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I am submitting herewith a thesis written by John E. Fritz entitled "Analysis of Diurnal Rhythms in *Gallus domesticus*." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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✓

ANALYSIS OF DIURNAL RHYTHMS IN *GALLUS DOMESTICUS*

A Thesis

Presented to

the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by

John E. Fritz

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ABSTRACT

The pineal of certain birds and mammals are known to contain biologically active amines which, with their enzymes, show circadian variations. In the rat, Quay *et al.*⁸ have shown diurnal cycles in levels of pineal melatonin and 5-hydroxytryptamine (serotonin) (Quay *et al.*⁹). Additionally, the activities of two pineal enzymes, hydroxy-indole-0-methyltransferase (Axelrod *et al.*²¹) and N-acetyltransferase (Klein *et al.*²⁰) have been described. Birds also possess circadian rhythms for those parameters described above.

This investigation is a comparative study of the response of *Gallus domesticus* to changes in environmental lighting conditions as well as to the effects of pinealectomy on chosen internal body parameters.

Various lighting and operative conditions were used, and the following materials were quantitatively analyzed: (1) blood glucose levels, (2) serum calcium, (3) liver glycogen, (4) N-acetyltransferase, (5) 5-hydroxytryptamine (serotonin), (6) hydroxy-indole-0-methyltransferase, and (7) 5-methoxytryptamine (melatonin).

The analysis included determining whether the animal possessed a circadian rhythm under control conditions (12 hours light - 12 hours dark) and if the rhythms might be upset, or varied, by changing the external light patterns.

Results from these experiments, although they are in somewhat disagreement with those of other investigators, strongly support a conclusion that the pineal gland acts as an extraretinal photoreceptor. The hydroxy-amines and those enzymes responsible for their formation afforded prime examples of circadian rhythms, and the control of those rhythms by

an external stimulus (light). The basis for the studies was to determine whether or not the response of animals to light is monitored by the pineal and what the response dictates to the various body parameters which have previously been mentioned. The results of our studies on the above mentioned are described fully in the following chapters.

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P_X	Pinealectomized	
$P_X \rightarrow$	Pinealectomized @ 10 days of age	
0	Continuous darkness (i.e., 24 hours)	
24	Continuous light (i.e., 24 hours)	
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P_X	Pinealectomized	
$P_X \rightarrow$	Pinealectomized @ 10 days of age	
0	Continuous darkness (i.e., 24 hours)	
24	Continuous light (i.e., 24 hours)	
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P_X	Pinealectomized	
$P_{X\rightarrow}$	Pinealectomized @ 10 days of age	
0	Continuous darkness (i.e., 24 hours)	
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P_X	Pinealectomized	
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P_X	Pinealectomized	
$P_X \rightarrow$	Pinealectomized @ 10 days of age	
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12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)	29

CHAPTER I

INTRODUCTION AND LITERATURE SURVEY

Out of darkness and into light: this temporal signal is needed for the synchronization of many body functions, and thus for the formation of a biological clock.

The relationship between various body parameters and the onset of a diurnal "circadian" cycle has been of interest to many investigators in recent years. Excellent reviews of the subject have been published.¹

The literature concerning biological periodicity deals with a wide variety of biological parameters as well as test systems.

Bonilla *et al.*^{2,3} using Swiss albino mice showed diurnal variations in the serum calcium levels. The results demonstrated that external conditions play important roles in fixing body functions.

Sayler and Wolfson⁴ were interested in the effects of manipulated photoperiods and/or pinealectomy on the development of sexual maturity in the female Japanese quail (*Coturnix coturnix Japonica*). These results show that the pineal gland in the female contributes to the sudden and rapid maturation of the ovaries a brief interval before the onset of the laying period. Pinealectomy delays maturation and egg production for several days.

Daily photoperiod lengths as previously stated regulate various body functions. However, what if the animals' main photoreceptors (i.e., eyes) are removed? Experiments have been carried out by various investigators which indicate that (both in mammals as well as in birds) the pineal gland acts as an extraretinal photoreceptor.

The pineal is unique in another way; it contains the hormone melatonin, which along with other methoxyindoles is formed as follows:

tryptophan $\rightarrow$ hydroxytryptophan $\rightarrow$ serotonin $\rightarrow$ N-acetylserotonin $\rightarrow$ melatonin.⁵

Axelrod *et al.*⁶ found that the weight of the pineal and the activity of the melatonin-forming enzyme hydroxy-indole-0-methyltransferase (HIOMT) were greater in chickens exposed to continuous light or to diurnal light than in those exposed to continuous darkness. Light stimulates avian gonadal activity⁷ as will be shown in the discussion of the following experiments.

The pineals of certain mammals and birds have been shown to contain biologically active amines which show 24-hour (circadian) variations. In the rat pineal diurnal variation of levels of melatonin⁸ and 5-hydroxytryptamine (serotonin) have been well documented for a 24-hour period. Additionally, the activities of two pineal enzymes, hydroxy-indole-0-methyltransferase¹⁰ (HIOMT) and N-acetyltransferase,¹¹ have been identified in several species of mammals and birds.

Hydroxy-indole-0-methyltransferase catalyze the methyl transfer of N-acetylserotonin (5-hydroxy-N-acetyltryptamine) to form melatonin. The enzyme shows an absolute requirement for s-adenosylmethionine (AMe). The K_m for N-acetylserotonin and s-adenosylmethionine are 5.4×10^{-5} m and 4.6×10^{-5} m, respectively. A number of hydroxylated indole amines other than N-acetylserotonin can act as substrates of hydroxy-indole-0-methyltransferase. However, the compounds (including 5-hydroxy-indole-acetic acid and serotonin) had a much higher K_m for hydroxy-indole-0-methyltransferase than N-acetylserotonin.¹²

N-acetyltransferase transfers an acetyl group from acetyl coenzyme A to the amino group of serotonin to form N-acetylserotonin. In rats and chickens the enzyme maintains a 24-hour rhythm. During a diurnal lighting schedule, N-acetyltransferase activity is considerably lower in the light than in the dark. In addition, if the animals are blinded or maintained in constant darkness, their rhythm is maintained, whereas if they are submitted to constant light, the enzyme becomes arrhythmic with a greatly reduced activity.¹³

A number of specific questions arise and have led to the following objectives: (1) does changing the lighting schedule change the physical body and gland weights? (2) do the enzymes, substrates and products in the pathway of melatonin formation vary rhythmically with or without a diurnal lighting regime? (3) can one draw on various parameters (i.e., blood glucose, serum calcium, liver glycogen) of the body to see if external stimuli (i.e., light, dark) affect their circadian fluctuation? (4) finally, can pinealectomy cause changes in the above parameters or is the biological clock of synchronism of bodily functions unrelated to this extraretinal photoreceptor? These are a few of the questions which prompted these investigations.

CHAPTER 11

MATERIALS AND METHODS

Description of *Gallus domesticus*

Six hundred one day old White Leghorn cockrels were used in all treatments. The birds were a gift of the Riverside Hatchery, Knoxville, Tennessee. They were given water and fed *ad libitum*.

