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In Vivo & In Vitro Effects of Light on Ommatidial Morphology in the Cockroach, Leucophaea Maderae

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IN VIVO AND *IN VITRO* EFFECTS OF LIGHT ON OMMATIDIAL MORPHOLOGY IN THE COCKROACH, *LEUCOPHAEA MADERAE*

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IN VIVO AND *IN VITRO* EFFECTS OF LIGHT ON OMMATIDIAL MORPHOLOGY IN THE COCKROACH, *LEUCOPHAEA MADERAE*

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This thesis is dedicated to my wife and best friend, Pearl, who has waited patiently; to my three daughters, Hayley, Tara, and Natalie, who have lovingly grown up with their dad's head in a degree program; and, to my parents, James E. and Ruth Yule, who never stopped encouraging me. Special thanks are in order to Grant and Kathleen Bingeman in support of their son-in-law.

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Channon C. Yule December, 1993 Pages 32

Directed by: Drs. Blaine R. Ferrell, Rudolph Prins, and Kenneth Balak

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Clock-driven endogenous circadian rhythms in ommatidial morphology have been reported in *Leucophaea maderae* . The parameters used to measure changes in ommatidial morphology over time were palisade layer and rhabdom areas and the organization of light-screening pigment granules about the rhabdom. The fact that the palisade layer area only partially returned to the light-adapted state *in vivo* during subjective midday was indicative of a passive mechanism, not involving the clock output. Complete return of the palisade layer area to the light-adapted state during the daytime in animals held under LD 12:12 indicated light might play an active role. Temporal changes in rhabdom area were not previously reported. Light-screening pigment granules changed in concentration about the rhabdom on a daily basis; however, this rhythm did not persist on a circadian basis. Therefore, it seemed that control of pigment granule movement resided, at least in part, within the photoreceptor itself. The focus of this investigation was to determine if control of photomechanical movement in response to light did, in fact, reside within the photoreceptor itself. The research was divided into two phases: 1) a preliminary component, Phase One,

where the effect of different lengths of light exposure on dark-adapted ommatidial structure was monitored *in vivo* in order to determine an effective length of light exposure; 2) Phase Two, the effect of light exposure for a length of 10 minutes, a duration found to be effective in Phase One, on ommatidial morphology in dark-adapted eyes was monitored *in vitro*. All eye samples were surgically removed from cockroaches entrained to a LD 12:12 photoperiodic cycle during the dark phase. The *in vitro* samples were maintained in physiological cockroach saline for ten minutes whether treated with light or not. All photoreceptive tissue was fixed, stained, dehydrated, embedded and sectioned in preparation for examination using a Zeiss transmission electron microscope. Palisade layer and rhabdom areas were determined from photomicrographs using a Jandel PC3-D computer program. Light-screening pigment granule organization was determined by counting the number of granules within a 13.5 μ diameter circle centered about the rhabdom. The palisade layer area changes *in vivo* from dark-adapted to light-adapted were consistent with previous reports; however, the *in vitro* response was the opposite of that expected. The rhabdom response was significantly different between *in vivo* and *in vitro* conditions, however, the trends in response to light for both palisade layer and rhabdom areas were similar. The results with respect to rhabdom area are not similar to those previously reported. The aggregation of light-screening pigment granules in response to light *in vivo* was similar to the *in vitro* response, but at a depressed level -- suggesting some level of control at the photoreceptor level and the possibility that some control resided in a central nervous system locus other than or inclusive of the optic lobe pacemaker. Support for separate pathways of control of palisade layer area changes and light-screening pigment granule movement was noted.

Introduction

Endogenous rhythms in biological systems in the most restrictive sense exhibit self-sustaining oscillations in conjunction with a constant energy supply. Exogenous rhythms, on the other hand, experience abatement when external signals are constant. A less restrictive endogenous rhythm displays clock-driven oscillations coupled to periodic energy input (Aschoff 1981). Circadian rhythms are endogenous rhythms regulated by a physically discrete pacemaker. Such rhythms are characterized by entrainability to environmental cycles and persistence with period lengths approximating 24 hours when isolated from the same environmental energy input. The molecular mechanism of the pacemaking oscillation is still disputable but physical localization of the clock(s) has been accomplished (Chiba and Tomioka 1987, Pittendrigh 1981). Circadian rhythms in metabolism and in behavior are exemplified, respectively, by cytochrome oxidase activity (Lavialle *et al*. 1989) and locomotor activity (Pittendrigh 1981) in the cockroach, *Leucophaea maderae*. Circadian rhythms in electroretinogram (ERG) amplitude (Wills *et al*.1985) and in cuticle deposition (Weber 1985) have also been observed in *L. maderae*. Surgical isolation of bilaterally paired neuropiles in the optic lobes provided evidence that each lobe contained a single pacemaker (Page 1989). It has been postulated that separate or multiple oscillators are located within the same lobular region (Page 1989, Chiba and Tomioka 1987). A pacemaker in this region was responsible for regulating the expression of both locomotor activity and ERG amplitude rhythms. Persistence of the cuticle deposition rhythm following optic lobe ablation in the cockroach, *Blaberus craniifer*, indicated that this rhythm was controlled independently from the locomotor activity and ERG amplitude rhythms (Page 1989). The concept of a master pacemaker consisting of multiple oscillators that regulate a hierarchy of rhythms has been supported by results from optic-lobe ablation studies in mosquitoes (Chiba and Tomioka 1987, Aschoff 1981).

Distribution of information from the biological clock(s) occurs principally via efferent neurons (Page 1989).

The geophysical cycle of day and night impacts a variety of endogenous rhythms in various invertebrates (Aschoff 1981). The onset of light triggers a rapid breakdown and rebuilding of photoreceptive membranes in the horseshoe crab, *Limulus polyphemus*, lateral eye retinula cells. Interestingly, pacemaker generated efferent impulses to the photoreceptors signaling membrane turnover must be in conjunction with the onset of light after a normal nighttime period of enhanced lateral eye sensitivity (Chamberlain and Barlow 1979). The grapsid crab, *Hemigrapsus sanguineus*, unlike *Limulus*, demonstrates a clock-controlled circadian rhythm of photoreceptive membrane and visual pigment turnover in the absence of light. Light apparently suppresses the clock activity when the grapsid crabs are kept under constant bright illumination (Arikawa *et al.* 1987). A current model for the regulation of circadian rhythms in *Limulus* lateral eye retinula cells emphasizes the role of natural lighting in magnifying structural rhythms. Daylight induces photomechanical movements that enhance the endogenous change of decreased photon catch during the day. An increased tendency for photon catch at night is regulated endogenously, but enhanced by the onset of darkness. When the optic nerve is cut, both endogenous and photomechanical structural changes are attenuated (Chamberlain and Barlow 1987). The model proposes a push-pull mechanism involving circadian efferent output and cyclic lighting, the effects of which are reciprocal but not equivalent. This endogenous rhythm of less restrictive conditions is believed to result from increased efferent activity that pushes the retinal structures to a more dark-adapted state, whereas light onset produces the reciprocal effect. The dual effects of clock control and cyclical lighting are not equivalent. Severing the optic nerve in the absence of light resulted in an attenuated rhythm in eye morphology and, in the presence of light, no rhythm (Barlow 1990, Chamberlain and Barlow 1987).

A role for neurotransmitters, circulating hormones, ions, and other humoral components as yet unidentified have not been ruled out in connection with the control of structural rhythms in *Limulus, L. maderae* and the crayfish, *Procambarus*. The neurotransmitter octopamine is effective in triggering cytoskeletal changes that modify photoreceptor shape and ion channel concentrations to enhance cellular response to photons in *Limulus*. It is likely that another neurotransmitter, as yet unidentified, works in concert with octopamine to activate the postulated push-pull mechanism (Barlow 1990, Chamberlain and Barlow 1987). Photoreceptor cell levels of Na^+ and Ca^{2+} may play a role in the dispersion or aggregation of screening pigment granules in peeled retinal photoreceptors of the crayfish (Frixione and Ruiz 1988). Screening pigment aggregation or disbursement is accounted for in the crayfish via changes in intensity or duration of excitation by light which in turn regulates intracellular Ca^{2+} indirectly by varying the amount of Na^+ entering the photoreceptor cytoplasm (Frixione and Ruiz, 1988). According to this model, pigment aggregation within the axon occurs when minimum levels of Na^+ leak into the photoreceptors in the dark. Smooth endoplasmic reticulum then favors maximum Ca^{2+} sequestration and resultant screening pigment aggregation. Conversely, full illumination leads to maximum amount of Na^+ influx, a rise in intracellular Ca^{2+}, and dispersion of screening pigments (Frixione and Ruiz, 1988). Ca^{2+} has been demonstrated to be an inhibitor of light-dependent channels of conductance in excised patches of the ventral photoreceptors of *Limulus*, whereas, the cyclic nucleotide, cGMP, is the suspected messenger that opens these channels (Bacigalupo *et al.*1991). The endogenous control of ionic involvement is currently under investigation. Other yet unidentified humoral factors could bear significantly on circadian output such as a blood- born factor in *Periplaneta americana* that drove several cycles of locomotor activity (Cymborowski and Brady 1972). The fact that optic lobes surgically isolated from *L. maderae* and cultured for up to ten days

in the absence of neural or humoral factors expressed a circadian rhythmicity of neural activity (Page 1983) indicates that neural output is the principal agent involved in regulating rhythmicity in *L. maderae*.

