B) did not have an effect on ecdysteroid release by ovaries from LL animals on Day 3 post blood meal. Similarly, on Day 4 following a blood meal, no significant effect on ecdysteroid release with 2.8×10^{-7} M (Fig. 2.9A) and 1.7×10^{-6} M (Fig. 2.9B) bovine

insulin was observed. The findings parallel what was seen in ovaries from unfed and newly fed LD animals incubated with bovine insulin (Fig. 2.11). Interestingly, *Bombyx* rPTTH at a concentration of 6×10^{-10} M did not affect ecdysteroid release on Day 2 (Fig. 2.10A) or Day 4 (Fig. 2.10B) post blood meal.

3.7 Effect of Bovine Insulin, Humulin-R, and Corazonin on Ecdysteroid Release of Ovaries from Entrained (LD) Animals in vitro

Bovine insulin at a concentration of 2.8×10^{-7} M did not affect ecdysteroid release by ovaries from unfed (Fig. 2.11A) or newly (2 hr) fed animals (Fig. 2.11B) *in vitro*. Similarly, Humulin-R at a concentration of 2.8×10^{-7} M had no significant effect on ecdysteroid release by ovaries from unfed (Fig. 2.12A) and newly (2 hr) fed (Fig. 2.12B) animals, and levels of ecdysteroids were almost undetectable. *R. prolixus* corazonin (10^{-6} M) did not stimulate the ovaries from unfed (Fig. 2.13A) and newly (2 hr) fed (Fig. 2.13B) animals to release ecdysteroids when incubated *in vitro.*

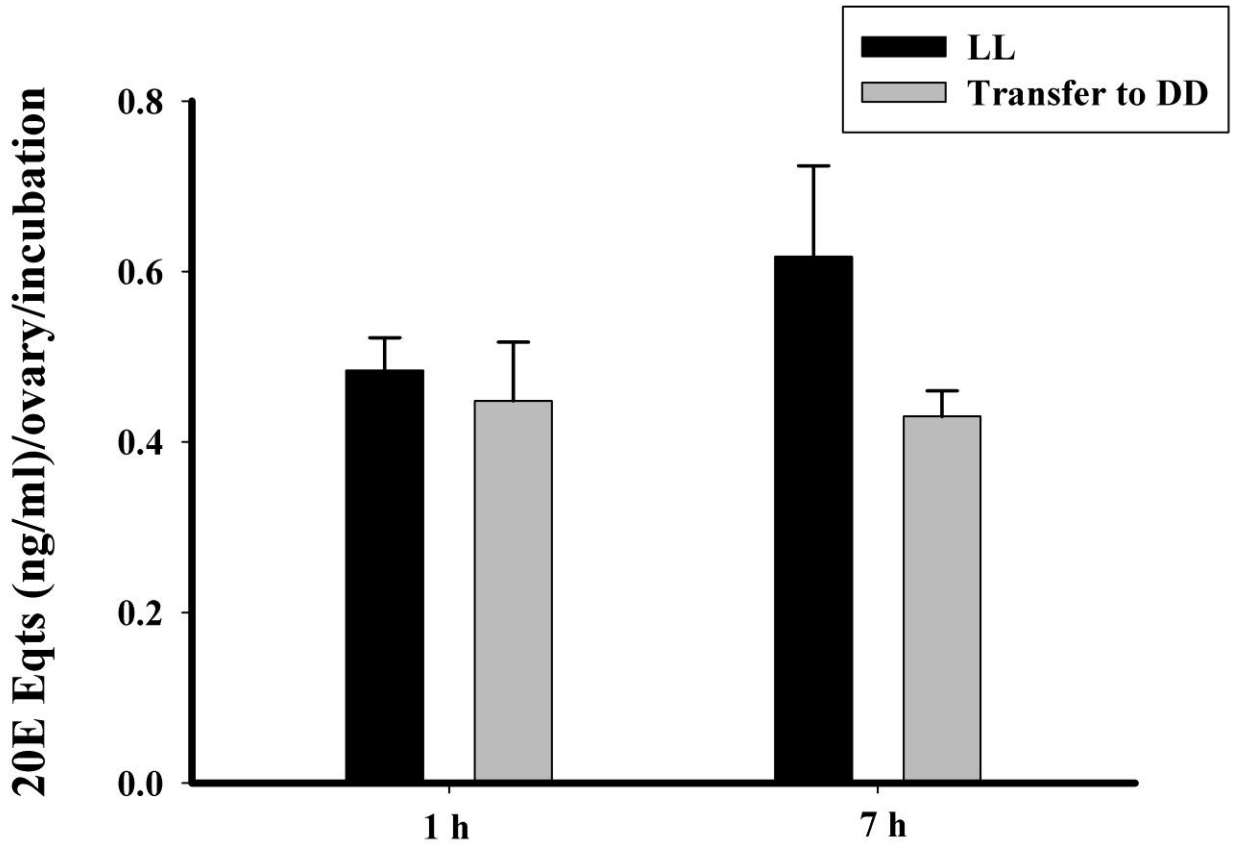

Figure 2.1 Ecdysteroid release of ovaries from LL animals transferred to DD on Day 5 following a blood meal, excised and incubated in saline, with one member of each pair transferred to DD immediately after excision. Medium was removed 1 hr and 7 hr after transfer, and stored at -20°C until assayed by RIA. No significant difference in levels of ecdysteroid release between ovaries incubated in DD and LL controls was observed. Data expressed as mean ± SEM (n=5-6).

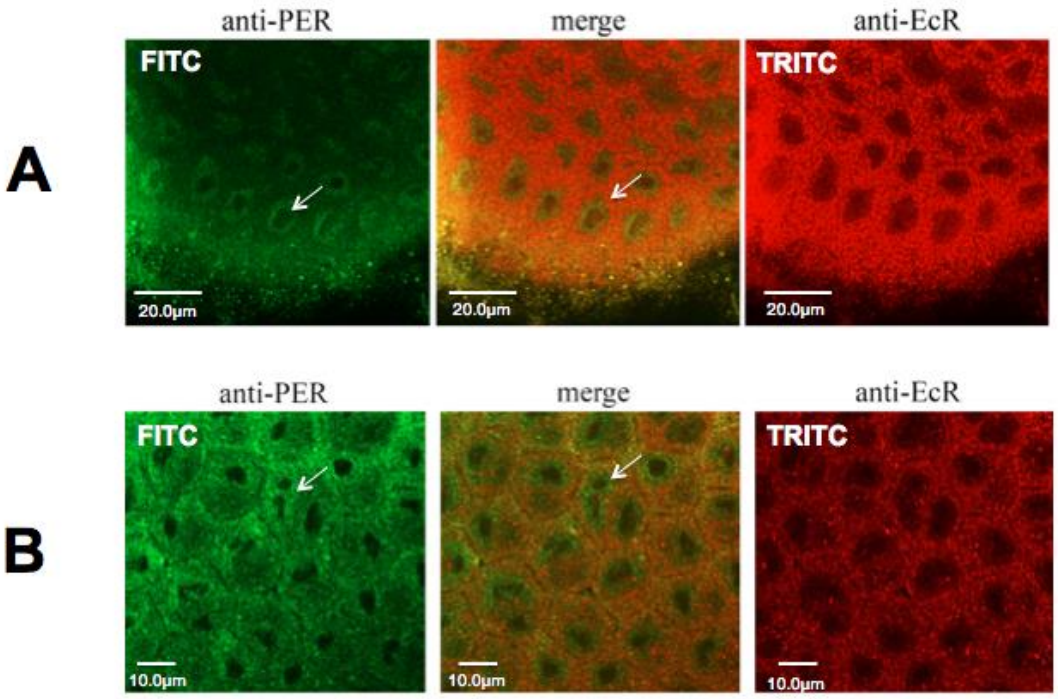

Figure 2.2 Developmental pattern of nuclear PER presence in ovarian follicle cells of terminal oocytes during vitellogenesis in entrained (LD) animals. Follicle cells were double stained with anti-PER (left column) and anti-EcR (right column). A merge of anti-PER and anti-EcR is shown in the middle column. **(A)** Nuclear PER localization in follicle cells of ovaries excised from animals during mid-photophase on Day 5 and **(B)** follicle cells of ovaries excised from animals during mid-photophase on Day 8 post blood meal (n = 70 ovarioles for each day).

