THE EOSINOPHIL CYCLES OF THE GENUS RANA

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Master of Science

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by

Harvey Weisman

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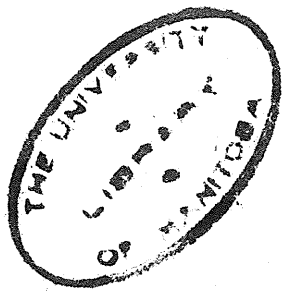

ACKNOWLEDGMENTS

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ABSTRACT

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It was the purpose of this study (1) to establish the eosinophil cycles in the circulating blood in the common leopard frog, Rana pipiens; (2) to trace the effects of laboratory controlled conditions such as light, age, diet, temperature, and humidity upon such cycles; and (3) to compare eosinophil activity in the amphibia with those of higher animals.

The presence of an endogenous eosinophil cycle has been established in the circulating blood in the common leopard frog, Rana pipiens. Light, age, diet, temperature, and humidity under controlled laboratory conditions play a significant role in the twenty-four hour cycle in which the phase and rhythm are measurably altered. The eosinophil periodicities in the frog are noted to compare in many respects with those of higher animals.

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CHAPTER I

THE PROBLEM AND DEFINITIONS OF TERMS USED

For many years a difference of opinion has existed regarding the phylogeny and function of eosinophil activity throughout the vertebrates. Eosinophilia has been established as symptomatic of nematode parasitism, however, in cases of health, variations in day to day eosinophil counts were an inexplicable phenomena.

I. THE PROBLEM

Statement of the problem. It was the purpose of this study (1) to establish the eosinophil cycles in the circulating blood in the common leopard frog, Rana pipiens; (2) to trace the effects of laboratory controlled conditions such as light, age, diet, temperature, and humidity upon such cycles; and (3) to compare eosinophil activity in the amphibia with those of higher animals.

Importance of the study. The function of the eosinophil in the higher animals has for many years been a controversial subject. The complex physical chemical system in such an animal body has added to the limitations to the scope of relevant experimentations. The affirmation of eosinophil activity in a less complex animal would facilitate future investigations in the solution of this problem. The presence of daily fluctuations between oral temperatures

and eosinophil levels in the higher animals seemed to indicate a direct causal interrelation. The presence of an eosinophil cycle in a poikilothermal animal would therefore indicate that the cycle is independent of changes in body temperature.

II. DEFINITIONS OF TERMS USED

Periodicity. Periodicity was interpreted as all repetitive changes occurring regularly regardless of the intrinsic mechanism involved in the periodic changes or the time period involved.

Cycle. Cycle is used to denote the entire specific change recurring within the organism in a correlated manner. It was employed in accordance with recognized terms, such as menstrual cycle and cardiac cycle.

Rhythm. The measurable manifestations that gave rise to a cycle in a repetitive quantitative change was referred to as rhythm.

Phase. Specified parts of a rhythm are referred to as a phase.

The demarcation in time of day was designated as 12:00 noon and 24:00 midnight with other hours in the day conforming to this type of nomenclature.

CHAPTER II

REVIEW OF THE LITERATURE

During the last fifty years there has been a difference of opinion in regard to the eosinophil count in various species of Rana. Friedsohn (1910) reported the variation in Rana esculenta to be 285 to 969 per cubic millimeter. Alder and Huber (1923) using the same species listed a variation of 250 to 950 per cubic millimeter while Klieneberger (1927) showed the variation as 295 to 1300 per cubic millimeter in the same species. Wintrobe (1933) reported an eosinophil count of 210 per cubic millimeter with no reference as to variation or species of Rana employed in the experiment.

Jordan (1919) first noted the migration of circulating eosinophils from the blood medium as a result of an environmental factor. He reported a larger eosinophil count in the fall with a corresponding decrease in the winter. Although the phagocytic movement of the eosinophil was well established, Jordan (1925) further designated a function to the cell's cytoplasmic inclusions. Jordan said:

> " The term segregation apparatus is here employed to designate a cytoplasmic collection of granules and globules combined in any numerical proportion.
> . . . . . .
>
> The volume of the segregation apparatus parallels in general the degree of ameboid activity."

A comparative study of eosinophil migration was

further experimented by Jordan in several two year old fish. He reported on the blood changes during estivation. The eosinophils were observed to undergo degeneration, while the granules became progressively less distinct and eventually took up no stain. The same changes were reported to occur in the cell's nucleus. On returning the animals to water, a normal blood picture returned within a few days.

Further experimentation on the migration of circulating eosinophils in dogs was reported by Halberg, Bobb, and Visscher (1950) in thirty-five dewormed dogs which were placed on an eighty-four hour fast. Their results showed a considerable eosinopenia of -51% of the starting value.

Halberg et al. (1951) studied the levels of circulating eosinophils in the blood of castrate mice as a possible indicator for the functional activity of the hyperplastic and metaplastic adrenal cortex. He reported a pronounced diurnal rhythm in circulating eosinophils of normal mice in that the minimum level occurred in the midnight hours and the maximum at mid-morning. Bander (1950) showed that the mouse is a nocturnal animal with a maximal activity period at about midnight. In his attempt to correlate the levels of circulating eosinophils and hormonal action, Halberg said:

> " . . . since there are wide fluctuations, from 21 to 168 per cubic millimeter in mean eosinophil counts as between midnight and morning in one strain of mice, it is at once obvious that there are large swings in the output or removal of these cells and correspond-

> ingly important shifts in the controlling factors. If these controlling factors are the adrenal cortical hormones, studies of the diurnal rhythm in eosinophil levels might yield important information about the output of such hormones."

Domarus (1931), Djavid (1935), and Appel (1939) reported significant declines in the numbers of circulating eosinophils in man between the hours 07:00 and 10:00, but failed to note the presence of a cycle. Rud (1947) gave the first account of an eosinophil cycle over a twenty-four hour period in man. Halberg et al. (1951) established statistically the presence of a circulating eosinophil cycle in man in which the lowest level was found to be at 09:30.

Halberg (1951) studied the eosinophil counts in patients with Addison's disease, bilateral adrenalectomy, and hypopituitarism and noted the absence of the characteristic 06:30 to 09:30 drop of these cells. The subcutaneous implantation of various 11- oxycorticoids by Halberg (1952) produced a qualitative change of maximal eosinopenia within ten hours. However, this factor was not utilized as a measure of corticoid activity since eosinopenia was noted to occur in the absence of exogenous corticoids.

Age was noted as a variating factor in the eosinophil cycle of human infants. Halberg and Ulstrom (1952) reported the absence of endogenous eosinopenia in the 06:30 to 09:30

period in the age group of from one day to seven months. This group was recorded by Jundell (1904) and Gofferje (1908) as exhibiting only slight deviations in the daily temperature curve as contrasted with the straight line during the neonatal period. Endogenous eosinopenia was found to be present in the fifteen month age group, a period in which many of the twenty-four hour physiological periodicities are featured.

The observation of eosinopenia as a result of pregnancy in the human to the first day post partum was made by Davis and Hulit (1949). This was established in several stocks of mice by Halberg and Bock (1953) and persisted for a period of forty-eight hours after delivery of the young. However the degree of variation observed in the mice were not sufficient to establish a statistical difference.

The critical role of light as an environmental factor was further studied in mice by Halberg, Visscher, and Bittner (1953) with constant light, continuous darkness and reverse lighting conditions. It was found that a reversal of the light cycle under controlled conditions showed a slight change at four and five days. The eosinophil cycle was reported to be completely reversed at the end of nine days. Mice kept in constant light with food available only during the day and those with food only during the night failed "to exhibit significant differences in mean number of eosinophils at certain definite periods of the day." [Halberg]. These

same mice kept in continual darkness for nine days failed to show any significant changes in eosinophil levels. However, mice kept in continuous darkness for thirteen days and previously subjected to twelve hour darkness alternating with twelve hour light maintained an eosinophil level of considerable amplitude.

It was found that a systematic selection of diet had a greater significance on the periodicities of eosinophil levels. The eosinophil cycle was reversed in mice restricted to a diet of carbohydrate and fat in accordance to the time of feeding despite the continuance of unchanged lighting conditions. Thus, mice fed on a calorie restricted diet in the morning displayed a lower count during the day. The contrast was found to be true.

The effective results of adrenalectomy on mice was limited by the survival rate. A total of 12% of those mice adrenalectomized were alive six weeks after the operation. However, it was suffice to observe that the amplitude of the cycle had been diminished or completely obliterated.

The persistence of an eosinophil rhythm after splenectomy in rats was observed by Halberg, Albrecht, and Lander (1954). It was noted that eosinophilia in the splenectomized rats occurred for a period of two months following the operation. Four months after splenectomy eosinophilia was no longer present and the rats exhibited a

twenty-four hour eosinophil cycle. The splenectomized mice were fed ad libitum and kept in twelve hour light alternating with twelve hour darkness. It was therefore concluded that the spleen was not essential for the maintenance of an eosinophil cycle.