The circadian rhythm of eye sensitivity to light as measured by ERGs in *L. maderae* (Wills *et al.* 1985) could be correlated with a circadian response in the three morphological structures of interest in this study. Analysis of structural variation within the retinula cells has of necessity been limited to changes in cross-sectional areas of the rhabdom and the palisade layer and of the number of light-screening pigment granules clustered about the rhabdom. Even though the cross-sectional area of rhabdoms in *Limulus* and some other invertebrates (Piekos 1989, Arikawa *et al.* 1988, Waterman 1982) generally increase under dark-adapted conditions, the rhabdom of cockroaches has shown no significant increase (Butler 1973, Ferrell and Reitcheck 1990). The cross-sectional area of the palisade that develops adjacent to the rhabdoms was apparent under dark-adapted conditions in *Limulus*, *P. americana*, and other invertebrates (Butler 1973, Frixione and Ruiz 1988, Waterman 1982, Fahrenbach 1968), as well as, *L. maderae* (Ferrell and Reicheck, 1990). The third parameter of circadian response under investigation in *L. maderae* eye morphology is the movement of light-screening pigment granules. The migration of pigment granules away from the rhabdom in nighttime eyes in *Limulus* was controlled by an endogenous clock, by light or an interaction of both (Barlow and Chamberlain 1980). Dispersion of screening pigments in dark-adapted photoreceptors allows for increased photon capture by photosensitive membranes. In the cockroach, *Periplaneta americana*, pigment particles are clustered about the rhabdom in the light-adapted state and are dispersed from the rhabdom as the palisade layer develops in the dark-adapted state (Butler 1973). Retinula cell visual pigments of the meal moth, *Ephestia kuchniella*, stimulated by ambient lighting, appear to be able to trigger the translocation of screening pigment granules in order to vary the light flux of photoreceptive structures (Weyrauther 1986). The sphingid moth, *Dielephila*

elpenor; however, has been used to demonstrate the possible control of screening pigment granules by some photopigment within an adjacent pigment cell or the adjacent cone cells distal to the rhabdom (Juse *et al.* 1987). The effects of external photic input on these morphological parameters in dark-adapted retinula cells from cockroaches acclimated to a LD 12:12 photoperiodic cycle and 25 $\pm$ 2°C with clock connections intact or surgically disconnected will be monitored in this study in order to determine whether regulation of morphological changes toward the daytime state in response to light requires central nervous system integration or simply occurs within the photoreceptor itself.

Materials and Methods

Adult male cockroaches, *L. maderae*, were obtained from colonies maintained in the laboratory. One week prior to each phase of the experiment, four to five animals were placed in a plastic cage with adequate food (*i.e.*, dog food) and water. This cage was then housed in an environmental chamber where temperature was controlled at 25±2 °C and the light/dark cycle was maintained at 12 hours of light and 12 hours of dark (LD12:12). One week of exposure to the LD12:12 photoperiod was previously deemed sufficient for entrainment of members of the colonies based on activity recordings. Only adult males were used to ensure experimental consistency.

Phase One, a preliminary investigation, served to establish representative morphological changes in *L. maderae* ommatidia in the absence of or in response to the presence of white light of two different durations *in vivo*. Eyes excised without stimulation by white light were surgically removed under red light with a wavelength > 640 nm, a wavelength determined to be without effect on eye sensitivity to light in another cockroach (Mote and Goldsmith 1983). The white light stimulated eyes were removed immediately after exposure or following 10 minutes of exposure to light. Phase Two differed in that tissue samples of both eyes from individual animals were surgically removed, one eye per animal was removed under red light and left in darkness for 10 minutes post-surgery (i.e., control eye tissue) and the other eye of the same animal similarly removed but stimulated by white light for 10 minutes post-surgery (i.e., experimental eye tissue). All eye tissue was excised from animals during the nighttime phase of the LD12:12 entrainment cycle between 2030h and 0130h. Phase Two eye tissue samples were immersed in a physiological cockroach saline bath in separate 35 mm x 10 mm plastic Petri dishes. Each bath was manually aerated with a pipette and suction bulb just prior to eye tissue removal.

All surgical procedures were initiated by transferring a cockroach to a 35 mm x 10 mm plastic Petri dish with a lid. The animals were anesthetized continuously with CO_2 from a pressurized cylinder, the CO_2 having been filtered through a water trap and conducted via rubber tubing to one of the two holes in the Petri dish lid. The other hole was triangular and after the head of the cockroach was passed through the hole, the head was stabilized with tape from behind. A second piece of tape anchored the body to the underside of the lid. Small strips of eye tissue were then cut from the anterior one third of each eye using a fractured razor-blade scalpel, surgical scissors and forceps. The eye tissue strips were then immediately or after ten minutes in cockroach saline placed in microviles with 4% gluteraldehyde and kept for 2.5 hours in the dark. All samples were then rinsed twice for 15 minutes each time with Sorensen's phosphate buffer, pH 7.2. Each sample was next stained with 1% osmium tetroxide for 1.5 hours, and again rinsed twice for 15 minutes each time with Sorensen's phosphate buffer. A serial ethanol dehydration followed in the order of 20%, 35%, 50%, 70%, 85%, 95%, and 100% twice. Every step in the dehydration sequence was for 10 minutes and the last 100% ethanol step immediately preceded embedding the tissue in Spurr's resin. The embedding process involved serial infiltration of the eye tissue with increasing concentrations of Spurr's resin. The first infiltration step consisted of placing the eye tissue in a mixture of one part Spurr's resin and three parts100% ethanol, followed by a one to one ratio, and a three to one ratio. The final exposure in the series was to 100% Spurr's resin. Each step in the series lasted 30 minutes. Prior to orienting each tissue sample and its label in a plastic Beem capsule, each capsule was heated in an aluminum holder at 65 °C for 1.0 hours to ensure no moisture remained in the capsules. One drop of 100% Spurr's resin was placed in the neck of each capsule, and all trapped air bubbles were dislodged. Each tissue sample was then oriented independently near the tip of its own capsule with the convex surface facing down. The

capsules were then filled completely with 100% Spurr's resin and along with the holder, placed in an oven at 65 °C for 11.5 to 12.0 hours. After cooling for approximately 24 hours each sample was carefully removed from the Beem capsule by slicing the wall of the capsule with a razor blade.

Each of the individual samples were prepared for sectioning by trimming away excess tissue, leaving a trapezoidal block of tissue containing 10 to 20 ommatidia. Each sample was mounted in a Reichert OMU2 ultramicrotome equipped with a glass knife. Sections refracting gold or silver light with an approximate thickness of 90 nm were retrieved with 200 or 300 mesh copper grids. The grids bearing sample sections were thoroughly examined using a Zeiss 9S2 transmission electron microscope at a magnification of 1950 times. Cross sections of ommatidia in the proximity of the crystalline cone apex were recorded on photomicrographs and later printed at an enlargement of 3.4 X. A total magnification of each ommatidial cross section at 6630 X served to provide uniformity in measurements.

Three morphological features of each ommatidial cross section were measured and recorded: (1) palisade layer area , (2) rhabdom area, and (3) the number of light-screening pigment granules. The perimeters of the areas of the palisade layer and the rhabdom were first traced with a 0.3 mm (extra fine point) Expresso pen on the photomicrographs.These perimeters were then traced separately into a Jandel PC3-D computer program,and the areas were calculated electronically.Light-screening pigment granule numbers were computed manually by counting all granules clustered within a 13.5μ diameter circle centered on each rhabdom. Data were analyzed using a 2X2 factorial analysis of variance,and differences were considered significant at the 95% confidence level or greater.