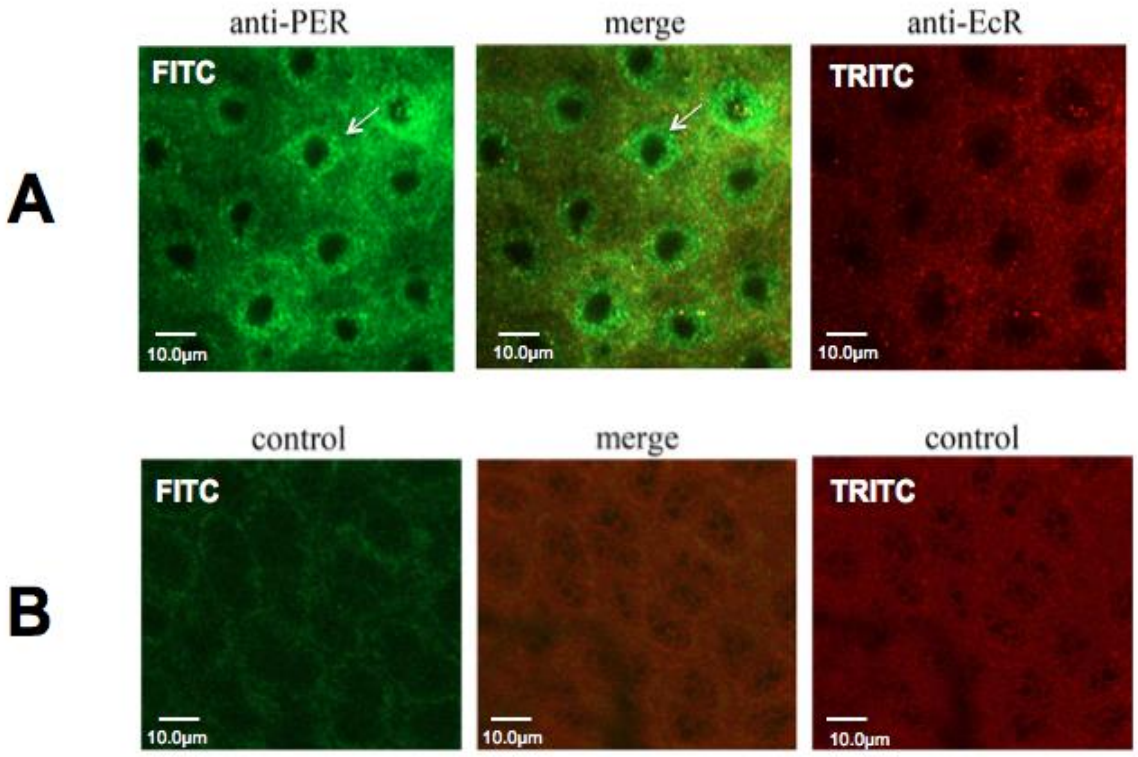

Figure 2.3 Nuclear PER in terminal oocytes from arrhythmic (LL) animals on Day 5 following a blood meal. Follicle cells were double stained with anti-PER (left column) and anti-EcR (right column). A merge of anti-PER and anti-EcR is shown in the middle column. **(A)** Nuclear PER surrounding condensed chromatin in follicle cells. **(B)** Negative controls in which 1° buffer and 2° antibody were used (n = 28 ovarioles). Negative controls are representative of all immunohistochemistry studies. White arrows point to the presence of nuclear PER (n = 70 ovarioles).

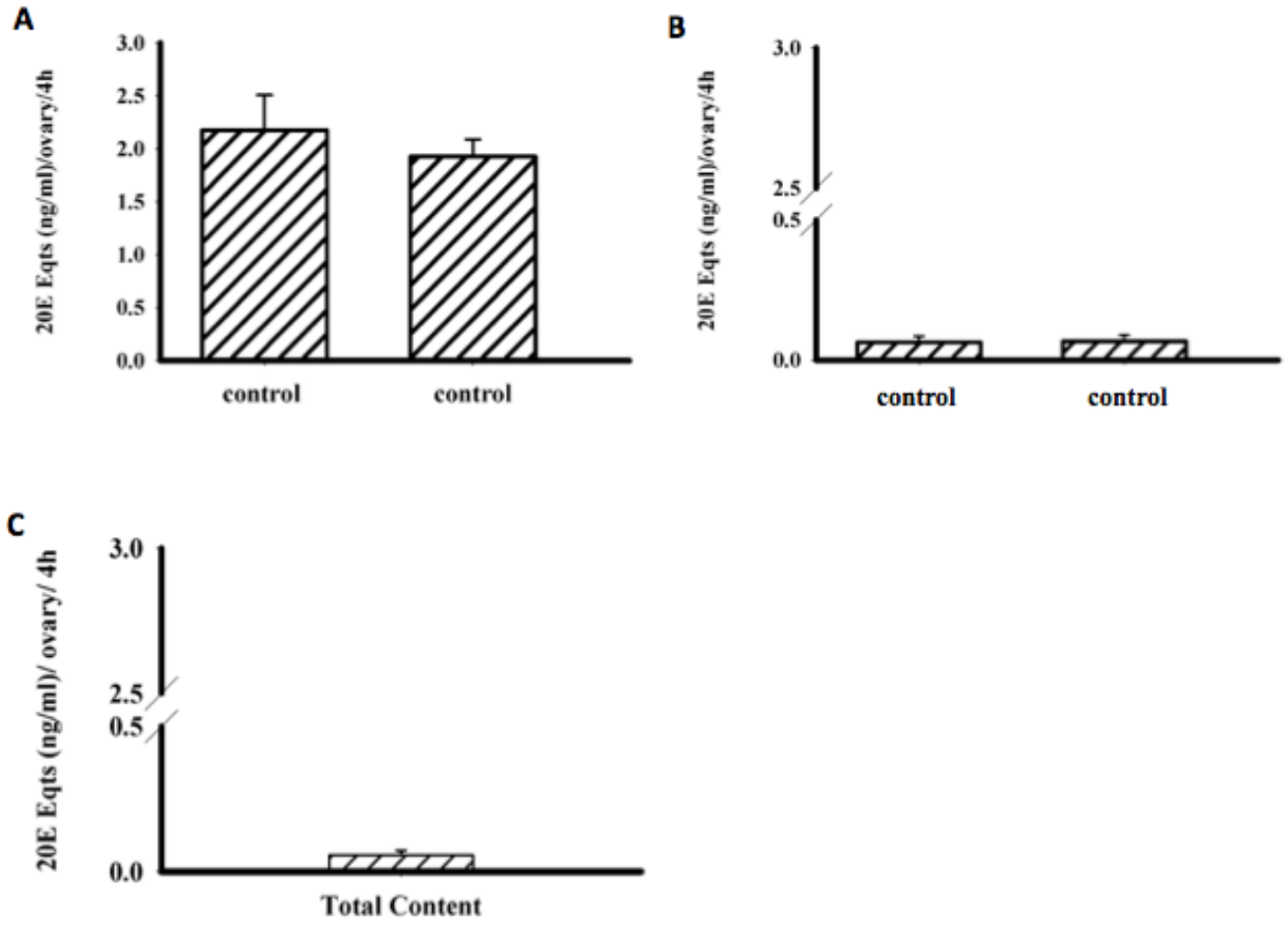

Figure 2.4 Ovarian ecdysteroid release *in vitro* during egg development. **(A)** Comparison of release between contralateral ovaries in entrained (LD) animals on Day 8 and **(B)** LL animals on Day 2 following a blood meal. **(C)** Total ovarian content of ecdysteroids in ovaries from unfed entrained (LD) animals. Dissections and incubations were carried out 2 hours after "lights on". Data expressed as mean ± SEM (n=5).

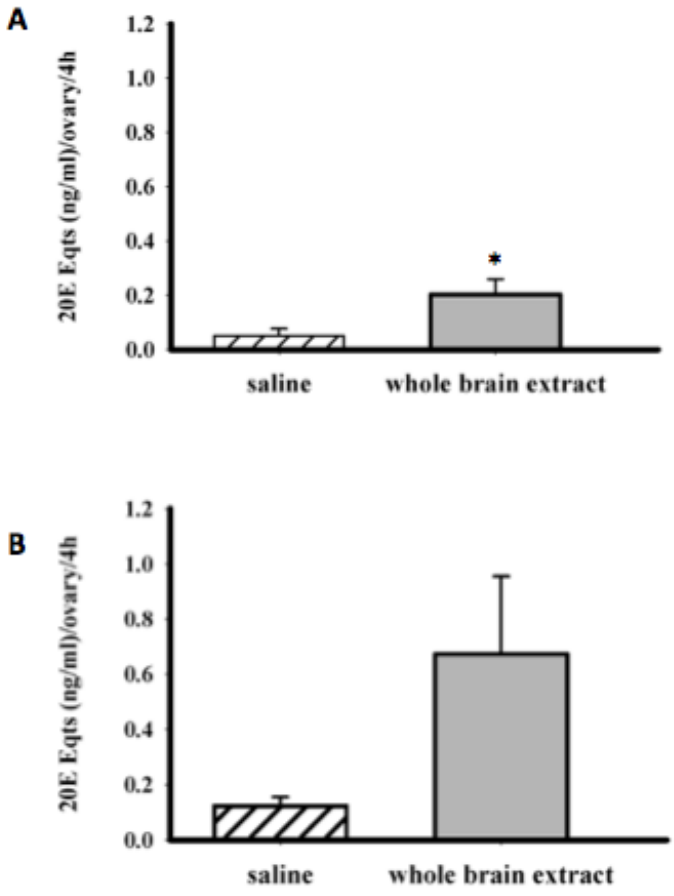

Figure 2.5 Effect of whole brain extract on ovarian ecdysteroid release *in vitro* by ovaries from LL animals on **(A)** Day 2 ($p < 0.05$) and **(B)** Day 4 post blood meal. Dissections and incubations were carried out 2 hours after "lights on". *: Significant, versus control. Data expressed as mean ± SEM (n=5). Results are shown as the activity of whole brain extract from one brain complex per experimental ovary

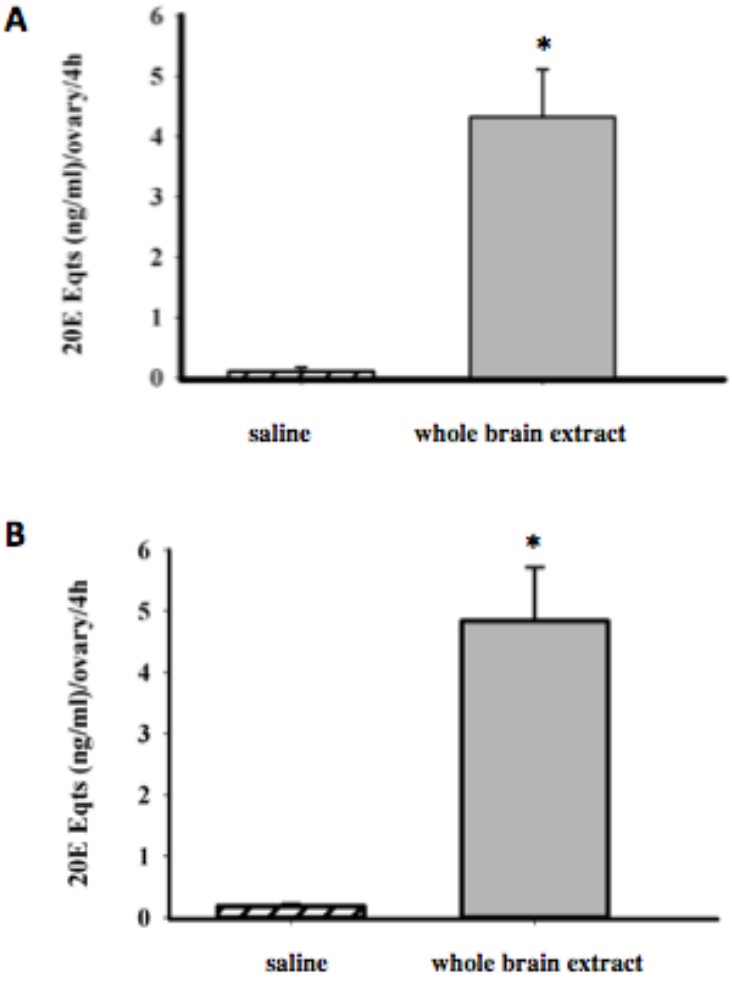

Figure 2.6 Ecdysteroid release from ovaries of LD animals incubated with whole brain extract. **(A)** Ecdysteroid release by ovaries from unfed LD animals ($p = 0.002$) and **(B)** ovaries from LD animals excised 2 hrs post feed ($p < 0.001$). A blood meal was given one hour after "lights on", and ovaries from unfed and newly fed animals were dissected during early photophase. *: Significant, versus control. Data expressed as mean ± SEM (n=5).

