# THE ROLE OF LIGHT AND THE BRAIN IN THE REGULATION OF RHYTHMIC STEROIDOGENESIS DURING DEVELOPMENT IN THE INSECT, *RHODNIUS PROLIXUS*

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

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## ABSTRACT

Prothoracicotropic hormone, PTTH, is released from the insect brain complex and is thought to activate steroidogenesis by the prothoracic glands (PGs) at one (or two) brief releases early in development. However, recent evidence in our laboratory using the hemipteran, *Rhodnius prolixus*, has shown that PTTH is released throughout the larval-adult stage in a circadian pattern regulated by a circadian oscillator located within the brain. Also, the PGs themselves have been shown to contain a circadian oscillator that regulates ecdysteroid synthesis and is also photosensitive *in vitro*. The focus of this thesis is on the functional relationship between the rhythm of PTTH and its only known target, the PGs. The significance of the rhythmic input of PTTH to the PGs *in vivo* has not been studied. The objectives of this thesis were two fold: 1) to establish the role of the circadian release of PTTH and how it interacts with the well documented oscillator found in the PGs of *Rhodnius prolixus* and 2) to examine the relation between the action of PTTH and light on the PG oscillator *in vivo*.

In order to accomplish these objectives, an *in vivo* study of the effects of decapitation and tetrodotoxin (TTX) was undertaken. TTX selectively and reversibly blocks voltage-dependent $Na^+$ channels in axons, inhibiting the generation of action potentials causing a flaccid paralysis that at the dose used lasted 92 hours. Since PTTH release is dependent on action potentials, TTX inhibits the release of PTTH. The effects of removal of the head and/or electrical activity in the nervous system (and hence PTTH release) on the rhythm of steroidogenesis by PGs was examined *in vivo*, together with

responses to light cues in the presence or absence of PTTH input.

Neither decapitation nor TTX injection eliminated rhythmic ecdysteroid synthesis by the prothoracic glands. This supports the view that an endogenous circadian oscillator resides in the PG themselves. In addition, the cycling amounts of ecdysteroid in the haemolymph also persisted in the absence of the brain and/or nervous activity and followed the pattern of PG synthesis. Hence it is concluded that the PG oscillator is the primary regulator of haemolymph ecdysteroid titre. However, removal of rhythmic PTTH input *in vivo* results in one major difference: the phase of the rhythms in PG ecdysteroid synthesis and haemolymph titre changes by 12 hours with respect to the normal pattern of oscillations previously reported in intact animals. It is inferred that the rhythm of PTTH acts as an entraining agent which regulates the phase of the ecdysteroid rhythms possibly through acting directly on the PG oscillator. This is a novel view of the neuropeptide which has pleiotropic effects: the classical view as activator of steroidogenesis and as regulator of phase of the PG oscillator after this initial activation period.

Photosensitivity of both the brain and PG oscillators was addressed by giving arrhythmic animals (reared in continuous light; LL) a photic cue *in vivo*. They were then transferred to continuous dark (DD) and both PTTH release from the brain and PG ecdysteroid synthesis was measured. Both PTTH release and PG ecdysteroid synthesis responded to the change in lighting conditions by producing similar rhythmic outputs. Specifically both rhythms peaked at a similar phase during the subjective day with a period length close to 24 hours. Animals reared in LL and treated with TTX prior to the transfer

to DD also showed induction of rhythmicity in PG ecdysteroid rhythm but with an inverted phase with respect to untreated animals. It is inferred that both the PTTH and PG oscillators are photosensitive, but with distinct properties such as different responses to light attributed to each oscillator. These individual characteristics support the view that separate oscillators regulate the two rhythms. However, *in vivo*, the PTTH oscillator appears to set the phase of the PG oscillator even in the presence of a conflicting photic cue. It is proposed that the circadian system in *Rhodnius prolixus* comprises multiple oscillators which regulate rhythmicity in the haemolymph ecdysteroid titre. This rhythm in the titre is regarded as the output of a complex timing system, whose main function appears to be regulation of temporal organization in developing cells and tissues. Similarities with other circadian systems are discussed, primarily with the more complex vertebrates, for which the *Rhodnius prolixus* model appears very useful. However, distinct differences between vertebrates and *Rhodnius prolixus* are revealed by this thesis which may lead to new and exciting discoveries about circadian organization in animals.

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# GENERAL INTRODUCTION

One of the most striking features of almost all living organisms is their ability to synchronize their behaviour, physiology and cellular events with the 24 hour solar day. An overt rhythm that persists in constant environmental conditions (such as continuous light or dark) with a temperature compensated period length close to 24 hours is termed circadian. Circadian rhythms are not direct responses to cyclical environmental cues but are driven by an internal timekeeping system or biological clock (Pittendrigh, 1960). Such 'clocks' become synchronized with the 24 hour cycle of the external environment through a process termed 'entrainment'. When entrained the biological clock maintains a fixed phase relationship to its external time cues (Zeitgebers) such as dawn or dusk; entrainment is a complex process by which the phase and/or period of the underlying oscillator(s) that comprise the 'clock' undergo daily adjustments in response to Zeitgebers to maintain synchrony with the external solar day (see Pittendrigh 1981, 1993).

These biological clocks are essential mechanisms for the organization of life processes so that an organism can anticipate and prepare itself in advance for the changes that are associated with its external environment (see Pittendrigh, 1993). This orchestration of functions from genes to behaviour has been termed temporal order. The ability to 'know' the time of day can be a matter of survival, especially when avoidance of predators is part of the organisms daily behavioural ritual. More importantly, organization of internal functions or what is known as internal temporal organization is not only needed for survival

but is a fundamental aspect for the maintenance of life in organisms as diverse as algae, fruit flies and humans.

Classical studies based mainly on formal analysis of the *Drosophila* eclosion rhythm (Pittendrigh, 1960; Pittendrigh and Daan, 1976), involved the concept of the pacemakers as the dominant entrainable oscillator within a timekeeping system. This pacemaker was visualized as relaying temporal information to the rest of the organism. The pacemaker concept was considered to be the temporal framework so that physiological and behavioural processes may be prepared for certain times of day when internal and external conditions are optimal (Pittendrigh, 1960). However, physiological studies with vertebrates have indicated that the role of pacemaker appears to be distributed among multiple oscillators which interact to produce the internal temporal order within the organism necessary for the maintenance of life (e.g. Ralph and Hurd, 1995).

## CIRCADIAN RHYTHMS IN VERTEBRATES

Rhythmic processes are found in all levels of organization in vertebrates. For example, rhythms exist in both cardiac and neuronal cells, sleep/wake cycles as well as in the timing of the onset of mitosis. In addition, many hormones show dramatic fluctuations in a circadian manner. Hormones play major roles in growth, development, and reproduction as well as maintaining the overall health and well being of almost all lifeforms. Consequently, circadian rhythms underlie important functions in humans and mammalian species such as respiration, nervous activity, adaptation to the environment, control of cell

division and the interactions between hypothalmus and pituitary that govern reproduction (Goldbeter, 1996). Although not all rhythms are controlled by biological clocks this thesis will confine itself to those that strictly fill circadian criteria.

The circadian systems of vertebrates are mainly distributed between three structures, the pineal, the suprachiasmatic nucleus (SCN) and the ocular retina with varying levels of relative importance depending on the species. The pineal and SCN have been termed pacemaking structures since one can transfer their properties through tissue transplantation (i.e. phase and/or period (see Jacklett, 1982)) from one organism to another which has had that particular structure removed previously (Zimmerman and Menaker, 1979; Ralph and Hurd, 1995). However, in most cases, a single structure is rarely the sole leader for the rest of the body. Hence, the classical pacemaker may be comprised of several oscillators, each with distinct properties, which feed back on one another and therefore can result in an altered overt rhythm being measured when all the different factors are taken into account. In such a 'distributed timing system', transplantation of circadian phase would not necessarily be exact to the donor animals but would depend on the relative strengths and phases of the individual components (Ralph and Hurd, 1995).

## The Multiple Oscillator System in Mammals

In mammals, the multiple oscillator system controlling locomotor activity in hamsters is comprised mainly of the hypothalamic SCN (for reviews see Lehman and Ralph, 1993; Ralph and Hurd, 1995) and retina. The role of the SCN has been shown by

the transference of period using the period mutation (*tau*) in the golden hamster. This mutation has three phenotypic groups: Wildtype with period length ($\tau$)=24hr, heterozygous with $\tau$=22hr and homozygous with $\tau$=20hr all of which do not overlap. Reciprocal transplantation experiments of SCN tissue among the three genotypes showed that the period of the restored rhythms generally fell within the range of the donor genotype. This shows that the SCN is the main oscillator controlling the period length (see Ralph and Hurd, 1995 for review).

However, the phase which is also considered a property of the endogenous biological oscillator controlling the overt behavioural rhythm has been shown not to be controlled by the SCN in the host animal, implying another oscillator may be present. Recent data implies that the host animal controls the phase of the implanted SCN, therefore an essential clock property lies outside the SCN, possibly in the retina or pineal (Serviere *et al.* 1994). The pineal gland is a well known source of melatonin which can produce phase shifts in SCN rhythms (Cassone *et al.*, 1993). Further, the mammalian retina also posses a circadian oscillator that drives rhythmic melatonin synthesis by the retina *in vitro* (Tosini and Menaker 1996). In addition, the neural input from the eye into the SCN is subject to circadian modulation by the retina (Serviere *et al.*, 1994). The pathways by which these various oscillators are coupled *in vivo* into a unified timing system are still unclear.

**The Multiple Oscillator System in Birds**

In contrast to mammals, the pineal in some birds is directly photosensitive and takes on a dominant role in their circadian organization. Older literature describes the pineal as a pacemaking element, since its properties, mainly phase (Robertson and Takahashi, 1988; Zatz *et al.*, 1988), can be transferred from one organism to the next. Specifically, the phase of perch-hopping behaviour in donor birds can be transferred following transplantation experiments of the pineal gland into pinealectomized sparrows which have arrhythmic behaviour (Zimmerman and Menaker, 1979). Thus, the implanted pineal both restores rhythmicity to the host and determines its phase.

Melatonin released from the pineal is viewed as an entraining agent to target tissues. The most intensively studied effect of melatonin is on the neuroendocrine-reproductive axis but it also is able to modulate many other structures and functions including the SCN (Weaver *et al.*, 1993; Turek, 1997) and hence can impose temporal order within the entire organism (see Webb and Puig-Domingo, 1995 for review). For example, the pineal, through the release of melatonin, regulates the rhythmicity in the SCN which, in turn, controls rhythmicity in hormone secretions such as gonadotropin releasing hormones and thus periodicity in seasonal reproductive cycles. However, the pineal does not serve as the main oscillator in all species of vertebrates (or even all birds), since in many species pinealectomy does not produce arrhythmicity (see Max and Menaker, 1992) and there is evidence for a second damped oscillator located in the SCN (Takahashi and Menaker, 1982). In addition, the retina in birds has also been shown to secrete melatonin which can

act as an entraining agent throughout the rest of the organism (Bernard *et al.*, 1997). In general, the avian circadian system has been suggested to be a neuroendocrine loop (Cassone and Menaker, 1984) with the pineal oscillator being affected by the periodic sympathetic input driven by the SCN and conversely, the SCN is affected by the periodic melatonin release from the pineal and retina.

From the above summary it is clear that multiple oscillator pacemaking systems are becoming well known in a variety of mammals and birds but their analysis is hampered by the structural and functional complexity of vertebrate systems. Hence, a much simpler model system is needed in which more complex circadian systems and their coupling mechanisms may be dissected. At present, very little is known about comparable systems in invertebrates.

## BIOLOGICAL CLOCKS IN INVERTEBRATES

The first evidence of biological clocks in an invertebrate was seen in the circadian control of ecdysis (Pittendrigh, 1960) in the fruit fly, *Drosophila*. Ecdysis behaviour is achieved through the use of gates in many insect species (reviewed by Truman, 1985) which is defined by Pittendrigh (1960) as the 'allowed zone' of the cycle, dictated by the circadian clock, through which insects may emerge. Hence, if an insect is not at the 'correct' morphological state to use the gate, it must wait 24 hours for the next available gate. Pittendrigh (1981) argued that in *Drosophila pseudoobscura* eclosion rhythms can be conceptualized as a result of the interaction of two oscillators. Since then a number of

studies have attempted to find the anatomical locations of circadian oscillators in invertebrates.

A number of circadian oscillators have been located in invertebrates all of which reside in nervous tissue. The eyes of several opisthobranch mollusks have been described as circadian pacemakers. The most intensively studied species are *Bulla gouliana* and *Aplysia californica*. The molluscan eye shows rhythmic firing of optic nerve compound action potentials (CAPs; see reviews in Block *et al.*, 1993; Block *et al.*, 1995) in a circadian manner. In insects, the optic lobes of cockroaches, beetles and crickets contain circadian oscillators controlling locomotor rhythms. Evidence of the optic lobe as a circadian oscillator was observed in *Leucophaea maderae*, where it is thought to be located in the lobula region (Page, 1978). While in *Periplaneta*, the optic lobe was shown to contain a circadian oscillator originally using transplantation experiments (Page, 1983). More recent and conclusive data have shown that the optic lobe can produce a robust circadian rhythm in the frequency of neuronal activity *in vitro* (Colwell and Page, 1990). In the beetles, *Carabus problematicus* (Balkenohl and Weber, 1981) and *Pachymorpha sexguttata*, lobectomy resulted in loss of rhythmicity in locomotor rhythms (Fleissner, 1982), indicating the optic lobes contained a circadian oscillator. Lobectomy also produced arrhythmicity in the behaviour of crickets (Wiedenmann, 1983). On the other hand, in moths and flies, a circadian oscillator responsible for behavioural rhythms was reported to reside in the cerebral lobes and not the optic lobes, since surgical removal of the optic lobes did not alter the flight activity rhythms while removal of the cerebral lobes resulted in

arrhythmicity (Truman, 1974; Helfrich, 1987; Cymborowski *et al.*, 1994).

In all these well known circadian oscillating systems, there is a close relationship between the photoreceptor and the clock, which emphasizes the dominant role that the light cycle plays in entraining the system (Pittendrigh, 1993). In summary, all the clearly documented photosensitive circadian oscillators have been located in nervous tissues prior to recent work in our laboratory, there has been no direct evidence of circadian rhythmicity in any non-neural endocrine gland (see below).

## INSECT GROWTH AND DEVELOPMENT

Wigglesworth (1934) was the first to demonstrate that in *Rhodnius prolixus* there exists a critical period during the moulting cycle after which the head becomes unnecessary for the process to continue. This period was found by decapitation experiments. Decapitation a few days after feeding prevented the formation of an underlying cuticle while decapitation after this head critical period (HCP) did not prevent new cuticle formation (Wigglesworth, 1934). The factor from the head, termed the 'brain hormone', was thought to be necessary prior to the HCP for the moulting process. Meanwhile, research by Hachlow (1931) showed evidence of the existence of a thoracic center involved in the endocrine control of moulting. Specifically, he showed that different combinations of body segments from pupae when isolated must contain the thorax for moulting to occur. The thorax was termed the source of 'moulting hormone'. The interaction between the sources of 'brain hormone' and 'moulting hormone' was shown by

Williams in 1947 who used isolated abdomens of diapausing *Hyalophora cecropia* and implanted them with brains of non-diapausing pupae and 2 prothoracic glands (PGs) which resulted in the initiation of development in the abdomen. The PGs of *Rhodnius prolixus* were described by Wigglesworth (1952) as the source of the 'moulting hormone' using implantation experiments into isolated abdomens. Wigglesworth clarified the brain-PG interaction using *Rhodnius prolixus* and showed that the secretion of the 'moulting hormone' from the PGs was stimulated by the 'brain hormone'. Since then, the 'brain hormone' has been know as prothoracicotropic hormone (PTTH) based on it's ability to stimulate the PGs. This is the endocrine axis of insect larval-adult development, a two step process whereby the brain stimulates the PGs to produce the 'moulting hormone'. The 'moulting hormone' has subsequently been identified as the steroid ecdysone and we now know that many ecdysteroids exist (Koolman, 1989).

The neuropeptide, PTTH, has been fully purified only from the silkmoth, *Bombyx mori* (reviewed by Ishizaki and Suzuki, 1994) where it was found to be a 30-kDa glycoprotein consisting of two identical subunits linked by a disulfide bond. *Bombyx mori* PTTH has more recently been found to be a member of the vertebrate growth factor superfamily (Noguti *et al.*. 1995). The PTTH *of Rhodnius prolixus* may possess conformational similarities with *Bombyx mori* PTTH since *Rhodnius prolixus* PGs respond to *Bombyx mori* PTTH *in vitro* (Vafopoulou and Steel. 1997) and that a single pair of neurosecretory cells is immunoreactive to the antiserum generated against PTTH of *Bombyx mori* (Nseiri and Steel. 1997) in *Rhodnius prolixus*.

PTTH is synthesized in specific neurosecretory cells in the insect brain where it is transported to the corpora allata which also act as neurohaemal sites in moths (Agui *et al.*, 1980). PTTH is the principal known regulator of the developmental changes in ecdysteroid synthesis. Classical indirect studies of the release times of PTTH were done using neck ligations (see Bollenbacher and Gilbert, 1985) which viewed PTTH as an 'activator' of PGs with release only occurring at one (or two) times early in each instar (Bollenbacher and Gilbert, 1981; Bollenbacher and Granger, 1985; Gilbert, 1989). Each release of PTTH was associated with the stimulation of ecdysteroid synthesis and an increase in haemolymph ecdysteroid titre (Bollenbacher and Gilbert, 1981; Steel *et al.*, 1982). Since removal of the head after the HCP does not prevent moulting, it has been generally assumed the PTTH was not released after that point. Hence, the role of PTTH has generally been thought to be limited to activation of ecdysteroidogenesis which then continues throughout development without further input from PTTH (see Gilbert, 1989 for review).