Results

Ommatidia of dark-adapted eyes used as an *in vivo* control in Phase One of the investigation (Table 1, Figure 1) had an average palisade layer area of $10.7 \pm 5.7 \mu^2$ (mean $\pm$ standard deviation), an average rhabdom area of $38.7 \pm 12.5 \mu^2$ (Table 2, Figure 1), and an average number of light-screening pigment granules of 169 ± 25 (Table 3, Figure 1). Ommatidia of dark-adapted eyes exposed to light and immediately surgically removed represented an *in vivo* experimental group. This group had an average palisade layer area of $12.2 \pm 3.5 \mu^2$ (Table 1, Figure 2), an average rhabdom area of $32.3 \pm 15.5 \mu^2$ (Table 2, Figure 2) and an average number of light-screening pigment granules of 195 ± 75 (Table 3, Figure 2). This experimental group was used only in Phase One for comparison with the response of ommatidia to the ten minutes of illumination to determine which treatment would yield a greater measurable morphological change, particularly in regard to the arrangement of the light-screening pigment granules (i.e.,195 ± 75 to 234 ± 62). This preliminary *in vivo* comparison was not used to generate data for statistical purposes. Using the statistical 2 X 2 factorial anova to analyze the morphological parameter of palisade layer area, there was a significant interaction at the 95% confidence level such that (1) light causes the palisade layer area to increase for the *in vitro* condition (i.e., $6.2 \pm 1.8 \mu^2$ to $11.5 \pm 4.4 \mu^2$; Figures 6 and 7) and (2) light causes it to decrease for the *in vivo* condition (*i.e.*, $10.7 \pm 5.7 \mu^2$ to $8.0 \pm 5.5 \mu^2$; Figures 6 and 7). In general, there is no significant difference in the effect of light compared to no light; and in general *in vitro* and *in vivo* conditions do not differ significantly (Table 1).

The 2 X 2 factorial anova applied to the morphological parameter of rhabdom area identified a significant interaction at the 95% confidence level, such that (1) light causes the rhabdom area to increase *in vitro* (i.e., $40.2 \pm 13.1 \mu^2$ to $56.8 \pm 18.3 \mu^2$; Figure 6 and 8) and

(2) light causes it to decrease *in vivo* (i.e.,38.7+12.5$\mu^2$to 29.5+10.1μ^2; Figure 6 and 8). There also was a significant difference between *in vivo* and *in vitro* conditions at the 99% confidence level with regard to changes in rhabdom area. On the other hand, there was no significant difference in the effect of light compared with no light in either condition (Table 2).

Light-screening pigment granules within a 13.5μ diameter circle centered about the rhabdom for ommatidia of dark-adapted eyes exposed continuously to white light for ten minutes was the treatment of choice determined in Phase One (Table 3, Figure 3) to be used in Phase Two (Table 3, Figure 5). When subjected to the 2 X 2 factorial anova the degree of increase in light-screening pigment number in response to light *in vivo* (i.e., 169$\pm$25 to 234$\pm$62; Figures 6 and 9) was similar to the response to light *in vitro* (i.e., 124$\pm$55 to 141$\pm$33; Figures 6 and 9). There was a significant effect of light overall and the trend was similar for both *in vivo* and *in vitro* conditions. A highly significant difference existed between samples treated with light and samples not treated with light. A significant difference existed between ommatidia treated *in vivo* compared with those treated *in vitro* (Table 3).

Not all observations of the cell samples were recordable in a quantitative way. Nearly all of the samples taken from cockroaches in the dark and left in the dark for ten minutes demonstrated some form of cellular disruption, or discontinuity, as observed under the transmission electron microscope. It was extremely tedious locating recordable data for Phase Two control groups.

Table 1. The *in vivo* and *in vitro* effects of different light durations on ommatidial palisade layer area

TREATMENT	N	PALISADE LAYER AREA (μ^2)
PHASE ONE (*in vivo*)		
A Control-1[3]	9[1]	10.7±5.7[2]
B IIL-1	6	12.2±3.5
C EIL-1	9	8.0±5.5
PHASE TWO *(in vitro)*		
D Control-2	9	6.2±1.8
E EIL-2	9	11.5±4.4

1. N is the number of ommatidia examined and these ommatidia are the same as those in Table 2 and 3.
2. Mean + the standard deviation about the mean.
3. Abbreviations: Control = dark-adapted; IIL = immediate illumination; EIL = illumination for 10 minutes; 1= Phase one; 2= Phase two.

2 X 2 Factorial ANOVA Summary Table

Source of Variance	SS	df	MS	F	Significance
In Vivo vs *In Vitro*	3.60	1	3.60	<1	NS
Control vs EIL	15.33	1	15.33	<1	NS
Interaction	143.31	1	143.31	5.99	p<.05
Error variance	765.47	32	23.92		

Figure 1. Electron photomicrograph of an ommatidial cross-section obtained during the dark-adapted state by excision under red-light (> 630 nm) of an eye from a cockroach maintained under LD 12:12 and 25$\pm$ 2°C (Control-1= *in vivo* control, P= palisade layer, R= rhabdom, and G= light-screening pigment granules).

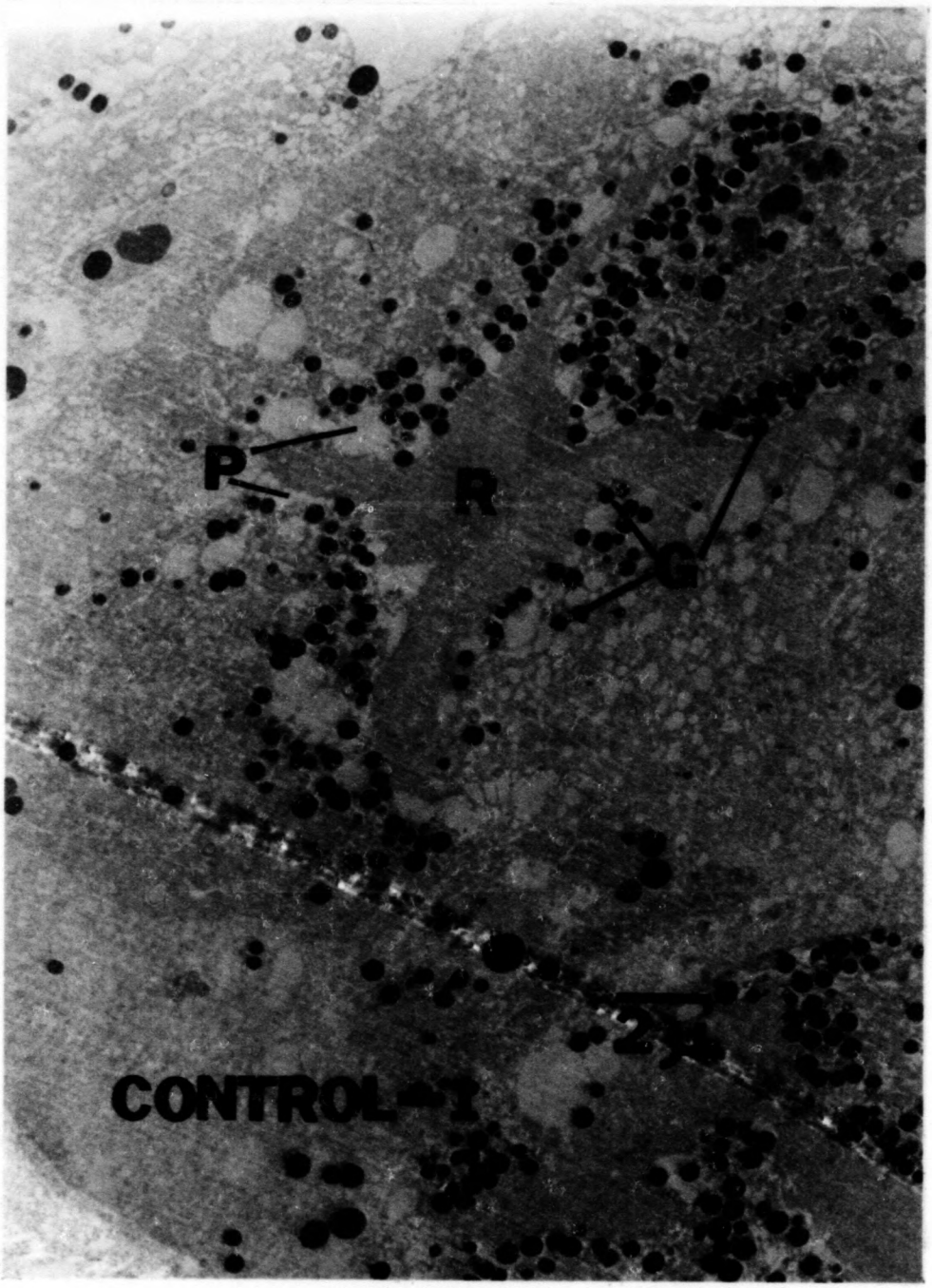

P
R
CONTROL

Figure 2. Electron photomicrograph of an ommatidial cross-section obtained during the dark-adapted state by excision under white light immediately following photostimulation of an eye from a cockroach maintained under LD 12:12 and 25$\pm$ 2^{o}C (IIL-1= immediate illumination *in vivo*, P=palisade layer, R=rhabdom, and G=light-screening pigment granules).