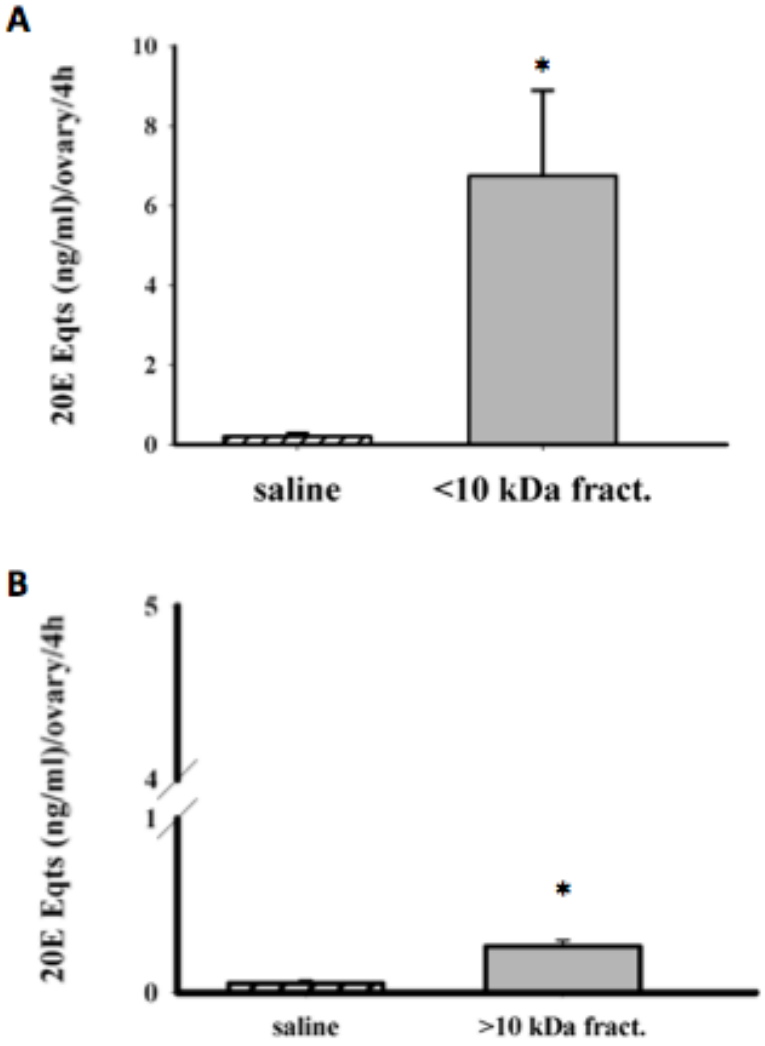

Figure 2.7 Ecdysteroid release from ovaries of LD animals treated with fractionated brain extracts 2 hr following a blood meal. **(A)** Treatment with <10kDa brain fraction ($p = 0.008$) and **(B)** treatment with >10kDa brain fraction ($p < 0.001$). A blood meal was given one hour after "lights on". *: Significant, versus control. Data expressed as mean ± SEM (n=5). Results are shown as the activity of fractionated brain extract from one brain complex per experimental ovary.

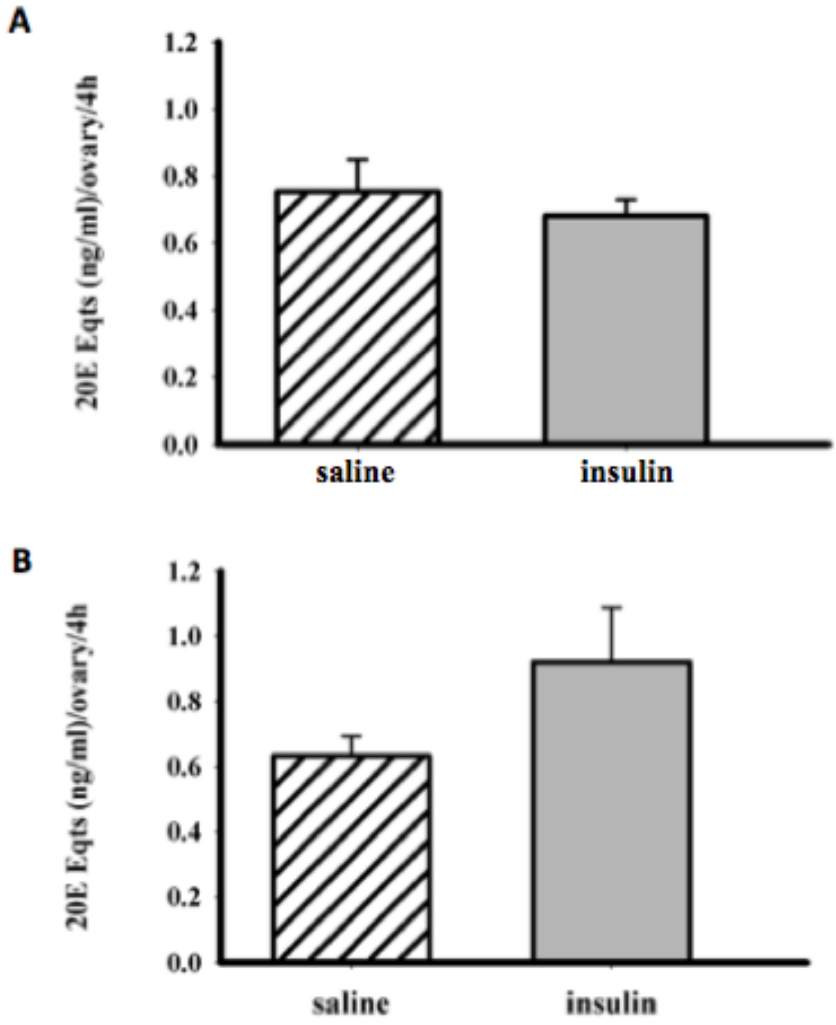

Figure 2.8 Effect of bovine insulin on *in vitro* ecdysteroid release in LL animals on day 3 following a blood meal. **(A)** Treatment with 2.8 x 10^{-7} M and **(B)** treatment with 1.7 x 10^{-6} M. Dissections and incubations were carried out 2 hours after "lights on". Data expressed as mean ± SEM (n=5).

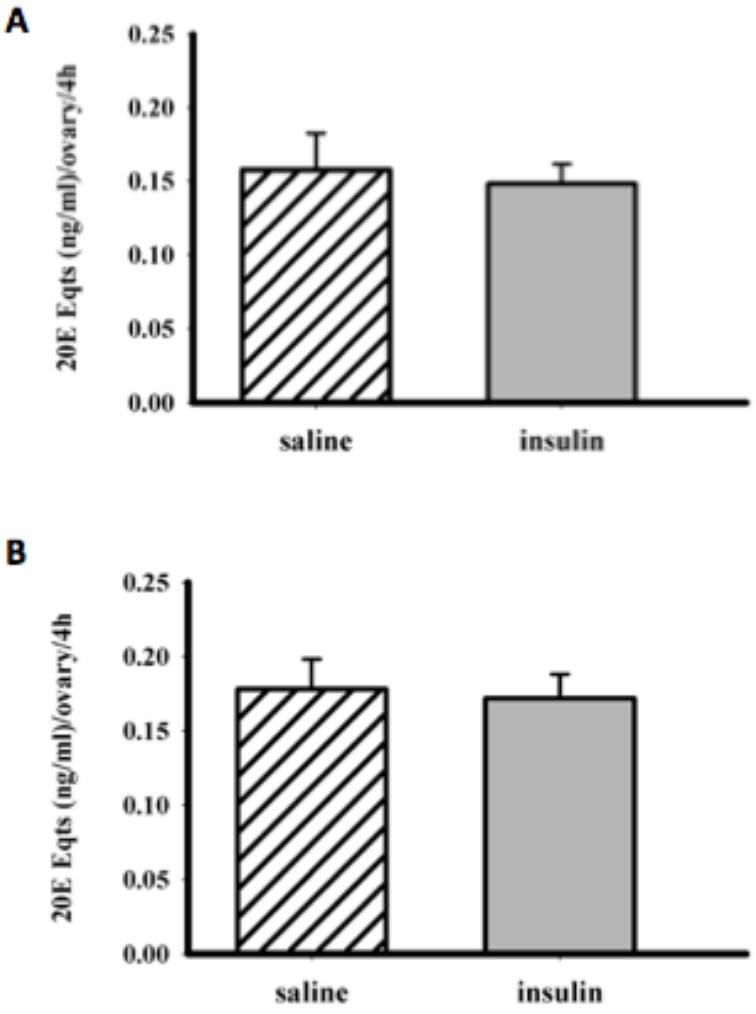

Figure 2.9 Effect of bovine insulin on *in vitro* ecdysteroid release in LL animals on day 4 following a blood meal. **(A)** Treatment with 2.8 x 10^{-7} M insulin and **(B)** treatment with 1.7 x 10^{-6} M. Dissections and incubations were carried out 2 hours after "lights on". Data expressed as mean ± SEM (n=5).