Jordan and Speidel (1925) reported the effects of splenectomy on frogs. At the end of sixty days following splenectomy, the frogs died of marked anemia. The number of erythrocytes noted at the time of death were one half the number prior to the operation. Jordan estimated the average age of the mature erythrocytes which showed a dark hemoglobin color prior to splenectomy as thirty days of age. There were relatively small numbers of erythrocytes produced in the bone marrow after splenectomy. Jordan and Speidel (1925) concluded that the only red blood cells present in the splenectomized frogs at the end of sixty days were mainly those produced just prior to the operation. It was therefore concluded that the spleen among other functions in the adult frog was as a main centre for erythropoiesis. Eosinophilia was not reported to have occurred in frogs after splenectomy.

CHAPTER III

MATERIALS AND METHOD OF PROCEDURE

Source of animals. The species of animal selected for this study was the common leopard frog, Rana pipiens, Schreber. The great majority of frogs used in this study were collected in the vicinity of Netley marsh, which is situated forty miles north of Winnipeg. The animals collected were immediately placed in wet sacks and transferred to glass battery jars upon return to the laboratory. This method did not permit a period of partial desiccation of the frogs. Of the three hundred animals employed in this study approximately forty-eight were received from a biological supply house in Wisconsin. Most of the frogs were collected in the spring and early summer.

Age determinations. The animals were grouped according to approximate age group. Body length, tip of head to end of rump, was the method employed for age determinations. Animals which had a body length not exceeding two centimeters were grouped as being one month of age from time of metamorphosis; four centimeters in body length as one year of age; six centimeters as two years of age; greater than seven centimeters as three or four years of age.

Light and temperature control compartment. The frogs

were kept in glass battery jars which measured five by seven inches at the base and twelve inches in height. One-quarter inch wire mesh was fastened at the top of the glass container. The jars were then transferred to a wooden compartment measuring three by eight feet at the base and four feet in height. It was equipped with six fluorescent bulbs of two feet in length. The compartment was divided into two chambers by two quarter-inch plate glass sheets which were fitted three inches from the light bulbs in order to prevent excessive heat entering the lower chamber. Entry into the larger chamber was gained by two doors, one of which was fitted with ventilating holes covered by six light baffles. A thermostat was installed to maintain constant temperature. An automatic light switch operated independently of the thermostat. Intensity of illumination striking the frogs was measured as one hundred Weston Meter units.

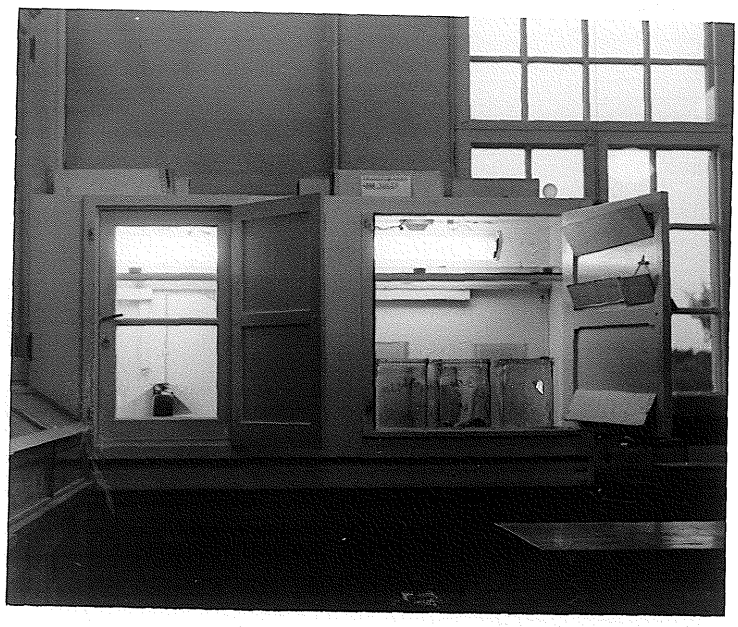


FIGURE 1

TEMPERATURE AND LIGHT CONTROL COMPARTMENT

Daily care of frogs. It was noted that in order to maintain a state of health in the frogs it was necessary to change daily the one-half inch of water in the jars. The temperature of the lower chamber was kept at 75.6° F. while the light cycle was established as twelve hours of light commencing at 06:00 followed by a twelve hour period of total darkness at 18:00. Those frogs which developed signs of infection, such as red-leg, were discarded and the containers cleaned with a detergent. A maintenance diet of

strained baby foods consisting of vegetables, beef, and liver, were fed orally twice a week by means of a hypodermic syringe with the needle removed. Amount fed was determined by the size of the oral cavity.

Blood extraction. Attempts to withdraw blood from the ventral abdominal vein by means of a hypodermic syringe were unsuccessful. The linea alba was used as a guide point for the needle. However, the venous blood extracted became diluted from the contents of the subcutaneous lymph spaces. The larger vessels in the web and those along the terminal digits did not produce appreciable amounts.

As a result blood samples from all frogs were obtained directly from the heart ventricle by two methods. The frogs which had an age of less than three years, were incised along the ventral body wall. The pericardium was removed and the apex of the ventricle elevated by means of an entomological pin. A small transverse incision into the ventricle was made and the pipette inserted. The total handling time of the frog was less than three minutes.

FIGURE 2

INCISED VENTRICLE WITH INSERTED PIPETTE

The second method employed was confined to the frogs of three and four years of age. A hypodermic syringe fitted with a #25 gauge needle was inserted obliquely behind the xiphoid cartilage and one-quarter to one-half cubic millimeter of blood was slowly drawn from the ventricle. The ventral body wall was swabbed with 95% alcohol, while the syringe and needle were sterilized with a 2% solution of Di-sol. These animals were not used again for a period of two weeks. The frogs were not anaesthetized during both methods of blood extraction. The animals were held immobile by two clamps fitted with soft rubber ends.

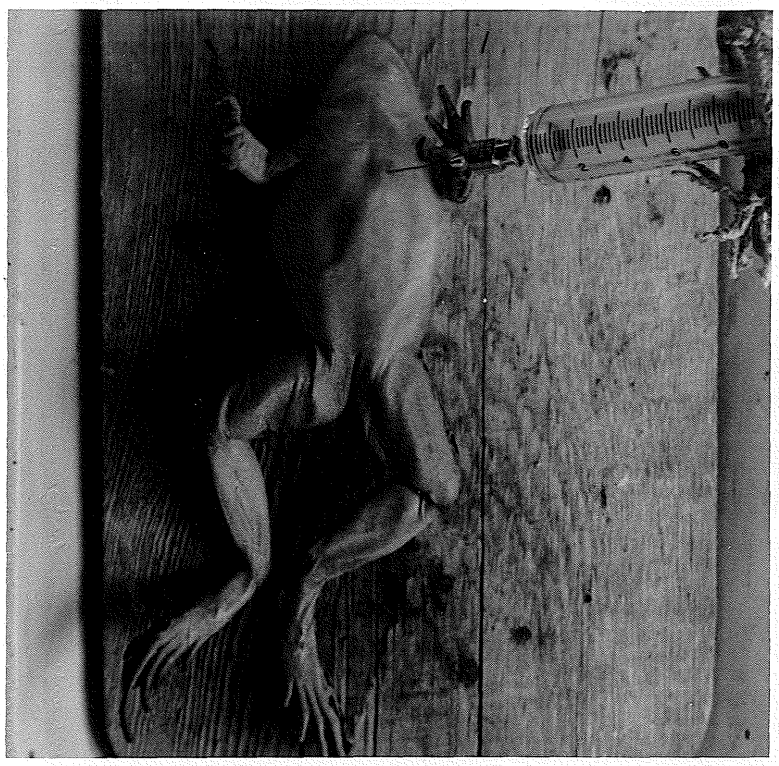


FIGURE 3

POSITION OF HYPODERMIC SYRINGE

FIGURE 4

MEDIAN SAGITTAL VIEW SHOWING BLOOD EXTRACTION FROM VENTRICLE

In cases where this method failed in the first attempt the former method was immediately employed since the pericardium had been punctured with possible damage to the ventricular wall.

The visceral organs of the animals were examined for parasites at a later date.