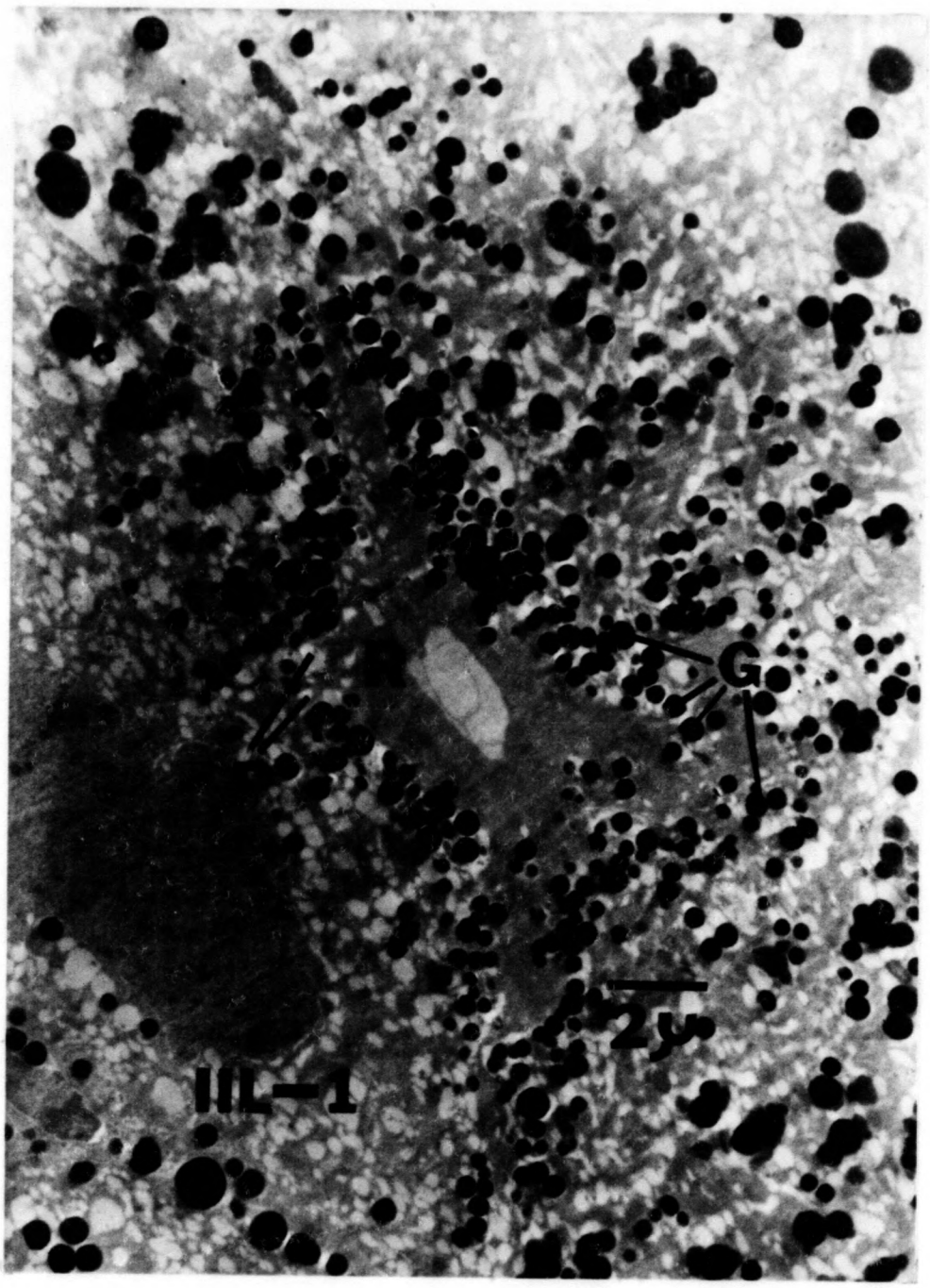

G
2μ
III-1

Figure 3. Electron photomicrograph of an ommatidial cross-section obtained during the dark-adapted state by excision under white light following 10 minutes of photostimulation of an eye from a cockroach maintained under LD 12:12 and 25$\pm$ 2^{o}C (EIL-1=extended illumination *in vivo*, P= palisade layer, R= rhabdom, and G= light-screening pigment granules).

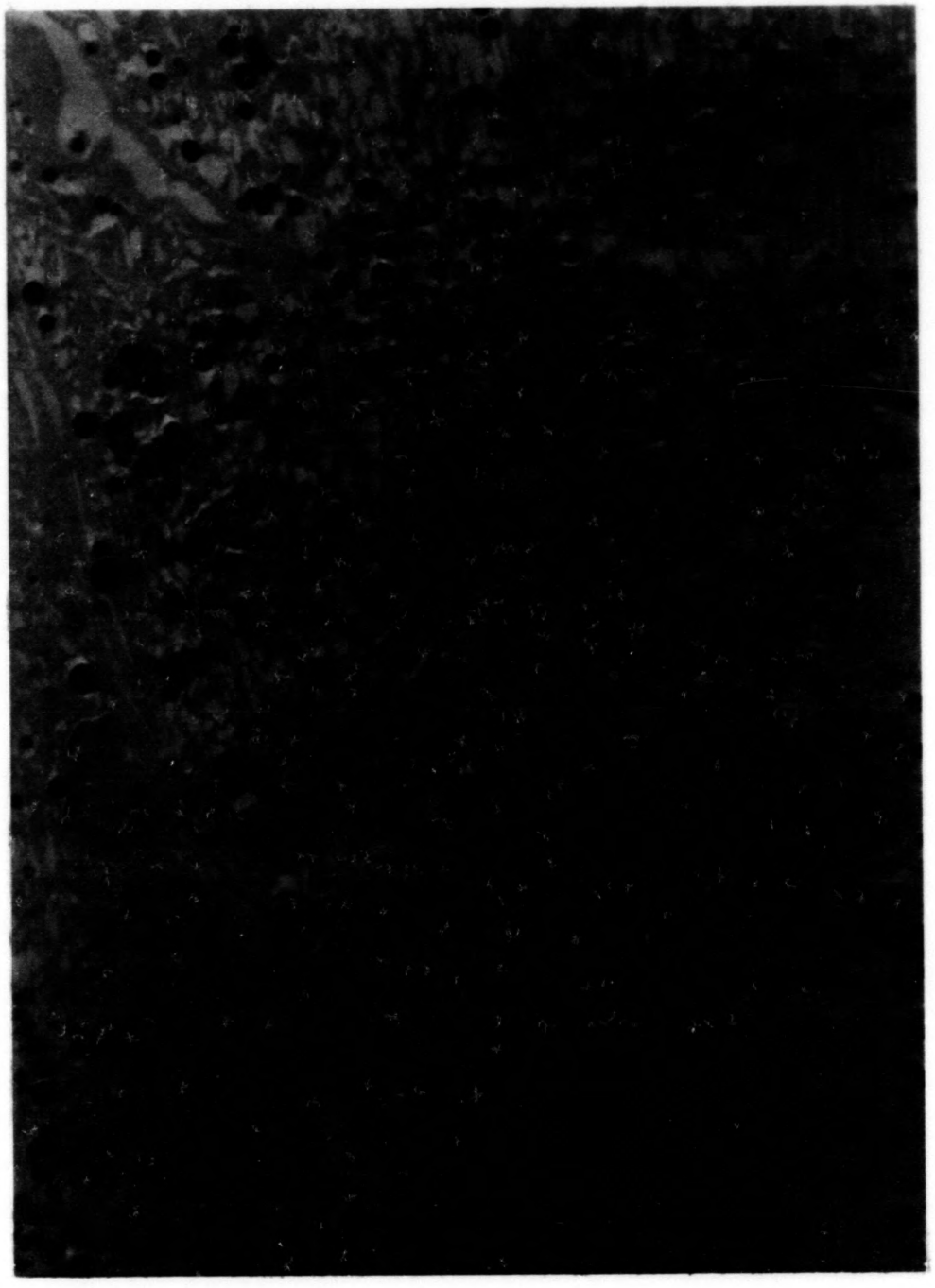


Table 2. The *in vivo* and *in vitro* effects of different light durations on ommatidial rhabdom area

TREATMENT	N	RHABDOM AREA (μ^2)
PHASE ONE (*in vivo*)		
A Control-1[3]	91	38.7±5.12
B IIL-1	6	32.3±15.5
C EIL-1	9	29.5±10.1
PHASE TWO (*in vitro*)		
D Control-2	9	40.2±13.1
E EIL-2	9	56.8±18.3

1. N is the number of ommatidia examined and these ommatidia are the same as those in Table 1 and Table 3.
2. Mean ± the standard deviation about the mean.
3. Abbreviations: see Table 1.