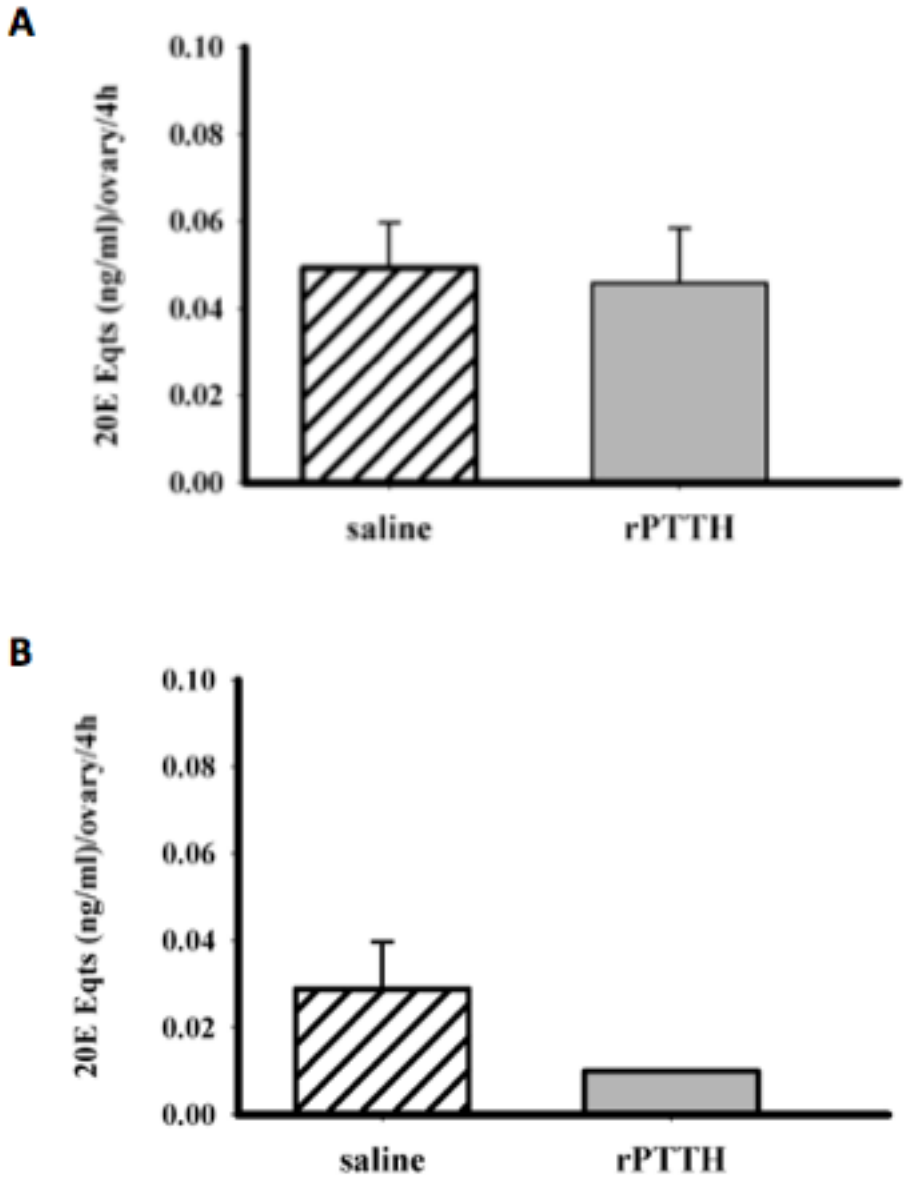

Figure 2.10 Effect of *Bombyx* rPTTH (6 x 10^{-10} M) on ovarian ecdysteroid release *in vitro* by ovaries from LL animals **(A)** Day 2 and **(B)** Day 4 post blood meal. Dissections and incubations were carried out 2 hours after "lights on". Data expressed as mean ± SEM (n=5).

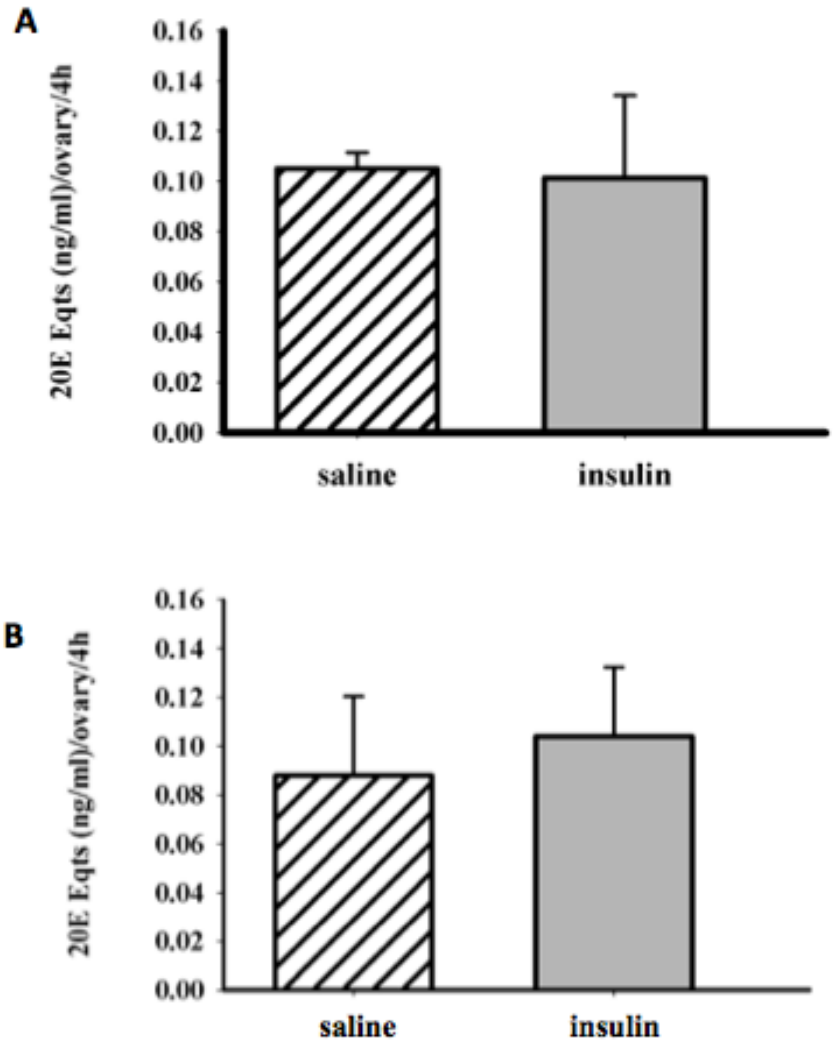

Figure 2.11 Effect of bovine insulin (2.8 x 10^{-7} M) on ecdysteroid release *in vitro* by **(A)** ovaries from unfed LD animals and **(B)** by ovaries excised from LD animals 2 hrs post feed. A blood meal was given one hour after "lights on", and ovaries from unfed and newly fed animals were dissected during early photophase. Data expressed as mean ± SEM (n=8).

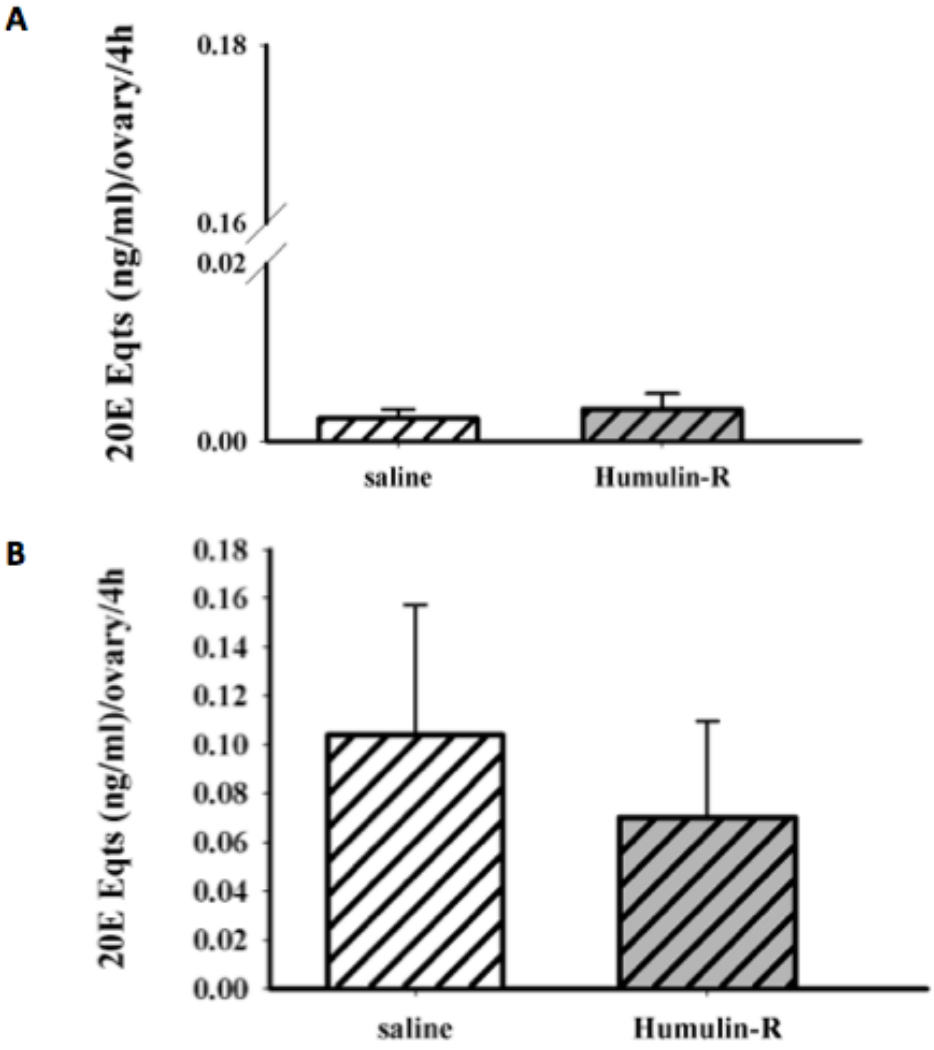

Figure 2.12 Effect of Humulin-R (2.8 x 10^{-7} M) on ecdysteroid release *in vitro* by **(A)** ovaries from unfed LD animals and **(B)** by ovaries excised from LD animals 2 hrs post feed. A blood meal was given one hour after "lights on", and ovaries from unfed and newly fed animals were dissected during early photophase. Data expressed as mean ± SEM (n=8).