The mechanism by which PTTH acts on the PGs has been extensively studied in moths. In *Manduca*, PGs incubated in $Ca^{2+}$-free medium, or with medium with a calcium chelator or a calcium channel blocker showed impeded production of ecdysteroids in response to PTTH (Smith *et al.*, 1985). In 1989, Smith and Gilbert showed that internal as well as external $Ca^{2+}$ may be mobilized in response to PTTH stimulation. Elevation of internal $Ca^{2+}$ is known to be followed by cAMP production which was also shown to increase steroidogenesis in *Manduca* PGs *in vitro* (Smith *et al.*, 1984; 1985). However,

$Ca^{2+}$ was shown not to be important in the transduction pathway distal to cAMP production since the absence of calcium in the medium did not affect ecdysteroid production when glands were incubated with a cAMP analog. Membrane associated calmodulin-sensitive adenylate cyclase has also been found in *Manduca* PGs which is activated through the binding of guanine nucleotide-binding protein (G-protein; Meller *et al.*, 1988). Although the overall scheme of PTTH stimulated ecdysteroid biosynthesis is still not clear, the generalized picture involves calcium, calmodulin, G-proteins and adenylate cyclase (see Gilbert *et al.*, 1996 for review). In summary, PTTH appears to mobilize calcium stores, and increases intracellular cAMP formation in PGs, which, in turn, can lead to the activation of cAMP-dependent protein kinases (PKA's) and subsequent protein phosphorylation (see Smith, 1993). PTTH-stimulated ecdysteroidogenesis in *Manduca* appears to require protein synthesis as well as RNA synthesis as noted by Rybczynski and Gilbert (1995). These events are very similar to vertebrate stimulated steroid synthesis (see Orme-Johnson, 1990) and support the view that the study of insect steroidogenesis can be used as a model system for higher organisms (Gilbert 1989; Gilbert *et al.*, 1996).

## ECDYSTEROID EFFECTS ON TARGET TISSUES

As described above, growth and development in insects is controlled mainly by the moulting hormones, ecdysteroids (Riddiford and Truman, 1978). Upon stimulation by PTTH, the PGs synthesize and simultaneously release 3-dehydroecdysone into the

haemolymph (Gilbert, 1989) where it is reduced by a keto-reductase to ecdysone. The ecdysone is quickly converted to the principal moulting hormone, 20-hydroxyecdysone (20E), in peripheral tissues by ecdysone 20-mono-oxygenases. (Gilbert, 1989). 20E evokes moulting responses in target tissues (Richards. 1981).

Almost all the cells of insects are targets of ecdysteroids. The principle target of ecdysteroids is the epidermis itself which secretes the cuticle for the next stage depending on the amounts of ecdysteroids in the haemolymph (Riddiford, 1985; 1989; also see General Discussion). The best known action of ecdysteroids is in stimulation and growth of imaginal discs (see Bayer *et al.*, 1996). Other roles of ecdysteroids include regulation of growth of motor neurons (Prugh *et al.*, 1992) as well as the initiation of the breakdown of larval structures during metamorphosis (Truman, 1996). Ecdysteroids are known to act on the genome (see Cherbas and Cherbas, 1996) where they act on ecdysone responsive genes. Since the expression of many such genes is both time and concentration dependent (Cherbas and Cherbas, 1996), it is possible that rhythmic changes in ecdysteroid titre could participate in the temporal sequencing of the expression of these genes (see General Discussion)

## BIOLOGICAL CLOCKS IN INSECT DEVELOPMENT

Early indirect experiments done on the Saturnid moths revealed the presence of circadian control in insect development. Truman (1972; 1974) suggested that the HCP represents the "gate" for the presumed PTTH release in *Manduca*. Fujishita and Ishizaki

(1981) speculated that in *Samia cynthia ricini* there appeared to be the involvement of a circadian oscillator controlling larval ecdysis during larval-larval development. This was determined using neck-ligations applied at different times of day and measured development through "gated" responses (Truman and Riddiford, 1974; Fujishita and Ishizaki, 1982). Although a clock controlling PTTH secretion was previously suggested (Truman, 1972; Fujishita and Ishizaki, 1981) this was never actually shown since there was no assay for PTTH at this time. No hormones were measured in these experiments and the existence of a maximum of two consecutive gates is not enough to demonstrate persistence under aperiodic conditions and thereby prove the existence of a circadian oscillator in the brain controlling PTTH release. These indirect experiments only suggested circadian control over insect development but were inconclusive.

More direct evidence on the clock-controlled PTTH release was obtained from studies of pupal diapause in *Manduca sexta* and *Antheraea pernyi*. Pupal diapause in these insects is photoperiodically induced (Denlinger, 1985). Pupae go into diapause when larvae experience short days. Diapause induction has been shown to be due mainly to the absence of PTTH, and is terminated by release of PTTH, which is induced through a change in daylength (see Denlinger, 1985). Brain transplantation experiments by Williams and Adkisson (1964) as well as *in vitro* experiments on the brain in relation to photoperiodism (Bowen *et al.*, 1984) were able to show that the brain is directly photosensitive and contains the photoperiodic clock that regulates the gated PTTH release that terminates diapause. Specifically, short day programs (hours of light: dark, 12L:12D)

during larval development programs the brain to forego the release of PTTH in the pupal stage and hence results in pupal diapause. while long days (18L:6D) experienced by the larva early in the last larval instar for 3 days will result in PTTH release and diapause is prevented (Bowen *et al.*. 1984). *In vitro* reprogramming of the photoperiodic clock in the *Manduca* brain-retrocerebral complex was shown by Bowen and colleagues in 1984. They studied the effect of exposing brains to 3 consecutive long days *in vitro* from Day 1 last instar larvae which were destined for diapause (i.e. reared under short days). They re-implanted the treated brains after 3 days into diapause destined larval hosts and tested the incidence of non-diapause in the pupae. They found that brains that were exposed to long days *in vitro* caused aversion of pupal diapause in recipient larvae. Their results show that the larval brain-retrocerebral complex is capable of light reception *in vitro*, that it stores this information and expresses it (through PTTH release) 9 days later in another stage of development (pupa). Therefore, Bowen and colleagues (1984) concluded that the photoperiodic clock is capable of being reprogrammed *in vitro*.

More recently, Saumann and Reppert (1996) showed that the 'clock gene' *per* is localized to two pairs of cells in each hemisphere of the brain of *Antheraea*. One pair of *per* expressing cells was found to be adjacent to the PTTH producing neurons and showed axonal arborizations with them suggesting that PTTH and *per* are very closely associated and that *per*-protein (PER) found in the axons may be the method of regulation of the PTTH cells (see General Discussion: Saumann and Reppert. 1996). Saumann and Reppert (1996) assumed that the function of *per*-expressing cells in *Antheraea* was the regulation

of diapause termination by stimulating PTTH release in response to photoperiodic cues. It has not been until more recently in our laboratory that it has become apparent that a comparable pattern of connections may also regulate a circadian rhythm of PTTH release (Vafopoulou and Steel, 1996a,b; see below).

The PGs of the saturniid moth, *Samia cynthia ricini* are thought to contain a circadian clock which controls the overt response of 'gut purge' as suggested by localized illumination and PG transplantation experiments on 5th instar larvae (Mizoguchi and Ishizaki, 1982). These experiments showed that the general region of the PGs was photosensitive *in vivo* and regulated the gated response of 'gut purge'. However, no hormones were measured in these indirect experiments.

Generally, insect development in moths is very difficult to study due to the fact that they are continuous feeders. Hence, synchronizing a population and conducting experiments without disrupting feeding is very demanding. In *Rhodnius prolixus*, the study of growth and development is simplified since a single large blood meal synchronizes the development of an entire population from one instar to the next (Uribe, 1927). Consequently, precise stages of development can be selected when using *Rhodnius prolixus* without disrupting feeding. Synchronization of development is very important for studying development related events because of their precise timing (Steel and Vafopoulou; 1989; 1990). In addition, there is a wealth of knowledge about the physiology and development of *Rhodnius prolixus* primarily due to the great contributions of Professor Sir Vincent B. Wigglesworth (see Locke, 1992 for biography)

## THE CIRCADIAN REGULATION OF ECDYSTEROID SYNTHESIS

The first evidence of the circadian regulation of development in *Rhodnius prolixus* was found by measuring haemolymph ecdysteroid titres in the fifth larval instar under 12L:12D conditions. Ampleford and Steel (1985) studied days 14-19 after feeding and later Vafopoulou and Steel (1989) analyzed titres for days 10-13, both of which showed massive daily increases and decreases in titre peaking in each night (scotophase). Real evidence of an endogenously controlled rhythm comes from studies done in continuous conditions such as continuous light or dark. In addition, the period length of the rhythm must be shown to be temperature compensated in order to be called circadian (see Saunders, 1977). Both of these criteria were satisfied in the ecdysteroid haemolymph titre which continued to oscillate in animals transferred from 12L:12D to continuous darkness (DD), and the free-running period length remained unchanged at 24°C and 28°C (Ampleford and Steel, 1985).

Vafopoulou and Steel (1991) showed that the PGs can synthesize and release ecdysteroids *in vitro*. The daily rhythm of ecdysteroid synthesis by PGs was also shown to be under circadian control with a rhythm that free-runs in DD which peaks in the subjective night and has a temperature-compensated period length close to 24 hours (Vafopoulou and Steel, 1991). In addition, PG's of *Rhodnius prolixus* are directly photosensitive *in vitro* (Vafopoulou and Steel, 1992). Recent evidence in our laboratory has shown that rhythmic steroidogenesis can be induced by light cues *in vitro* showing that a photosensitive oscillator resides in the glands themselves (Vafopoulou and Steel, 1998).

Since PGs are not innervated (Wigglesworth, 1952), they qualify as the first non-neural endocrine circadian oscillator (versus the list mentioned above which are all found in nervous tissue), driving rhythms in target tissues.

## CIRCADIAN REGULATION OF PTTH RELEASE

While most of the recent research on PTTH has been aimed at its purification (Ishizaki and Suzuki. 1992; 1994) and the biochemistry of its action on the PGs (Smith. 1993; Rybzynski and Gilbert, 1994 ), our laboratory has concentrated its efforts towards understanding the regulation of PTTH release. Indirect studies of PTTH release used cytological (Steel, 1982) electrophysiological (Orchard and Steel, 1980) and surgical (Knobloch and Steel, 1989) techniques during larval development. These showed two distinct release times. The first release occurs within the first two hours following a blood meal. while the second release occurs during the HCP on days 5-6 of larval development. Later, Vafopoulou and Steel (1993) used an *in vitro* assay of PTTH released from brain-retrocerebral complexes during larval-adult development to confirm these predicted release times of PTTH. Using the *in vitro* assay a third novel release time of PTTH after the HCP was also discovered, which occurred during days 11-13 of larval-adult development in *Rhodnius prolixus* reared in continuous light (Vafopoulou and Steel. 1993).

This novel release of PTTH after the HCP was the precedent for the current evidence of circadian release of PTTH throughout development (Vafopoulou and Steel.

1996a). Using an *in vitro* bioassay, Vafopoulou and Steel (1996b) found rhythmic PTTH release from intact animals which free-runs in DD and peaks in the subjective night with a period length close to 24 hours. Rhythmic PTTH release normally closely resembles the pattern of ecdysteroid biosynthesis by the PGs *in vivo*. Hence, a second circadian oscillator was discovered in *Rhodnius prolixus* and is thought to be located in the brain retrocerebral complex (Vafopoulou and Steel, 1996b) which follows a similar pattern to that described above for vertebrates.

It is generally thought that steroid rhythms in vertebrates are slaves to neuropeptide input. This view is seriously challenged by the fact that the PGs contain their own photosensitive oscillator *in vitro* (Vafopoulou and Steel, 1998). Therefore the question remains, what is the purpose of a circadian rhythm of PTTH release *in vivo*? Since decapitation after the HCP does not prevent the moulting process (Wigglesworth, 1934), this repeated release of PTTH is not qualitatively necessary for the continuation of development and currently the function of the continued release is unknown. As mentioned earlier, PGs are not-innervated (Wigglesworth, 1952), hence they are thought to be coupled to the brain oscillator via a humoral agent. Since PTTH released from the brain complex is the principal humoral agent that acts on the PGs, it is possible that the prolonged, rhythmic release of PTTH described above plays a role in the regulation of the daily activities of the PGs. This idea implies that the role of PTTH release is clearly more complex than just the initial 'activation' and that these daily signals from PTTH are conveyed to the PGs. The purpose of these daily inputs is currently unknown and will be

addressed in this thesis. It is postulated that the PG and brain oscillators comprise a multiple oscillator system controlling the overt rhythm of haemolymph ecdysteroid titre which, in turn, affects rhythms in target tissues and the overall process of moulting in insects.

## OBJECTIVES

The objectives of the present study were two fold:

1) Attempt to establish the role of the circadian release of PTTH throughout larval-adult development and how it interacts with the well documented oscillator found in the PGs of *Rhodnius prolixus*.

2) To determine photosensitivity of PGs *in vivo* and discuss the possible role of this photosensitivity in the circadian interaction with the brain and on the overall development of *Rhodnius prolixus*.

# CHAPTER 1

# RHYTHMIC STEROIDOGENESIS BY THE PROTHORACIC GLANDS OF THE INSECT, *RHODNIUS PROLIXUS*, IN THE ABSENCE OF RHYTHMIC NEUROPEPTIDE INPUT: IMPLICATIONS FOR THE ROLE OF PROTHORACICOTROPIC HORMONE

D.PELC AND C.G.H. STEEL

## INTRODUCTION

The cerebral neuropeptide prothoracicotropic hormone (PTTH) plays a central role in insect development (Bollenbacher and Granger, 1985; Gilbert *et al.*, 1996) by stimulating the prothoracic glands (PGs) to synthesize and release the steroid moulting hormones (ecdysteroids; Koolman, 1989). Ecdysteroids in turn act on target tissues, in which they elicit the temporally precise sequences of gene expression that underly development (Cherbas and Cherbas, 1996). Until very recently, it was understood that the release of PTTH occurred at only one (or two) discrete moments early in the development of each larval instar and induced sustained activation of steroidogenesis in the PGs (Bollenbacher and Gilbert, 1981; Bollenbacher and Granger, 1985). PTTH has therefore been understood to activate the endocrine cascade in insect development but to play no apparent role in its progression. This classical picture has recently become complicated by two discoveries; first, PTTH release is not confined to initial activation of the PGs, but continues throughout development (Vafopoulou and Steel, 1996a) and second, both PTTH and ecdysteroids exhibit pronounced circadian rhythmicity (Vafopoulou and Steel 1991;1996b). These findings raise many previously uncontemplated questions concerning the function(s) of this prolonged release of neuropeptide and the significance of rhythmicity throughout the endocrine axis regulating insect development.

The PGs are the only known target of PTTH, so it seems likely that rhythmic PTTH release is associated with their regulation. However, classical experiments employing decapitation and neck ligation have shown that PTTH is not essential for the continuation

of development after the first few days (Wigglesworth, 1964, 1985; Bollenbacher and Granger, 1985). Indeed, a basically normal profile of changes in the haemolymph ecdysteroid titre during development is seen in decapitated larvae (Dean and Steel, 1982). Therefore, it is unlikely that steroidogenesis by the PGs is a passive slave rhythm that is maintained by continuous rhythmic input from PTTH. A more subtle role of rhythmic PTTH release is implied. The present paper addresses the functional relationship between these two hormonal rhythms. The approach adopted has been to examine the rhythm of steroidogenesis by PGs when rhythmic input from PTTH is removed. We report that the PTTH rhythm is not required to sustain rhythmic steroidogenesis; rather, it appears to regulate the phase of the rhythm. This conclusion implies that PTTH functions as an entraining agent to the rhythm of steroidogenesis. This is a novel and unprecedented conceptualisation of the function of this developmental neuropeptide, in which it is assigned a key role in the regulation of temporal order within the endocrine system during the course of development.

## MATERIALS AND METHODS

Fifth (last) larval instar *Rhodnius prolixus* were reared at 28±0.5°C in a 12 hr light: 12 hr dark (12:12) regime. Larval-adult development was initiated by a large bloodmeal. Development was timed from the day of feeding, which was designated as day 0. Only male larvae were used. Ecdysis to the adult is under circadian control (Ampleford and Steel, 1982) and the median day of ecdysis in a population of males is day 21 (Vafopoulou

and Steel, 1989). The pigmented area of the compound eye begins to spread beyond the periphery of the eye of the fifth instar on day 14 in males (for details see Vafopoulou and Steel, 1989). The time of ecdysis and of onset of the spreading of eye pigment were recorded in order to determine if delays in visible development occurred in treated animals.

Decapitation was performed one hour before lights-off on day 9, with a noose of fine surgical silk as described by Knobloch and Steel (1987), but without anaesthesia. Tetrodotoxin (TTX) was injected into a hind tibia using a calibrated glass capillary, as previously described (Knobloch and Steel, 1988). All animals received 4 µl of $2.0 \times 10^{-6}$M TTX in 1% sodium chloride. The symptoms and time-course of both the onset of, and recovery from flaccid paralysis have been detailed elsewhere, as has their relation to the electrical activity of the retrocerebral complex (Knobloch and Steel, 1988) from which PTTH is released. In brief, electrical activity in the retrocerebral complex ceases 30 min after injection and flaccid paralysis appears complete at 60 min; recovery of behaviour occurs progressively over a 12 hr period and electrical activity in the retrocerebral complex reappears over the same period. Using the present dose of TTX, complete flaccid paralysis lasted 92 hr. Injections were given one hour before lights-off on day 9;therefore paralysis was complete by the time of lights-off on day 9 and the first signs of recovery were detectable during the late photophase of day 13.