2 X 2 Factorial ANOVA Summary Table

Source ofVariance	SS	df	MS	F	Significance
In Vivo vs *In Vitro*	1,873.73	1	1,873.73	8.72	P<.01
Control vs. 10 Min.	121.81	1	121.81	<1	N.S.
Interaction	1,502.09	1	1,502.09	6.99	P<.05
Error variance	6,872.66	32	214.77		

Figure 4. Electron photomicrograph of an ommatidial cross-section obtained during the dark-adapted state by excision under red light (>630 nm), followed immediately by 10 minutes in darkness in a physiological saline bath, of an eye from a cockroach maintained under LD 12:12 and 25±2 °C. (CONTROL-2=internal *in vitro* control, P=palisade layer, R= rhabdom, and G= light-screening pigment granules).

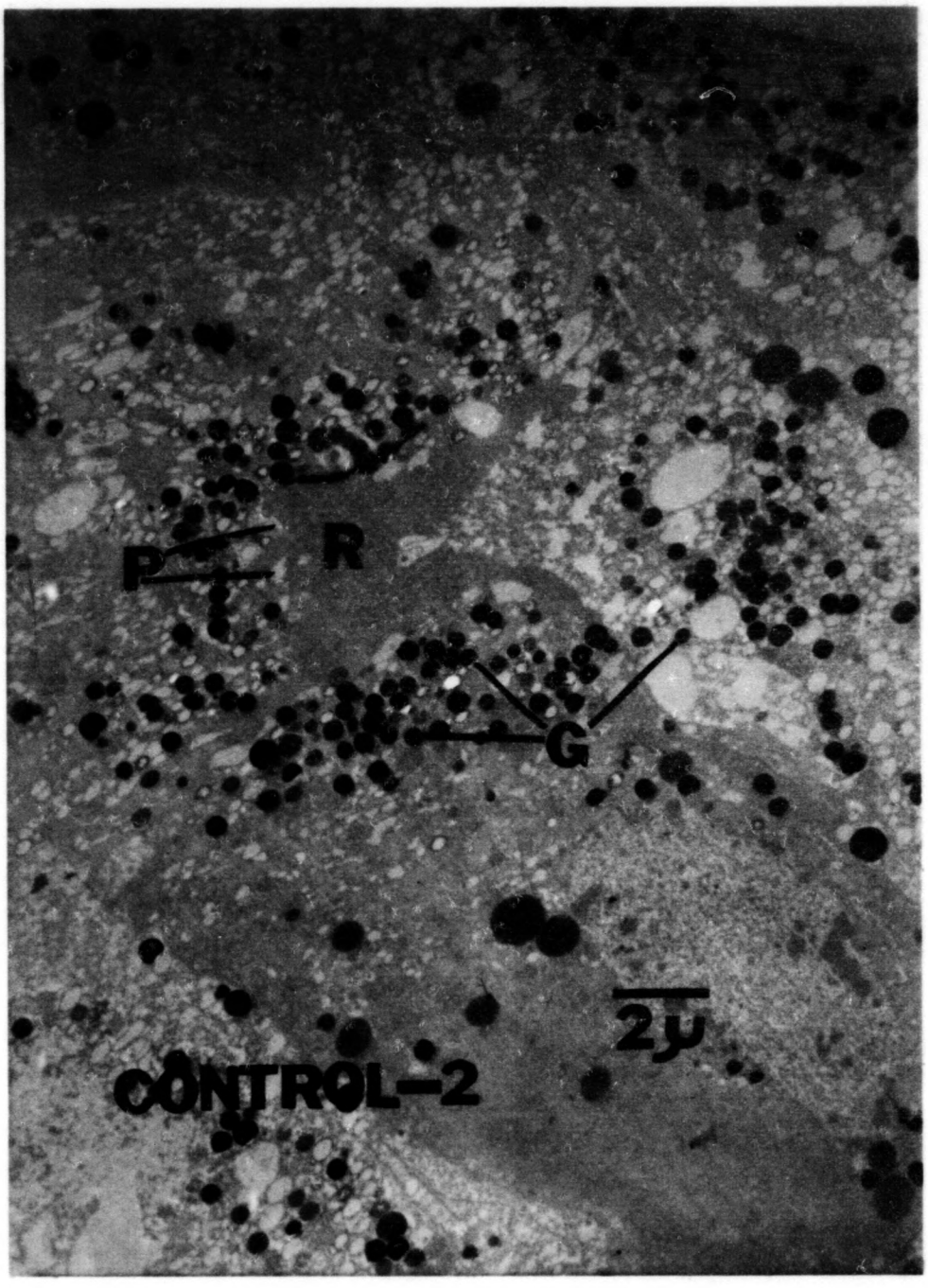

P
R
G
2μ
CONTROL-2

Figure 5. Electron phototmicrograph of an ommatidial cross-section obtained during the dark-adapted state by excision under red light (>630 nm), followed immediately by 10 minutes in white light in a physiological saline bath, of an eye from a cockroach maintained under LD 12:12 and 25±2 °C. (EIL-2= extended illumination *in vitro*, P= palisade layer, R= rhabdom, and G=light-screening pigment granules).