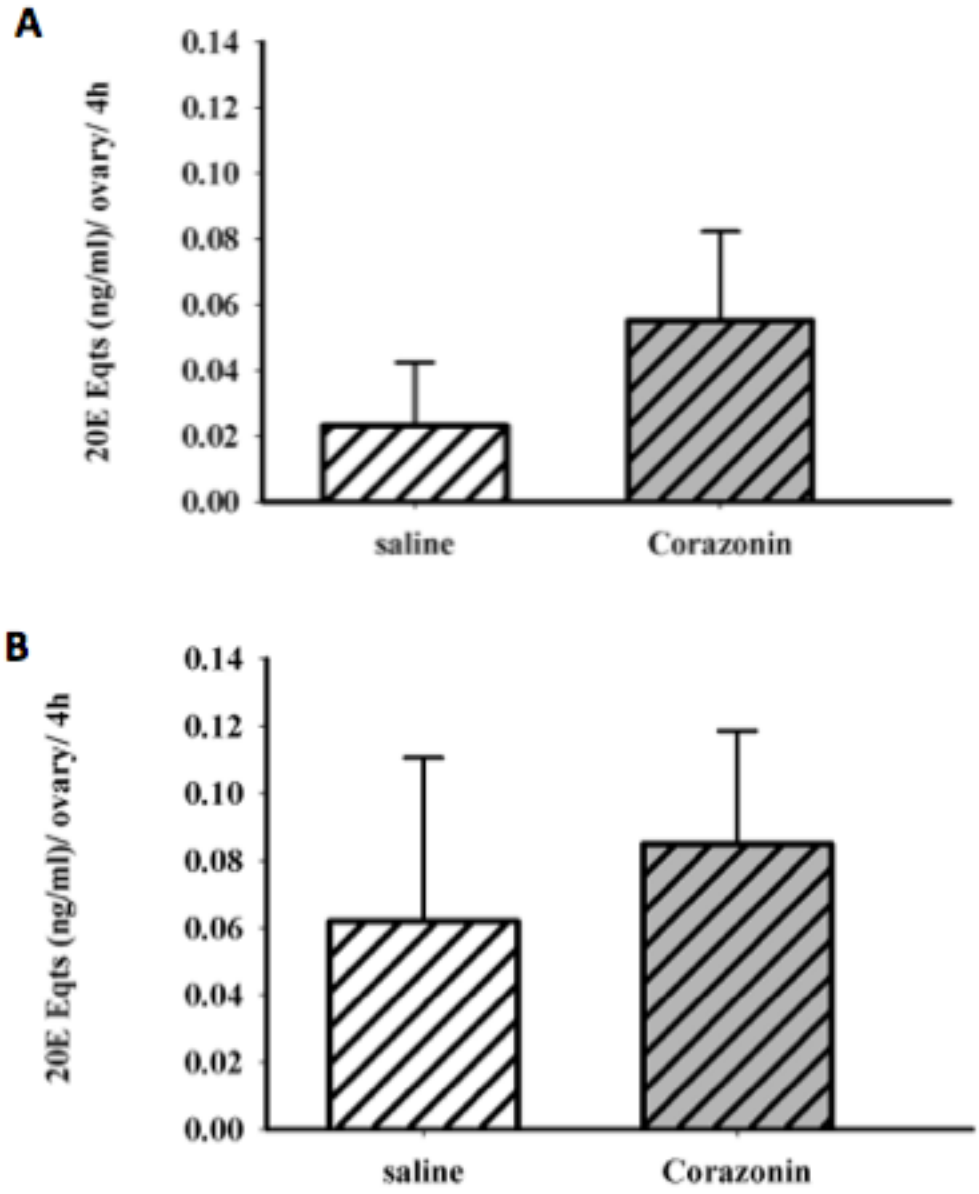

Figure 2.13 Effect of *R. prolixus* corazonin (10^{-6} M) on ecdysteroid release *in vitro* by **(A)** ovaries from unfed LD animals and **(B)** by ovaries excised from LD animals 2 hrs post feed. A blood meal was given one hour after "lights on", and ovaries from unfed and newly fed animals were dissected during early photophase. Data expressed as mean ± SEM (n=8).

IV. DISCUSSION

4.1 *Discussion of Results*

4.1.1 No Induction of Ovarian Ecdysteroid Release Upon Transfer to DD in vitro

The present data demonstrates that the ovaries *in vitro* do not release ecdysteroids in response to a signal of darkness. If the ovaries contained an endogenous circadian clock, it is expected that ecdysteroid release would be stimulated by transfer to DD, as has been demonstrated in other systems. PGs from larval fifth instar maintained in LL and transferred to DD *in vitro* elicited a rhythm of ecdysteroid release immediately, with a phase reversal of ecdysteroid release (peak release occurring during photophase, instead of scotophase) (reviewed by Steel and Vafopoulou, 2006). This rapid rate of ecdysteroid synthesis was not observed when PGs from DD animals were transferred to LL, demonstrating that the PGs respond to a "lights off" signal but not a "lights on signal". It was concluded that PTTH entrains the clock in the PGs to synthesize ecdysteroids at night, as PTTH is naturally released during the night (Vafopoulou and Steel, 1998). In the present study, transfer to DD of ovaries from arrhythmic animals failed to stimulate ecdysteroid release. In fact, the level of release was constant after 7 hours, in comparison to the release of ovaries from LL animals, which fluctuated. This suggests that unlike the PGs, the ovaries may not respond to light cues to drive rhythmic ecdysteroid release *in vitro*.

Oviposition (or laying of ovulated eggs) occurs with a circadian rhythm in *R. prolixus*, approximately 12 h after "lights on" on successive days during a reproductive cycle (Days 4-8, approximately) (Ampleford and Davey, 1989). Rhythmic egg laying is a gated phenomenon

(Pittendrigh, 1966), as it is observed in individuals and whole populations and it occurs near the light to dark transition (Ampleford and Davey, 1989). Furthermore, rhythmic egg laying persisted following transfer to DD and was temperature compensated. The results demonstrated that a circadian clock was controlling the egg laying rhythm, but the location of the clock was not elucidated. A myotropic ovulation hormone is released from the brain at the time of oviposition when eggs are in the ovary, and assists the expulsion of eggs from the ovary by increasing the power of ovarian contractions (Kriger and Davey, 1983). It was hypothesized that the release of the ovulation hormone could be a gated event that drives the rhythmicity of egg laying, but nervous control of the muscles in the ovary regulating egg release is also likely (Ampleford and Davey, 1989). Because a daily rhythm of ovulation and oviposition occurs in *D. melanogaster*, and persists in mutant flies (per^l, per^s, per^0) (McCabe and Birley, 1998), it was suggested that an endogenous circadian clock for ovarian rhythms in flies was located outside of the ovary (reviewed in Sellix, 2015). It is possible that a similar method of circadian timekeeping occurs in *R. prolixus*, with ovarian ecdysteroid rhythms (and probably ovulation and oviposition) being driven by a clock outside of the ovary (See Section 4.1.5).

4.1.2 Developmental pattern of nuclear PER in ovarian follicle cells in R. prolixus

Recent work has demonstrated non-cyclical PER expression throughout early to mid-egg development (days 1-6) in previtellogenic and vitellogenic follicle cells, as well as trophocytes in the tropharium (Hajia Yakubu, unpublished). Around day 5 following a blood meal, the first cycle of vitellogenic oocytes and mature oocytes are observed (see Results)

(Hajia, Yakubu, Andrea Durant, Xanthe Vafopoulou, unpublished observations). These observations coincided with the presence of nuclear PER in follicle cells during mid to late scotophase (Hajia Yakubu, unpublished).

The present study confirms this finding that around the fifth day following a blood meal, widespread nuclear PER is observed in some terminal oocytes, appearing at a specific stage of development before the deposition of the chorion layers. However, in this study widespread nuclear PER was observed during mid-photophase and not at other time points examined (i.e. scotophase), indicating that the presence of nuclear PER is not dependent on light cues but rather on a specific developmental phenomenon. Furthermore, widespread nuclear PER was localized to follicle cells of terminal oocytes during photophase on Day 8 following a blood meal. PER expression in follicle cells of terminal oocytes during the last few days of a reproductive cycle had not been examined previously. Again, terminal oocytes (presumably the second or third cycle of vitellogenic follicles within each ovariole during a reproductive cycle) have ended vitellogenesis at this point, and nuclear PER expression was observed in follicle cells that had not yet deposited the chorion. This confirms that PER expression is brief, non-rhythmic, and occurs in many terminal oocytes, which are at a specific developmental stage regardless of the time of day.