Enumeration of eosinophils. The diluting fluid employed was specific for eosinophils. The diluent consisted of 1 c.c. of a 1% eosin solution, 2.2 c.c. of acetone and

6.8 c.c. of distilled water. The method employed conformed to the technique by Halberg et al. (1951):

> " Certified leukocyte pipettes were used. The blood was drawn to the 0.5 mark, the tip of the pipette wiped clean with a piece of gauze and diluting fluid was immediately drawn to the 11 mark. The pipette was rotated during the aspiration of the fluid and then vigorously shaken for about twenty-five seconds during which the fluid in the bulb was inspected for gross particulate matter.
> . . . . . .
>
> It is imperative to discard samples with thread-like or dotlike impurities.
> . . . . . .
>
> Upon completion of the mixing, the first three drops of diluted blood were discarded and the standard Levy Counting Chamber with Fuchs-Rosenthal Double Ruling was immediately filled using the fourth and fifth drops of blood for the two chambers of the slide."

Three criteria were used in distinguishing the eosinophils from other cells and extraneous matter: the granules are spheroidal and resemble rings due to a rarefied central portion, the presence of pseudopodia, the absence of a cytocentrum-like structure found in neutrophils. It was found that the diluent did not act as a complete lytic agent on nucleated erythrocytes, however, they remained visible as ghost-forms. New diluent was prepared every four hours due to the volatility of acetone.

Due to the impossibility of serial dependent blood sampling in these experiments, it was necessary to employ a serial independent sampling. This method entailed a greater number of animals and the method of enumerating eosinophil levels

are in accordance to the statistical method employed by the Division of Biostatistics, United States Public Health Service, Minneapolis.

Mean relative eosinophil levels in serial independent sampling were calculated in the following manner: all values obtained from a given number of subjects at a definite time period are totalled and their mean calculated. The mean values of successive samplings, which in these experiments were every four hours, were totalled and their mean calculated. The individual mean value received at a definite time period is expressed as a per cent of the twenty-four hour mean.

The second method was to express each individual sampling as the number of eosinophils per cubic millimeter and their mean value at a definite time period recorded. The latter method was employed in view of the variations recorded by previous investigations on the Genus Rana.

Experiments on frogs one month old. Blood samplings were taken from six different frogs every four hours with the starting time at 12:00. The frogs were kept in the control compartment for seven days prior to the experiment. The lights were on for a twelve hour period commencing at 06:00 and off for an equal time starting at 18:00. The temperature was recorded at $80^{\circ} \pm 2^{\circ}$ F.

Another group was kept in the control compartment with no water in the glass battery jar. Sampling was taken from three frogs every four hours commencing at 12:00 for a period of twenty hours. This desiccation experiment was discontinued at 08:00.

Experiments on one year old frogs. Blood samples were taken from six different frogs every four hours commencing at 12:00. The light cycle corresponded to an alternating twelve hour period as in the above experiment. The diet consisted of strained vegetables, beef and liver. The temperature was recorded at a constant 75.6° F.

Experiments on two year old frogs. The alternating light cycle was the same as in the previous experiment. Frogs were fed a maintenance diet of strained foods. The temperature recorded was a constant 75.6° F. Blood samples were taken every four hours.

A second group was kept under the same laboratory conditions as above. The diet consisted of a daily dosage of 1 to 1½ c.c. of a 20% casein solution for seven days prior to the experiment.

A third group was fed 1 to 1½ c.c. of a 20% solution of Analar dextrose for seven days prior to sampling. The controlled conditions remained constant in accordance to

the previous experiment.

Experiments on three and four year old frogs. Three experiments were conducted in accordance to specific diets: strained foods, 20% casein, and 20% dextrose. The controlled laboratory conditions remained constant as in previous experiments. The daily amount fed to these frogs varied from two to three cubic centimeters for a period of seven days.

The light cycle was reversed for a period of ten days on frogs previously kept in an alternating light cycle. The lights in this experiment were on for twelve hours commencing at 18:00 and off for the same time period starting at 06:00. The animals were fed a maintenance diet twice a week.

The light cycle was discontinued on two groups of frogs. One group was kept in continuous light for a period of ten days while the other group was kept in constant darkness for the same period. The temperature remained constant at 75.6° F. in both experiments. The frogs were fed the maintenance diet.

A further experiment was conducted in two temperature extremes. Three frogs from each age group were placed at a temperature of 42° F. for forty-eight hours at constant darkness. A similar number from each age group was kept in 110° F. for a period of ten hours at constant light.