Ecdysteroid synthesis by PGs was quantified using an *in vitro* assay (Vafopoulou and Steel, 1989). PGs were removed from animals at the times of day stated (see Results) and incubated in saline *in vitro* for 4hr. The ecdysteroid content of incubation medium was

quantified by RIA (see below). It has been shown that ecdysteroids are not stored by the PGs, hence all ecdysteroid in the medium results from synthesis (Vafopoulou and Steel, 1989). Synthesis of ecdysteroids is linear for at least 4 hr; thus, changes in amounts of ecdysteroid synthesized by different PGs reflect changes in relative rates of synthesis. At the time of PG explantation, a sample of haemolymph was collected in a calibrated glass capillary. The haemolymph ecdysteroid titre was determined by RIA of methanol extracts of haemolymph (see below). Groups of 8 - 12 PGs were examined at intervals of 5-6 hr throughout the period from 24-96 hr after decapitation or injection of TTX. This period corresponds to days 11, 12 and 13 of development. PGs from animals during the scotophase, or during continuous darkness (DD), were incubated in darkness; PGs from the photophase, or during continuous light (LL) were incubated in the light. The procedure for obtaining samples during darkness is described by Vafopoulou and Steel (1991).

The RIA for ecdysteroids in haemolymph has been described by Steel *et al.* (1982) and in incubation medium by Vafopoulou and Steel (1989). The ligand, a-[23,24(N)-$^{3}$H]-ecdysone was purchased from New England Nuclear (sp.act. 89 Ci/mmol). 20-hydroxyecdysone (20E) was used as the standard in the assay, thus all amounts are expressed as 20E equivalents.

The amplitude of observed rhythms was determined using two procedures, both of which gave similar results. In the first, all the individual data measurements for time points within a 24 hr period were totalled and the mean calculated; amplitude was calculated as

amounts by which the mean peak and trough values for that period differed from this mean. In the second, amplitude was measured as the vertical distance between a line connecting consecutive peaks and a line connecting consecutive troughs.

## RESULTS

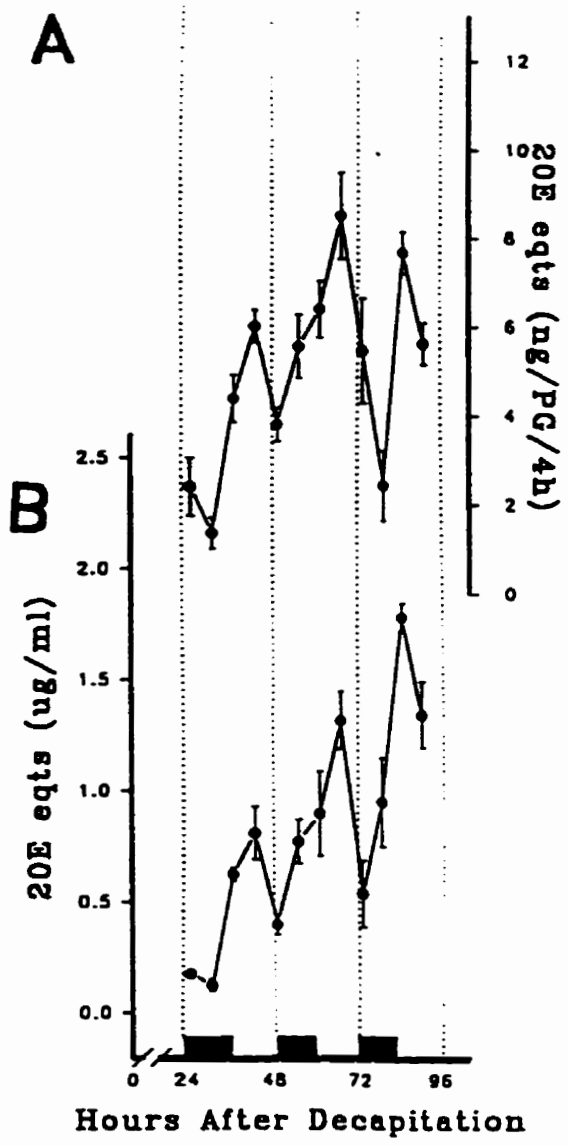
Initial experiments to examine the role of the head in regulation of the rhythm of steroidogenesis by PGs employed decapitation. Decapitation was performed at the end of the photophase of day 9 of larval-adult development. Commencing 26 hr after decapitation, 8 - 12 PGs were excised every 6 hr for 3 consecutive days (days 11-13 of development) and the level of ecdysteroid synthesis determined *in vitro*. The haemolymph ecdysteroid titre of each animal was also determined from an aliquot of haemolymph expressed at the time of dissection.The results showed that rhythmicity was maintained throughout the sampling period in both ecdysteroid synthesis by the PGs (Fig. 1A) and haemolymph ecdysteroid titre (Fig. 1B). Further, the close coupling of the two rhythms seen in intact animals (Vafopoulou and Steel, 1991) is retained in headless animals. However, both rhythms display peaks in the photophase and troughs in the scotophase, the reverse of their positions in intact animals (see Discussion). Decapitation obviously causes injury to various tissues and it is well documented that insect development is sensitive to injury (Bulliere and Bulliere, 1985). Ecdysteroid titres are altered by injury in several insects, including *Rhodnius prolixus* Knobloch and Steel, 1988). In decapitated animals,

the effects due to injury cannot be distinguished from those due to the removal of PTTH since there is no control procedure for decapitation.

Therefore, analysis of these ecdysteroid rhythms was conducted in animals paralyzed with a large sub-lethal injection of TTX. The severence of a tibia required to perform the injection has no detectable effect on ecdysteroid levels if performed after day 6 of development (Knobloch and Steel, 1988). The dose of TTX employed eliminated action potentials from the retrocerebral complex for 92 hr (see Methods). Since the axon terminals of PTTH cells are located in this complex (Agui et al., 1980), we conclude that release of PTTH is abolished for at least 92 hr.

The rhythms of ecdysteroid synthesis and titre of animals paralyzed by TTX in 12:12 is shown in Fig. 1C and 1D. Both rhythms are maintained and show the same reversal of phase seen in decapitated animals. Therefore, the reversal seen in decapitated animals is not due to injury or damage to the PGs. However, the rhythms of paralyzed animals have more precisely defined peaks and troughs, confirming the suitability of paralyzed animals for their analysis. If the rhythm of steroidogenesis by PGs was a passive slave rhythm driven by rhythmic PTTH release, some evidence of damping might be expected, but this is not seen. The amplitude of the rhythms (see Methods) is sustained. Further, the rhythms maintain a stable phase relation to the light cycle. These findings suggest that the rhythm of steroidogenesis is driven by an oscillator within the PGs (see Discussion).

FIGURE 1: (A) Entrained rhythm of synthesis of ecdysteroids by PGs from animals that remained left in 12 hr light:12 hr dark (12L:12D) conditions after decapitation at the end of the photophase of day 9. Results are shown for days 11-13 of larval-adult development (separated by vertical dotted lines). Synthesis is measured as the quantity of ecdysteroid produced during 4 hr *in vitro* incubation following explantation at the times indicated (see Methods). Points are means of 8-12 incubations $\pm$SEM. (B) Haemolymph ecdysteroid titre of the same animals as in (A). Note the close relationship between the rhythms of steroidogenesis and haemolymph titre. (C) Rhythm of synthesis of ecdysteroids by PGs from animals that remained in 12L:12D after induction of paralysis with tetrodotoxin (TTX) at the end of the photophase of day 9. Same technical details as in (A). (D) Haemolymph ecdysteroid titre of the same animals as in (C).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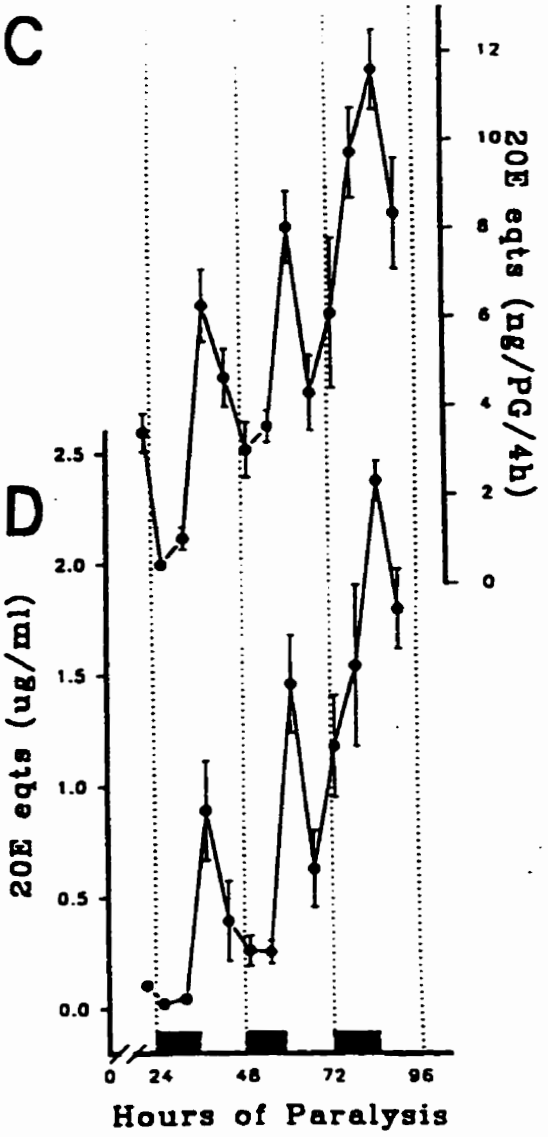
Hours After Decapitation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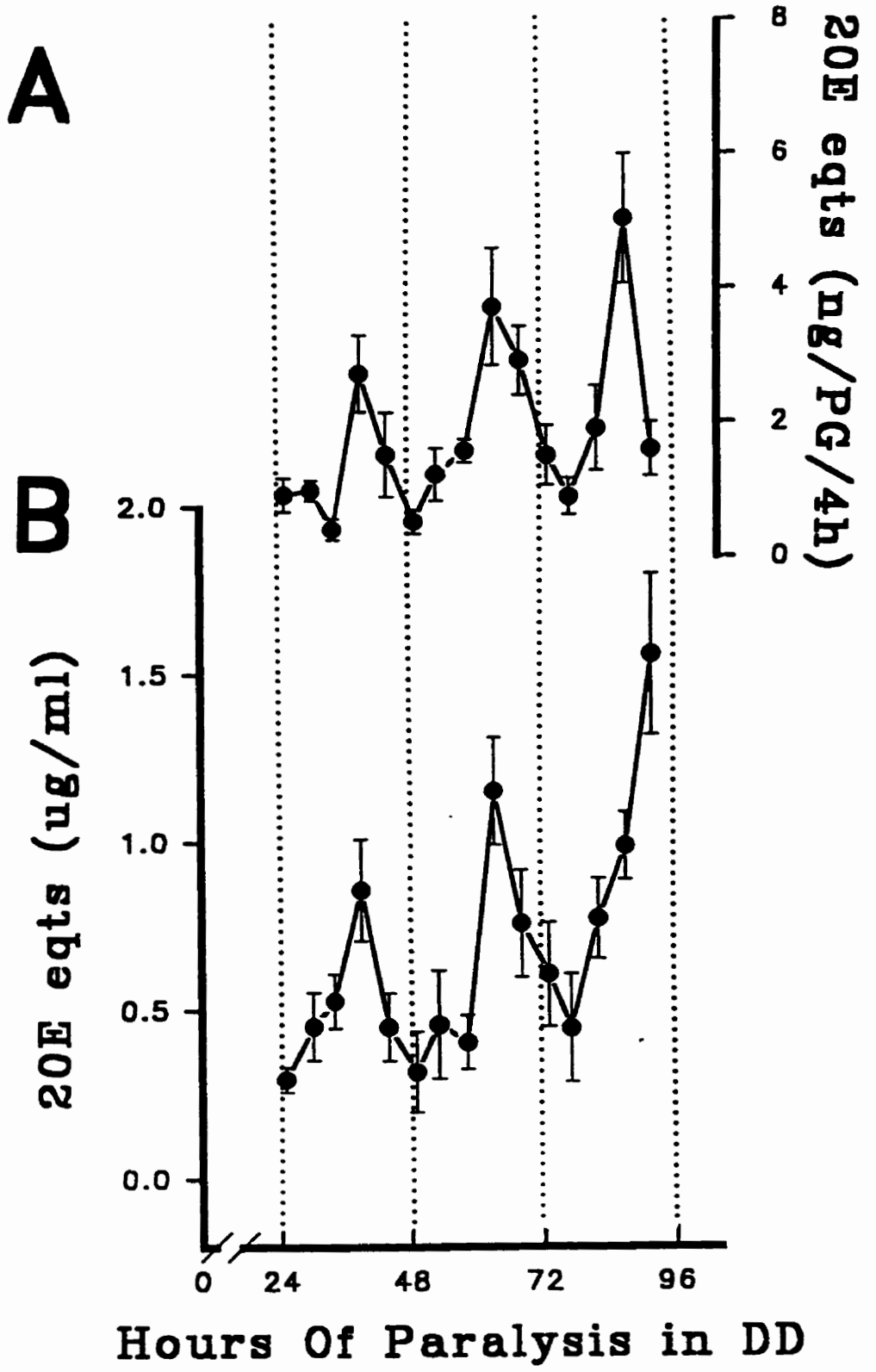
Hours of Paralysis

FIGURE 2: (A) Free-running rhythm of synthesis of ecdysteroids by PGs from animals transferred to continuous dark (DD) after induction of paralysis with tetrodotoxin (TTX) at the end of the photophase of day 9. Vertical dotted lines depict "subjective days" in each of which the first 12 hours is "subjective scotophase" and the second 12 hours is " subjective photophase". Technical details as in Fig. 1(A). (B) Haemolymph ecdysteroid titre of the same animals as in (A).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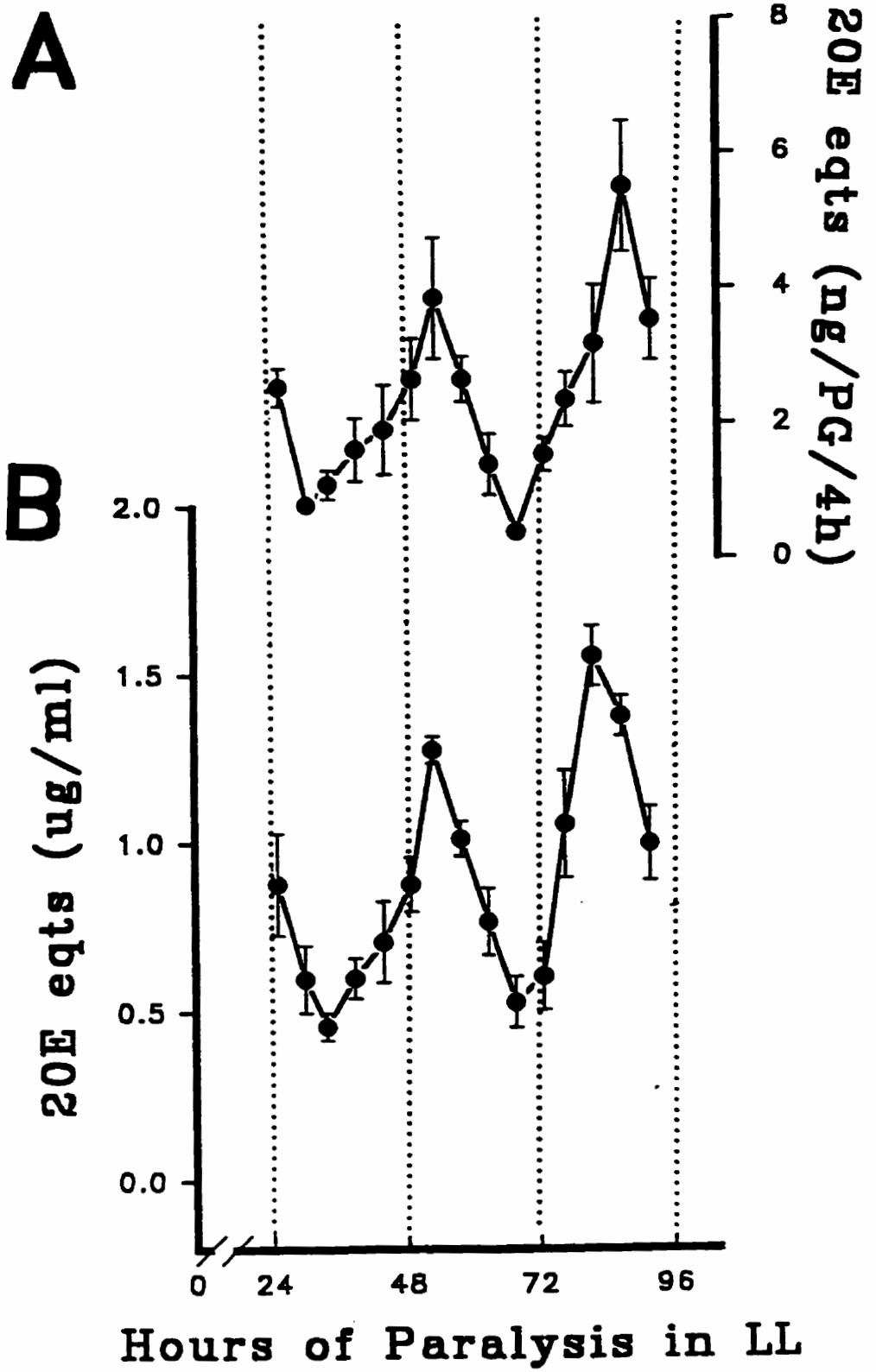
24
48
72
96
Hours Of Paralysis in DD

FIGURE 3: (A) Free-running rhythm of synthesis of ecdysteroids by PGs from animals maintained in continuous light (LL) after induction of paralysis with tetrodotoxin (TTX) at the end of the photophase of day 9. Therefore animals had been in light 12 hr prior to induction of paralysis. Vertical dotted lines depict "subjective days" in each of which the first 12 hours is "subjective scotophase" and the second 12 hours is "subjective photophase". Technical details as in Fig. 1(A). (B) Haemolymph ecdysteroid titre of the same animals as in (A). Note the reversal of phase of both rhythms relative to Figs. 1 and 2.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Hours of Paralysis in LL

We then examined whether rhythmic steroidogenesis retained circadian properties in paralyzed animals. Paralyzed animals were transferred from 12L:12D to DD at the habitual time of lights-off on day 9 and ecdysteroid synthesis and titre measured throughout subjective days 2-4 after transfer to DD. The rhythm of ecdysteroid synthesis is maintained (Fig. 2A) and remains coupled to the haemolymph ecdysteroid titre rhythm (Fig. 2B). Both rhythms show peaks in the subjective photophase and appear to be free-running with a period length close to 24hr. Therefore, PGs continue to exhibit rhythmic ecdysteroid synthesis when rhythmic inputs from both PTTH and the light cycle are removed. However, the oscillations are more parallel to the X-axis (compare Figs. 1 and 2), indicating that the ascending baseline associated with development is reduced (see below). However, the amplitude of the rhythm (see Methods) shows no evidence of damping, even after 4 cycles in DD.