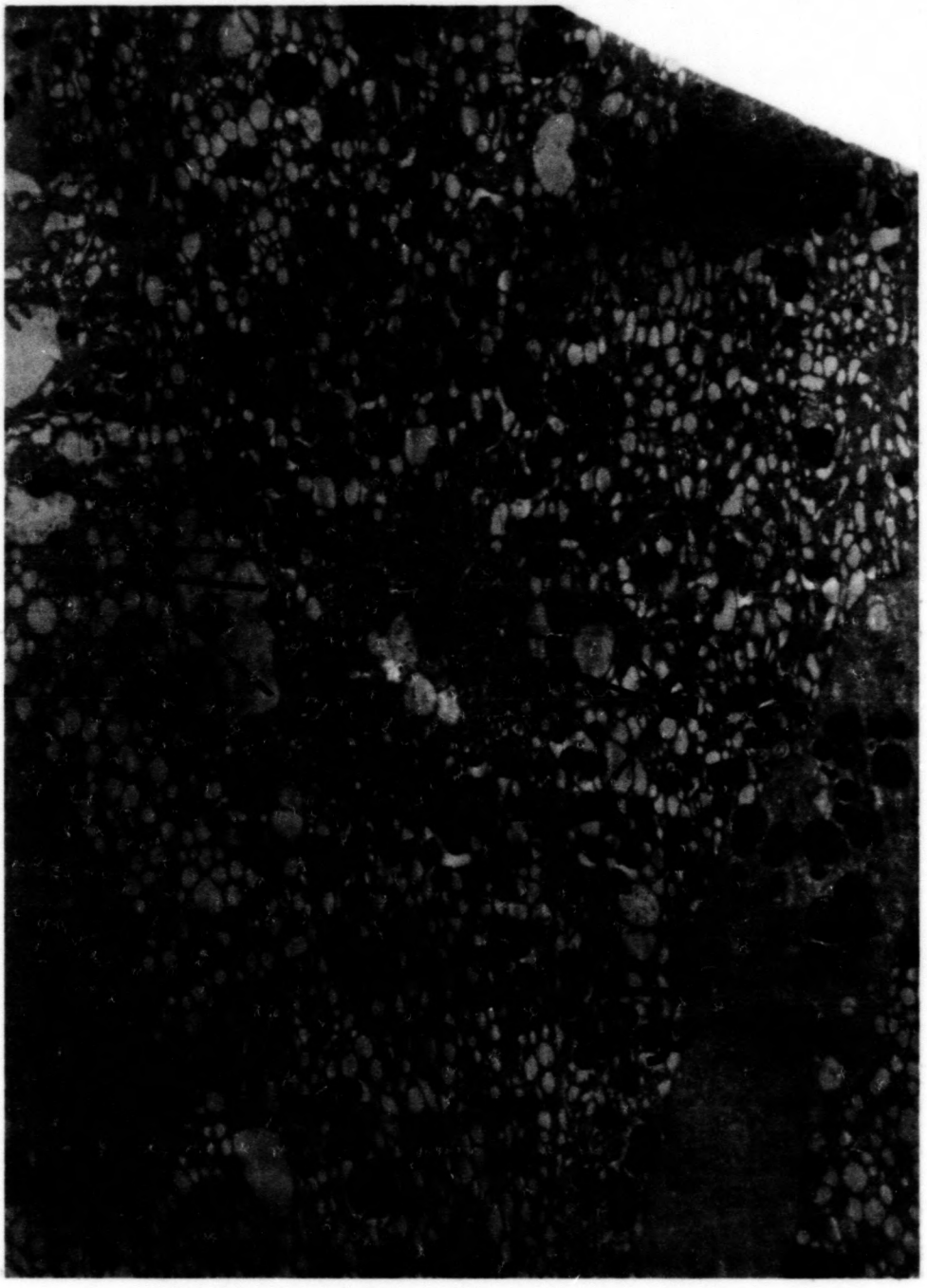


Table 3. The *in vivo* and *in vitro* effects of different light durations on ommatidial light-screening pigment granule (LSPG) number

TREATMENT	N	LSPG
PHASE ONE (*in vivo*)		
A Control -1[3]	9[1]	169±25[2]
B IIL-1	6	195±75
C EIL-1	9	234±62
PHASE TWO (*in vitro*)		
D Control-2	9	124±55
E EIL-2	9	141±33

1. N is the number of ommatidia examined and these ommatidia are the same as those in Table 1 and Table 2.
2. Mean ± the standard deviation about the mean.
3. Abbreviations: see Table 1.

2 X 2 Factorial ANOVA Summary Table

Source of Variance	SS	df	MS	F	Significance
In Vivo vs. In Vitro	42,093.36	1	42,093.36	17.29	p<.01
Control vs. 10 Min.	15,088.03	1	15,088.03	6.20	p<.05
Interaction	5,256.25	1	5,256.25	2.16	N.S.
Error variance	77,927.33	32	2,435.23		

Figure 6. Mean palisade layer areas, mean rhabdom areas, and mean light screening pigment granule numbers of dark-adapted ommatidia determined following *in vivo* or *in vitro* treatment (Control-1, *in vivo*--eyes removed under red light, IIL-1, immediate illumination *in vivo*-- eyes removed under white light, EIL-1, extended illumination *in vivo*-- eyes removed under white light following 10 minutes of photostimulation, Control-2, *in vitro*-- eyes removed under red light and bathed in physiological saline in darkness for 10 minutes, EIL-2, extended illumination *in vitro*--eyes removed under red light and bathed in saline in white light for 10 minutes) in cockroaches maintained under LD12:12 and 25$\pm$2 ^{o}C (SD=standard deviation).

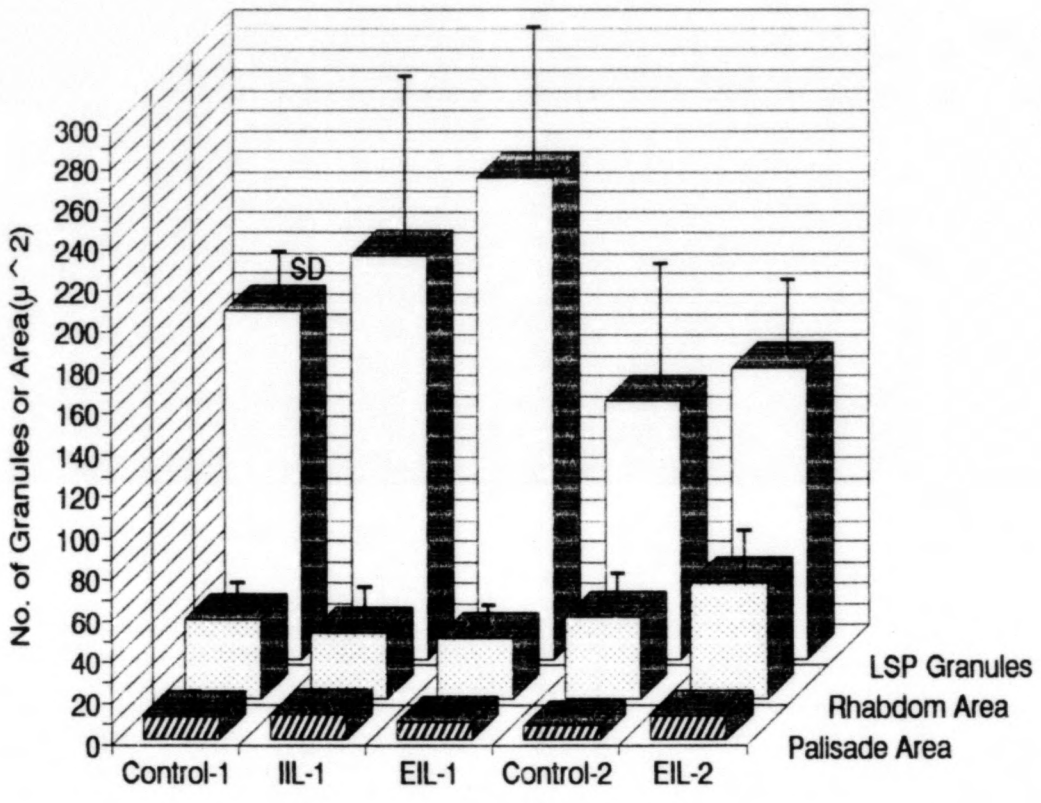
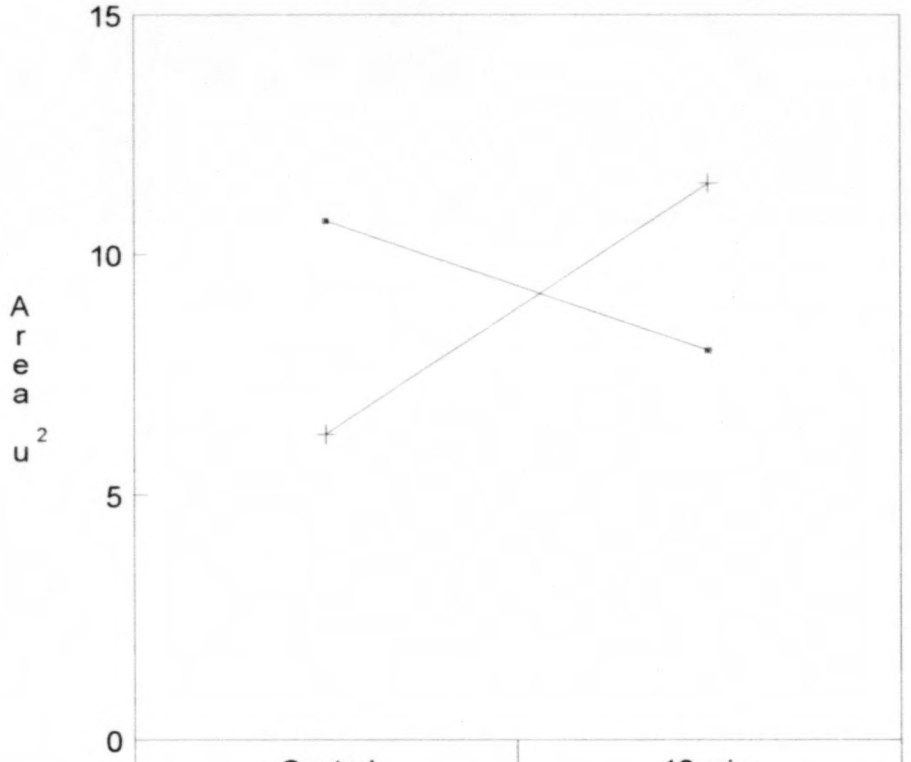

No. of Granules or Area(μ ^ 2)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
SD
Control-1
IIL-1
EIL-1
Control-2
EIL-2
LSP Granules
Rhabdom Area
Palisade Area

Figure 7. Summary graph of a 2 X 2 factorial ANOVA comparing mean palisade layer areas between no light and light exposure *in vivo* and *in vitro*.