Treatments

Treatments were started immediately; for those treatments requiring diurnal lighting, time clocks were set to start on at 6 a.m. and end automatically at 6 p.m. The birds were maintained in environmentally controlled rooms with dimensions of 5 feet x 5 feet. Each treatment consisted of two rooms, each room containing fifty (50) birds. In most of the determinations at least four birds were used per two hour time periods. Pinealectomies were performed as described by Dr. H. Shirley.¹⁴ The following is a description of the treatments and abbreviations used:

Treatment

- (1) P_X^0
- (2) 0
- (3) P_X^{24}
- (4) 24
- (5) $P_X^{12-12 \rightarrow 24}$
- (6) $12-12 \rightarrow 24$
- (7) $P_X^{12-12 \rightarrow 0}$
- (8) $12-12 \rightarrow 0$

(9) P_X 12-12

(10) 12-12

(11) 12-12(PX)→0

(12) 12-12(PX)→24

The → (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow. The birds were maintained on a treatment for a period of 5 weeks.

P_X Pinealectomy

0 Continuous darkness (i.e., 24 hours)

24 Continuous light (i.e., 24 hours)

12-12 Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

Treatment #11 shows that the animals were raised on a diurnal lighting schedule for the first 10 days at which time they were pinealectomized and raised in the dark. In Treatment #12 the birds were maintained in 12 hours light-12 hours dark for the first 10 days, pinealectomized and then put on continuous light.

Whole Blood Glucose

The determination of blood glucose levels was by the procedure of Dubowski.¹⁶ Preparations of protein free filtrates of whole blood were made by bringing the appropriate dilution to 3.0 percent trichloroacetic acid, and filtering through Whatman No. 2 filter paper. To 1.0 mL. of the protein free filtrate was added 3.0 ml of 6.0 percent v/v o-Toluidine in glacial acetic acid. The tubes were mixed and immersed in a water bath @ 100°C for 10 minutes. After cooling the transmittance was measured on a Coleman Model 60 Jr. Spectrophotometer at 630 or 635 nm.

Liver Glycogen

The estimation of liver glycogen was based on the work of Seifter *et al.*¹⁷ Immediately after excision from the five (5) animals, 1 gram of

liver per bird was added to a previously weighed test tube containing 3 ml. of 30 percent KOH solution. The tissue was then digested by heating the tube for 20 minutes in a boiling water-bath after which the digest was cooled and appropriately diluted with water to give a solution of glycogen of approximately 3-30 ug/ml. A 5 ml. aliquot was removed and to it was added 10 ml. of a 0.2 percent anthrone reagent. The tubes were covered with glass marbles and heated for 10 minutes @ 100°C. They were then cooled and read in the colorimeter at 620 nm. Calculations can be made using the formula

$$\text{ug of glycogen in aliquot} = \frac{100 \times U}{1.11 \times S}$$

U = the optical density of the unknown test solution

S = the optical density of the 100-ug glucose standard

1.11 = the factor determined by Morris²⁶ for the conversion of glucose to glycogen

Determination of Serum Calcium

The spectrophotometric method of Farese and Mager¹⁸ for the direct determination of microgram quantities of calcium in serum was used. In this method glyoxal bis (2-hydroxyanil) (GBHA) forms a complex with calcium which is assayed spectrophotometrically at 520 nm. Samples consisted of four (4) separate serum determinations and were taken every two (2) hours for a twenty four hour period.

Fluorescence Assay for Tissue Serotonin

A sensitive and specific method for the estimation of serotonin in biological materials has been described.¹⁹ The method involves the

extraction of serotonin from the pineal gland into 1-butanol from a salt-saturated solution at pH 10.0. Serotonin was then returned to an aqueous solution and reacted with ninhydrin to yield a fluorescent product, which was excited at 385 nm and measured at 490 mμ on a spectrophotofluorimeter described by Longworth and Battista.¹⁵ The fluorescence intensity was proportional to serotonin concentration over a range of 5 μg/ml to 500 μg/ml. Each time point consisted of four (4) separate pineal assays.

Diurnal Rhythmic Activity of N-acetyltransferase

N-acetyltransferase activity was determined by the procedure of Klein *et al.*²⁰ on four (4) separate pineal glands. Measurements of the (¹⁴C) N-acetylserotonin and (¹⁴C) melatonin formed by the homogenate of an individual pineal gland incubated for 10 minutes @ 37°C in 0.1 molar sodium phosphate (pH 6.8) - (¹⁴C) serotonin, acetyl coenzyme A assay mixture was made. Radiolabeled N-acetylserotonin and melatonin were isolated by two dimensional thin layer chromatography on cellulose. The cellulose contained a fluorescent dye which under ultraviolet light showed the position of the amines. They were then scraped off into a scintillation vial and counted using a toluene-2, 5-bis-2- (5-tert-Butylbenzoxazyolyl)-Thiophene cocktail.

Five (5) percent or less of the (¹⁴C) N-acetylserotonin was converted to (¹⁴C) melatonin.

Estimation of Melatonin

Melatonin may be separated from N-acetylserotonin and other hydroxyindoles by the procedure of Axelrod and Weissbach.²¹ Pineal gland homogenates were extracted with chloroform. The organic phase was extracted with water and an aliquot of the chloroform phase was evaporated to dryness. The residue was dissolved in 3 N HCL. The melatonin was measured

fluorometrically in the instrument described above (activation 300 mμ, fluorescence 340 mμ).²² This method is sensitive to levels of 1.0 μg melatonin. Our experiments, however, have uncovered a very important and possibly controversial point, that of the wavelength at which the melatonin extracted from the pineal fluoresces on the spectrofluorimeter. In the procedure of Axelrod and Weissbach²¹ the melatonin was measured fluorometrically (activation 300 mμ, fluorescence 540 mμ). However, repeated tries in this laboratory have proved unsuccessful in obtaining fluorescence at 540 mμ. After our futile attempts standard of known melatonin were made and instead of activating at 300 mμ and reading at 540 mμ (which did not work), we scanned each concentration of melatonin and found that the maximum fluorescence was at 340 mμ, not 540 mμ. After the standards were run, the samples were also read at 340 mμ, Fig. VII being the results. The reason for this discrepancy is not within the scope of this paper. At first, it was assumed to be a misprint in the literature; however, checking back further shows that 540 mμ has been used by these investigators in the past. The results were compared with a standard curve of melatonin in 3 normal hydrochloric acid.

Measurement of Hydroxy-indole-O-methyltransferase

Hydroxy-indole-O-methyltransferase was determined by the methodology of Axelrod and Weissbach.²¹

N-acetyl-2-(¹⁴C)-serotonin was added to a single pineal homogenate which contains 12 μmoles S-adenosyl methionine and 0.1 μmoles sodium phosphate pH 7.9. The reaction mixture was incubated 30 minutes @ 37°C. The final mixture was extracted with chloroform and aliquot was removed and dried. The residue was resuspended in ethanol and was assayed in a (Packard) scintillation counter after the addition of 10 mls of a toluene based scintillation fluid.