In a complementary experiment to the above, ecdysteroid synthesis and titre were examined in paralyzed animals transferred to LL at the habitual time of lights-off on day 9. It is again clear that rhythmicity is maintained in both ecdysteroid synthesis (Fig. 3A) and the haemolymph ecdysteroid titre (Fig. 3B) and the two rhythms remain closely coupled. The ascending baseline associated with development is again reduced. However, striking differences from the rhythms in both 12L:12D and DD are apparent. The peaks initially occur in the subjective night rather than the subjective day; also, the period length of the rhythm appears longer, such that by the end of the sampling period, the peaks appear to drift into the next subjective day. Thus, both ecdysteroid rhythms in LL exhibit a reversal

of phase relative to 12L:12D or DD (compare Fig. 3 with Figs. 1 and 2). In comparison with unparalyzed animals, the ecdysteroid rhythms of paralyzed animals are always shifted by about 12 hr under all the lighting regimes examined (see Discussion).

In light of the finding that the ascending baseline of the rhythms in ecdysteroid synthesis and titre associated with development is impaired in paralyzed animals, we examined whether overt developmental events regulated by ecdysteroids showed a related impairment. The pigmented area of the compound eye begins to increase on day 14 in normal animals, which is the day during which paralyzed animals undergo behavioural recovery (see Methods). However, animals recovering from paralysis on day 14 showed no expansion of their eye pigment; the expansion which is seen in normal animals on day 14 occurred in these animals on day 15. Similarly, the median day of ecdysis was delayed one day from day 21 to 22. Therefore, paralysis for four days resulted in a delay in overt development by only one day.

## DISCUSSION

The original report (Vafopoulou and Steel, 1991) of circadian regulation of ecdysteroid synthesis in *Rhodnius prolixus* employed intact animals. The complexity of the free-running behaviour of the rhythms of ecdysteroid synthesis by PGs and the haemolymph titre led to the postulation that two oscillators participated in their regulation. One was assigned to the PGs themselves and it was speculated that the second might involve PTTH from the brain. However, the prevailing understanding of PTTH release was

that release occurred only at the onset of development (see Introduction) and therefore was presumed to be absent from the animals used in the above study. Recently, it has been shown that release of PTTH in *Rhodnius prolixus* occurs with a daily rhythm throughout most of larval-adult development and that a rhythm of PTTH is seen in the haemolymph (Vafopoulou and Steel, 1996a). The rhythm of release of PTTH is under circadian control (Vafopoulou and Steel, 1996b). Therefore, the rhythms of ecdysteroid synthesis reported by Vafopoulou and Steel (1991) were occuring in the presence of a rhythm of PTTH.

In the present study, decapitation or paralysis with TTX were employed to reveal the properties of the ecdysteroid synthesis rhythm in the absence of rhythmic neuropeptide release from the head. The close similarity between the effects of decapitation and paralysis on rhythmic steroidogenesis by PGs (Fig. 1) shows that the effects of paralysis can be interpreted in terms of loss of electrical activity in the head alone. The PGs of *Rhodnius prolixus* are not innervated (Wigglesworth, 1952); therefore, the head influences the PGs by a humoral route. The only known cerebral neurohormone that acts on the PGs is PTTH. PTTH is the principle known regulator of steroidogenesis by PGs (see Bollenbacher and Granger, 1985). Accordingly, we interpret the effects of decapitation or paralysis on rhythmic steroidogenesis by PGs in terms of removal of rhythmic PTTH release from the head. While it is theoretically conceivable that some currently unknown cerebral neuropeptide also participates in regulation of the PGs. this view is not required by the present findings. The TTX paralysis paradigm was preferred over decapitation for several reasons. First, it minimizes surgical injury, which itself can

disrupt ecdysteroids in *Rhodnius prolixus* (see Knobloch and Steel, 1988). Second, its effects are reversible: flaccid paralysis and the absence of action potentials at the PTTH release sites lasted for 92-104 hr, but the animals recovered and completed development with a delay of only one day. Third, *in vitro* experiments have shown that TTX has no detectable effect on steroidogenesis by PGs or on their responsiveness to light (Vafopoulou and Steel, 1992).

Following either decapitation or paralysis, a robust rhythm is maintained for at least four cycles in both ecdysteroid synthesis by PGs and the haemolymph ecdysteroid titre. The rhythm showed no evidence of damping or of loss of entrainment in 12L:12D, both of which might be expected if the rhythm of steroidogenesis was a passive slave to rhythmic neuropeptide input. Further, the rhythm free-runs in both DD and LL.Therefore, rhythmic steroidogenesis is sustained when inputs from both PTTH and the light cycle are removed. These experiments support the view that the PGs contain an endogenous circadian oscillator that regulates ecdysteroid synthesis, as suggested by Vafopoulou and Steel (1991), and does not support the possibility that the rhythmicity in ecdysteroid synthesis of intact animals is driven by rhythmic PTTH release. This conclusion contrasts with comparable systems in vertebrates, where rhythms in steroidogenesis are regarded as passive slaves driven by rhythmic neuropeptide input (see Turek, 1994). Further, the tight coupling of the rhythms of ecdysteroid synthesis and the haemolymph ecdysteroid titre reported in intact animals (Vafopoulou and Steel, 1991) is retained in paralyzed animals, indicating that the PTTH rhythm is not necessary for this coupling. The PG oscillator is

therefore the principal regulator of the rhythmic increases in the haemolymph titre. The finding that the daily decreases in haemolymph ecdysteroid titre are also unaffected in paralyzed animals implies that the mechanisms involved in removal of ecdysteroids from the haemolymph each day are also TTX-resistant.

Classical studies have demonstrated that apparently normal development can continue in insects in the absence of the head (see Steel and Davey, 1985). Although, it has been generally assumed that ecdysteroid production can continue in such animals, there have been no previous studies of ecdysteroid synthesis by PGs of headless animals. The present findings suggest that development is continous in headless animals because steroidogenesis is sustained by the PG oscillator. Moreover, rhythmicity in steroidogenesis is also maintained without the head. It has been suggested that rhythmic steriodogenesis imposes temporal order on development (Vafopoulou and Steel, 1996a, b), presumably by orchestrating sequential patterns of gene expression in target tissues (see Huet *et al.*, 1995). Thus, the apparently normal development of headless animals may be due to the maintenance of internal temporal order by the PG oscillator, which would become directly entrained by light when the PTTH rhythm is removed (see below).

In paralyzed or decapitated animals, the rhythm of ecdysteroid synthesis was always about 12 hr out of phase with the rhythm in intact animals (Vafopoulou and Steel, 1991) where rhythmic PTTH is present. This was true in entrained conditions, in DD and in LL. Therefore, removal of rhythmic input from PTTH causes the rhythm of steroidogenesis to undergo a phase shift of about 12 hr irrespective of the conditions of illumination. We

therefore suggest that a function of rhythmic PTTH release is to set the phase of the rhythm of steroidogenesis generated by the PG oscillator. However, steroidogenesis by PGs is directly photosensitive *in vitro* (Vafopoulou and Steel, 1992) and rhythmic steroidogenesis showed stable entrainment to a light cycle in both decapitated and paralyzed animals. Therefore, the PG oscillator is also entrainable by light. We infer that the PG oscillator appears to be entrainable both by light and by PTTH. But in intact animals, the rhythm of steroidogenesis is in phase with the rhythm of PTTH release under all conditions of illumination examined (Vafopoulou and Steel, 1996 b). We conclude that in the intact animal, entrainment by PTTH appears to dominate entrainment by light; photic entrainment of steroidogenesis is revealed only when rhythmic PTTH imput is removed.

Several examples are now known in which circadian systems can be entrained by both light and neurochemical agents; these include the actions of neuropeptide Y(Huhman and Albers, 1994) and melatonin (Lewy *et al.*, 1995) on the mammalian circadian system and the action of serotonin on the circadian system in the eye of the mollusc *Aplysia* (Corrent *et al.*, 1982; Block *et al.*, 1993). In each of these cases, it is striking that the phase response curve (PRC) for neurochemical entrainment is displaced by about 12 hr relative to that for light. Similarly, light and PTTH appear to entrain the steroidogenesis rhythm to phases that are about 12 hr apart. For example, PGs exposed to light cues but no PTTH cues (paralyzed animals in 12:12) show peak steroidogenesis in the photophase, whereas PGs exposed to PTTH cues but no light cues (intact animals in darkness, Vafopoulou and

Steel, 1991) show peak steroidogenesis in the subjective scotophase. A PRC for the action of PTTH on the rhythm of steroidogenesis would yield valuable information, but would be extremely laborious to construct due to the huge numbers of animals required and the complexity of the assay.

The above view of PTTH as an entraining agent to the PGs represents a novel conceptualization of the function of this hormone. It is therefore striking that this function of PTTH could be mediated by the same intracellular pathway by which it has been described as mediating stimulation of steroidogenesis in PGs which lack apparent rhythmicity. In the moth *Manduca*, PTTH appears to regulate a $Ca^{2+}$ channel in the PGs and the resulting $Ca^{2+}$ influx appears to stimulate a calmodulin-sensitive adenylate cyclase (reviewed by Gilbert, 1989; Smith, 1993). $Ca^{2+}$ and/or cAMP are known to induce phase shifts in many circadian systems and frequently produce PRCs which are 12 hr displaced from the PRC for light i.e. similar to those for neurochemical entrainment (for discussion, see Edmunds, 1988). This signal transduction pathway is therefore an appropriate pathway by which PTTH could convey phase control information to the PG oscillator of *Rhodnius prolixus*.

The above findings indicate that the role of PTTH in development is not confined to an initial activation of the PGs early in development but that it plays a continuing role throughout development as a key component of the circadian system that regulates the synthesis of the moulting hormones.

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# CHAPTER 2

# CIRCADIAN RHYTHMICITY IN THE NEUROENDOCRINE AXIS REGULATING DEVELOPMENT IN THE INSECT, *RHODNIUS PROLIXUS*: INDUCTION OF RHYTHMICITY BY LIGHT CUES *IN VIVO*

X. VAFOPOULOU, D. PELC and C.G.H.STEEL

## INTRODUCTION

The cerebral neuropeptide prothoracicotropic hormone (PTTH) plays a pivotal role in insect development. PTTH release triggers a cascade of neuroendocrine and endocrine phenomena which lead to moulting and metamorphosis. Its primary target of action is a pair of endocrine glands, the prothoracic glands (PGs), which it stimulates to synthesize and release the steroid hormones, ecdysteroids. Ecdysteroids from the PGs are quickly converted in peripheral tissues to 20-hydroxyecdysone (20E) which is the active form (see Gilbert, 1989;Gilbert *et al.*, 1996). Therefore, stimulation of steroidogenesis by PTTH leads to dramatic and distinct changes in the haemolymph ecdysteroid titre within a larval instar that eventually program the temporal pattern of ecdysone-responsive gene expression in target tissues (see Henrich and Brown, 1995; Cherbas and Cherbas, 1996).

Our laboratory has focussed on the role of circadian timing systems that control the neuroendocrine system responsible for ecdysteroid synthesis in the hemipteran, *Rhodnius prolixus*. We have found that synthesis of ecdysteroids by the PGs occurs with a daily rhythm which is regulated by a circadian system (Vafopoulou and Steel, 1991) and drives a circadian rhythm of changes in the haemolymph ecdysteroid titre (Ampleford and Steel, 1985). Initial analysis of the regulation of the ecdysteroid rhythm suggested the involvement of multiple coupled oscillators (Vafopoulou and Steel, 1991), analogous to the dawn (M) and dusk (E) oscillators originally described by Pittendrigh (1981a, b; Pittendrigh and Daan, 1976). The oscillators constituting this timing system were apparently located in anatomically separate sites: one oscillator appeared to reside in the

head (see Discussion), and thus it implicated PTTH, while the other was located in the PGs. In an attempt to dissect the components of this multiple oscillator system responsible for the regulation of rhythmicity of ecdysteroid synthesis, we have found that release of PTTH from the brain occurs with a daily rhythm throughout development (Vafopoulou and Steel, 1996a) and is under circadian control (Vafopoulou and Steel, 1996b). We have also recently demonstrated *in vitro* that a photosensitive oscillator resides within the PGs (Vafopoulou and Steel, 1998). This PG oscillator generates the rhythmicity seen in the haemolymph ecdysteroids *in vivo* and is also responsive to light cues *in vitro* (Vafopoulou and Steel, 1998). However, *in vivo*, it behaves as if entrained by the PTTH rhythm rather than by light (See Chapter 1). These findings collectively imply that the rhythmic output of ecdysteroids to which target tissues are exposed reflects the combined output of (at least) two oscillators; One located in the brain regulating rhythmicity in PTTH release and the other located in the PGs. The study of the individual properties of these oscillators and their functional relationship to one another therefore requires specific criteria by which to distinguish and separate their actions. In the present paper we report that rhythmicity can be induced in both oscillators by light cues *in vivo*. However, the two oscillators possess distinct characteristics. It is shown that PGs are photosensitive *in vivo*, despite the thick overlying cuticle; however when both light and PTTH are present, entrainment occurs preferentially to PTTH. It is concluded that regulation of the circadian endocrine system underlying insect development involves the coordinated expression of at least two oscillators which respond differentially to light but are coupled *in vivo* by rhythmic cues

from the neuropeptide PTTH to PGs. This notion assigns a new functional role to PTTH which is now seen as a hormonal coupling agent between anatomically distinct circadian oscillators that comprise the timing system that underlies the hormonal regulation of development.

## MATERIALS AND METHODS

Male fifth instar larvae of *Rhodnius prolixus* were maintained in continuous light (LL) at 28 $\pm$ 0.5$^0$C. Transfer to LL occured 2-3 weeks before feeding. A blood meal at day 0 initiated larval-adult development. Ecdysis to the adult is under circadian control (Ampleford and Steel, 1982) with a population median at day 21. At the end of subjective day 9 the larvae were divided in three groups. One group was maintained in LL and served as control. A second group of larvae was treated was injected with 4ul of 2x10$^{-6}$M tetrodotoxin (TTX) in 1% sodium chloride. TTX at this concentration abolished all action potentials in peripheral nervous tissue within 30 min from administration (Knobloch and Steel, 1988) and induced temporary flaccid paralysis of larvae for approximately 5 days (See Chapter 1). TTX injections inhibited all neuropeptide release from the brains of affected larvae (See Chapter 1). At the conclusion of subjective day 10 the group of TTX-treated larvae as well as a third group of untreated larvae were transferred to continuous dark (DD). PTTH release and ecdysteroid synthesis by both TTX-treated and untreated larval groups in DD were then monitored for several days as described below.

***In vitro* assays for PG ecdysteroid synthesis and brain-retrocerebral complex PTTH release:**

PG dissection began 1hr after transfer to DD and was carried out at regular intervals every 6hr for the total of 72hr, according to the methods of Vafopoulou and Steel (1989). PGs from a group of control LL animals at the same age as larvae in DD were also dissected out at the same time periods. PGs were incubated individually for 4hr at room temperature in *Rhodnius prolixus* saline and the ecdysteroid content of the media was assayed by RIA (see below). PGs from larvae in DD were incubated in the dark and PGs from larvae in LL were incubated in the light. Statistical analysis was carried out with the Student's *t* test.