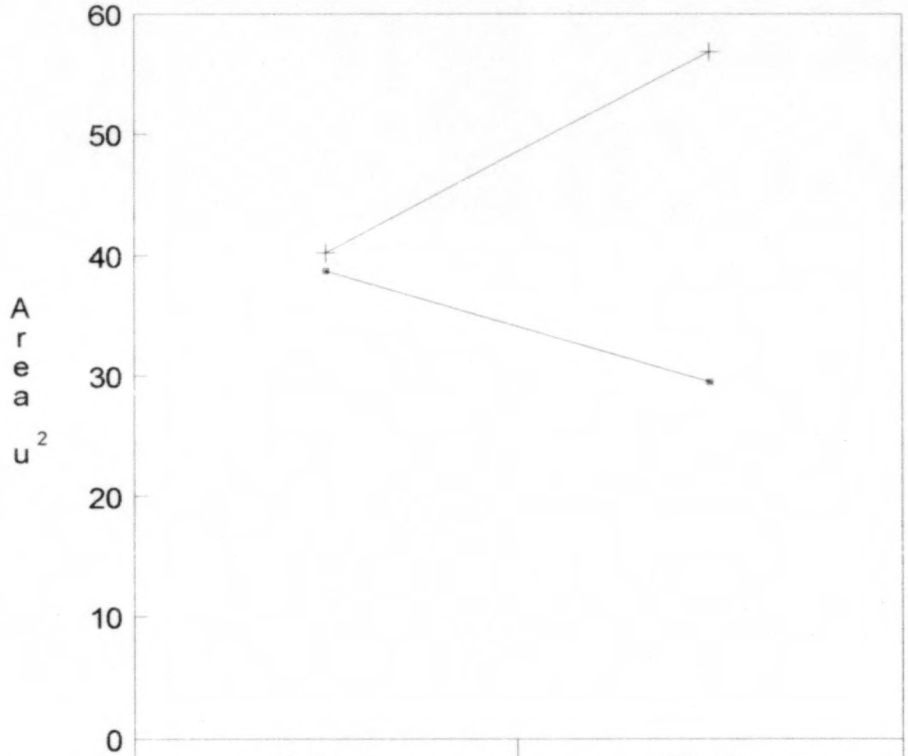
Mean Palisade Layer Area



	Control	10 min.
in vivo	10.7	7.98
in vitro	6.24	11.49

Figure 8. Summary graph of a 2 X 2 factorial ANOVA comparing mean rhabdom areas between no light and light exposure *in vivo* and *in vitro*.

Mean Rhabdom Area

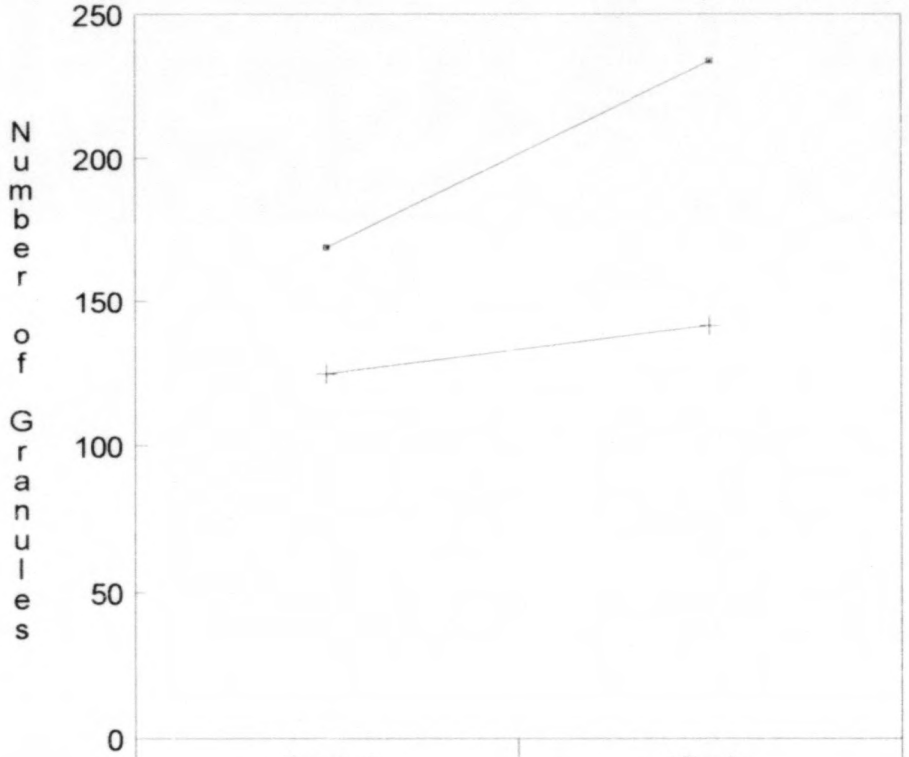


	Control	10 min.
in vivo	38.72	29.48
in vitro	40.23	56.83

in vivo in vitro

Figure 9. Summary graph of a 2 X 2 factorial ANOVA comparing mean light-screening pigment granule numbers between no light and light exposure *in vivo* and *in vitro*.

Mean LSPG Number



	Control	10 min.
in vivo	168.67	233.78
in vitro	124.44	141.22

Discussion

Cross-sectional area of the palisade layer in dark-adapted ommatidia changed in response to light whether or not the eye was connected to or disconnected from the clock(s), however in opposite directions. The palisade layer area decreased or increased after ten minutes of illumination in ommatidia connected to or disconnected from the clock(s), respectively . The *in vivo* condition produced results consistent with those demonstrated in *P. americana* (Butler 1973) and in previous research on *L. maderae* (Ferrell and Reitcheck 1990). The *in vitro* analysis of morphological parameters in *L. maderae* was apparently without precedent. However, a similar investigation carried out in *Limulus*, the horseshoe crab, demonstrated the absence of a rhythm normally present in retina aperture length (Chamberlain and Barlow 1987). This lack of structural rhythm in *Limulus* suggests that the dual effects of circadian efferent activity and natural lighting are not simply additive. The apparent divergent effect of natural light on *L. maderae* palisade layer area within *in vitro* dark-adapted eyes compared to the *in vivo* condition is at present without meaningful explanation, except to say that it could share a common mechanism of control with changes in rhabdom area given similar treatment.. Rhabdom area did not change *in vivo* , a finding consistent with previous results (Ferrell and Reitcheck 1990). The increased rhabdom area found in *L maderae in vitro* was without corroboration in previous research and is therefore difficult to interpret. Perhaps membrane turnover triggered by light could have affected rhabdom area in eyes disconnected from clock influence. The effect of *in vitro* compared with *in vivo* treatment with respect to ten minutes of illumination is significant. As discovered in *Limulus*, photoreceptive membranes of rhabdoms appear to be under the control of ambient light conditions. Photoreceptive membrane turnover in the grapsid crab, *H. sanguineus* appears to be under the control of a circadian efferent clock (Arikawa *et al.* 1988). Not knowing how alterations in photoreceptive membranes affect the dimensions of

the rhabdoms, hence, cross-sectional areas, the data of rhabdom areas in this and previous investigations in *L. maderae* (Ferrell and Reitcheck 1990) provide no meaningful agreement as to the mechanism(s) of control. Uncertainty concerning the mechanism of control by natural lighting and/or endogenous oscillations is the consensus. The inability to section uniformly through all ommatidia at precisely the same depth and the lack of uniformity in rhabdom shape (Chamberlain and Barlow 1987) presents anticipated obstacles to verification of rhabdom area change and to the most reasonable control mechanism.

Accumulation of light-screening pigment granules within a 13.5μ diameter circle, centered about the rhabdom, was greater in light-adapted compared with dark-adapted photoreceptors, whether *in vivo* or *in vitro*. This finding supports the idea that light plays a role in driving the daily rhythm of light-screening pigment granule organization. The degree to which light is responsible for the movement of light-screening pigment granules against a background of endogenous clock control has yet to be established. The positive effect of light on light-screening pigment granule aggregation about the rhabdom in the *in vivo* condition and the *in vitro* condition deserves explanation.