FIGURE 5

MEAN RELATIVE EOSINOPHIL LEVEL IN ONE YEAR OLD FROGS
ON MAINTENANCE DIET IN NORMAL LIGHT CYCLE

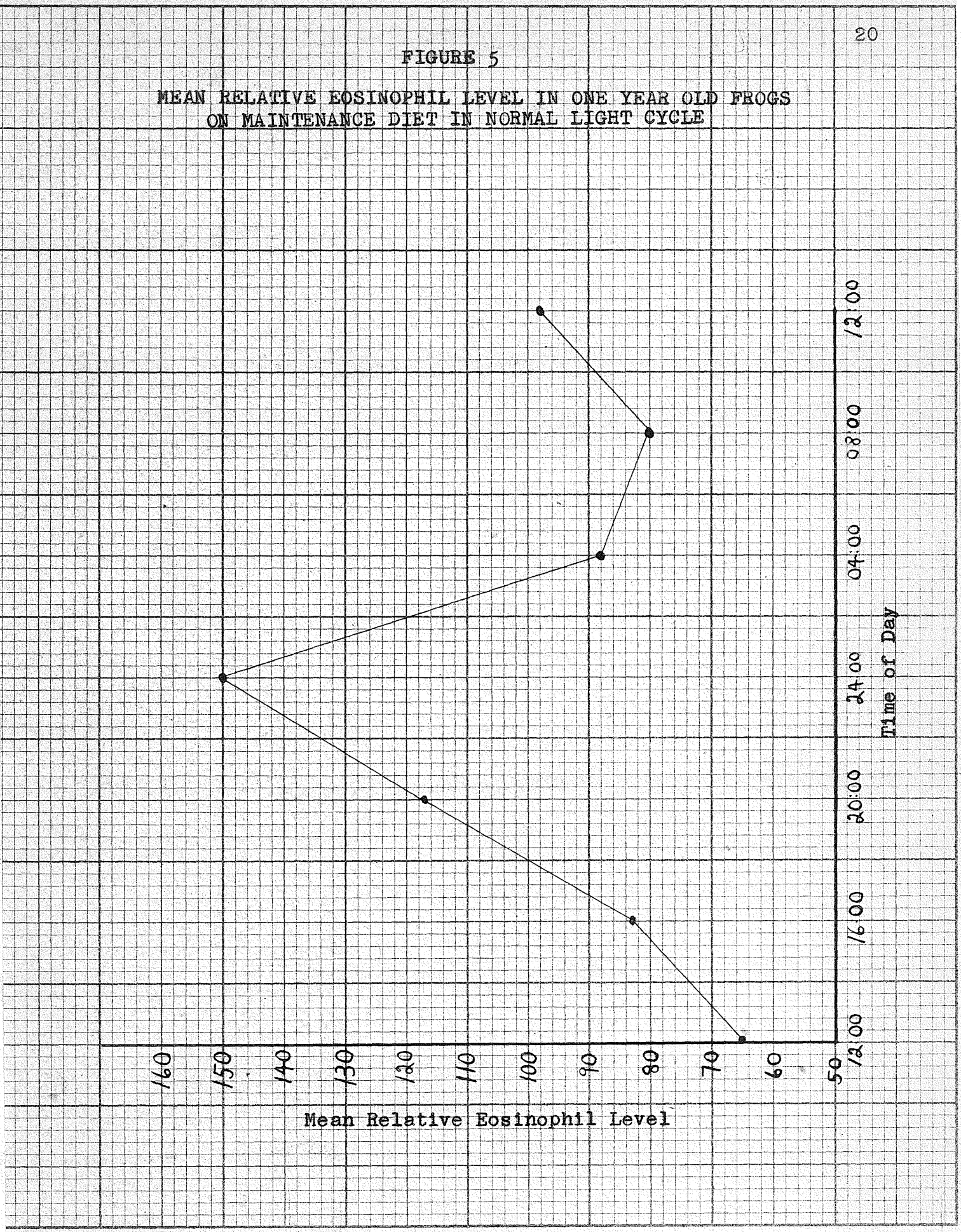


FIGURE 6

MEAN RELATIVE EOSINOPHIL LEVEL IN TWO YEAR OLD FROGS
ON MAINTENANCE DIET IN
NORMAL LIGHT CYCLE

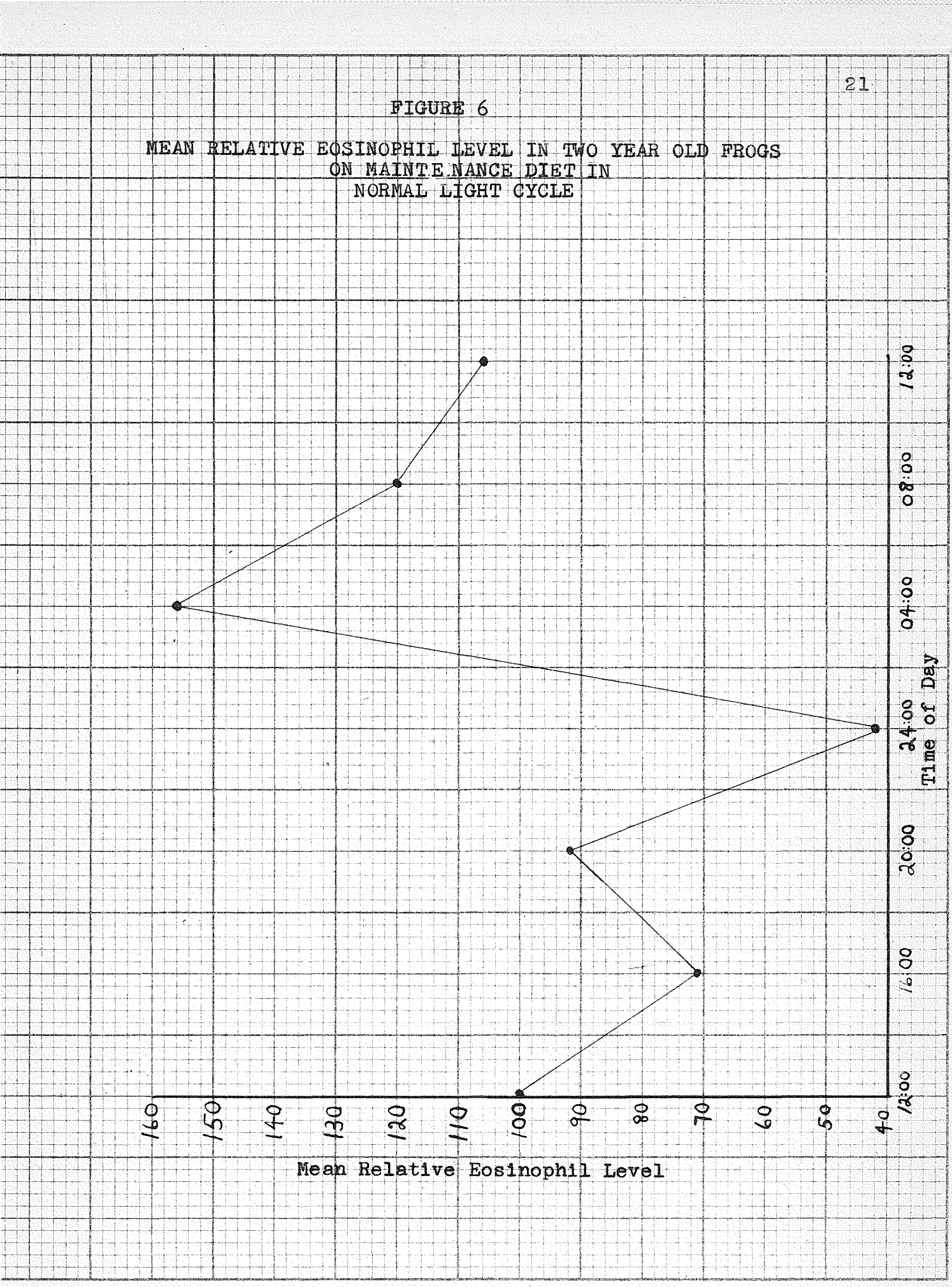


FIGURE 7

MEAN RELATIVE EOSINOPHIL LEVEL IN TWO YEAR OLD FROGS
ON CASEIN DIET IN
NORMAL LIGHT CYCLE

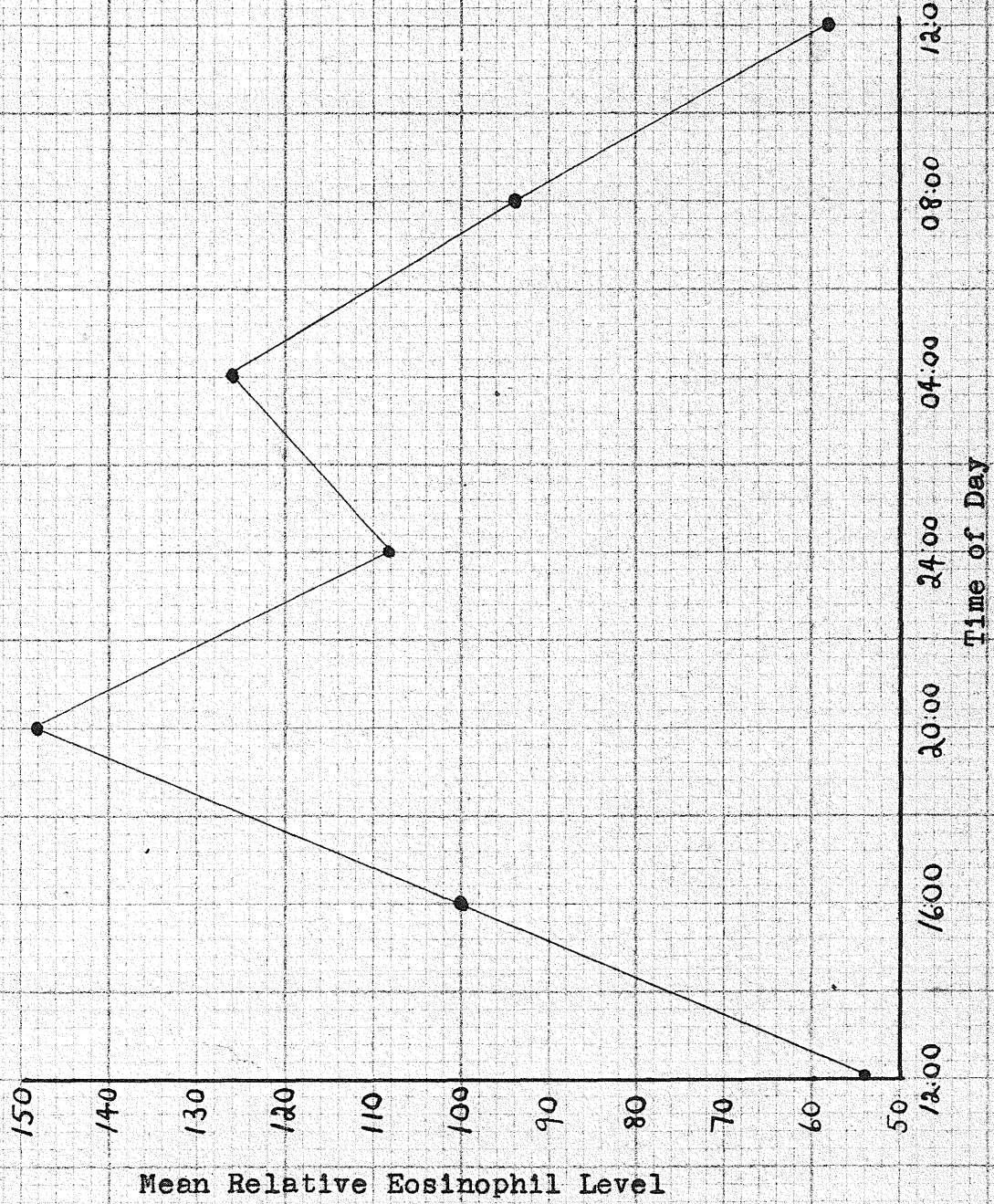


FIGURE 8