PTTH release from extirpated brain-retrocerebral complexes (brain complexes) was assayed with an *in vitro* assay established by Vafopoulou *et al.*, (1996). Brain complexes were incubated individually in 25ul *Rhodnius prolixus* saline for 4hr at room temperature. All media were retrieved individually, heated at 100$^0$C for 2min and stored at -80$^0$C. Brain complexes from larvae in DD were incubated in the dark and brain complexes from larvae in LL were incubated in the light. PTTH content of the medium was assayed by its capacity to stimulate ecdysteroid synthesis in day 7 LL PGs. Day 7 larvae were chosen as PG donors because they exhibit low basal levels of ecdysteroid synthesis and can be maximally stimulated with PTTH *in vitro* (for details see Vafopoulou *et al.*, 1996). PGs were incubated individually in PTTH containing medium previously derived from individual brain complexes. Stimulation of synthesis was measured by comparing the

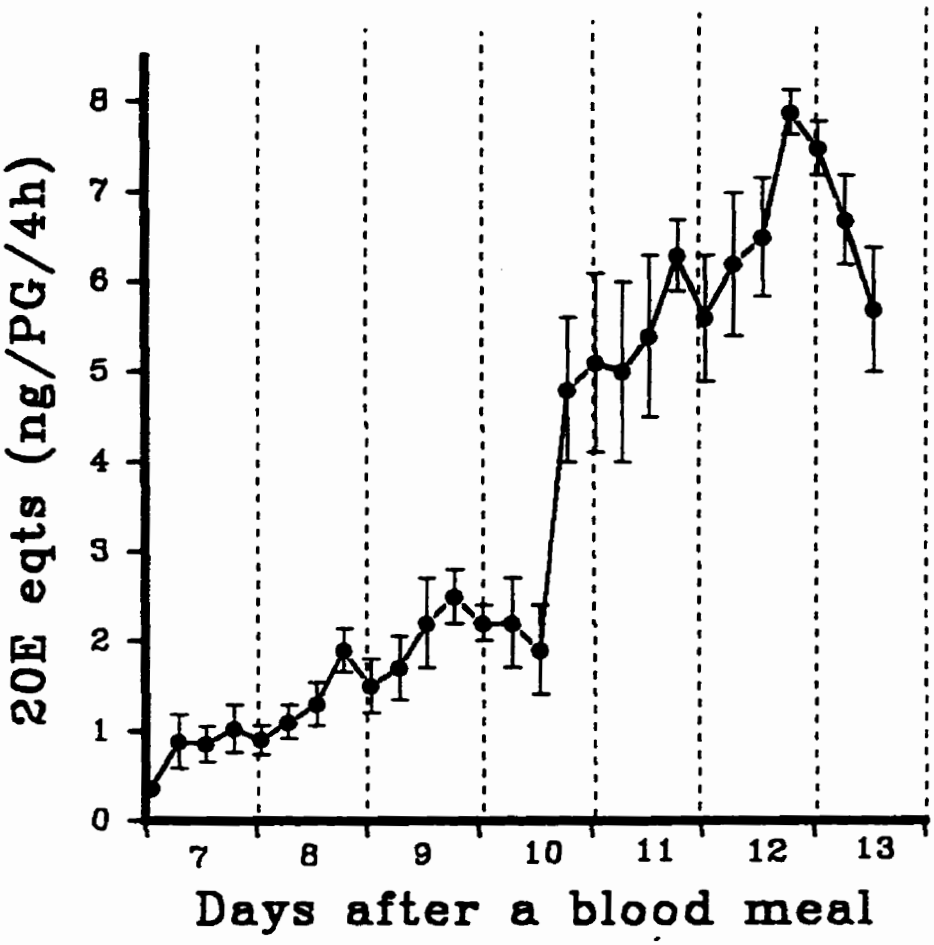
amount of ecdysteroid synthesis by one member of a PG pair in the presence of PTTH containing medium to that of its contralateral member which was incubated in saline (i.e. in the absence of PTTH: control). Stimulation was expressed as the mean of differences (± SEM) (in ng 20E equivalents) in amounts of ecdysteroid between stimulated and control PGs for a group of pairs. Statistical analysis was carried out with the paired-sample *t*-test (two tailed) and the resulting *t* values were plotted as Stimulation Index (SI) in order to reveal the level of significance of stimulation (Vafopoulou *et al.*, 1996).

For the RIA, α-[23,24(N)-$^3$H]ecdysone (NEN) was used as a ligand (sp. act, 89Ci/mmol). 20-hydroxyecdysone (20E) was used as standard, thus all amounts are expressed as 20E equivalents (±SEM).

## RESULTS

PGs were sampled at regular intervals for 7 consecutive days (subjective days 7-13) from LL larvae in order to confirm that chronic exposure to LL abolished rhythmic ecdysteroid synthesis. As shown in Fig. 1, the profile of ecdysteroid synthesis in LL exhibited a progressive increase until day 10. At day 10, synthesis increased abruptly, reached a peak at day 12 and then began to decline. Synthesis of ecdysteroids in LL thus exhibited a developmental pattern similar to that of normal animals in 12hr light:12 hr dark (12L:12D) conditions (Steel *et al.*. 1982). but it clearly lacked all daily circadian oscillations characteristic of the entrained state (Vafopoulou and Steel. 1991). When LL larvae were transferred to DD for 72hr (subjective days 11-13) the

**FIGURE 1:** Ecdysteroid synthesis by PGs of *Rhodnius prolixus* larvae maintained in continuous light (LL) during days 7-13 of the larval-adult development. Points are means of 12 cultures ±SEM.


20E eqts (ng/PG/4h)
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2
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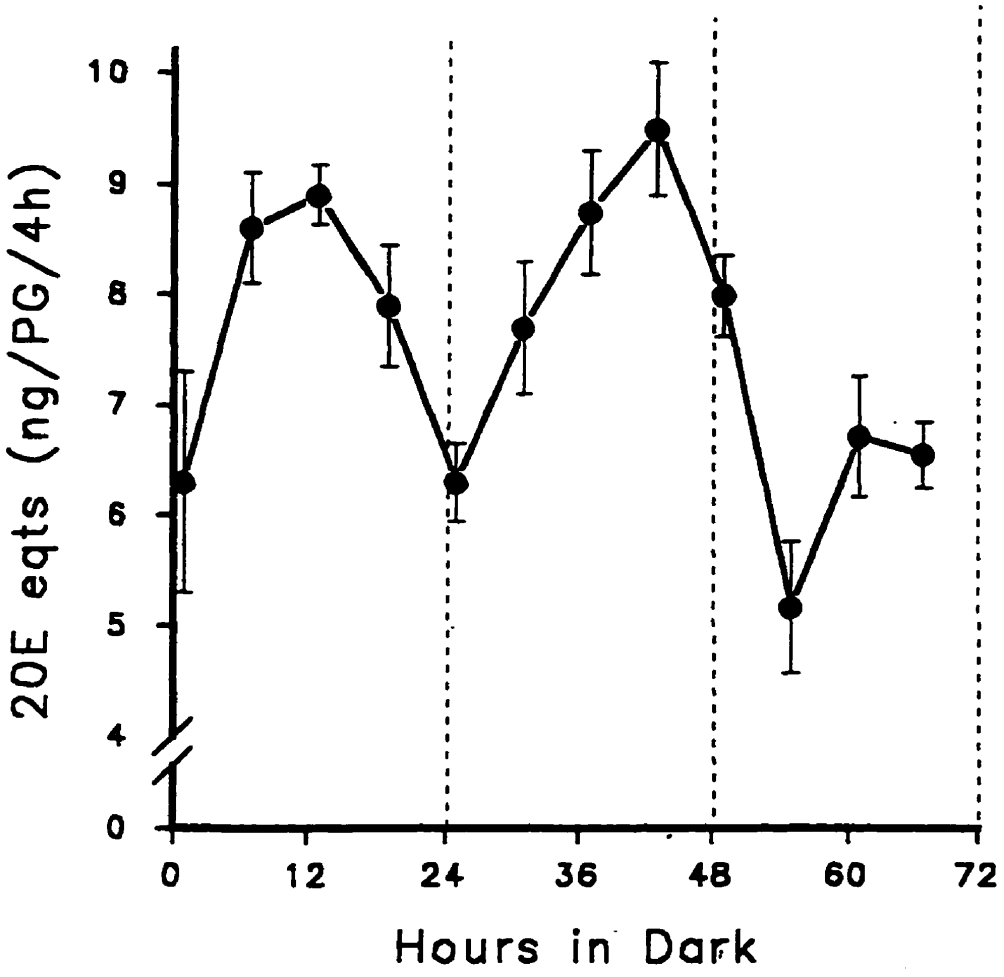
12
13
Days after a blood meal

**FIGURE 2:** Induction of rhythmicity in ecdysteroid synthesis by PGs from continuous light (LL) larvae which were transferred to dark on day 11 and maintained in continuous dark (DD) for 72hr. Synthesis was measured at 6hr intervals. Points represent means of 10 cultures ±SEM. Peaks of synthesis occur approximately every 24hr (compare with Fig. 1).


20E eqts (ng/PG/4h)
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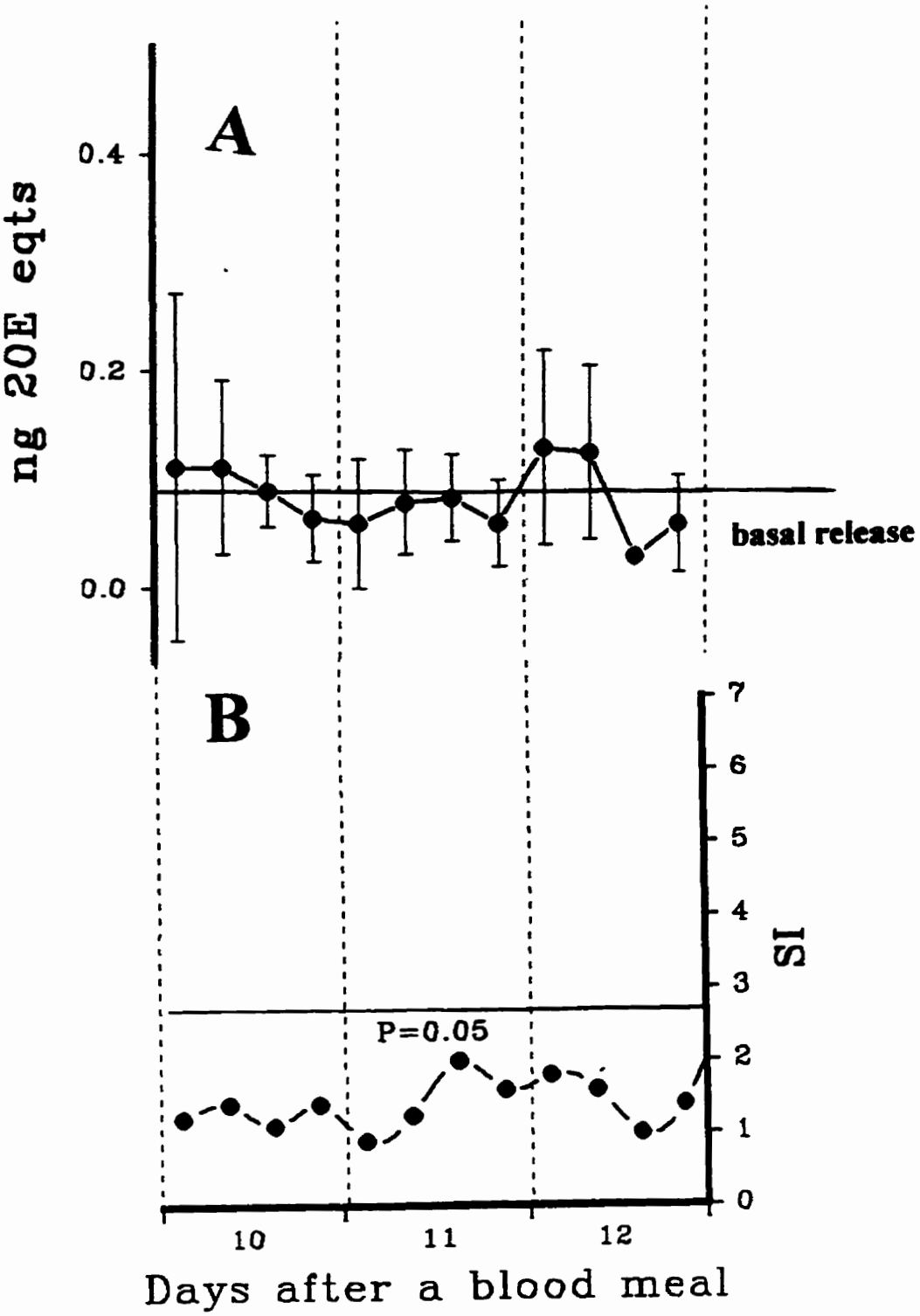
48
60
72
Hours in Dark

**FIGURE 3:** Stimulation of ecdysteroid synthesis by PTTH released from the brain-retrocerebral complexes removed at 6hr intervals from days 10-12 continuous light (LL) larvae. (A) Stimulation is expressed as the numerical difference in synthesis between stimulated and unstimulated (contralateral) members of each PG pair plotted as mean difference ±SEM of 6 separate determinations. (B) SI values derived from the statistical analysis of the data in A. Data points above the line at P=0.05 represent significant stimulation. Note the absence of release on days 11 and 12. Basal release indicates release of PTTH by brain complexes from unfed larvae, which do not release PTTH *in vivo* (Vafopoulou and Steel,1993), but contain large quantities of biologically active PTTH (Vafopoulou *et al.*, 1996). Any release from these brain complexes is artifactual, probably induced by trauma or by the *in vitro* experimental conditions, and is substracted from the mean values used in B.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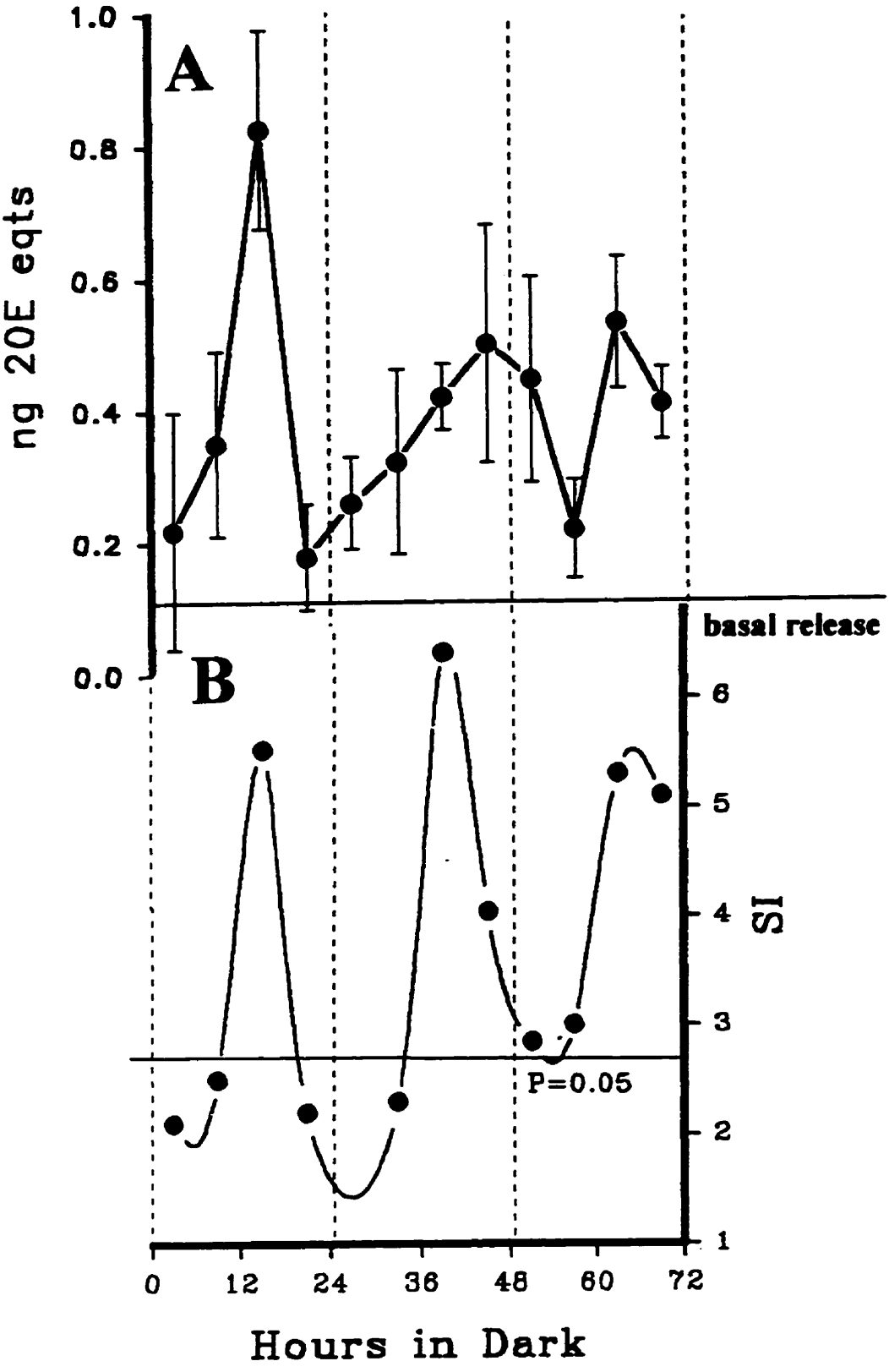
SI
P=0.05
10
11
12
Days after a blood meal

FIGURE 4: Induction of a rhythm of PTTH release by continuous dark (DD). Continuous light (LL) larvae were transferred to dark at day 11 and were maintained in DD for 72hr. PTTH release was assayed at 6hr intervals. (A) Amounts of stimulation were determined as in Fig.3. (B) Levels of significance of stimulation shown in A were calculated as in Fig. 3. Note that the DD-induced rhythm shows highly significant peaks of release approximately every 24hr.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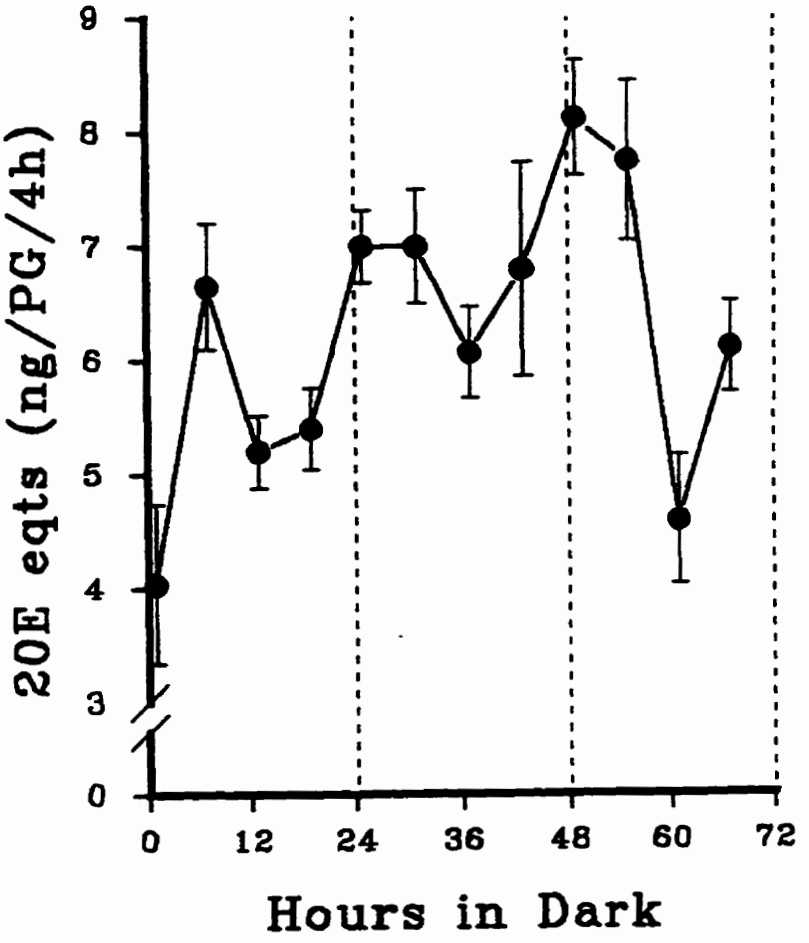
Hours in Dark

profile of ecdysteroid synthesis changed dramatically when compared to the profile in LL (Fig. 2). DD induced the generation of a robust rhythm in synthesis with significant increases occuring at regular intervals of approximately 24hr ($P<0.05$ comparing lowest point to highest point within a day). Peaks occured every subjective photophase and troughs every subjective scotophase. This rhythm was stable during the 3 days of sampling.