Interestingly, widespread nuclear PER expression was also observed in ovarian follicle cells from arrhythmic (LL) animals during the middle of a reproductive cycle (subjective Day 5). If PER had a circadian function in follicle cells, and was part of a molecular oscillator, it might be expected that PER expression would be severely reduced or abolished in these cells in arrhythmic animals. This depletion of PER as a result of chronic exposure to light was demonstrated in PG cells of *R. prolixus* (Vafopoulou and Steel, 2014). Additionally, the idea

that the high abundance of PER in some cells, such as the follicle cells in this study, is unnecessary for its role as a transcriptional regulator of the steroidogenesis pathway leads to the notion of a multifunctional protein (Vafopoulou and Steel, 2005). Non-circadian functions of clock genes have been reported, such as cocaine sensitization in *D. melanogaster* (Andretic *et al*., 1999). It is quite possible that PER in ovarian follicle cells of *R. prolixus* has a function that is not related to timekeeping.

In *D. melanogaster*, developmentally regulated clock gene expression was shown (see Section 1.8) (Kotwica *et al*., 2009). In previtellogenic follicle cells, PER expression is high, cytoplasmic, and non-rhythmic, and is co-localized with TIM (Beaver *et al*., 2003). DOUBLETIME (DBT), a kinase that phosphorylates and destabilizes PER, was absent from previtellogenic follicle cells. At the onset of vitellogenesis, follicle cells exhibit nuclear PER localization, which also corresponds to cytoplasmic DBT localization (Kotwica *et al*., 2009). The finding that nuclear PER in follicle cells is only observed during the early vitellogenic phase, and the nuclear phase of PER appears to be very brief, supports the idea in the present study that PER in follicle cells of insects appear to have a non-circadian role in egg development.

In the oocytes of the mammalian ovary, it was shown that clock gene mRNA fluctuated with a developmental pattern of expression, in contrast to the rhythmic clock gene expression and mRNA abundance found in the GCs (see Section 1.4.5) (Amano *et al*., 2009). It was postulated that this expression suppresses rhythms of clock controlled gene expression during oocyte maturation and embryonic development, indicating a role for clock genes in oocyte maturation that are oscillator independent (Amano *et al*., 2009). The idea that the SCN may coordinate the timing of reproductive events independently of an ovarian clock, or more

possibly that a peripheral oscillator in the ovary is partially entrained by the SCN and that the molecular clock is "turned on" in developing follicle cells at some point (Sellix, 2015), illustrates the uncertainties of how the timing system regulates ovarian physiology.

4.1.3 A factor in the brain stimulates ovarian ecdysteroid release

The finding that whole brain extract stimulates ecdysteroid release by the ovaries *in vitro* from unfed and newly fed animals is good evidence of neurohormonal control of the ovarian ecdysteroid rhythm. The present data supports the notion that the ovary does not appear to contain an endogenous circadian clock controlling the ovarian ecdysteroid rhythm. This is the first demonstration of regulation by the brain of ecdysteroid release from the ovary of adult *R. prolixus*. Not only did whole brain extract stimulate ecdysteroid release in unfed and newly fed insects, whole brain extract also stimulated ecdysteroid release in LL animals a few days into egg development, suggesting that a factor in the brain is important for the initiation and regulation of ecdysteroid release throughout an entire reproductive cycle.

In unfed adult *R. prolixus*, the brain is not releasing PTTH and ILPs (Vafopoulou and Steel, 2001). During egg development, the adult brain contains and releases PTTH and ILPs in high quantities with a circadian rhythm (Vafopoulou *et al*., 2012, Vafopoulou and Steel, 2012). The rhythm of neuropeptide release occurs in concert with the rhythm of ecdysteroid release by the ovaries (Fig. 1.5), suggesting a possible parallel axis of control as was shown in larval development. In the present study, when the ovaries from unfed adult R. prolixus were exposed to whole brain and fractionated brain extract *in vitro*, ecdysteroid release was significantly greater than the levels of detectable ecdysteroids in ovaries from unfed animals.

Therefore, it appears that the unfed ovary responds to brain hormones by synthesizing and/or releasing ecdysteroids. However, it is also possible that the ovaries store conjugated ecdysteroids that are then deconjugated and released upon stimulation with brain hormones.

It was previously shown that the content of ecdysteroids in ovaries several days after feeding was 7-8 fold higher than levels found in the hemolymph (Cardinal-Aucoin *et al*., 2013), but it was not known whether immunoreactive ecdysteroids were synthesized *de novo* within the ovary or were produced by deconjugation of stored ecdysteroids that had been previously taken up by the developing oocyte (Cardinal-Aucoin *et al*., 2013). Ovarian follicle cells in other insects have been shown to synthesize ecdysteroids *de novo* (Zhu *et al*., 1983), and some ecdysteroids are incorporated into the developing oocyte (Lagueux *et al*., 1979). However, it is also possible that the ovaries from unfed animals store high levels of conjugated, non-immunoreactive ecdysteroids, which are then deconjugated and released following a blood meal (Lagueux *et al*., 1979). This would require a suitable site of storage for high levels of conjugated ecdysteroids in the unfed insect, as well as the rapid deconjugation of free ecdysteroids in response to brain factors. Conjugation and deconjugation of ecdysteroids with a circadian rhythm could be driven by rhythmic neuropeptide action from the brain in the absence of an ovarian clock. While both scenarios seem possible to account for the high quantities of ecdysteroids released by ovaries from unfed animals when stimulated with brain extract, the present study demonstrates that a factor in the brain does, in fact, effect ecdysteroid release by ovaries in adult *R. prolixus*.

Brain fractions with ILP-like or PTTH-like activity, which have been shown to have ILP-like and PTTH-like activity on *R. prolixus* PGs, also have a stimulatory effect on ecdysteroid release by the ovaries. Stimulation of ecdysteroid release by the ILP-like fraction

was 10 fold greater than that of PTTH-like fraction, suggesting a greater role for ILP involvement in insect reproduction. Bombyxin, an ILP from *B. mori*, has ecdysiotropic activity on *R. prolixus* PGs in high doses. Similar results of ecdysteroid secretion in response to ILPs were found by the PGs of *B. mori* (Gu *et al*., 2009). In larval *B. mori*, bombyxin induced meiosis in ovaries cultured *in vitro* (Orikasa *et al*., 1993). It was proposed that the ovaries were stimulated by bombyxin to produce ecdysteroids, because the ovaries from fourth instar larvae secrete ecdysteroids, and 20-hydroxyecdysone (20E) induces meiosis at low concentrations (Orikasa *et al*., 1993). In the blowfly, *Phormia regina*, extracts from the median neurosecretory cells (MNCs) of brains, which contain endogenous ILPs, effectively stimulated ovarian ecdysteroidogenesis (Maniere *et al*., 2004). In the mosquito, *Aedes aegypti*, three of eight encoded ILPs (ILP1-8) are expressed in the adult female brain, and ILP3 stimulated ovarian ecdysteroid production in a dose-dependent manner in much lower concentrations compared to bovine insulin (Brown *et al*., 2008).

This study demonstrates that ILP-like activity appears to play a major role in the regulation of ovarian ecdysteroid release in *R. prolixus*. Additionally, it is the first to show a possible role for PTTH activity as an ovarian ecdysiotropin in an adult insect. PTTH-like activity had a much smaller (in comparison to ILP-like activity) but significant effect on ecdysteroid release. To date, no other studies have examined the ecdysiotropic activity of PTTH on ovaries during reproduction. It is important to note that the insect brain contains a wide variety of neuropeptides, and it is therefore possible that other factors (in addition to those examined) in the brain may be influencing the ovarian ecdysteroid rhythm (see Section 4.1.4). Furthermore, brains from larval *R. prolixus* were used in this study and it is conceivable that the adult brain contains other factors that are not present in larvae. However,

the use of larval brain fractions in which PTTH-like and ILP-like activity have been demonstrated, gives us a potential insight into the nature of stimulation by each brain fraction observed.

4.1.4 Regulation of Ovarian Ecdysteroid Release by Vertebrate Insulins, Bombyx rPTTH, and Corazonin

In *A. aegypti*, bovine insulin stimulated ecdysteroid production and release by ovaries in adult females (Brown *et al*., 2009). Significant stimulation was observed in much higher concentrations than that of ILP3 (up to 5 nmol vs 100 pmol, respectively), which suggested that mammalian insulins have weak ecdysiotropic activity in comparison to endogenous ILPs (Brown *et al*., 2009). This study demonstrates that high concentrations (2.8×10^{-7} M and 1.7×10^{-6} M) of bovine insulin do not stimulate ecdysteroid release by ovaries from unfed or newly fed LD animals, or ovaries from LL animals three or four days following a blood meal. In *P. regina*, high concentrations of bovine insulin (10^{-5} M) have ecdysiotropic activity on the ovaries, but in an age-dependent manner following adult ecdysis (Maniere *et al*., 2004). Insulin at a concentration of 10^{-6} M or less was ineffective at stimulating ovarian ecdysteroid release (Maniere *et al*., 2004). It is conceivable that bovine insulin at other doses would have an effect on ecdysteroid release by *R. prolixus* ovaries. The concentrations of bovine insulin that stimulated ovarian ecdysteroid release in *A. aegypti* and *P. regina*, which differed significantly, could have the same effect on ovaries of *R. prolixus.* Very little information is known about the hormonal regulation or functional significance of ecdysteroids in adult insects. Only very recently was it demonstrated in *R. prolixus* that the ovary is the primary

source of ecdysteroids in adult females, and that there is an important circadian element involved (Cardinal-Aucoin *et al*., 2013).