MEAN RELATIVE EOSINOPHIL LEVEL IN TWO YEAR OLD FROGS
ON DEXTROSE DIET IN
NORMAL LIGHT CYCLE

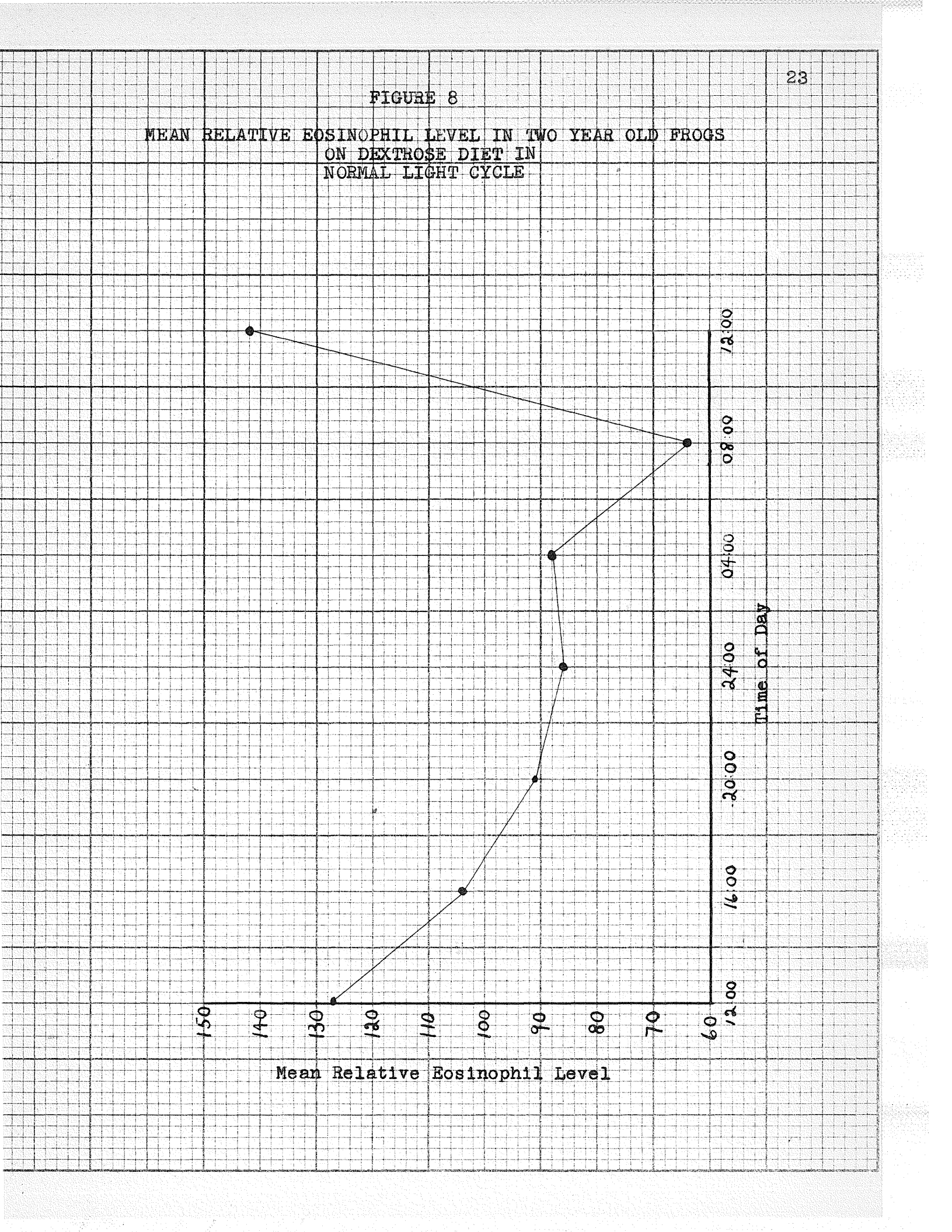


FIGURE 9

MEAN RELATIVE EOSINOPHIL LEVEL IN THREE--FOUR YEAR OLD FROGS ON MAINTENANCE DIET IN NORMAL LIGHT CYCLE

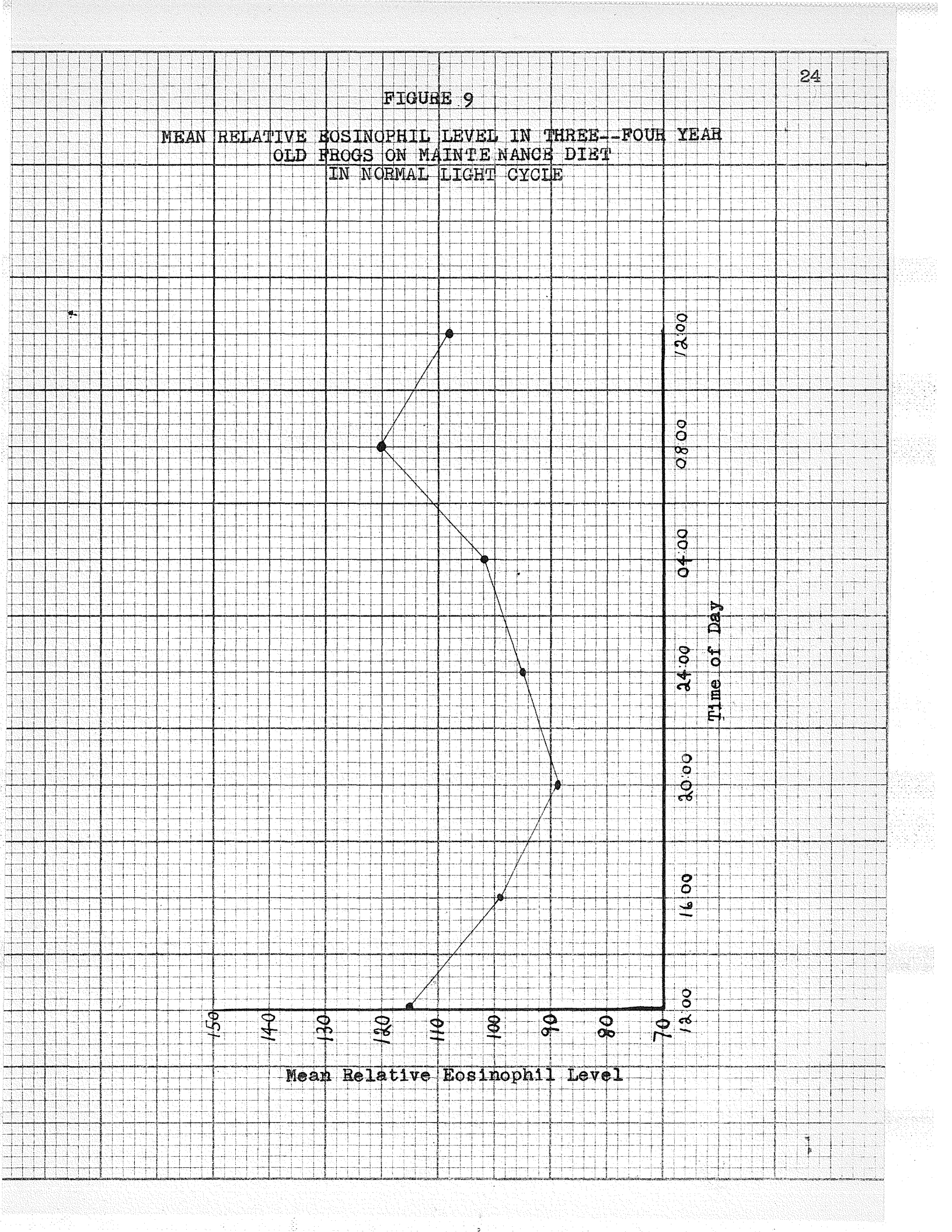


FIGURE 11

MEAN RELATIVE EOSINOPHIL LEVEL IN THREE--FOUR YEAR OLD FROGS ON DEXTROSE DIET IN NORMAL LIGHT CYCLE

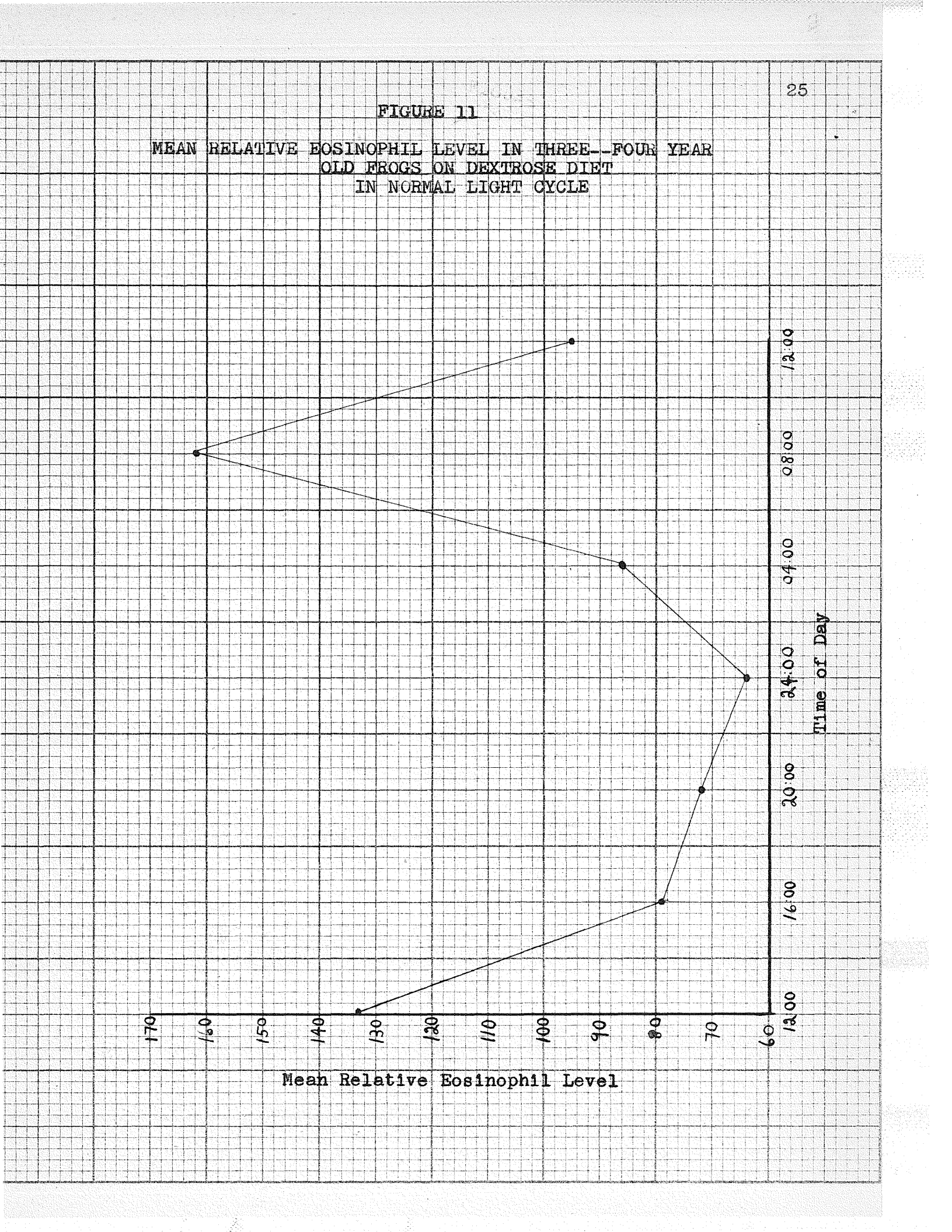


FIGURE 10