Chronic exposure to LL also abolished the daily rhythmicity of PTTH release, which is normally present throughout larval-adult development (see Vafopoulou and Steel, 1996a) (Fig. 3a and b). The level of PTTH release at subjective days 10-12 was insignificant and close to 'basal release'. In contrast, transfer from LL to DD resulted in rapid induction of a strong daily rhythm of release (Fig. 4a and b). The numerical values of the means exhibited a pattern of peaks of release at each subjective photophase alternating with troughs at each subjective scotophase (Fig.4a). Peaks indicated times when release was highly significant ($P<0.01$), whereas little or no release occured at the troughs (Fig. 4b). The period length of the DD-induced release rhythm was aproximately 24hr. The peaks of PTTH release coincided with the peaks of ecdysteroid synthesis in animals in DD (compare Figs. 2 and 4). Thus transfer from LL to DD induces synchronous rhythms in both PTTH release and ecdysteroid synthesis.

The question therefore arises of whether the induced rhythm in the PGs is a response to the induced PTTH rhythm or whether the PGs respond independently to light. This was examined in animals from LL which were injected with TTX to abolish

FIGURE 5: Induction of rhythmicity of ecdysteroid synthesis by PGs from larvae reared in continuous light (LL) which were treated with tetrodotoxin (TTX) on day 10 and were transferred to DD at day 11. Larvae were maintained in DD for 72hr. Points represent means from 10 PGs ±SEM. Note that the rhythm is 12 hours out of phase from the continuous dark (DD)-induced synthesis rhythm of PGs from normal LL larvae (compare Figs. 2 and 5).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Hours in Dark

rhythmicity in PTTH (see Methods). 24 hrs later, these animals were transferred to DD at the end of subjective day 10. Since PTTH release in intact animals in LL is minimal (Fig. 3) these animals, there was no significant PTTH release prior to the injection of TTX and the PTTH release that occurs after transfer to DD was blocked by TTX. This paradigm permits study of the response of PGs to transfer from LL to DD without the influence of PTTH. DD elicited a stable rhythm of ecdysteroid synthesis in TTX-treated animals which was characterized by a period length of about 24hr (Fig. 5). However, peaks of synthesis occured each subjective scotophase and troughs at each subjective photophase ($P<0.05$ when compare lowest and highest points within a day). Therefore, the DD-induced rhythm of synthesis in the absence of PTTH is approximately 12hr out of phase from the DD-induced rhythm in normal larvae (compare Figs 2 and 5). Therefore PGs repond to the light-dark transition in the absence of PTTH, but the resulting rhythm exhibits a reversed phase.

## DISCUSSION

In this paper, we have demonstrated that a lights-off cue *in vivo* induced stable rhythms in both PTTH release from the brain-retrocerebral complex and in ecdysteroid synthesis by PGs of previously arrhythmic fifth instar larvae of *Rhodnius prolixus*. Both rhythms were induced simultaneously and run in synchrony for several cycles peaking at the same time of the day. Synchronization of expression of both rhythms is a characteristic of the entrained state where the rhythms always remain tightly coupled, even when they

free run in DD or LL (Vafopoulou and Steel, 1996a). However, the rhythms could be successfully dissociated with the use of TTX that inhibited cerebral neuropeptide release. In this instance, a lights-off cue induced the generation of a stable rhythm of ecdysteroid synthesis in previously arrhythmic animals but this rhythm ran in antiphase to that of intact animals. This observation shows that the photosensitive oscillator shown previously to reside in the PGs (Vafopoulou and Steel, 1998) is capable of responding to light cues *in vivo* despite the presence of a thick overlying cuticle. The induction of rhythmicity by light cues in the PGs is therefore not dependent on PTTH input to the PGs. Lights-off induces rhythmicity in both PTTH release from the brain and in ecdysteroid synthesis by the PGs. However, the induced rhythms free-run in antiphase, indicating that separate photosensitive oscillators regulate the two hormonal rhythms.

The brain is the presumed anatomical location for the oscillator responsible for the generation of the rhythm of PTTH release. This is an established condition in vertebrates where a photosensitive brain oscillator controls the rhythmic release of neuropeptides (see Turek, 1994). In insects, there is also some compelling evidence that photosensitivity of PTTH release resides in the brain. In moths, earlier indirect evidence showed that release of PTTH from the head necessary for moulting was gated (Truman, 1972). More direct studies later showed that the photoperiodic clock controlling PTTH release during diapause was indeed located in the brain (Bowen *et al.*, 1984). Recently, only 2 pairs of cells expressing the period (*per*) gene were localized in each hemisphere of the brain of a moth: one of these pairs was adjacent to neurons from the cells producing PTTH and formed

axonal arborizations with them (Sauman and Reppert, 1996). These *per*-expressing neurons were regarded as "clock cells" and the presence of *per*- protein (PER) in their axons adjacent to PTTH cells led to the suggestion that PER might be secreted and act as a regulator of the PTTH cells (Sauman and Reppert, 1996). Similar to the existence of only 2 pairs of *per*-expressing cells, PTTH is produced in moths by only 2 pairs of bilaterally located cells in the protocerebrum of the brain (Kawakami *et al.*, 1990; Yagi, *et al.*,1995; Sauman and Reppert, 1996). A similar arrangement is found in *Rhodnius prolixus* in which a single pair of neurosecretory cells in immunoreactive to the antiserum generated against PTTH of *Bombyx mori* (Nseiri and Steel, 1997). Collectively, these findings suggest that the oscillator regulating PTTH release in *Rhodnius prolixus* is likewise located in the brain.

The present findings show that the circadian rhythm of PTTH release reported previously (Vafopoulou and Steel, 1996b) can be induced by a light cue. We infer that the above 'PTTH oscillator' is also photosensitive. However it is unlike the PG oscillator in several ways. First, the PTTH rhythm initiated by 'lights-off' peaks in the subjective day and is therefore in antiphase to that initiated by the same cue in PGs. Second, in prolonged LL, the PTTH rhythm not only damps out but is suppressed to insignificant levels; by contrast, the rhythm of ecdysteroid synthesis also damps but is not suppressed. These distinct properties support the view that separate oscillators regulate the two rhythms.

The earlier suggestion that PTTH is able to entrain the PG oscillator (see Chapter 1) is supported and extended by comparison of the properties of the PG oscillator in intact

and paralyzed animals. In intact animals, the rhythm of ecdysteroid synthesis induced by 'lights-off' is in phase with the induced rhythm of PTTH release and 12 hours out of phase with the rhythm induced in the absence of PTTH. These findings imply that the PG oscillator rapidly acquires the phase of the PTTH oscillator, even in the presence of a conflicting photic cue. We infer that the PTTH oscillator is the dominant component of this system *in vivo*. However, the relationship between them is not consistent with classical relationships between 'pacemaker' and 'slave' (see Saunders, 1977) because the PG oscillator damps only slowly, is photosensitive and rapidly accomodates phase shifts.

We conclude that two interacting photosensitive oscillators regulate the circadian rhythm of ecdysteroid synthesis, one in the brain and the other in the PGs. The two oscillators appear to communicate with each other via the rhythmic release of PTTH. In this relationship, the PTTH release oscillator conveys information about the phase of the oscillation to the PG oscillator. In addition, it may be possible that a feedback mechanism exists between these oscillators whereby the PG oscillator, via the ecdysteroid titre rhythm, conveys some information back to the brain. There is considerable physiological and biochemical evidence for feedback regulation of ecdysteroids on the brain (see Marks et al, 1972; Steel, 1973, 1975; Agui and Hiruma, 1977) which could be an integral part of the pathway of communication between the two oscillators.

Thus, the emerging picture is that regulation of rhythmicity in steroidogenesis in *Rhodnius prolixus* is similar to that in mammals based on two fundamental similarities. First, a brain oscillator regulates the rhythm of release of the neuropeptide and second,

rhythmic neuropeptides influence the steroid rhythms. However, there is a major difference between the two systems. The steroid rhythm in *Rhodnius prolixus* is not a passive 'slave' to neuropeptide input as they are thought to be in mammals (Klein *et al.*, 1991; Turek, 1994).

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# GENERAL DISCUSSION

## MULTIPLE OSCILLATORS WITHIN THE NEUROENDOCRINE AXIS

Pittendrigh (1960) was first to describe the general concept that one oscillator cannot explain the complex formal properties of timekeeping systems. He noted that in Arctic ground squirrels the control of a circadian program required not only one, but possibly many oscillators. Originally Pittendrigh described these multiple oscillators as dawn (M) and dusk (E) oscillators (Pittendrigh and Daan, 1976; Pittendrigh, 1981) based on their differential responses to light. In view of more recent literature, Pittendrigh's theory can also be interpreted as two distinct oscillators with individual properties including differing light sensitivities. This multiple oscillator model has been invoked in many systems from plants to insects to mammals (Tyschenko, 1966; Hoshizaki *et al.*, 1974 and Pittendrigh and Daan, 1976). The concept first set by Pittendrigh (1960; 1974; 1981) is becoming well documented in the 'distributed timing system' in mammals (Ralph and Hurd, 1995; Reuss 1996; see General Introduction). In our laboratory, we are only beginning to see physiologically identifiable correlates of the concept derived by Pittendrigh (1960) in the insect model system that controls the haemolymph ecdysteroid titre. It is argued in this thesis that a multiple oscillator system exists in *Rhodnius prolixus* whose role appears to be to impose internal temporal order on development by controlling the rhythmic synthesis and release of ecdysteroids in the haemolymph.

This thesis has attempted to dissect the interactions between two well documented rhythms in *Rhodnius prolixus* which are, in some way, communicating *in vivo*. The

oscillators in *Rhodnius prolixus* occupy different anatomical locations and are coupled by a humoral factor. The first oscillator resides in the prothoracic glands (PGs) which rhythmically synthesize ecdysteroids in constant conditions in a circadian fashion (Vafopoulou and Steel, 1991) with a temperature compensated period length of close to 24 hours. Recent studies using an *in vitro* assay have confirmed that the second oscillator resides in the brain complex and controls the circadian release of PTTH (Vafopoulou and Steel, 1996b). It is well established that PTTH is the principle known regulator of the PGs (Bollenbacher and Granger, 1985; Steel and Davey, 1985; also see General Introduction). Since the PGs are not-innervated (Wigglesworth, 1952), the only possible form of communication is via a humoral route between the two oscillators. This implies PTTH as the coupling agent (Steel and Vafopoulou,1990). PTTH release after the head critical period (days 5-6) of larval-adult development is not qualitatively necessary for developmental modulation of ecdysteroid titre since decapitated larvae maintain basically normal profiles of haemolymph ecdysteroid titre (Dean and Steel, 1982). The role of rhythmic PTTH release after the head critical period (HCP) is being addressed in this thesis. A novel function for the neuropeptide is uncovered which is believed to be more involved in circadian modulation of the PG oscillator than stimulation of ecdysteroidogenesis.

This thesis employed decapitation or injection of a sublethal dose of tetrodotoxin to eliminate rhythmic PTTH input to the PGs after the HCP. TTX selectively and reversibly blocks voltage-dependent $Na^+$ channels in axons, inhibiting the generation of action

potentials without affecting resting membrane potential (Narahashi, 1974). PTTH release is dependent on action potentials; therefore, TTX inhibits the release of PTTH. The results show a drastic alteration but not the elimination of rhythms in both ecdysteroid synthesis and haemolymph ecdysteroid titre which persisted in the absence of the brain and nervous activity (see Chapter 1). In addition, it was shown that induction of rhythmicity by light cues in arrhythmic brains as well as PGs is immediate and occurs simultaneously (see Chapter 2), but the response differs. PGs were also shown to be able to respond to light/dark cues *in vivo* in the absence of PTTH input (see Chapter 2). This finding shows that the photosensitivity of the PGs previously reported *in vitro* (Vafopoulou and Steel, 1998) occurs also *in vivo* despite the presence of a thick overlying cuticle. It is concluded that there are at least two separate oscillators with different properties or characteristics which communicate *in vivo*. Hence, a multiple oscillator system for the control of the timing of ecdysteroidogenesis and the overt rhythm of ecdysteroids in the haemolymph in *Rhodnius prolixus* is proposed which, in turn, controls the overall timing of development. The possibility of a feedback mechanism of ecdysteroids on the brain will also be addressed.

## COMPONENTS OF THE INSECT MULTIPLE OSCILLATOR SYSTEM

### The PG Oscillator

Upon utilizing TTX we found that rhythmicity in the synthesis and release of ecdysteroids by the PGs persists, with a period length of approximately 24 hours under all

lighting conditions examined (see Chapter 1). The fact that PGs remain rhythmic supports previous results showing an endogenous circadian oscillator exists within the PGs themselves which has been shown to also operates *in vivo* in the absence of the head or nervous activity by the present study. In both sets of experiments we found that the PG oscillator responds to a change in lighting conditions in the absence of PTTH release, which implies that the PGs are directly photosensitive *in vivo*. Specifically, in Chapter 1, the peaks of the rhythms of ecdysteroid synthesis and haemolymph titre in both 12L:12D and DD occurs in the real or subjective photophase, respectively; however, when animals are placed in LL, the peak occurs in the subjective scotophase. In Chapter 2, arrhythmic animals maintained in prolonged LL conditions then treated with TTX also showed rhythmic ecdysteroid synthesis when exposed to DD. Direct photosensitivity of the PGs has been recently confirmed in our lab *in vitro* by Vafopoulou and Steel (1998), but measurement of phase (ie. time of a re-occuring peak) was not possible due to the limited amount of time PGs could be maintained *in vitro*.

In all the cases examined in this thesis the phase of the rhythm in TTX injected animals is the same in the haemolymph ecdysteroid titre and in the synthesis by the PGs. Hence, the PG oscillator drives the rhythmicity seen in the haemolymph titre (i.e. rhythmicity in titre is generated by PG oscillator and not by the brain). However, the properties of the PG oscillator with and without input from the brain are different, indicating that the brain plays a role in regulating the PG oscillator *in vivo* (see below).

**The PTTH Oscillator**

Recent studies in our lab have shown that the PTTH rhythm from normal animals free runs in a circadian manner with a peak in aperiodic conditions which occurs in the subjective night and period length of close to 24 hours (see Vafopoulou and Steel, 1996a). PTTH release rhythm from the brain complex of *Rhodnius prolixus* is normally very similar to PG synthesis rhythm *in vivo* in intact animals. Specifically, both PTTH release and PG ecdysteroid synthesis are in phase with each other under all lighting conditions (Vafopoulou and Steel, 1996b). Both rhythms not only free-run in continuous conditions, but both have similar phases to that on the entrained state in DD, while both rhythms show an abrupt phase reversal with a somewhat shorter period length when they are placed in LL. In contrast, under all the conditions examined in this thesis, TTX injected animals showed phases in ecdysteroid synthesis and haemolymph titre that were consistently different from those of normal animals (approximately 12 hours apart). The difference in phase is attributed to the lack of PTTH. This supports the view that *in vivo*, PTTH acts as an entraining agent to the PG oscillator and hence entrains the PG oscillator to a phase 12 hours different than from that seen for light. Hence, PTTH is shown in this thesis as having pleiotropic effects in insect development. It is not limited to its classical role of stimulating ecdysteroidogenesis (see General Introduction) but also acts as a regulator of the phase of the PG oscillator *in vivo*. Most of the PTTH released during development occurs after the HCP (days 5-6 of larval-adult development: see Vafopoulou and Steel, 1996a): thus, most of the PTTH released during development is involved with the circadian modulation of the

PG oscillator rather than just the initial activation of ecdysteroid synthesis. This fact emphasizes the importance of circadian organization of the endocrine system in development.