Bombyxin and vertebrate insulin do not have similar amino acid sequences, and three-dimensional models show that bombyxin does not form dimers or hexamers that are characteristic of mammalian insulins (Ebberink *et al*., 1989). Furthermore, the first insect ILP three-dimensional structure to be resolved was Bombyxin II, and it was shown that the structure resembles relaxin more than insulin (Nagata *et al*., 1995). *D. melanogaster* bombyxins consist of a heterodimer of A and B chains linked by two disulfide bridges (Ishizaki, 2004), but it is not known if they are cleaved into A and B chains like vertebrate insulins (Gronke and Partridge, 2010). Based on this information, it is possible that bombyxin would have a different effect on ecdysteroid release by ovaries from *R. prolixus* than vertebrate insulins (which was no effect). Additionally, human insulin differs from bovine insulin by only three amino acids. Therefore, it was presumed that human insulin (Humulin-R isoform used in this study) would exhibit the same effect on ovaries as bovine insulin. As expected, 2.8 x 10-7 M Humulin-R did not stimulate ecdysteroid release by ovaries from unfed and newly fed LD animals.

Corazonin is a neuropeptide in insects that is stored and released from the corpora cardiaca (CC), and only one isoform of the peptide is found in all insects examined to date (Predel *et al*., 2007). With the exception of its heart rate-accelerating properties (Veenstra, 1991), the regulation of color polymorphisms in locusts (Tawfik *et al*., 1999), and the control of ecdysis behaviour (Kim *et al*., 2004), the general role of corazonin in insects has not yet been elucidated. Interestingly, corazonin immunoreactive cells in the brains of two cricket species, *Dianemobius nigrofasciatus* and *Allonemobius allardi*, co-localizes with locations of

presumed circadian clock components in optic lobes and protocerebrum (Sehadova *et al*., 2007). However, no difference in the distribution and intensity of immunoreactivity was observed when animals were reared at two different photoperiods (Sehadova *et al*., 2007). Corazonin at high concentrations (10^{-6} M) exhibits ecdysiotropic activity on fifth instar *R. prolixus* PGs (Vafopoulou and Orchard, unpublished). However, no stimulation of ecdysteroid release was seen when ovaries from unfed and newly fed LD animals were incubated with corazonin. Perhaps this neurohormone plays a larger role in larval development than in reproduction.

Surprisingly, *Bombyx* rPTTH did not have ecdysiotropic activity on the ovaries from LL animals on days 2 and 4 after a blood meal. The ovaries release ecdysteroid rhythmically, with maximal release occurring during the night (Cardinal-Aucoin *et al*., 2013), correlating with peak release of PTTH from the brain during the night (Vafopoulou *et al*., 2012). Furthermore, transfer of insects from 12hr light:12hr dark to LL abolishes the rhythmic release of PTTH from the brain of larvae and ecdysteroid release from the ovaries (Cardinal-Aucoin *et al*., 2013; Vafopoulou and Steel, 2012). In larval *Rhodnius*, PGs from LL animals respond to treatment with PTTH in a manner similar to transfer to DD, initiating abrupt synthesis of large quantities of ecdysteroids (Vafopoulou and Steel, 1996). From this, it was expected that treatment with rPTTH would stimulate ecdysteroid release by ovaries *in vitro*. In the PGs of *R. prolixus*, stimulation of ecdysteroid synthesis and release was seen using concentrations of 1ng/ml and up, with significant stimulation occurring above 5 ng/ml (Vafopoulou and Steel, 1997). Because the adult brain possesses double the amount of immunoreactive PTTH cells (Vafopoulou *et al*., 2007), it is possible that much higher concentrations of PTTH are released following a blood meal, and higher concentrations than

used in this study would elicit an ovarian response of ecdysteroid release. The effect of rPTTH in increasingly larger doses on ovarian ecdysteroid release would be an interesting focus of future studies.

In addition to those examined, other neuropeptides have been shown to have an ecdysiotropic function during egg development in insects. In *A. aegypti*, the egg development neurosecretory hormone (EDNH) was discovered as one of the first invertebrate gonadotropins (Lea and Handel, 1982). EDNH is produced by the medial neurosecretory cells and is stored in the CC and is released into the hemolymph following a blood meal, where it stimulates the ovarian follicle to synthesize and secrete ecdysteroids. It was suggested that EDNH and PTTH were the same peptide (Kelly *et al*., 1986). One study examined the action of purified brain extract from *A. aegypti* heads on the PGs of larval *Manduca sexta in vitro,* which demonstrated that PTTH and EDNH may have different physiological functions. Partially purified EDNH from *A. aegypti* did not possess prothoracicotropic activity in the *in vitro* PG assay, and partially purified PTTH from *M. sexta* did not activate *Aedes atropalpus* ovaries to synthesize ecdysteroids *in vitro* (Kelly *et al*., 1986). Therefore, in different insects PTTH and EDNH appear to regulate ecdysteroid synthesis in different tissues at different stages of the life cycle (Kelly *et al*., 1986).

Neurohormones that stimulate the ovaries to secrete ecdysteroids were termned "ovary ecdysteroidogenic hormones" (OEHs), including EDNH (Matsumoto *et al*., 1989). The first fully characterized ovary ecdysteroidogenic hormone, OEH I, was purified from *A. aegypti* heads (Brown *et al*., 1998). OEH I shares sequence similarity with neuroparsin A, a neuropeptide identified in *Locusta migratoria* shown to be involved in insect reproduction, as well as the N-terminal part of vertebrate insulin-like growth factor binding proteins (IGFBPs)

(Janssen *et al*., 2001). Interestingly, some IGFBPs have been shown to regulate steroidogenesis in mammalian gonads (Monget *et al*., 1996). In *B. mori*, a *Bombyx* IGF-like peptide (BIGFLP) was purified from hemolymph, and was found to have high homology with bombyxins. Like IGFs, BIGFLP is secreted into the hemolymph as a single chain peptide (Okamoto *et al*., 2009). The dominant source of circulating BIGFLP in the hemolymph is the fat body, but the gonads of adult insects also synthesize and secrete BIGFLP. Therefore, IGFs in insects (in addition to OEH) may play a role in the synthesis and secretion of ecdysteroids, as they do in mammals. B-type allatostatins (Grb-AST B1, specifically) were shown to inhibit ecdysteroid synthesis *in vitro* by adult cricket *G. bimaculatus* ovaries (Lorenz *et al.*, 1997). In the cockroach, *Blaptica dubia*, Grb-AST B1 had the opposite effect, stimulating ovarian ecdysteroidogenesis (Lorenz *et al.*, 2004).

4.1.5 Is Rhythmicity in Ovarian Ecdysteroids a Driven Rhythm or Controlled by an Ovarian Clock?

While PER is present in ovarian follicle cells in insects (see Section 4.1.2), it appears briefly at a specific developmental stage, and non-rhythmically. If the follicle cells were clock cells, it would be expected that the appearance of nuclear PER would be rhythmic. The ovaries *in vitro* did not respond to a "lights-off" signal by releasing ecdysteroids, in which this signal of darkness has been shown to influence ecdysteroid release by circadian clocks in other systems (see Section 4.1.1) (described Vafopoulou and Steel, 2006). The probable absence of an ovarian clock suggests that the rhythmic release of ecdysteroids occurs in response to the rhythmic release of brain factors (or even factors found elsewhere). Numerous possibilities exist for the regulation of the ovarian ecdysteroid rhythm. The clock in the brain

controls the rhythmic release of neurohormones such as PTTH and ILPs in larvae and adults (Vafopoulou *et al*., 2012; Vafopoulou and Steel, 2012). Therefore, the rhythm of ecdysteroid release could be driven by rhythmic hormonal stimulation. In the present study, it was demonstrated that whole brain extract stimulates ecdysteroid release by the ovaries, indicating that a factor in the brain may regulate rhythmicity in the ovaries. The questions that remain are what exactly are these brain factors that are influencing ecdysteroid release by ovaries, and how they function to cause the release from the ovary. Brain factors could be initiating *de novo* synthesis of ecdysteroids, or controlling rhythmic deconjugation of ecdysteroids. Because of the lack of evidence of the latter, control of ecdysteroid rhythms most likely occurs at the level of *de novo* synthesis by follicle cells.

4.2 General Discussion

4.2.1 New Insights into the Presence of PER in Follicle Cells of R. prolixus Ovaries During Egg Development

The present study demonstrates a non-circadian role for the clock protein PER in reproduction. Similar findings of a non-circadian function and regulation of PER, as well as TIM, in *D. melanogaster* oogenesis have been described (Beaver *et al*., 2003). PER and TIM were detected in ovarian follicle cells of females maintained in LL (similar to findings in this study), suggesting a role in temporally regulated but non-circadian processes (Beaver *et al*., 2003). Therefore, the notion that the ovaries of *D. melanogaster* lack a functional circadian clock is very possible (Beaver *et al*., 2003).

In mutant mice that are *mPer2* deficient, an increase in tumor development and reduced apoptosis of cancer cells was reported (Fu *et al*., 2002). Additionally, the disruption of cell division in *mPer2* mutants was demonstrated by the deregulation of temporal expression of genes involved in cell cycle regulation and the DNA damage-responsive pathways (Fu *et al*., 2002). In human cancer cells, inhibition of *hPer1* expression resulted in reduced apoptosis, which was associated with the altered expression of key cell cycle regulators (Gery *et al*., 2006). It was suggested that the core clock factors function in tumor suppression by direct interactions with cell cycle checkpoint proteins (ATM kinase and its downstream target *Chk2*), in addition to transcriptional regulation of other cell-cycle related genes, indicating a non-circadian function of *mPer1* in tumor suppression (Gery *et al*., 2002). Interestingly, *mPer1* expression in tumor tissues (breast and lung cancers) was significantly reduced compared to normal tissues (Gery *et al*., 2006), and *hPer2* expression was reduced in lymphoma and leukemia patient samples (Gery *et al*., 2005). Therefore, tissue-specific functions of *hPer1* and *hPer2* in suppression of tumorigenesis were proposed. It is likely that there are many non-circadian and tissue specific functions of PER in insects in addition to those previously mentioned (see Section 1.8), as demonstrated by the development patterns of nuclear PER presence in the ovaries of *R. prolixus* and *D. melanogaster*.