MEAN RELATIVE EOSINOPHIL LEVEL IN THREE--FOUR YEAR OLD FROGS ON CASEIN DIET IN NORMAL LIGHT CYCLE

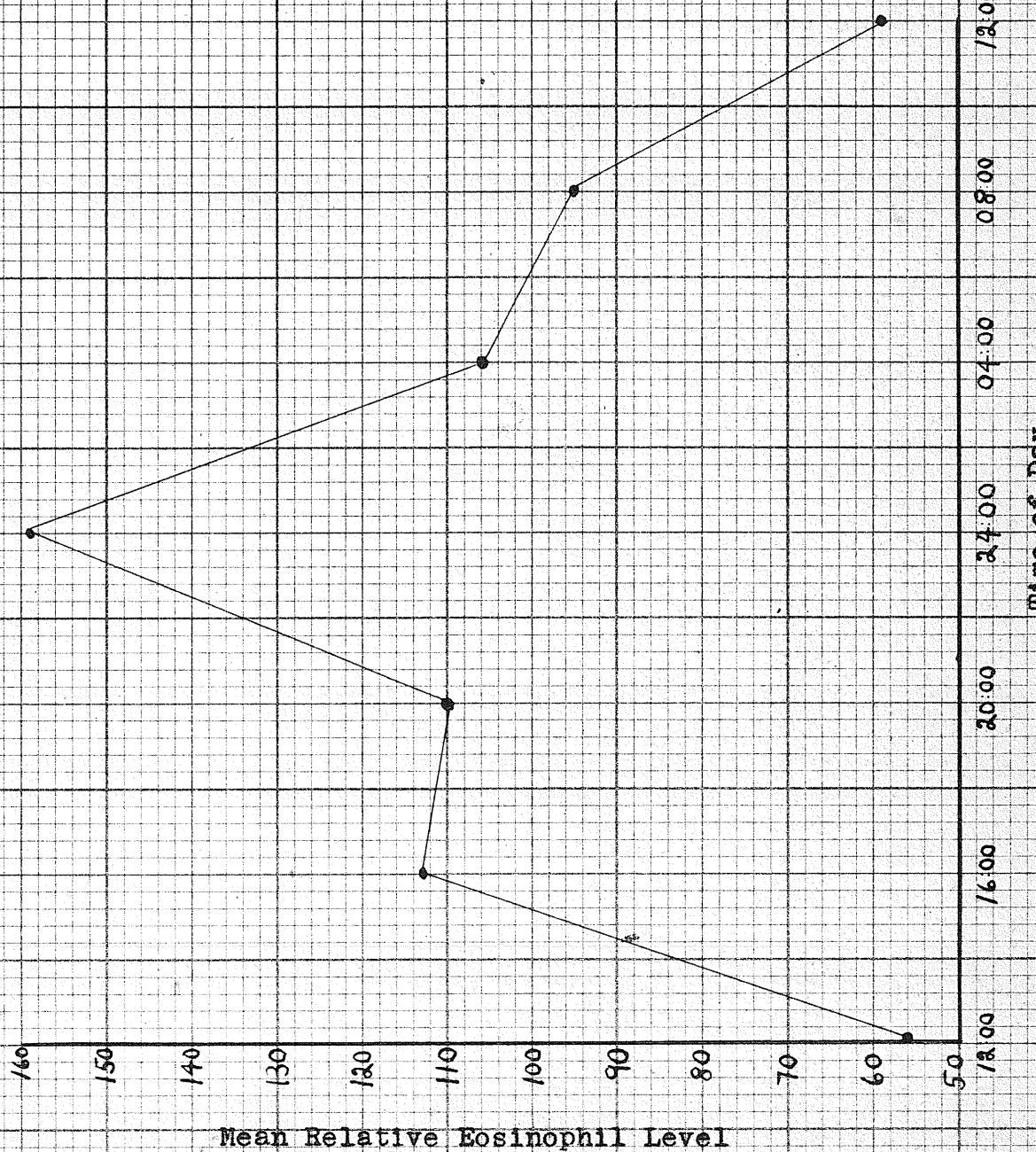


FIGURE 12

MEAN RELATIVE EOSINOPHIL LEVEL IN THREE--FOUR YEAR OLD FROGS KEPT IN REVERSED LIGHT CYCLE

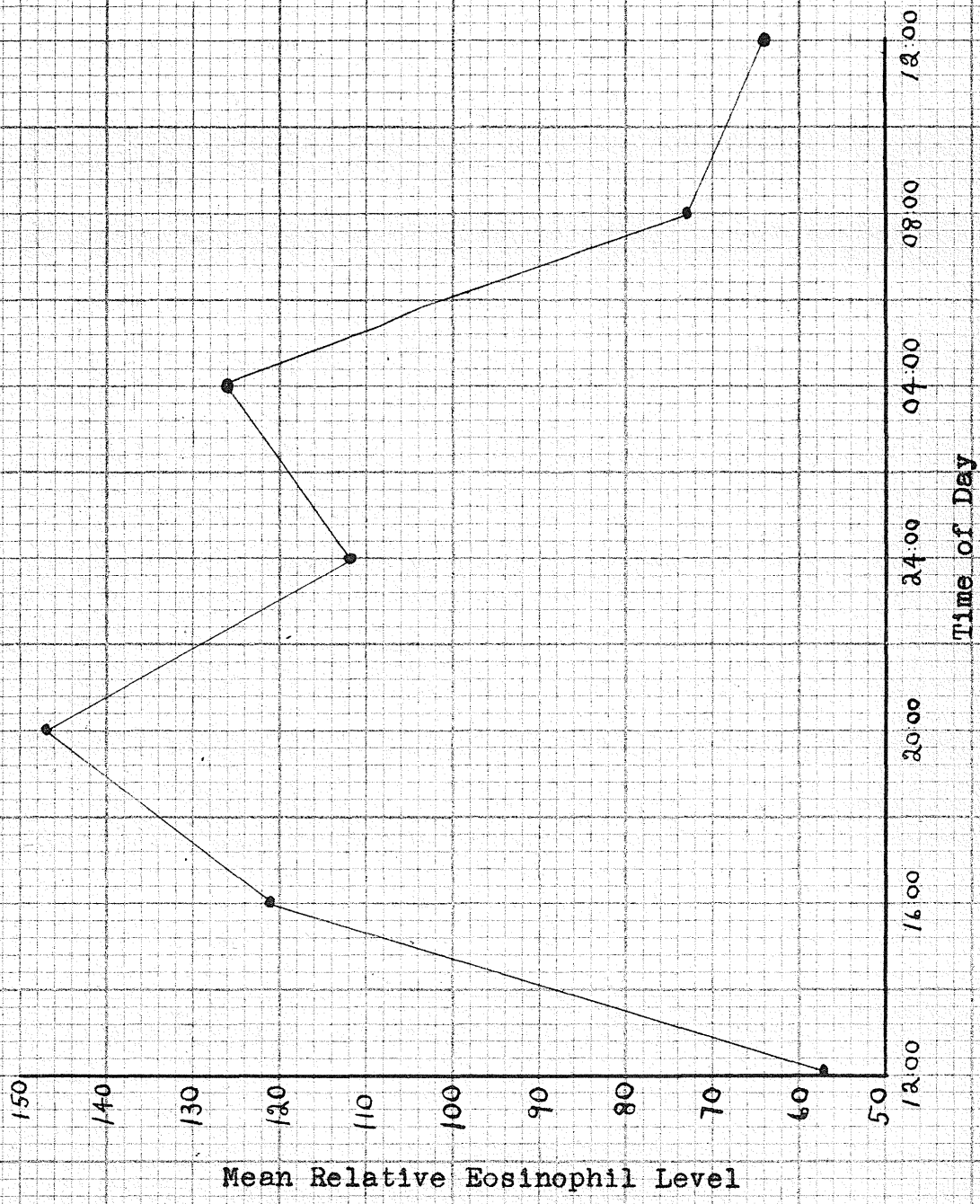


FIGURE 13

MEAN RELATIVE EOSINOPHIL LEVEL IN THREE--FOUR YEAR OLD FROGS KEPT IN CONTINUOUS LIGHT

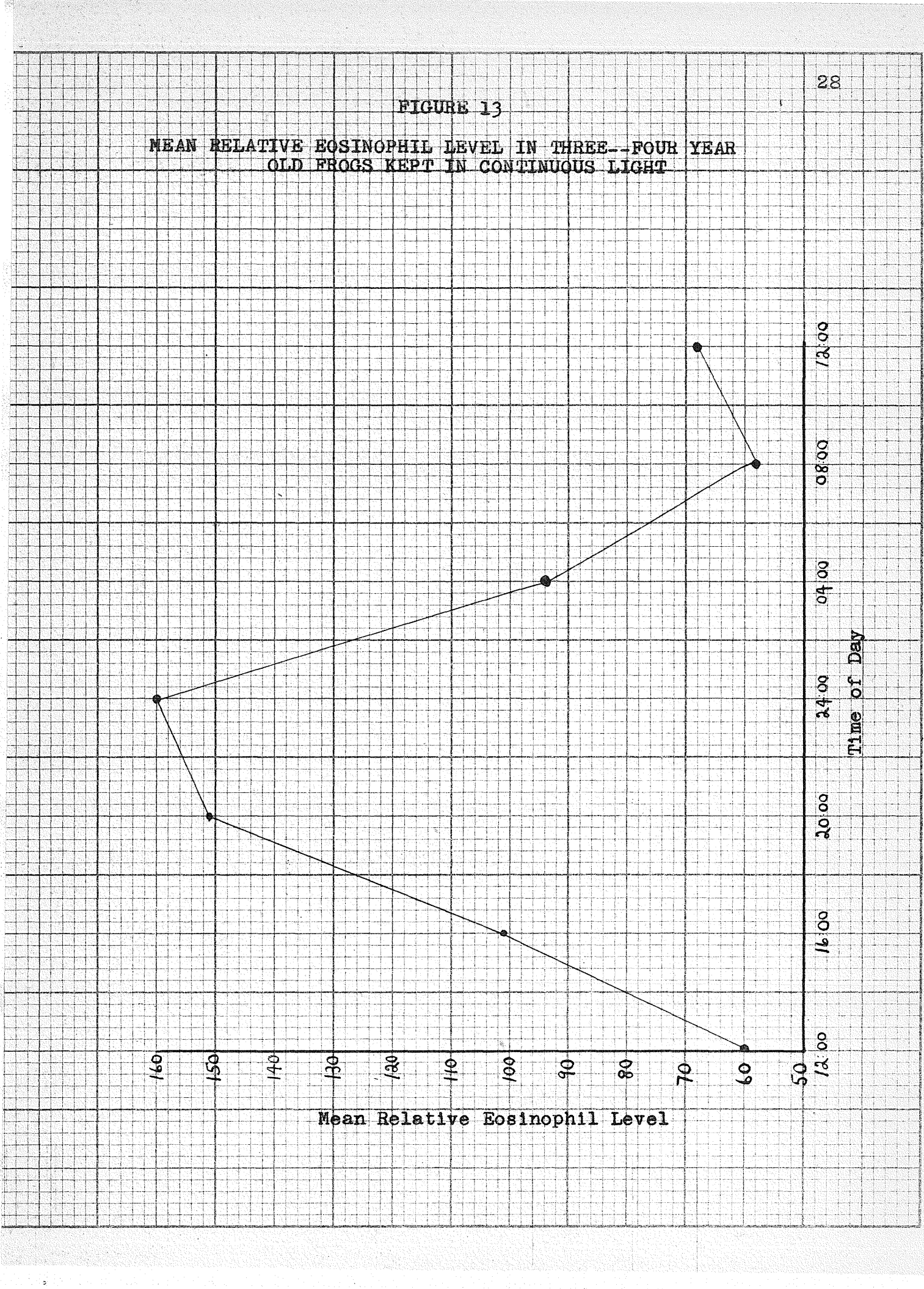
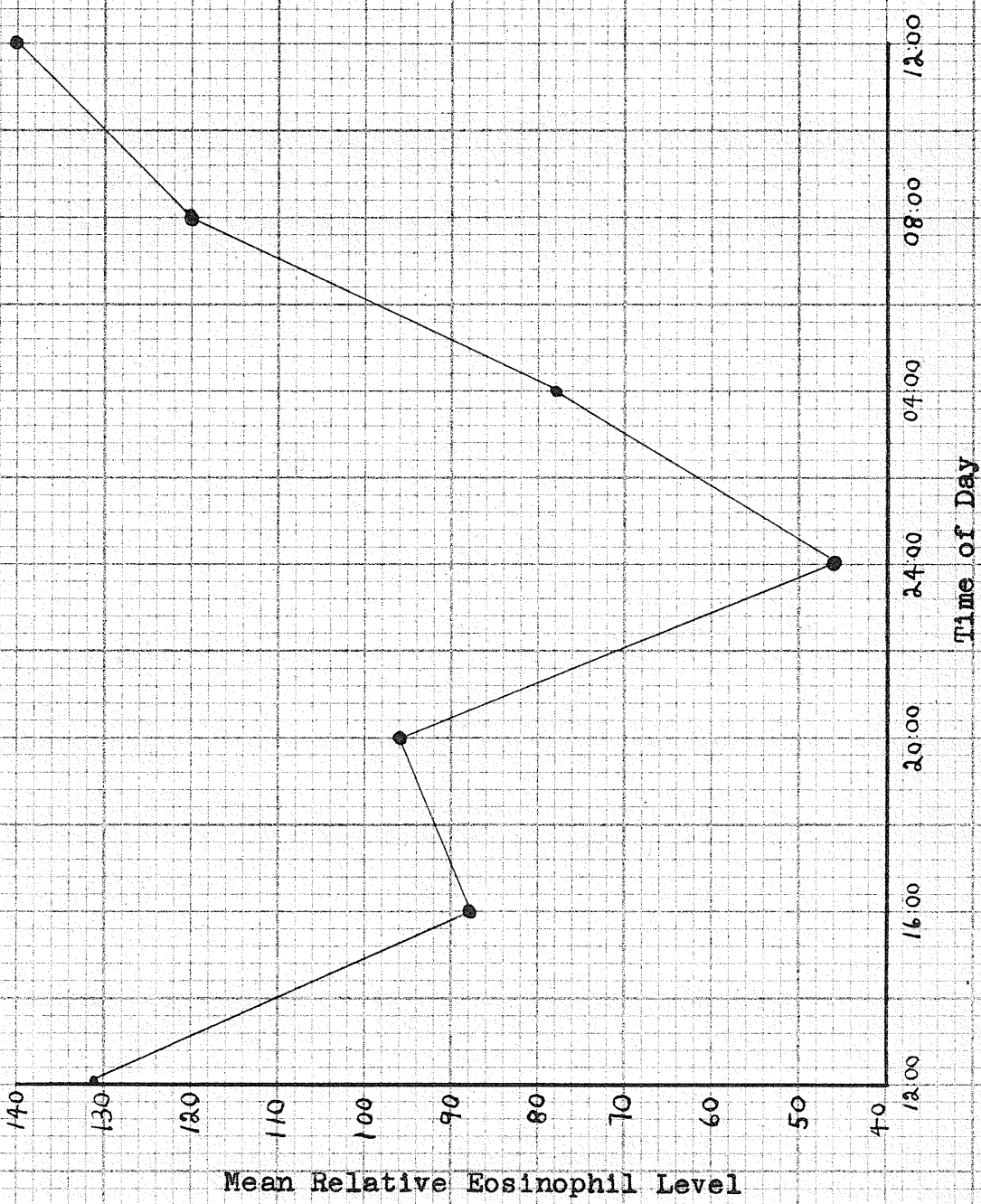


FIGURE 14

MEAN RELATIVE EOSINOPHIL LEVEL IN THREE--FOUR YEAR OLD FROGS KEPT IN CONTINUOUS DARKNESS



CHAPTER IV

RESULTS OF THE INDIVIDUAL EXPERIMENTS

Mean relative eosinophil levels and counts are presented in figures 1 to 10 while table 1 presents the numbers of circulating eosinophils per cubic millimeter in the various age groups under controlled laboratory conditions. The information here presented serves to show the great variability encountered in the periods chosen for blood sampling.