. The question arises then, if PTTH entrains the PG oscillator *in vivo*, why have light entrain the PGs as well? The phase set by PTTH (a non-photic hormonal cue) is 12 hours different from that for light; therefore, light and PTTH do not entrain the PG oscillator to the same phase. These two entraining agents appear to conflict, therefore they are not merely reinforcing one another. We do not know what these signals signify to the insect but we do know that this phenomenon has also been observed in other animals. Neuropeptide phase shifting effects have also been observed in mammals (Albers and Ferris, 1984; Piggins *et al.*, 1995). Gastrin releasing peptide (GRP), peptide histidine isoleucine (PHI) and vasoactive intestinal polypeptide are all capable of producing phase shifts in the mammalian SCN (Piggins *et al.*, 1995). More importantly, Neuropeptide Y (NPY) in hamsters (Albers and Ferris, 1984) is capable of phase shifting circadian rhythms in hamsters almost 12 hours when compared to the phase effects achieved using light pulses. Specifically, the light phase response curve (PRC; Takahashi *et al.*, 1984) for the SCN versus NPY PRC (Albers and Ferris, 1984) are in 'anti-phase' with respect to the measurement of locomotor activity. Other non-photic hormonal cues have been shown to also phase shift the circadian system. For example, in molluscs, the light PRC (Eskin *et al.*, 1984) versus the PRC for the neurotransmitter serotonin (Eskin, 1982) for the ocular pacemaker of *Aplysia* are also in 'anti-phase' with respect to the firing of compound action

potentials (CAPs). Therefore, two classes of entrainment pathways have been found in a number of systems but the significance of this arrangement is not yet clear in any system. Hence, light and hormonal cues are capable of entraining circadian rhythms to different phases as in *Rhodnius prolixus*, making the entrainment pathways quite diverse and probably communicating different time related information.

**Phase Shifting Effects of Second Messengers**

The role of PTTH as phase regulator of the PG oscillator is a novel concept in insect endocrinology. However, phase control could be accomplished through the same second messenger system described for triggering synthesis (i.e. $Ca^{2+}$ and cAMP; see General Introduction). This hypothesis is consistent with both the established role for cAMP in mediating the action of PTTH on the PGs (see Gilbert *et al.*, 1996 for review) and with the widely documented role of second messengers in phase shifting circadian rhythms (see Block *et al.*, 1993 for review; see also below).

In the marine mollusc, *Bulla gouldiana*, light depolarizes the ocular circadian pacemaker located within the basal retinal neurons (BRNs). Elevated concentration of extracellular $K^+$ can mimic the effects of light as well as direct depolarization. Low extracellular $Na^+$ or hyperpolarization can stop the phase shifting effects of light as shown by McMahon and Block (1987). Reduction of extracellular calcium or extracellular application of the calcium channel blocker, $Ni^{2+}$, can prevent phase shifts due to light (McMahon and Block, 1987; Khalsa and Block, 1988). Hence, it was suggested that

during entrainment a flux of the second messenger $Ca^{2+}$ into the BRNs mediates phase shifts of the BRN's.

The calcium flux hypothesis was directly tested by Khalsa and Block (1990) using pulses of lowered calcium/EGTA seawater. They found a very similar phase shifting response as to hyperpolarizing treatments. They suggest that phase shifts in the neuronal oscillator are achieved through a reduction in transmembrane calcium (see Block *et al.*, 1993). In addition, both hyperpolarizing pulses and low calcium pulses can be blocked by the concurrent use of anisomycin, a protein synthesis inhibitor (Khalsa and Block, 1990). Serotonin is also known to produce phase shifts in the eye of *Aplysia* (Eskin *et al.*, 1984) which can be blocked by anisomycin. These results suggest that the phase shift is achieved through a decrease in transmembrane calcium followed by protein synthesis (Block *et al.*, 1993).

More importantly, in the molluscs, *Bulla* (McMahon and Block, 1987; Khalsa and Block, 1990) and *Aplysia* (Eskin, 1982) hyperpolarizing treatments appear to generate phase shifts during the subjective day that are approximately 180° (or 12 hours) different from that caused by light. Similarly, in mammals the second messenger system has also been shown to be able to phase shift the SCN pacemaker. Specifically, cAMP can shift the mammalian SCN with a very similar PRC to that of hyperpolarizing treatments to BRN's (see Block *et al.*, 1993). Since all the cases observed in *Rhodnius prolixus* lacking PTTH appear to produce a phase shift of 180 degrees it is very likely that the same steps mentioned above for both mollusks and mammals can also be applied to *Rhodnius prolixus*

PGs. In summary, cAMP dependent entrainment in most organisms appears to result in dark-type responses whereas light cues do not use cAMP and result, obviously in light type PRC's. Collectively, these findings suggest that phase-shifting effect of PTTH may be mediated by the described second messenger pathway for PTTH action on PGs, but imply that the cAMP pathway provides an input to the PG oscillator itself and is not solely an input to the enzyme pathway for ecdysteroid biosynthesis.

## PER IMMUNOREACTIVITY - THE CLOCK GENE IN PGS AND BRAINS

*Per* gene and *timeless* gene as well and their protein products are believed to play central roles in the circadian system that controls rhythms of eclosion and locomotor activity in flies (Konopka and Benzer, 1971). Recently, *per* gene expression of *per* protein (PER) has been shown to be rhythmic in cultured *Drosophila* ring glands by Emery *et al.* (1997). This finding supports the view that an endogenous oscillator resides in the PGs. However no rhythmic steroid synthesis or photosensitivity has been observed in *Drosophila* ring glands whereas both are established in *Rhodnius prolixus* PGs. Anti-*per* immunoreactivity has also been shown in the brain of the beetle, *Pachymorpha sexguttata* (Frisch *et al.*, 1996) as well as the giant silkmoth, *Antheraea pernyi* (Sauman and Reppert, 1996) which exhibit overt circadian rhythmicity in locomotor and eclosion rhythms, respectively. Sauman and Reppert (1996) showed that PTTH and PER are located in the same region of the brain but in different cells. They suggest that the close proximity may be the routes to communication between these two cell populations which may be important

for the photoperiodic termination of diapause. The present studies suggest this arrangement may also be involved in the circadian regulation of PTTH release in *Rhodnius prolixus*.

## ECDYSTEROID FEEDBACK ON THE BRAIN

This thesis shows that the PGs and the brain complex interact after the HCP in *Rhodnius prolixus*. The coupling agent is thought to be PTTH through its action on the PGs. It is also possible that PGs may be acting on the brain via a feedback loop through rhythmic release of ecdysteroids. Ecdysteroids have been shown to feedback and affect nervous tissue in many insects including *Rhodnius prolixus*; Specifically, ecdysone was shown to be a feedback regulator for the neurosecretory brain cells in *M. brassicae* (Agui and Hiruma, 1977) *in vitro* while Steel (1973, 1975) showed the same phenomenon to be true in *Rhodnius prolixus in vivo.* Ecdysteroid feedback on the release of molt inhibiting hormone (MIH) from the crab, *Cancer antennarius* S., has also been observed *in vitro* (Mattson and Spaziani, 1986). It is then possible that ecdysteroid feedback on the brain in *Rhodnius prolixus* affects not only the release of PTTH but its rhythmicity. This notion raises the possibility that the brain and PG oscillators are coupled bidirectionally, with ecdysteroids acting as a second coupling agent between PGs and brain.

In vertebrates, steroids have also been shown to exert feedback action on production and/or release of neuropeptides. Negative feedback effects have generally been seen in adrenal glucocorticoid inhibition of corticotropin-releasing factor from the hypothalmus as well as adrenocorticotropin (ACTH) release from the pituitary (see

Keller-Wood and Dallman, 1984 for review). Hence, bi-directional coupling where the brain affects the glands and vice-versa is probable and testable in the context of the circadian system of *Rhodnius prolixus*.

## COMPARISON WITH VERTEBRATES

The circadian timing system regulating neurohormone release in *Rhodnius prolixus* shows some functional similarities with vertebrate neuroendocrine systems (Klein *et al.*, 1991; Turek, 1994). Specifically, in both *Rhodnius prolixus* and mammals a brain oscillator regulates the rhythm of release of neuropeptide which, in turn, at least influences steroidal rhythms. In addition, there are structural analogies between the insect retrocerebral complex and the pituitary which have been known for quite some time (Scharrer and Scharrer, 1944; Hanstrom 1949). However, in almost all vertebrate endocrine glands, the brain contains the 'master' oscillator controlling the output of steroid hormones (see Turek, 1994). For example, steroidogenesis in the adrenal cortex has been described as being driven by rhythmic stimulation by the peptide, ACTH (see Vinson *et al.*, 1992).

Contrary to vertebrate models, the PG clock is entrainable, but not driven by, PTTH in *Rhodnius prolixus*. This is the first clear evidence of a steroid rhythm that is not driven by neuropeptide. However, the adrenal cortex has, in a few cases, also been suggested to contain a circadian oscillator controlling steroidogenesis (Andrews 1971; Shiotsuka *et al.*, 1974). Insects and mammals have been shown to be greatly similar in

other aspects of the regulations of steroidogenesis (see Gilbert 1989; Gilbert *et al.*, 1996). Hence. the general view that steroidal rhythms are driven by neuropeptide input in vertebrates may need to be reassessed based on the evidence presented in this thesis showing that synthesized steroids by an insect endocrine organ can be controlled by a circadian clock located within themselves.

. Our insect multiple oscillator model appears to share many functional similarities with the avian muliple oscillator system. In birds, internal synchronization is achieved from the interaction of the SCN, pineal and ocular retina (see General Introduction) while in our insect model the interaction is at least between the brain and PG oscillators. In addition, both the pineal and the PG oscillators are photosensitive, supporting the insect analogy with vertebrates. In the avian pacemaking system, melatonin is produced in the pineal gland and retina in a circadian manner and appears to convey information about the time of day to other tissues of the organism (see Takahashi *et al.*, 1989 for review; Bernard *et al.*, 1997) as ecdysteroids synthesized by the PGs of *Rhodnius prolixus* can convey messages to target tissues (see below). Recent evidence suggests that calcium and cAMP act to regulate melatonin (Nikaido and Takahashi, 1996); ecdysteroids are also regulated by calcium and/or cAMP. Melatonin is viewed as a possible coupling agent between the multiple oscillators in vertebrates (Cassone *et al.*, 1993); ecdysteroids may also have a similar role in insects (see below). Collectively. these functional similarities between the avian and our insect multiple oscillator systems mentioned above supports the view that the study of the interactions of vertebrate multiple oscillators *in vivo* can benefit from the use and

knowledge achieved by using an insect system as a model.

Although our insect model shares many functional similarities with the avian system, there are some striking differences between the avian pineal gland compared to the *Rhodnius prolixus* PG oscillators which also need to be mentioned. In contrast to the avian pineal gland, the PG oscillator in our insect model appears not to be dominant in the multiple oscillator system. Specifically, in birds, pineal melatonin regulates the phase of the SCN oscillator (Gwinner *et al.*, 1997) as opposed to the PG oscillator in *Rhodnius prolixus* whose phase is regulated by the brain oscillator. Also, as mentioned earlier, the PGs are not innervated which contrasts with all other photosensitive endocrine oscillators known to date. It is also necessary to mention that in *Rhodnius prolixus*, the PGs are paired and each is able to independently oscillate *in vitro* (Vafopoulou and Steel, 1998). Similarly, the PTTH oscillator appears to be bilaterally duplicated by specific cells in each side of the brain (see Nseiri and Steel, 1997). Hence, we infer that there are at least 4 oscillators which comprise the pacemaking system controlling haemolymph ecdysteroid titre in *Rhodnius prolixus*.

## MULTIPLE OSCILLATORS WITHIN THE PGS?

PGs are made of a homogeneous cell type (Wigglesworth, 1952; Beaulaton *et al.*, 1984) it has been thought that one cell type is unlikely to contain more than one oscillator (Edmunds, 1988). However, a number of more complex systems have shown that single cells in preparation are capable of producing circadian rhythms *in vitro*. The closest

comparison to *Rhodnius prolixus* PGs are the avian pineal photoreceptor cells (Pickard and Tang, 1994) which have been shown to contain receptor, clock and effector all in one cell (Nakaliora *et al.*, 1997). These cells entrain the rhythm of melatonin synthesis to the light/dark cycle (Robertson and Takahashi, 1988; Zatz *et al.*, 1988). Similarly, individual neurons dissociated from the mammalian SCN (Welsh *et al.*, 1995) as well as isolated *Bulla* neurons (Michel *et al.*, 1993) can also oscillate *in vitro.* In the molluscan ocular (see Block *et al.*, 1993) pacemaker, a number of cells that are electrically coupled by gap junctions (Block and McMahon, 1984) and oscillate to produce the overt rhythm seen in the firing of CAP's. PG cells are also coupled by gap junctions (Dai *et al.*, 1994) but they are not innervated. Hence more than one oscillator may exist within the PGs themselves, possibly in the individual PG cells. Multiple oscillators within the PGs themselves implies a role for PTTH as synchronizing the synthesis rhythm between the two members of a PG pair.

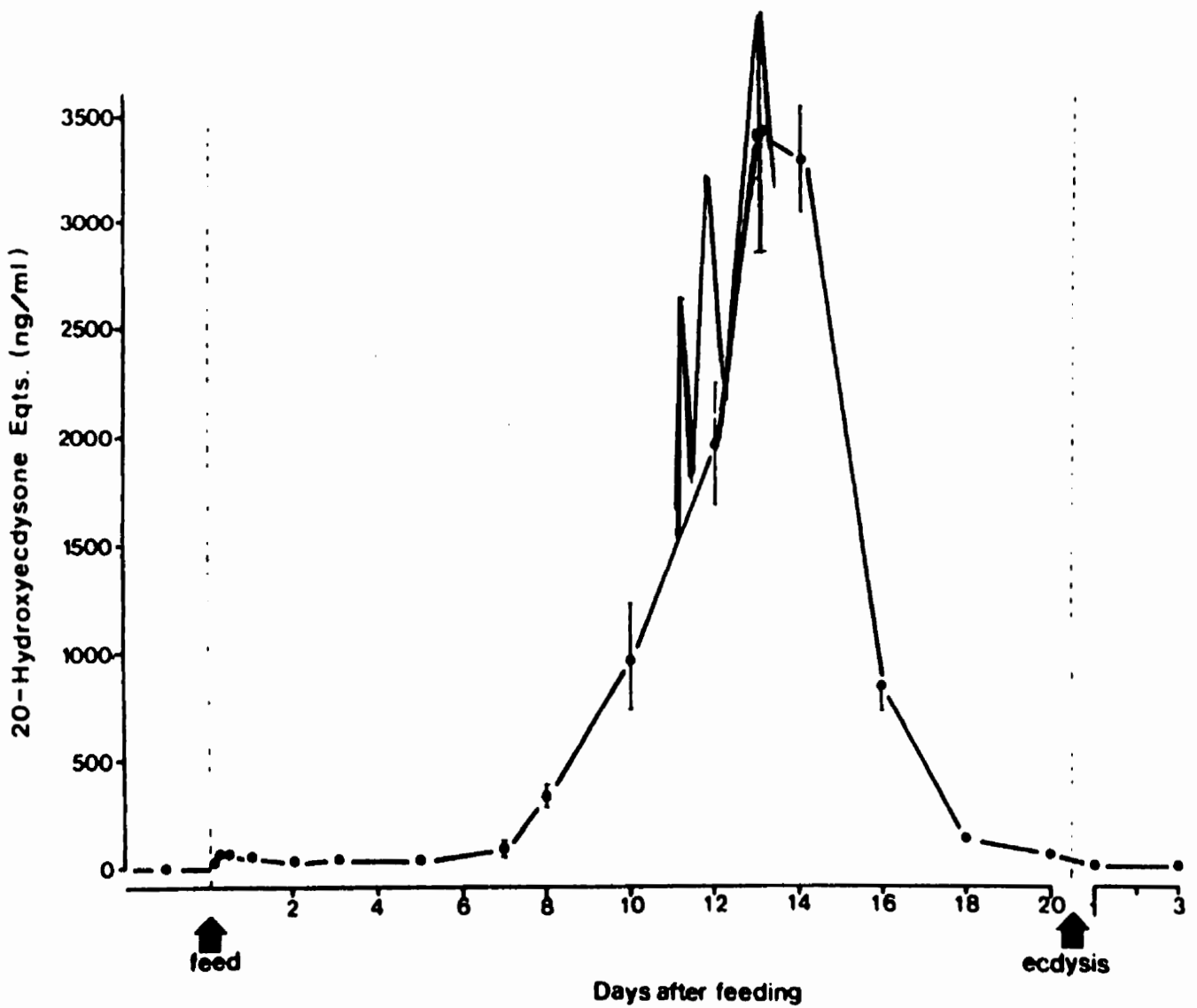
Takahashi and Menaker (1984) suggested that three components are necessary for a pacemaker: i) photosensitivity, ii) circadian oscillator generating the output rhythm of and iii) which is coupled to rhythmic hormone synthesis. All these pacemaking qualities appear to reside in individual pineal cells which may also be shown true for individual PG cells. In order to determine the existence of multiple oscillators within the PGs one must be able to produce a dispersed PG cell in culture with a gap junction inhibitor and be able to measure small quantities of ecdysteroids in media. With the current advancement in reverse haemolytic plaque assay (see Pickard and Tang, 1994) used on pineal photoreceptor cells

such experiments are feasible and may provide the answers on whether multiple oscillators can exist within one endocrine organ.