TABLE I

MEAN ORGAN AND BODY WEIGHTS FOR THE VARIOUS LIGHTING REGIMES

TREATMENT ¹	(gms) EYE	(mgs) THYROID	(mgs) ADRENAL	(mgs) TESTES	(mgs) PITUITARY	(gms) BODY WTS.
(1) P _X 0	2.5 ±0.32	28.1 ±0.15	26.7 ±1.43	69.9 ±1.09	2.1 ±0.13	540.6 ±15.7
(2) 0	2.7 ±0.27	31.9 ±0.22	28.9 ±1.27	98.6 ±0.87	2.0 ±0.09	654.1 ±18.4
(3) P _X 24	1.7 ±0.19	24.9 ±0.29	17.5 ±0.50	84.6 ±1.73	2.0 ±0.10	612.8 ±13.2
(4) 24	1.9 ±0.23	20.0 ±0.14	45.7 ±1.39	142.9 ±2.90	2.2 ±0.19	710.3 ±15.5
(5) P _X 12-12→24	1.8 ±0.29	37.1 ±0.19	27.1 ±1.41	103.6 ±1.24	2.0 ±0.08	556.3 ±18.9
(6) 12-12→24	1.9 ±0.45	20.1 ±0.31	35.1 ±1.28	132.8 ±1.91	2.0 ±0.10	561.7 ±11.1
(7) F _X 12-12→0	2.3 ±0.30	24.5 ±0.27	31.1 ±1.14	76.1 ±1.86	1.6 ±0.20	574.1 ±16.7
(8) 12-12→0	2.3 ±0.43	32.4 ±0.32	36.0 ±1.54	77.1 ±2.30	2.0 ±0.20	631.2 ±13.0
(9) P _X 12-12	1.7 ±0.47	25.2 ±0.29	25.2 ±1.18	82.3 ±1.29	2.3 ±0.18	539.6 ±14.7
(10) 12-12	1.6 ±0.28	24.9 ±0.43	30.9 ±1.60	104.8 ±1.73	2.6 ±0.24	560.4 ±10.0
(11) 12-12(PX)→0	2.2 ±0.20	25.2 ±0.27	33.1 ±1.48	94.8 ±1.50	2.5 ±0.31	537.7 ±9.0
(12) 12-12(PX)→24	1.8 ±0.31	23.0 ±0.30	24.3 ±1.45	70.2 ±2.39	2.0 ±0.08	596.2 ±7.4

TABLE 1. (Continued)

1. Relative mean weights for the various lighting regimes. Each value represents the mean $\pm$ standard error of 24 replicates. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X Pinealectomized

$P_X \rightarrow$ Pinealectomized @ 10 days of age

0 Continuous darkness (i.e., 24 hours)

24 Continuous light (i.e., 24 hours)

12-12 Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

FIGURE 1. Results on comparative studies of the effects of changes in environmental light on blood glucose rhythms. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_X \rightarrow$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

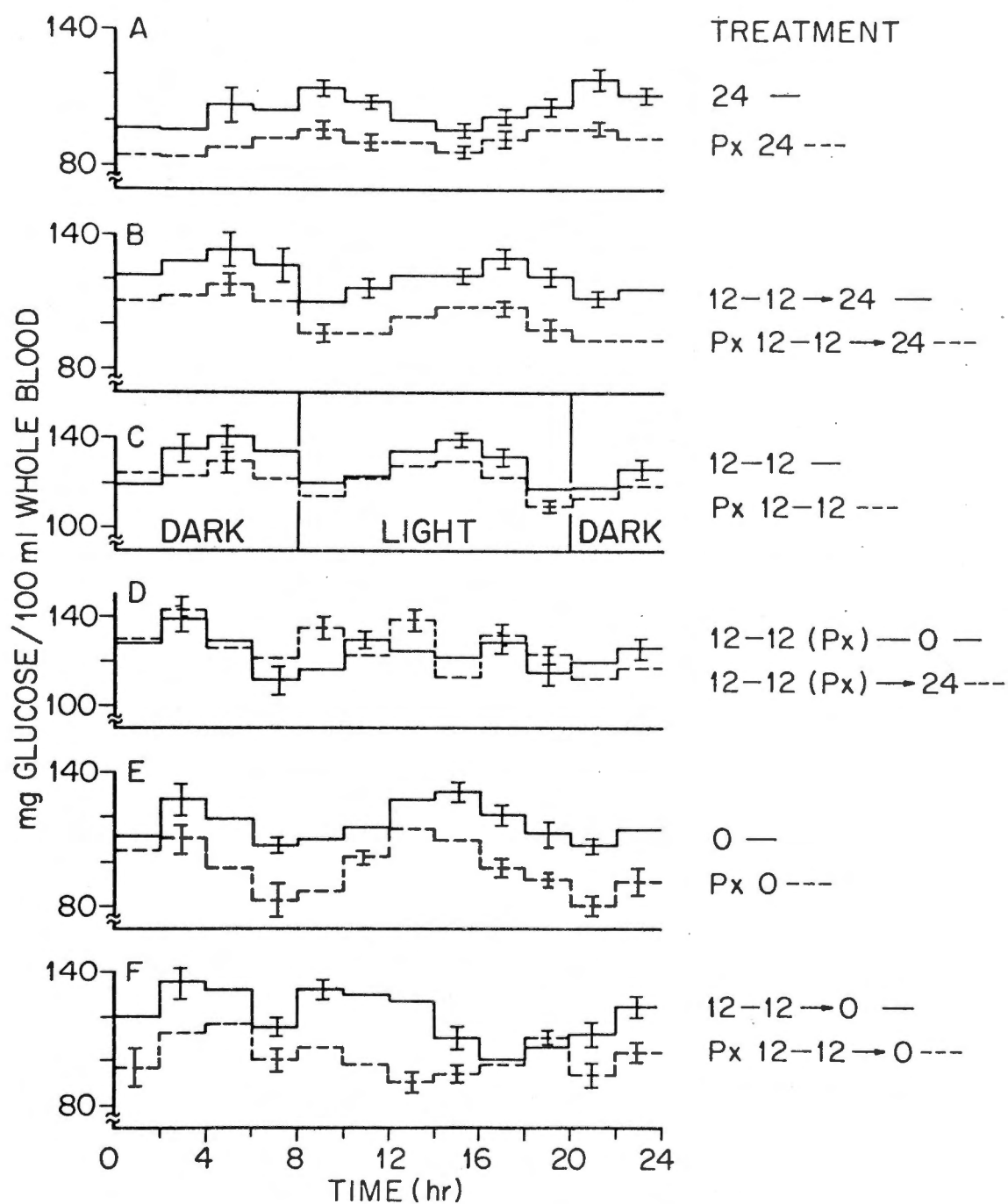


FIGURE 1

A-B-C-E-F) the glucose level of birds which were pinealectomized the absolute milligram quantity was statistically lower than those animals with intact pineals. Figure 1, Point D showed no differences in milligram quantities of glucose. Pinealectomized animals maintained in constant light demonstrated no significant difference over those maintained in constant darkness. From these results it seems likely that the pineal may have a regulatory effect on blood glucose levels. The mean 24 hour values for glucose are shown in Table II.

Estimation of Liver Glycogen

A partial rhythm was seen with most treatments except in those birds maintained in constant light. Glycogen measurements on individual animals varied so drastically that even when 5 sets of birds were used per time point the P value > 0.01 .

Fig. II shows the liver glycogen values of pinealectomized versus non-pinealectomized animals during the various lighting regimes. The mean values (μg glycogen/mg liver) is shown in Table II.