In experiment 1, it was noted that the numbers of circulating eosinophils in one month old frogs did not present a cyclic phenomenon. The variations within the samplings taken every four hours ranged from 576 to 124 eosinophils per cubic millimeter. Individual samplings taken during the day varied within each group to the same magnitude as the night samplings.

Experiment 2, using one month old frogs under conditions of desiccation, showed a general trend towards the migration of eosinophils from the blood. Samplings taken at 12:00 revealed these cells to be present in diminishing numbers. The initial count was recorded as 93 per cubic millimeter while the final count was noted at 24 per cubic millimeter. The experiment was discontinued after twenty hours since the blood sample in the pipette congealed to form

a gross particulate matter. The subcutaneous lymph spaces were void of fluid. The highest rate of mortality was noted during this condition. The eight frogs which survived at the end of twenty hours were returned to the water and their eosinophil count was noted to be normal after twenty-four hours.

In experiment 3, the one year old frogs displayed an eosinophil cycle. However, it will be noted in figure 5, that these animals exhibited a cycle similar to the seven month old period of the human infant. The periodicity of circulating eosinophils displayed a fourteen hour cycle in which the amplitude of the highest value exceeded the lowest point by one hundred per cent. It must be noted, however, that the variation in the starting value to the final value indicated the cycle may be of shorter duration with a diminished amplitude.

Figure 6 of experiment 4 showed that a cycle is present in two year old frogs in an inverse proportion to the general activity of the animal. A night low of circulating eosinophils was recorded to be coincidental with the peak of nocturnal activity. The diet consisted of strained food.

The results of a highly select diet were given in figure 7 of experiment 5. It was noted that a 20% casein solution administered daily to frogs of two years of age

resulted in an inverted eosinophil cycle after seven days. A night high was recorded together with a day low although the activity of the animal and the alternating light cycle remained unchanged. The numbers of circulating eosinophils was noted to rise prior to the lights being shut off at 18:00 hours.

The data obtained in experiment 6 revealed the amplitude of the eosinophil cycle was greatly diminished in two year old frogs as a result of feeding of a 20% dextrose solution. As seen in figure 8 a night low was present, however, the low point was reached after the lights had been on for two hours. A comparison of figure 6 and figure 8 exhibited a variation due to a restriction of diet. The heart of these animals was observed to be greatly enlarged in comparison to other frogs of similar chronological age. No effort was made to serial section the heart, however, upon gross examination the ventricular wall appeared thinner.

The effect of age on the amplitude of the cycle was revealed in figure 9 of experiment 7. The three year old frogs were fed the same type of strained foods as those given in figure 6. The amplitude of the cycle had been diminished while a night low was recorded at 20:00 hours.

The effect of 20% casein solution was further evidenced in figure 10 of experiment 8. The cycle was completely reversed in the three year old frogs with a night

high recorded at 24:00 hours. The frogs employed in this experiment were previously fed the maintenance diet for a period of two weeks prior to the administration of the 20% casein solution. The animals in this experiment were not utilized in previous blood samplings.

The result of feeding a 20% solution of dextrose on three year old frogs previously fed a maintenance diet was given in figure 11 of experiment 9. The comparison of figure 8 and figure 11 showed that the dextrose solution produced a greater variation in the night low and morning high of circulating eosinophils in frogs three years of age. The validity of this effect was further given by the comparison of figure 11 and figure 9.

The role of light in the rhythms presented were further shown in figure 12 of experiment 10. Blood samples from three year old frogs kept in a reversed light cycle for a period of ten days revealed the eosinophil cycle to be completely reversed. The frogs were kept on a maintenance diet and free from excessive disturbance during this period. A comparison of figure 12 and figure 7 showed the inverted cycles to resemble each other. The probability here presented indicated the intrinsic mechanism of eosinophil periodicities were similarly endogenously stimulated. However, the age differential between the two experiments and the comparison of figure 12 and figure 9 negate the supposition.

The secondary importance of light as an exogenous factor was further revealed in figure 12 of experiment 11. The frogs kept in a reversed light cycle were transferred to a continuous source of light for a period of ten days. It was noted that the eosinophil cycle continued as an inverted rhythm. A night high and day low count were still present in these animals.

In experiment 12, frogs kept in continuous darkness exhibited a periodicity in the numbers of circulating eosinophils. The increased measurable manifestation of the cycle was given in figure 14. These animals were previously employed in an experiment of maintenance diet which enabled the animals to continue on an uninterrupted food schedule. Frogs employed in figure 14 were previously studied in figure 9.

The cooling effect at 42° F. on frogs from each age group is as follows:

one year old	148 eosinophils per cubic millimeter
two years old	43 eosinophils per cubic millimeter
three years old	97 eosinophils per cubic millimeter

The effect of heat at 110° F. on the numbers of circulating eosinophils is listed:

one year old	279 per cubic millimeter
two years old	530 per cubic millimeter
three years old	682 per cubic millimeter

The parasites found in the visceral organs upon post-mortem were:

Opalina sp., Pneumonoeces sp., and an unidentified nematode. None of these parasites were recorded as having contributed to a condition of eosinophilia.

TABLE I

THE NUMBER OF EOSINOPHILS PER CUBIC MILLIMETER IN RANA PIPIENS

1 year old frogs

Time of day	Maintenance Diet No. of cells/cmm
12:00	155
16:00	198
20:00	303
24:00	359
04:00	210
08:00	189
12:00	235

2 year old frogs

Time of day	Maintenance Diet No. of cells/cmm	20% Casein Diet No. of cells/cmm	20% Dextrose No. of cells/cmm
12:00	86	74	347
16:00	62	136	285
20:00	80	204	248
24:00	37	138	235
04:00	136	173	241
08:00	105	130	179
12:00	93	80	384

3-4 year old frogs

Time of day	Maintenance Diet No. of cells/cmm	20% Casein Diet No. of cells/cmm	20% Dextrose No. of cells/cmm
12:00	793	279	229
16:00	744	576	136
20:00	669	558	124
24:00	713	812	111
04:00	768	539	148
08:00	899	483	279
12:00	812	297	167

TABLE I (continued)

THE NUMBER OF EOSINOPHILS PER CUBIC MILLIMETER IN *RANA PIPIENS*

3-4 year old frogs	Diet - Maintenance		
Time of day	Reversed light cycle No. of cells/cmm	Continuous light No. of cells/cmm	Continuous darkness No. of cells/cmm
12:00	322	347	508
16:00	682	582	341
20:00	830	868	372
24:00	632	1,017	179
04:00	713	539	303
08:00	415	334	465
12:00	359	390	545

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary. The presence of an endogenous eosinophil cycle has been established in the circulating blood medium in the common leopard frog, Rana pipiens. Light, age, diet, temperature, and humidity under controlled laboratory conditions play a significant role in the twenty-four hour cycle in which the phase and rhythm are measurably altered. The eosinophil periodicities in the frog are noted to compare in many respects to those of higher animals.

It should be noted that the controlled conditions in the laboratory are unlike those of the natural ecological factors affecting the frogs. Factors such as population pressure, source of food, predators, temperature, and humidity may cause an altered eosinophil periodicity considerably different from that noted in the laboratory.

The effect of humidity and temperature on the numbers of circulating eosinophils in the frog has exhibited a trend in the phylogenetic relationship of these cells in one month old frogs to the adult of fishes.

Conclusions.

1. The endogenous eosinophil cycle is not present in frogs one month old, a period corresponding to the neonatal period of the human.

2. The numbers of circulating eosinophils diminishes with the severity of desiccation to a minimal point.
3. Frogs one month of age display an eosinophil cycle corresponding to the seven month old human.
4. A twenty-four hour cycle is present in adult frogs with a recorded night low and morning high.
5. The amplitude of the twenty-four hour cycle in frogs is influenced by the age of the animal.
6. A concentrated carbohydrate diet of 20% dextrose solution in three and four year old frogs increases the amplitude of the twenty-four hour cycle and decreases in two year old frogs.
7. A concentrated protein diet of 20% casein solution in adult frogs results in an inverted eosinophil cycle with a recorded night high and morning low.
8. The influence of light on the cycle is of secondary importance in animals fed a restricted diet.
9. The number of circulating eosinophils per cubic millimeter in the frog varies with time of day, age, diet and environmental temperature.
10. The eosinophil cycle in frogs appears to be the result of endogenous hormonal activity.

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