Even though the expression of daily differences observed in the organization of light-screening pigment granules does not appear to be regulated endogenously by a pacemaker (Ferrell and Reitcheck 1990), biological rhythms result from complex, non-linear outputs from pacemaker systems; therefore extreme caution should be exercised in making any generalization (Enright 1981). Several hypotheses have been advanced to explain the mechanisms of cellular interactions responsible for biological rhythms in arthropods whether strictly endogenous, exogenous, or endogenous in a less restrictive sense. One hypothesis proposes a push-pull mechanism of circadian efferent activity and cyclic lighting as demonstrated in attenuation of photomechanical structural changes in *Limulus* when the optic tract is severed in the presence of light (Barlow 1990, Chamberlain and Barlow 1987 1979). Evidence for this hypothesis in *L. maderae* has been in regard to the active

endogenous push toward the nighttime morphology of increased palisade layer area and the more passive, partial drift back towards its daytime morphology (Ferrell and Reitcheck 1990). Even though the movement of light-screening pigment granules does not appear to be circadian in a strictly endogenous sense, neither does it appear to be completely exogenous (Aschoff 1981). The photomechanical movements of the granules were similar under both *in vivo* and *in vitro* conditions; however, the change was greatest under *in vivo* conditions. Based on the results of this investigation, it appears that light stimulates motility within the photoreceptor cells directly, but could require a slave oscillator or local feedback loop (Pittendrigh 1981) in the distal portion of the optic lobe that functions in conjunction with the pacemaker and or its efferent neurons. It is reasonable to assert that palisade layer area and light-screening pigment movement are not coupled to the same pathway in that palisade layer area changes in response to light *in vitro* are opposite to those expected, whereas the movement of light-screening pigment granules in *in vitro* is as anticipated. A second hypothesis suggests that some humoral factors originating outside the optic lobes of *L. maderae* could be involved in the establishment of biological rhythms (Page 1983) but the analysis of this postulate is beyond the scope of this investigation. A third hypothesis proposes that the smooth endoplasmic reticulum of photoreceptor cells has a Na^+ - sensitive capacity for regulating concentrations of Ca^{2+} in these photoreceptors through the control of pigment granule transport in light/dark adaptation as seen in the crayfish, *Procambarus* (Frixione and Ruiz 1988). This hypothesis, coupled with the observation that the inhibition of photon-dependent membrane channels by Ca^{2+} occurs in Limulus ventral photoreceptors (Bacigalupo *et al*. 1991), may well provide an explanation for the cellular mechanisms of granule motility in *L. maderae*, but that too is beyond the scope of this investigation. Yet another hypothesis proposes that the photomechanical reaction in retinular screening pigments is triggered by visual pigments as demonstrated in the meal moth, *Ephestia keuhniella*. (Weyrauther 1986). Even though it is not known

absolutely whether the pigment controlling the retinular screening pigment migration is located in the retinula or in the pigment cells, it seems more likely that the independent control is within the retinula cells themselves since the function of the screening pigments is to control the light flux to the visual pigments in the microvillar membranes of the retinula cells (Weyrauther 1986). This hypothesis can provide insight into the similar response to light of *in vitro* dark-adapted eyes in *L. maderae* when comparedwith the more elevated response in the *in vivo* condition. The fact that there was a significant response at all in *in vitro* supports the conclusion that some control exists within the photoreceptors themselves. A conflicting interpretation exists if the clock's multiple effects on the retina extend over an entire day even after efferent activity has ceased as demonstrated in *Limulus* (Chamberlain and Barlow 1987). If it is possible for the efferent control to extend beyond the time of pacemaker activity, then the ten minutes after the photoreceptors have been severed from the optic lobe could still be sufficient time for the clock to have some effect on the migration or dispersion of the light-screening pigment granules, even in conjunction with light. Another hypothesis suggests that the involvement of light-screening pigment granules could be controlled within the accessory screening pigment cells or adjacent cone cells distal to these pigment cells as recorded for the sphingid moth, *Deilephilia elpenor* (Juse *et al.* 1987). Once again, the results from this investigation support this hypothesis in part, in that the photoreceptors isolated from the clock(s) in *L. maderae* are not separated from accessory pigment cells and, thus, could be controlling their granule movement within photoreceptors to some extent, even though at a depressed level. The mechanism currently accepted in cockroach research indicates inhibitory control through general modulation by the circadian oscillator(s) in the optic lobe(s) over the excitability of the central nervous system, including the photoreceptors (Page 1989). The influence of the clock(s), the other central nervous system sites of oscillation, and the photoreceptors could all be involved in the total hierarchy of control (Kasai and Chiba 1987) as *L. maderae* interacts with its environment.

The fact that the photoreceptors serving as *in vitro* controls (i.e. dark-adapted eyes with no photostimulation) had light-screening pigment granules less densely packed around the rhabdom, and also at a depressed level compared to the *in vivo* control, suggests that factors other than the optic lobe clock(s) and light are influencing granule movement.

One factor that was given consideration as a depressor of values in the *in vitro* condition was the trauma of surgery. Although this is not unreasonable, *L. maderae* has demonstrated remarkable abilities to withstand radical surgeries and express behaviors reflective of intact animals or tissues. The regeneration of rhythmicity in transplanted *L. maderae* optic lobes (Page 1982), the persistence of a rhythm for up to ten days in surgically isolated *L. maderae* optic lobes (Page 1987), and sectioning of the optic nerve in *Limulus* at noon leaving the structure of the retina in the daytime state for up to twenty-four hours (Chamberlain and Barlow 1987) are examples of the hardiness of *L. maderae* and the horseshoe crab, *Limulus*. This hardiness can help explain the retention of the trend in the *in vitro* condition to parallel *in vivo* pigment granule movement. Nevertheless, the depressed values of pigment granule movement could be partially in response to the trauma of surgery. Another factor to be considered is the ten-minute period in which the excised eyes for both dark and light adaptation were maintained in physiological saline. Portions of invertebrate nervous systems have been kept alive with relative ease for days or even weeks, *in vitro*, either in a physiological saline (Page 1981) or in culture medium (Page 1981, Levi-Montalcini *et al.* 1973) with the successful identification of neuronal circadian pacemakers. The most thoroughly researched example in terms of circadian rhythms has been in the isolated eye of the marine gastropod, *Aplysia*.. A circadian rhythm persisted for several days in the eye while they were maintained in either culture medium or filtered seawater (Page 1981). Thus, the impact of the physiological saline on the depression of values for the movement of light-screening pigments could involve interference with normal activity, but that does not seem to be the case as reported in similar research. The method

of providing oxygen for the excised eyes could have contributed to the lowered *in vitro* values, but there is no way of confirming this, short of repeating the investigation and using a system of oxygen supply that is continual, not simply at the beginning of the ten minute time frame. Another important factor that could be responsible for the depression of values *in vitro* is the time during the dark-adapted state that the eyes were removed. Since the maximum activity of axons in the optic tract is during the subjective day in intact animals that are free running in constant darkness (Page 1989), the fact that eyes were removed for *in vitro* treatment during night (2030h-0130h) and not during day (*i.e.*, light on set or 0600h) did not allow for maximum programming of all cellular components for daytime morphology. A recommended best time to maximize the contribution of the endogenous pacemaker would be during free running conditions of constant darkness at subjective light onset in order to maximize the effect of light on the movement of light-screening pigment granules independent of the clock(s). Another factor that could influence the lowering of values *in vitro* light treatment could be the intensity and/or the duration of the light (Frixione and Ruiz 1988). The effect of light intensity and duration, or total photic energy, impinging on photoreceptors in the preliminary investigation of Phase One gave evidence supporting an increased aggregation of light-screening pigment granules with increased duration and constant intensity. The intensity and the duration in Phase Two (*i.e.*, *in vitro*) were kept consistent with Phase One (i.e., *in vivo*); therefore, there is no reason to expect the depressed values of numbers of light-screening pigment granules to be lower than in the *in vivo* condition. Also, the fact that eyes excised under red light *in vivo* and *in vitro* both responded with light-screening pigment granule aggregation in response to ten minutes of light suggests that lack of variance in the total photic energy from *in vivo* to *in vitro* could not be responsible for the depressed values. However, the lack of light and the absence of active efferent control from the optic lobe did tend to allow for discontinuity and disruption

of cellular integrity in the Phase Two control group, suggesting the involvement of both pacemaker and/or photoreceptors in maintaining cellular integrity.

Location of control centers and descriptions of models of the control mechanisms within the optic tract of *L. maderae* can be finalized only after the multiple factors such as the effects of surgery, *in vitro* physiological saline, oxygen supply, timing of analysis to coincide with the maximum control of clocks and photoreceptors, and the intensity and duration of light, are all minimized or maximized for the best revelation of endogenous and/or exogenous biological rhythms. The terminology that defines and describes the hypothetical mechanisms of control are plethoric: from the role of light as a trigger (Chamberlain and Barlow 1979), an inhibitor, accelerator, or suppressor (Arikawa *et al*. 1988), an inducer, synchronizer, enhancer, magnifier, amplifier, or primer (Chamberlain and Barlow 1987) to the clock(s) as a pacemaker, oscillator (Page 1989) or modulator (Kasai and Chiba 1987). The search for permissive conditions under which a persistent rhythm can be observed in a given organism is often a frustrating exercise with no assurance of success (Enright 1981).

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