Photometric Analysis of Serum Calcium Levels

Animals which were pinealectomized from the onset of treatment showed an arrhythmic pattern of serum calcium levels. Similar results were seen in those animals which were non-pinealectomized but were maintained in constant darkness. These results are shown in Fig. III. Birds which were started on a diurnal lighting regime (i.e., 12 hours light, 12 hours dark) maintained a rhythmic but not circadian calcium cycle throughout the testing period no matter if they were pinealectomized and/or changed to a different lighting schedule after the first 10 days. Table II shows the mean values (mg ca/100 mls serum) over a 24 hour period.

TABLE II

MEAN VALUES FOR GLUCOSE, GLYCOGEN, CALCIUM AND SEROTONIN
OVER A 24 HOUR PERIOD

TREATMENT ¹	$\bar{X}$ mg glucose/100 mls blood	$\bar{X}$ μ g glycogen/mg Liver	$\bar{X}$ mg Ca/100 mls serum	$\bar{X}$ mg serotonin/mg Pineal
(1) P _X 0	98.31 $\pm$ 0.39	7.07 $\pm$ 2.34	25.90 $\pm$ 0.33	
(2) 0	118.20 $\pm$ 2.40	7.38 $\pm$ 1.70	4.80 $\pm$ 0.16	45.04 $\pm$ 5.3
(3) P _X 24	104.58 $\pm$ 1.87	4.80 $\pm$ 0.89	13.83 $\pm$ 0.48	
(4) 24	109.52 $\pm$ 1.31	14.20 $\pm$ 1.37	14.38 $\pm$ 0.37	38.82 $\pm$ 4.1
(5) P _X 12-12-24	119.95 $\pm$ 0.98	7.39 $\pm$ 1.62	36.88 $\pm$ 0.20	
(6) 12-12-24	104.90 $\pm$ 1.69	10.89 $\pm$ 1.19	12.47 $\pm$ 0.49	43.05 $\pm$ 3.9
(7) P _X 12-12-0	101.08 $\pm$ 2.20	12.04 $\pm$ 1.26	3.71 $\pm$ 0.29	
(8) 12-12-0	121.20 $\pm$ 2.00	12.01 $\pm$ 1.41	20.86 $\pm$ 0.63	30.36 $\pm$ 7.8
(9) P _X 12-12	119.45 $\pm$ 2.87	9.29 $\pm$ 1.53	6.56 $\pm$ 0.51	
(10) 12-12	129.15 $\pm$ 3.70	12.57 $\pm$ 0.98	23.81 $\pm$ 0.38	12.24 $\pm$ 2.7
(11) 12-12(PX)-0	124.55 $\pm$ 1.91	12.82 $\pm$ 2.05	23.61 $\pm$ 0.26	
(12) 12-12(PX)-24	128.76 $\pm$ 1.41	9.05 $\pm$ 1.91	13.55 $\pm$ 0.40	

TABLE 11. (Continued)

1. Shows the mean values over a 24 hour period. Each value represents the mean $\pm$ standard error of 24 replicates. The serotonin estimation was performed twice with essentially similar results. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_X \rightarrow$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

FIGURE 11. Variations in glycogen rhythms through changes in environmental lighting regimes. Each point represents the mean value $\pm$ standard error for 5 chickens. The results are a composite of 2 separate experiments. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_X \rightarrow$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

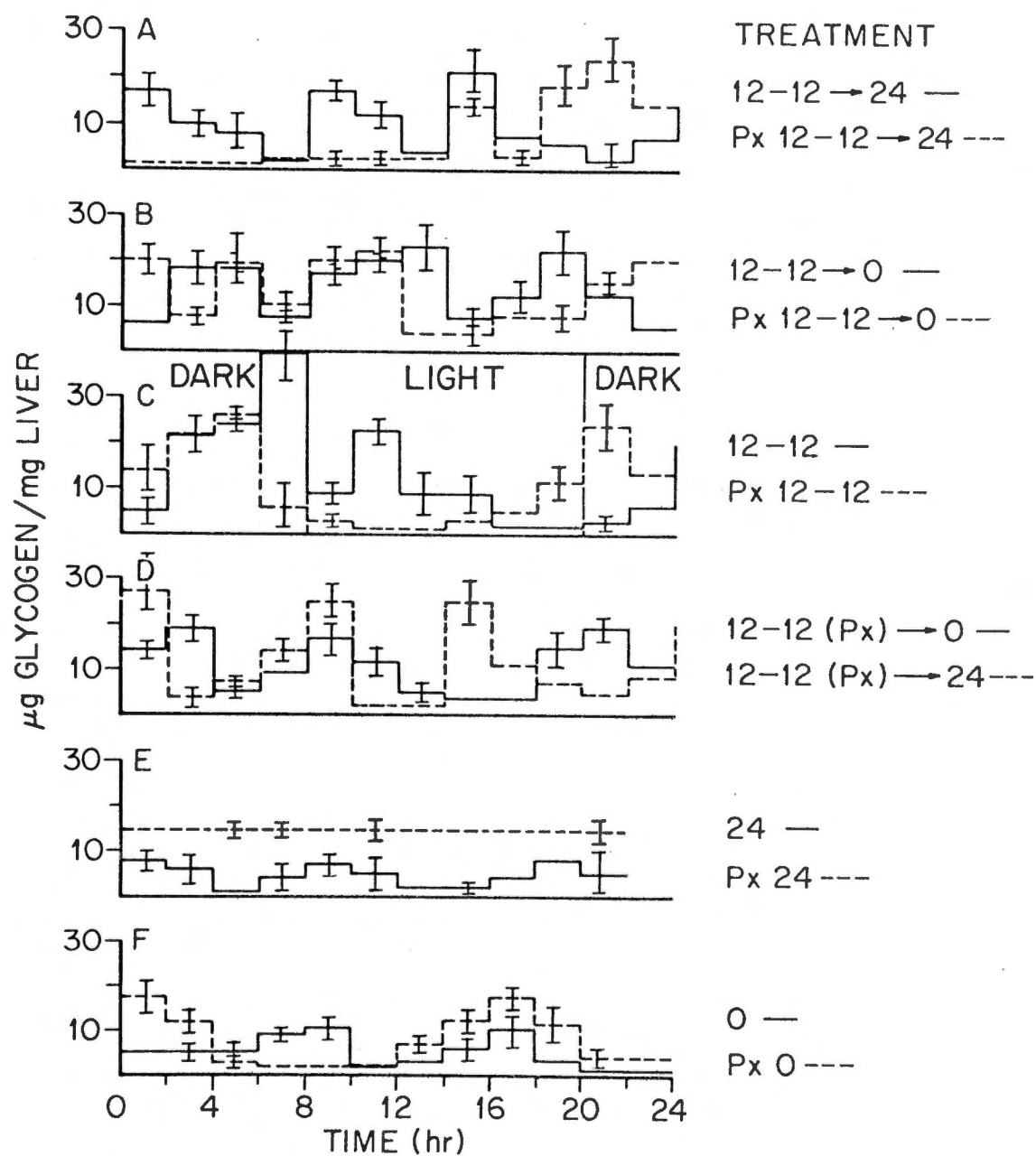
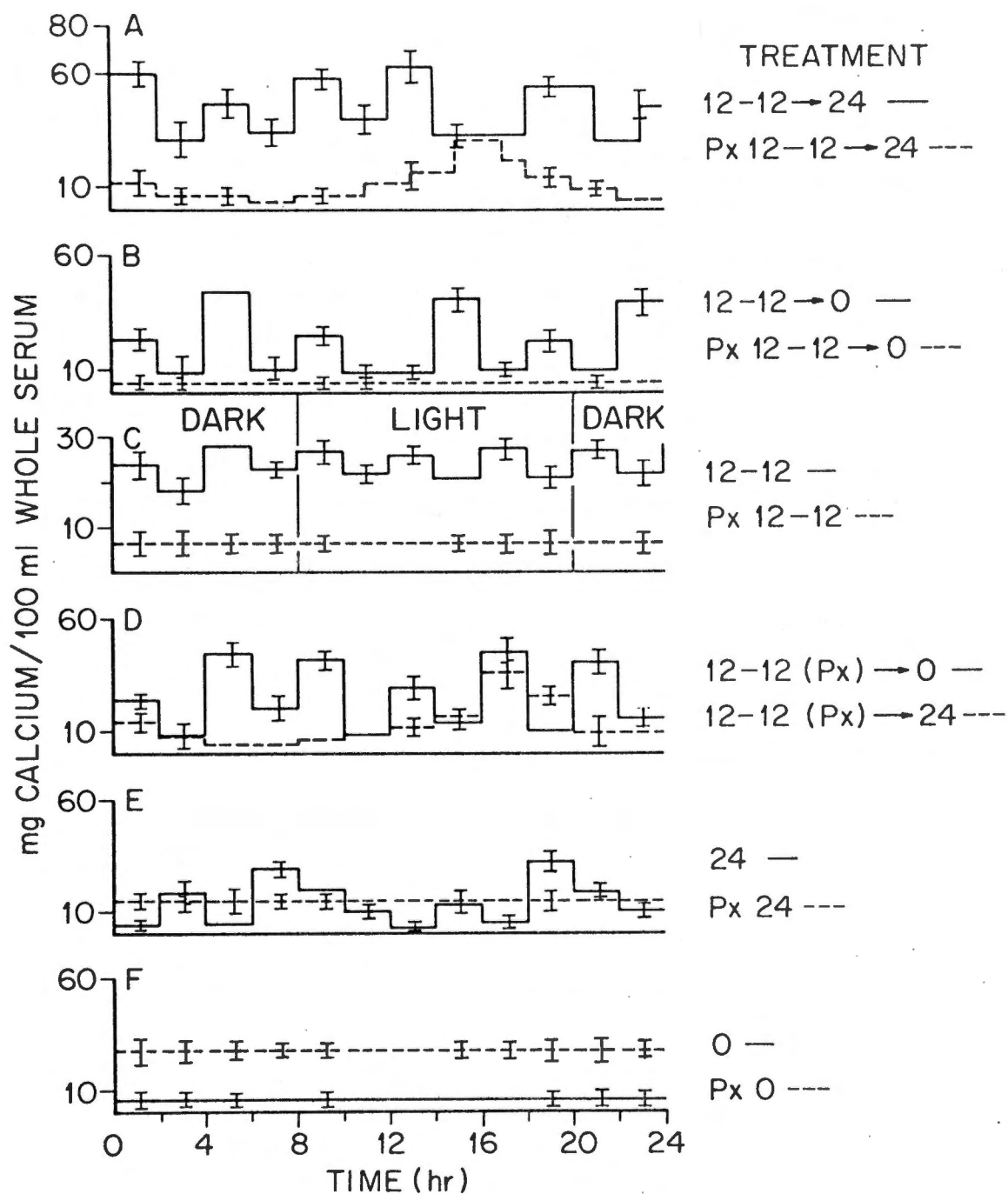