4.2.2 Emerging Roles of Insulin and ILPs in Insect Reproduction

In vertebrates, insulin is known for its diverse biological functions related to growth, metabolism, and reproduction. Similarly, emerging roles for ILP signaling in insect reproductive physiology have been demonstrated in several species. In three lepidopteran

species, specific binding sites for bombyxin on ovarian follicle cells were identified (Fullbright *et al*., 1997), and a high level of bombyxin in the hemolymph of female *B. mori* was detected (Iwami *et al*., 1996). Furthermore, insects possess an insulin receptor, which is structurally and functionally similar to mammalian insulin receptors (reviewed in Claeys *et al*., 2002). However, neither insulin nor relaxin, which is another member of the insulin family with diverse roles in reproduction, were strong competitors for the binding of bombyxin to its receptor in ovarian cell lines from *Spodoptera frugiperda* and *B. mori* (Fullbright *et al*., 1997). Structural comparisons of bombyxin with the mammalian ovarian hormone, relaxin, suggest a closer relationship between bombyxin and relaxin than between insulin and bombyxin (Nagata *et al*., 1995). It is likely that bombyxin would have a stimulatory effect on ecdysteroid release, which was not mimicked by vertebrate insulins in this study.

Extensive studies in Dipterans demonstrated the steroidogenic functions of insulin and insulin-like growth factors in ovarian physiology. In *A. aegypti*, bovine and porcine insulin stimulated ecdysteroid synthesis and release in a dose-dependent manner (Riehle and Brown 1999; Brown *et al*., 2008; 2009). Furthermore, the mosquito insulin receptor (MIR) has been localized in follicle cells surrounding developing oocytes, the primary location of ecdysteroidogenesis in adult females (Riehle and Brown, 2002). Similar findings in the blowfly, *P. regina*, demonstrate the steroidogenic activity of bovine insulin, albeit at physiologically high concentrations, and endogenous ILPs on ovaries in another Dipteran species (Maniere *et al*., 2004). Even though only a few doses were examined, the present finding that vertebrate insulin did not have an apparent ecdysiotropic effect on the ovaries from *R. prolixus* may suggest that this response is limited to dipterans.

The physiological significance of ILPs in the regulation of insect reproduction has been demonstrated with the use of genetic studies in *D. melanogaster*. The onset of vitellogenesis and the rate of egg production in response to nutritional changes require an intact insulin-signaling pathway in *D. melanogaster* ovaries (Drummon-Barbosa and Spradling, 2001). In female flies lacking the insulin receptor, *InR* mutants, ovarian development was arrested at the previtellogenic stage (Tatar *et al*., 2001). In mammalian studies, transgenic mice with decreased insulin signaling showed reduced numbers of follicles, reduced plasma concentrations of gonadotropins and sex steroids, and decreased pituitary size (Burks *et al*., 2000). Therefore, in both insects and mammals, an intact insulin-signaling pathway is necessary for fertility.

4.2.3 PTTH Signaling in Adult Insects

Recent findings of PTTH synthesis and release from the brain in adult female *R. prolixus* following a blood meal led to the suggestion of a parallel axis of control of ecdysteroidogenesis by the ovaries as has been described in the PGs during larval development (Vafopoulou and Steel, 2006; Cardinal-Aucoin *et al*., 2013). However, possible targets of PTTH signaling in adult females have yet to be elucidated. In the present study, brain fractions with PTTH-like molecular weight had a stimulatory effect on ovarian ecdysteroid release, but with much lower levels of stimulation than brain fractions with ILP-like molecular weight. Furthermore, rPTTH did not have an apparent effect on ovarian ecdysteroid release at the concentration used.

In *Manduca sexta*, it was found that PTTH is contained in the brain at all stages of adult life (reviewed in Rybczynksi *et al*., 2009). The possibility of other undescribed ecdysiotropic factors in the adult brain, in addition to PTTH was discussed, although the PTTH was found to be present in all brain extracts from adult brains (Rybczynksi *et al*., 2009). Target tissues of PTTH in adult female *M. sexta* were examined, and it was found that ovaries incubated with ^{3}H-cholesterol and rPTTH for varying amounts of time did not synthesize ecdysteroids or ecdysone precursors (Rybczynksi *et al*., 2009). Additionally, of the tissues examined only extracts of ovary and male accessory gland tubules of adult animals exhibited any changes in response to PTTH treatment. In the ovary, these changes were localized to the accessory gland reservoirs and not the ovariole (Rybczynksi *et al*., 2009). The significance of these changes in the accessory glands is not known. However, a role for PTTH in non-steroidogenic tissues and other reproductive processes is implied.

4.2.4 Conclusions

This study demonstrates that the ovaries do not release ecdysteroids in response to a "lights off" signal, and the demonstration of non-cyclical expression of PER in entrained and arrhythmic animals reinforces the notion that PER is a multifunctional protein, with functions in circadian timekeeping as well as non-circadian functions in other tissues. Similar patterns of nuclear PER in the follicle cells of ovaries from other insects and some mammalian systems have been observed. The ecdysteroid rhythm during egg development in *R. prolixus* is most likely driven by a factor in the brain, rather than an endogenous ovarian clock, or coordination of multiple circadian clocks as is achieved in the PGs of larvae. This study showed that low

molecular weight peptides from the brain (with ILP-like activity) stimulate ecdysteroid release from ovaries. In contrast, it was shown that high molecular weight peptides (with PTTH-like activity) had a relatively low stimulatory effect on ecdysteroid release. The data suggests that *R. prolixus* ILPs are the major regulators of ecdysteroid release, and PTTH plays a minor role. Additionally, evidence that brain factors stimulate *de novo* synthesis of ecdysteroids in ovaries was demonstrated in this study. While PTTH may not act on ecdysteroid producing tissues in adult insects, studies in other insects have shown a non-steroidogenic role in reproduction. Additional targets of PTTH signaling in *R. prolixus* have yet to be elucidated.

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APPENDIX A: Trypan Blue Exlcusion Assay

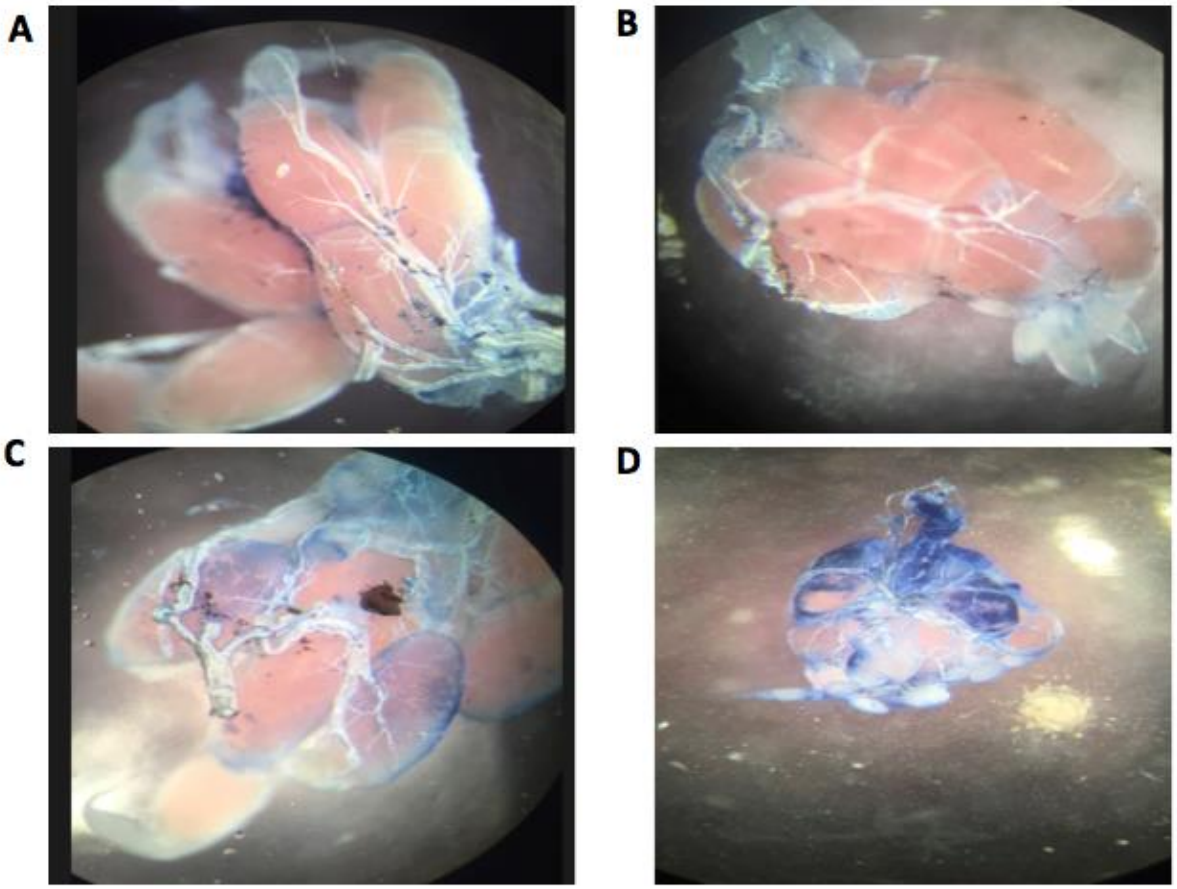

Figure 19. Trypan blue follicle cell viability assay. Ovaries from adult female *Rhodnius prolixus* on Day 5 following a blood meal incubated in *R. prolixus* saline immediately after excision for (A) 1 hour (B) 4 hours (C) 12 hours and (D) 24 hours. Ovaries were excised, incubated in saline for the specified time periods (see Section 2.9 for Methods) and were then incubated with Trypan Blue for 30 seconds. The dye will penetrate dead cells, but not live cells. Around approximately 12 hours *in vitro*, the ovariole sheath and the follicular epithelium begins to take up the dye, indicating the presence of dead cells.