## POTENTIAL FUNCTIONS OF THE TIMING SYSTEM

Many rhythms in target tissues that occur at specific times within the day are either driven, regulated or somehow influence by haemolymph ecdysteroids. The most obvious is ecdysis behaviour which is gated in many insect species (Truman, 1985) including *Rhodnius prolixus* (Ampleford and Steel, 1982). The ecdysis rhythm in *Rhodnius prolixus* receives input from the rhythm of ecdysteroids in the haemolymph (Ampleford and Steel, 1982; Steel and Ampleford, 1984). The multiple oscillator described by this thesis drives the circadian rhythm of ecdysteroids in the haemolymph titre of ecdysteroids. In addition 20E can actually act as a hormonal cue in arrhythmic animals which can result in the rhythmic gated ecdysis observed in *Rhodnius prolixus* (Ampleford and Steel, 1986). Rhythms of nuclear volume in *Drosophila* salivary glands can be phase shifted *in vitro* with 20E as well as other tissues which are synchronously affected by ecdysteroids such as *Drosophila* PGs (ring glands; Rensing 1969). Epidermal cells have also been shown to only respond to pulsatile 20E *in vitro* to deposit cuticle (Fristrom *et al.*, 1982). Endocuticle secretion (Neville, 1975), oxygen consumption (Taylor, 1977) as well as many other behaviours (Brady, 1982) also occur at certain times of day and maybe at least influenced by the varying amounts of ecdysteroids within the 24 hour cycle. In summary, varying

**Figure 1:** Haemolymph ecdysteroid titres during larval-adult development. Standard errors for some of the samples are smaller than the point symbols. Time axis is interrupted at ecdysis and the samples taken thereafter are grouped by time since ecdysis. An exerpt from Steel *et al.* (1982) with the addition of daily ecdysteroid titres for days 11-13 of larval-adult development added from Vafopoulou and Steel (1991). The daily oscillations in haemolymph ecdysteroid titre occur throughout larval adult development but are only shown here for days 11-13.


20-Hydroxyecdysone Eqts. (ng/ml)
3500
3000
2500
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1500
1000
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2
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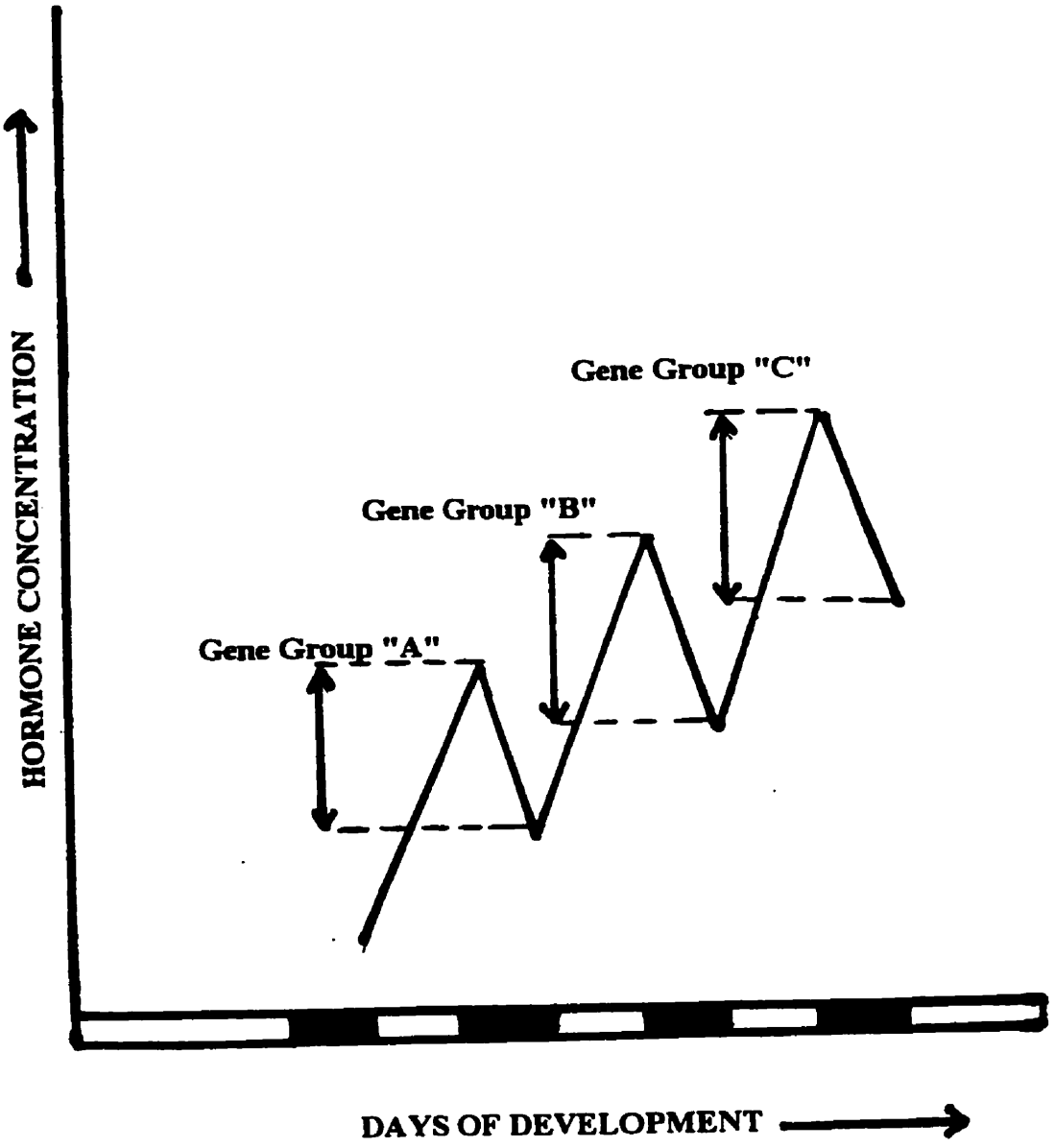
8
10
12
14
16
18
20
3
feed
ecdysis
Days after feeding

**Figure 2**: Schematic diagram of the possible effects of the ouput rhythm of the PG oscillator which is coupled to rhythmic ecdysteroid synthesis and the possible effects these different hormone concentrations during a day and throughout development can have on gene expression. Solid bars on X-axis represent the scotophase and unfilled bars represent the photophase within 24 hours. Note that the daily hormone (or ecdysteroid) pulses occur within a different range of hormone concentrations on consecutive days of development which may turn on different ecdysone responsive genes.


HORMONE CONCENTRATION
Gene Group "C"
Gene Group "B"
Gene Group "A"
DAYS OF DEVELOPMENT

amounts of ecdysteroids are necessary to either drive or regulate the processes underlying growth and development in insects.

**Internal Temporal Order**

Daily ecdysteroid 'pulses' can convey time cues to target cells which allows gene expression to be patterned into sequences within a 24 hour cycle as well as being synchronized with the external world. Using the developmental profile of *Rhodnius prolixus* as an example (see Figure 1), we see the titre of haemolymph ecdysteroids slowly rise from the day of feeding (day 0) to a peak on about days 13-14 and slowly declines towards day 21, the median day of ecdysis. Superimposed on the haemolymph developmental profile in figure 1 are the daily ecdysteroid oscillations (only shown for days 11-13 of larval adult development) which are present throughout the entire moult. The rise and fall in ecdysteroids are not only present throughout the developmental cycle but occur on each day as well, but on a different part of the developmental curve (see Figure 1) representing different ecdysteroid concentrations. Hence, throughout the day and throughout development different ranges of hormone concentrations are experienced by target tissues with ecdysteroid receptors to possibly turn on different groups of genes (see Figure 2 and see also below).

Daily sequences of ecdysteroid in the haemolymph titre play a large role throughout development. It is well known that target tissues show temporal sequences of developmental changes in response to haemolymph ecdysteroids. During development,

expression of many ecdysone responsive genes is both time and concentration dependent. Such developmental sequences includes secretion of cuticle proteins in specific sequences, daily deposition of layers. Riddiford (1985) showed that during the rising phase of ecdysteroids throughout the developmental cycle the cells undergo preparation for the production of a new cuticle which may include DNA synthesis and/or cell division which may differentiate into a totally new type of epidermal structure. This preparation includes the shutting off of synthesis in many cells. During the peak of ecdysteroid titre apolysis (detachment of the epidermis from the overlying cuticle) and moulting fluid secretion occur which is then followed by epicuticle deposition. When ecdysteroids begin to fall, exocuticle is then deposited which is followed by lamellate endocuticle, pre-ecdysial pigmentation which leads to ecdysis and post-ecdysial sclerotization (Riddiford, 1985). In summary, the work descibed on epidermal cells done by Riddiford (1985; 1989) show that these cells respond to 20E by producing different proteins at different times, which implies gene expression in response to haemolymph ecdysteroids occurs in temporal sequences.

The importance of studying the circadian control of ecdysteroids and how they are regulated is essential for the understanding of how temporal order within and between tissues is maintained within the organism throughout its development. These haemolymph ecdysteroids could not be as effective for ensuring proper growth and development in insects if they were present in constant amounts. The pulse duration for many hormones is related to the rate of adaptation of receptors (see Goldbeter, 1996). This implies that receptor blocking or desensitization does occur if constant amounts of hormone are

present. Therefore a hormone present in constant amounts fails to initiate a response and hence excess hormones are wasted or eliminated from the system. Therefore, pulsatile or periodic signaling is more effective in eliciting sustained responses in target cells which are subjected to reversible desensitization in the presence of constant ligand (Goldbeter, 1996).

In was found in the early 1980's using *in vitro* studies using 20-hydroxyecdysone showed that "pulses" were necessary to elicit biological responses in target tissues (e.g. Fristrom, 1982). Since the "pulse" occurs *in vivo* within a different range of hormone concentration every day (see Figure 2) it is possible that different sets of genes are being turned on by the sequences of hormone pulses in different ranges of hormone concentrations. These pulses of ecdysteroids are also required to convey complex messages on temporal order of development as opposed to a simple signal. Different concentrations of ecdysteroid can turn on certain genes and repress others (see Ashburner *et al.*, 1974; Cherbas and Cherbas, 1996), hence actually synchronize development in target tissues leading to internal temporal organization. Since, rhythms in ecdysteroids are detectable by all cells with ecdysteroid receptors, ecdysteroids can synchronize events in disparate cells and tissues and thereby synchronize development throughout the insect.

The focus of literature for decades has been on internal temporal organization within cells (e.g. Ashburner model and its derivatives; see Ashburner *et al.*, 1974). Possibly, a reason for this focus is the apparent lack of any extracellular signals around which orchestration can occur. This thesis puts a whole new emphasis on regulation of cellular events during development. Specifically, the emphasis has been switched more

onto extracellular signals such as light and PTTH rather than just intracellular co-ordination. This is a novel view in endocrine regulation in general which has been shown in this thesis to be comprised of the interaction of multiple oscillators which may act to regulate gene expression.

## CONCLUSION

### A Distributed Timing System in an Insect

Multiple oscillators within an endocrine system governing development have been revealed by the experiments presented in this thesis. Takahashi and Menaker (1984) visualized that three components are necessary for a pacemaker: i) photosensitivity, ii) circadian oscillator generating the output rhythm of and iii) which is coupled to rhythmic hormone synthesis. Ralph and Hurd (1995) added that transplantation of either phase or period of the underlying oscillator between animals revealed a structure as having pacemaking properties (see General Introduction). While both the brain and PG oscillators are photosensitive it is the PG oscillator, and not the brain, which is coupled to a hormone rhythm (i.e. the ecdysteroid rhythm in the haemolymph titre as seen in TTX animals in Chapter 1) which satisfies the first three requirements of a pacemaker set above by Takahashi and Menaker (1984). However, in whole animals the presence of PTTH in the system alters the phase of the PG clock. hence its phase is not transferable in the presence of the head. Therefore. neither the brain or PG oscillator alone appear to have all the qualities of a pacemaker. The emerging picture of the circadian system in *Rhodnius*

*prolixus* appears to be that both the brain oscillator (phase controller) and the PG oscillator (rhythm generator) are necessary to fulfill all the criteria described above. This circadian system functions to control rhythmicity in ecdysteroid titre found in the haemolymph. This, in turn, is capable of conveying time signals to all ecdysone-responsive cells and thereby regulating the expression of ecdysone-responsive genes during development. Consequently, these multiple oscillators appear to constitute a master timing system underlying the orchestration of development.

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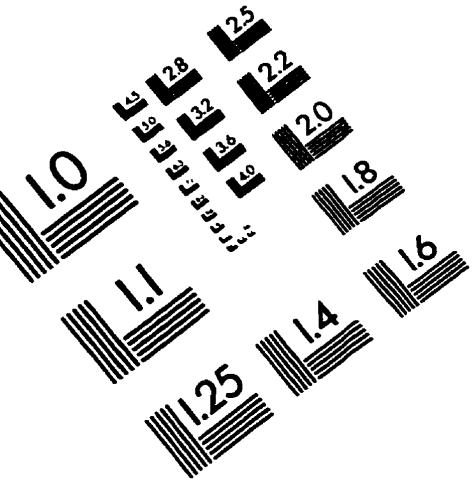
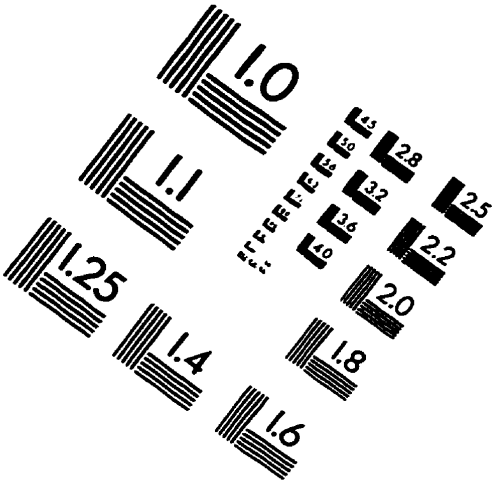
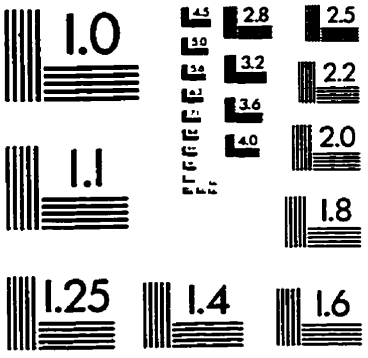
## CONTRIBUTIONS TO MULTI-AUTHORED WORK

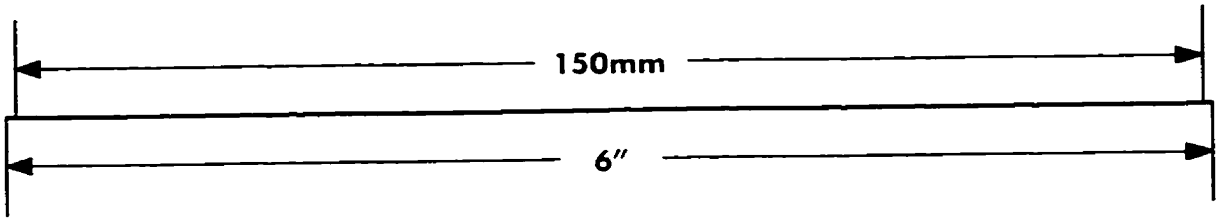
In Chapter 1, all experiments were performed and the text was written by myself. The experiments in Chapter 2 on intact animals were all done by Dr. Vafopoulou; My contribution was the experiment revealing PG synthesis rhythm in TTX injected animals. The text in Chapter 2 was mainly written by Dr. Vafopoulou with minor alterations done on my behalf. Dr. Steel aided in the final preparations of both Chapters and provided the funding that made the experiments possible.

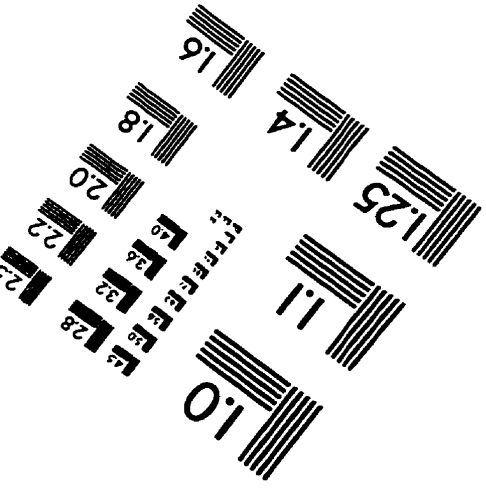
# IMAGE EVALUATION
# TEST TARGET (QA-3)

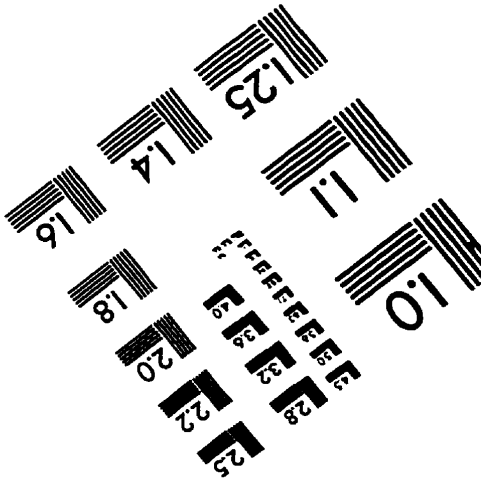
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