FIGURE 11

FIGURE III. Diurnal variations in the serum calcium level in *Gallus domesticus*. Each point shows the mean $\pm$ standard error determined with a sample size 4 chickens. Sampling was 12 times (2 hour intervals) over a 24 hour period. Each estimation was performed twice with similar results. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_X \rightarrow$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)



Fluorescence Assay for Tissue Serotonin

In the experiments conducted (Fig. IV) rhythmic variations persisted in all treatments. The birds which were under a diurnal light-dark cycle showed the greatest and most responsive change in serotonin level immediately following the dark cycle. Those animals maintained in diurnal lighting were observed to have significantly less serotonin on an average over a 24-hour period than those of the other treatments. These results are shown in Table II; the other four treatments did not statistically vary from one another.

Table II also shows the mean values over a 24-hour period for the above mentioned parameters (i.e., serum calcium, liver glycogen, and blood glucose).

N-acetyltransferase Activity

In our studies all birds except those (Fig. V B) which were maintained in constant light showed a rhythmic activity for N-acetyltransferase. Those animals which were maintained on a diurnal cycle had an enzyme content greater in the dark phase than the light. It is of interest to note (Fig. V A) that in those animals which were changed over to continuous light from a light from a lighting schedule of 12 hours light, 12 hours dark seem to be approaching a non-fluctuating level of enzymatic activity as that seen in Fig. V B for constant light.

Spectrofluorometric Assay for Melatonin

In our studies all birds except those (Fig. VI B) which were maintained in constant light showed circadian changes in pineal melatonin content. Examination of Fig. VI C indicates that melatonin content is highest during the dark phase and lowest in the median portion of the

FIGURE IV. The presence of a circadian rhythm in serotonin content of the chicken. Points are presented as the mean $\pm$ standard error for 4 pineals. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_{X\rightarrow}$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

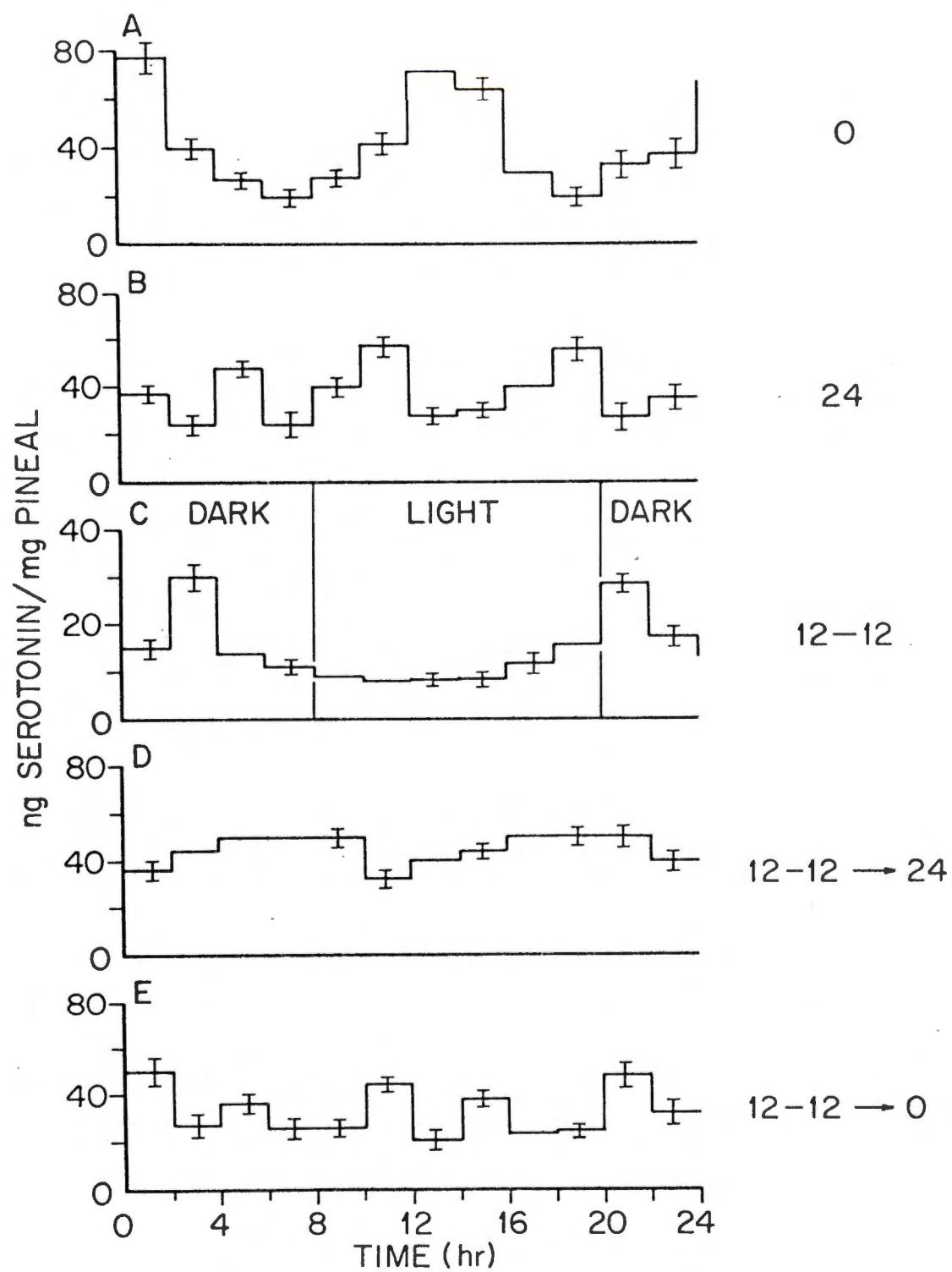


FIGURE IV

FIGURE V. Pineal N-acetyltransferase activity. Each point is the mean of 4 pineals. The ordinate is determined by measuring (^{14}C) N-acetyltransferase formed. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_{X \rightarrow}$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

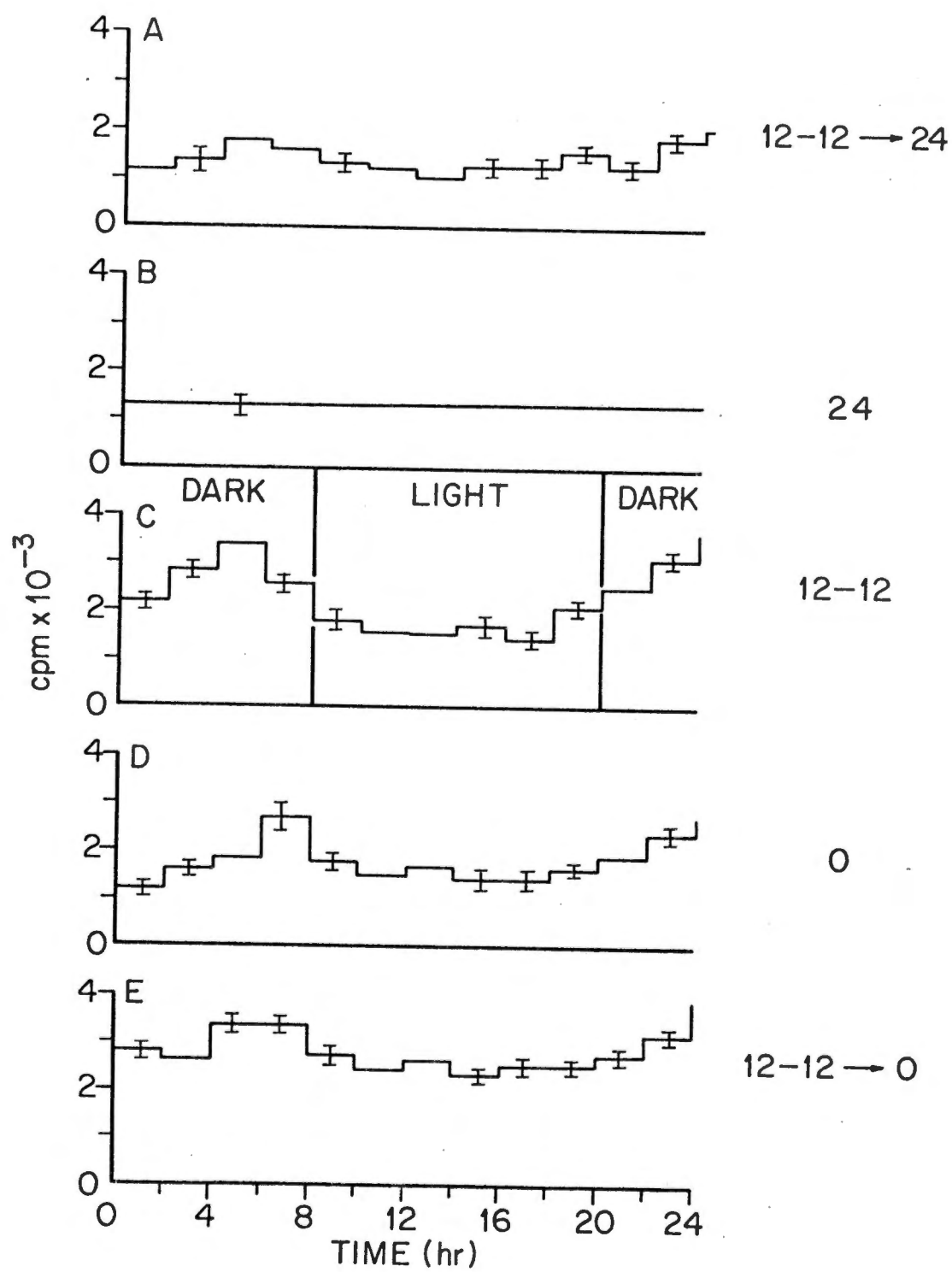


FIGURE V

FIGURE VI. Circadian rhythm of melatonin in the presence and absence of external stimuli (light). Values are means $\pm$ standard errors of 4 pineal glands. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_{X \rightarrow}$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

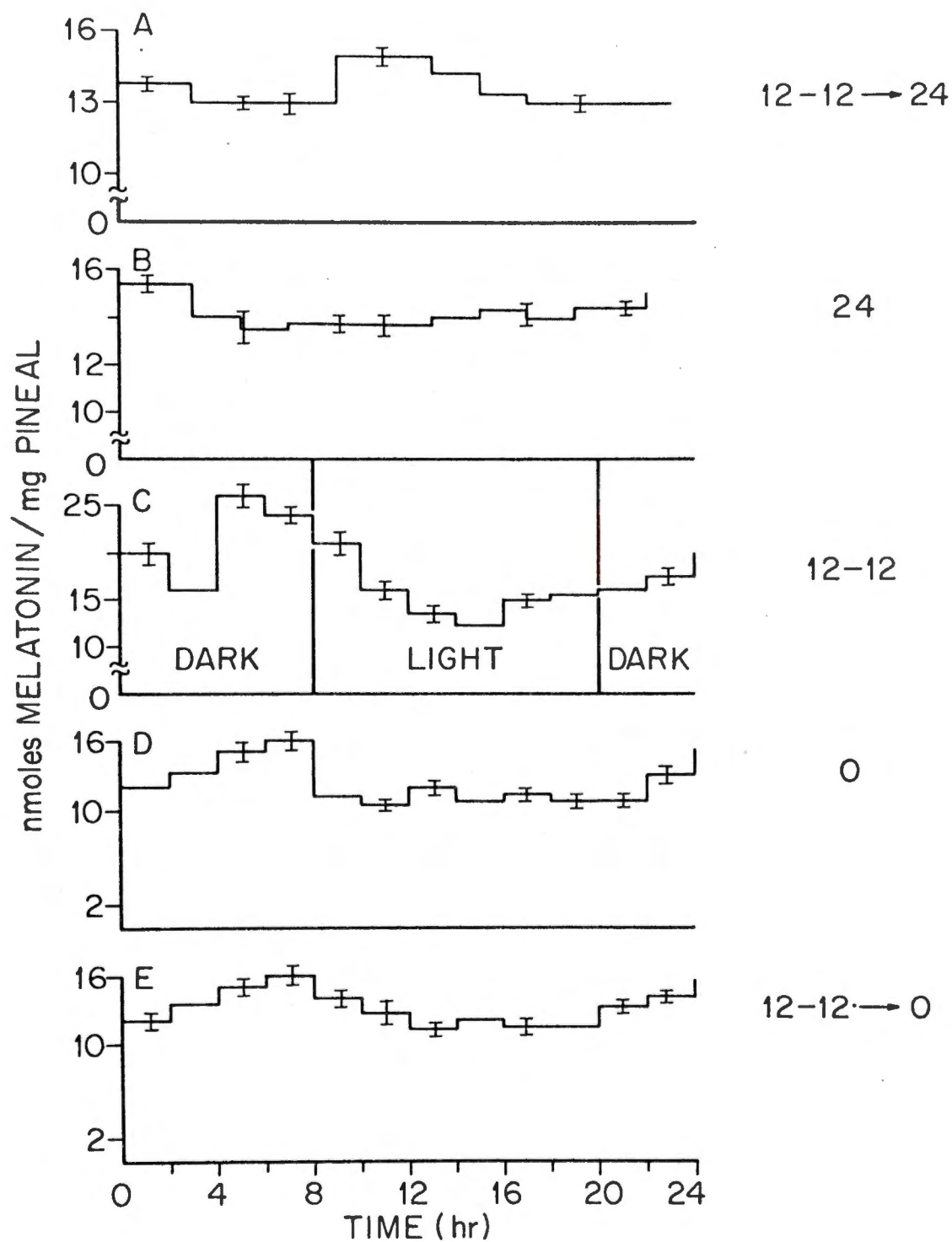


FIGURE VI

light phase. Previous investigators have estimated the quantities of pineal melatonin in chickens to fall within 20 n moles or less per pineal. Our findings are in accord with these.

Hydroxy-indole-0-methyltransferase (HIOMT) Activity

The hydroxy-indole-0-methyltransferase cycle in the bird appears to be dependent on the pineal gland functioning as an extraretinal photoreceptor.

Our experiments demonstrate (Fig. VII) that the hydroxy-indole-0-methyltransferase reaches a peak of maximum activity during the light phase in a diurnal cycle, an observation which is in discord with some earlier work by other investigators; this will be discussed in depth in Chapter IV.

Birds maintained in continuous light possess almost no rhythmic variation in their HIOMT activity, whereas those animals in the other treatments (Fig. VII, Figures A, D, E) show rhythmicity. Investigations recorded in Fig. VII, Figures D-E, show similar plots for the circadian fluctuations in enzyme activity.

FIGURE VII. Hydroxy-indole-0-methyltransferase activity as a function of the time of day in chicken pineal. The ordinates represent (^{14}C) N-acetyltransferase incorporated into (^{14}C) melatonin. Each point is the mean $\pm$ standard error of 4 pineals. The $\rightarrow$ (arrow) indicates that the treatment was changed at 10 days of age to the treatment immediately following the arrow.

P_X	Pinealectomized
$P_{X \rightarrow}$	Pinealectomized @ 10 days of age
0	Continuous darkness (i.e., 24 hours)
24	Continuous light (i.e., 24 hours)
12-12	Diurnal light-dark (i.e., 12 hours light - 12 hours dark)

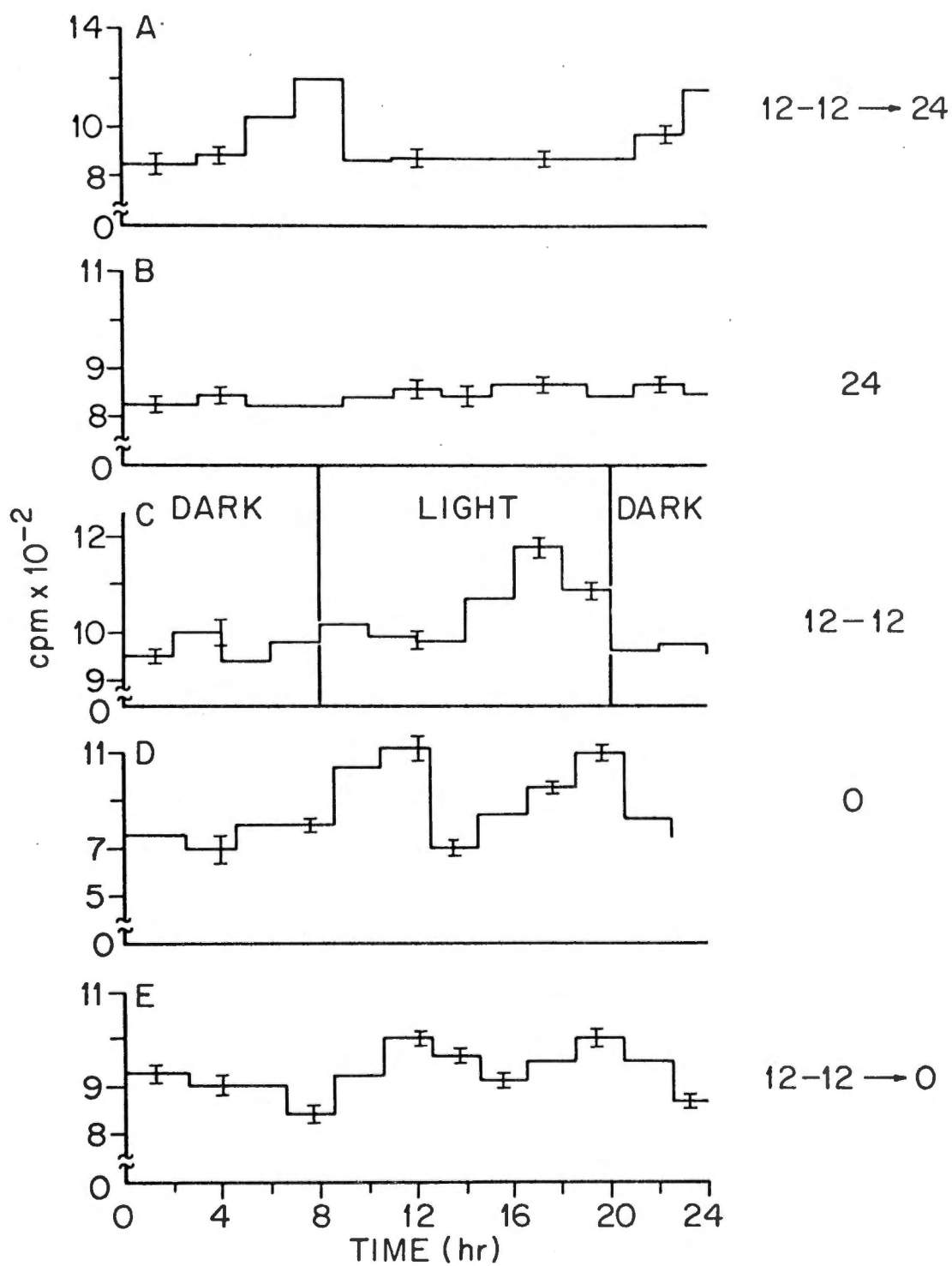


FIGURE VII

CHAPTER IV

DISCUSSION

As already cited, previous work has suggested that the pineal gland plays an active role in extraretinal photoreception, both in birds as well as mammals. Whether external light is required and/or some unknown internal condition causes the onset of circadian rhythms is still unknown.

As outlined on Page 3 of the Introduction, one of the questions to be answered by these investigations was, what effect does changing the lighting schedule have on body and glandular weights? As seen in Table I body weights remain constant except for those birds in continuous light. Table I also shows that there is no significant change in the weights of the thyroid, adrenal and pituitary glands. The eyes, on the other hand, show in all cases when the animals are maintained in constant darkness to be significantly larger than those birds in other treatments. The opposite is true for the testicular weights which show greater development and size in birds maintained in constant light.

Studies by Bonilla and Stringham²⁵ using Swiss albino mice, showed that the mice when kept on a light-dark cycle of 16 and 8 hours, respectively, demonstrated statistically no variation over a 24 hour period of the serum calcium levels. These results are different from those obtained in these experiments.

Our studies showed rhythmic variations (Fig. 1) in animals either started on or changed over to light-dark cycles and also those birds maintained in constant light. These differences may be in part due to

the type of animal system used, but this seems unlikely. The significant difference, I believe, may be in part due to the time intervals used in blood sampling (i.e., Bonilla's 4 samplings per 24 hours, as compared to our 12 samplings per 24 hours), also the number of animals studied (i.e., Bonilla, 30 mice versus 600 chickens).

The pineal 5-hydroxy- and 5-methoxyindoles of domestic fowl have been under investigation for many years. The daily rhythms of 5-hydroxytryptamine (serotonin) and 5-methoxytryptamine (melatonin) have been investigated.³⁰

According to our studies the circadian rhythm of serotonin content in the pineal gland is probably endogenous (controlled by an internal source) and does not seem to be directly affected by changes in external stimuli (i.e., light, dark), since it persists in the absence of environmental lighting. Studies of Snyder *et al.*²⁸ on the circadian changes of pineal serotonin in rats found that in constant light the changes in levels of serotonin were abolished and became free running. In contrast to this suppressive effect, rats maintained in constant darkness keep their rhythmic levels of serotonin.

Experiments of Ralph *et al.*²⁹ using three species of African weaver birds and the Japanese quail show melatonin to be present in greater amounts during the dark phase than during the light phase of a 24-hour cycle. In rats the synthesis of melatonin is reduced when the animals are exposed to constant light (Quay⁸). The quantities of pineal melatonin found by Quay fall within the ranges of those found in the chickens (20 ng or less per pineal).

It has been suggested that melatonin is either released or combined chemically almost as rapidly as it is formed (Quay⁸) thus accounting for

the small amounts found in both mammal and bird pineal glands. Also, the synthesis of melatonin is reduced in pigeons (*Columba livia*) when they are in the light cycle (Quay³⁰) and this effect seems to be mediated by the sympathetic nervous system (Wurtman *et al.*,³⁴).

Our experiments are in complete accord with those stated above. When the birds were maintained under continuous light, the pineal level of melatonin remained at a constant level. When a diurnal lighting regime was used, the melatonin concentration was highest in the dark phase and lowest in the light.

Axelrod and Wurtman³⁵ demonstrated that the activity of the enzyme hydroxy-indole-0-methyltransferase is higher in constant light than in darkness in chickens. In our experiments the enzyme maintains a rhythm which does not differ significantly whether the animals are maintained in constant dark or light. Observations by the same investigators³⁵ also indicate that hydroxy-indole-0-methyltransferase has maximum activity during the dark phase in a diurnal lighting schedule. The apparent paradox that under diurnal lighting HIOMT is higher in the dark yet in the constant conditions it is greater in the light, may be due to the fact that in constant lighting or dark conditions the animal maintains a constant level of the enzyme. Thus, depending on the time at which samples are taken, changes in the pineal hydroxy-indole-0-methyltransferase content may vary. Another possible reason for the discrepancy is simply that constant condition may not be related to those of diurnal lighting regimes. Hydroxy-indole-0-methyltransferase activity has been related to the rates of melatonin formation. However, in our hands there is no direct relationship between the enzyme activity and the melatonin content of the pineal.

Axelrod *et al.*⁶ reported that exposure to light increased hydroxy-indole-0-methyltransferase activity in chickens. This is completely out of phase with the results reported above (Table II, Fig. VI). If the pineal contents of melatonin and hydroxy-indole-0-methyltransferase are directly related, then in the birds we have investigated the hydroxy-indole-0-methyltransferase would be less active in the light than in darkness.

N-acetyltransferase activity in our set of experimental conditions shows little or no response to external lighting conditions under the diurnal conditions (Fig. V C). The enzyme reaches a maximum during the dark phase and a minimum in the light. Klein *et al.*¹³ also found in rats an increase in enzyme activity after the onset of darkness. The rhythm is maintained after the animals are placed in constant darkness whereas the exposure of the birds to constant light suppresses the rhythm and results in continually lower free running activity.

These characteristics indicate that N-acetyltransferase rhythms are like many other circadian rhythms, which can be altered by environmental lighting.

The mechanisms by which light and/or dark acts is not clear. One possibility is that light-induced electrical signals leaving the eye blocks an endogenous signal going to the pineal gland. Only in darkness would this anti-signal be reduced thus allowing this endogenous signal to reach the pineal and stimulate N-acetyltransferase.¹³ N-acetyltransferase rhythm is of physiological importance to the pineal. It appears that this enzyme plays a major role in the regulation of the serotonin → melatonin pathway. By our experiment it seems likely the circadian rhythm of serotonin is controlled by the rhythm of N-acetyltransferase.

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VITA

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