ASSAYS OF ADRENAL GLAND ACTIVITY IN THE WHITE MOUSE

by

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INTRODUCTION

Biologists have been aware of and interested in population fluctuations since the turn of the century. These fluctuations are noted most spectacularly in voles, lemming and hares, as seen in arctic and subarctic populations. The time intervals between peak densities appear to be dependent on fluctuations in the density of the prey species. These more or less regular peaks in numbers are usually followed by a "crash" of the population to levels of scarcity. Many theories have been proposed to explain the causes of these so-called "cycles" in the abundance of animals, involving such factors as food scarcity, climatic events, disease, and sunspots (11, pp.1-38). All these factors, at one time or another, could play an important role in explaining population fluctuations under natural conditions.

A number of investigations have been made on some of the physiological aspects of the foregoing phenomena. Using voles, rats, and house mice it has been demonstrated that there is a proportional decline in the animals reproductive capacity as the population density increases (28, pp.701-712; 9, pp.258-273; 10, pp.517-529). This is marked by a slight decrease in litter size, survival of the young, and an increased adult mortality. From this

it was then inevitable that after the population density reached a certain point, there would be a decline in numbers (28, pp.701-712). Louch found (op. cit.), using the field vole (Microtus pennsylvanicus), that restoration of reproductivity was not seen until an optimal low population density was reached.

Christian (11, pp.1-38) suggests that disturbances in the mechanism of endocrine balance are responsible for the reduced reproductive potential. He hypothesizes that high population pressures constitute a stress factor eliciting a response from the adrenopituitary system, the assumption being that population pressures act as "stressors" to the individual members of a population. He suggests further that the degree of activity of the pituitary adrenal axis is proportional to the density of the population. It is well known that almost any sort of "stress" stimulates the release of adrenocorticotropin (ACTH) from the anterior pituitary via pathways involving the higher brain centers and the hypothalmus, resulting in an increase in adreno-cortical secretion, primarily in the so-called gluco-corticoids (carbon-11 oxygenated) (ibid.). On the basis of these arguments, an increase in population density might be expected to cause increased adreno-cortical activity, especially with respect to the gluco-corticoids.

A number of criteria have been employed to measure the activity of the adrenopituitary system, including blood eosinophil levels, adrenal gland weights and zona fasciculata width, liver glycogen concentration, adrenal cholesterol concentration, seminal vesicle weight and length, testicular weight, thymus weight, preputial gland weight and adrenal ascorbic acid concentration. The measurements used in the present study were adrenal cholesterol concentrations, liver glycogen concentrations, blood eosinophil levels and paired adrenal gland weights. Two of these, eosinophil count and liver glycogen, may be considered as rough indicators of changes in gluco-corticoid production.

A most striking action of adrenal gluco-corticoids, whether injected or produced endogenously, is to diminish the number of eosinophils (eosinopenia) in the peripheral circulation (23, pp.755-768; 27, pp.668-671; 32, pp.536-544). It is felt that the magnitude of eosinopenia is proportional to the amount of hormone in the body. This is one of the most sensitive bioassay methods. Compounds such as cortisone, cortisone acetate, and 17-hydroxy-corticosterone (Compound F) may be assayed between the limits of 0.5 and 6 μg. of steroid by this method. Such steroids as testosterone, estradiol and progesterone are inactive in this test.

Also, only a partial eosinopenic response is detectable with desoxycorticosterone (mineralo-corticoid) and only when administered in quantities over 100 µg. (13, pp.105). Three explanations have been offered to account for the eosinopenia: (1) inhibition of the bone marrow may prevent their proliferation and release; (2) the hormone may cause the eosinophils to segregate in such organs as the spleen and lungs; and (3) the hormone may exert a destructive effect on formed eosinophils after they have entered the body fluids (41, pp.177).

It is well known that the formation of glycogen in the liver (but not in muscle) is affected by cortisone and other so-called gluco-corticoids from the adrenal cortex. Adreno-cortical insufficiency is characterized by low levels of liver glycogen and blood sugar (41, pp.173). Injection of cortisone elevates hepatic glycogen and promotes increased catabolism of proteins. It is believed that the increased liver glycogen is brought about by the accelerated conversion from blood glucose (ibid.). A quantitative approach to the problem of using liver glycogen as an assay for gluco-corticoids was reviewed by Verzar who stated that any corticoids which increased the glycogen content of the liver in a 6-hour test in the rat or mouse could be designated as "carbohydrate active" (42, pp.299). Evans (15, pp.301-303)

found that at low oxygen pressure the glycogen content of the liver remains relatively high in normal, but not in adrenalectomized animals. This observation was confirmed by Langley and Clark (26, pp.535) who obtained the following mean values for liver glycogen content in intact rats: fed rats, 3.32%; rats fasted for 24 hours, 0.14%; rats fasted for 24 hours at a 20,000 foot altitude, 2.53%. They also reported that liver glycogen values were invariably higher in adrenalectomized rats exposed to low atmospheric pressure than in unexposed adrenalectomized animals when they both were maintained on a constant amount of adrenal cortical extract.

Another criteria for measuring adrenal cortical activity is based upon adrenal cholesterol concentration. Situations which may be expected to increase the secretory activity of the adrenal cortex are associated with a reduction in the concentration of cholesterol in the adrenal. Sayers et al. (33, pp.390) reports that ACTH depleted the cholesterol content of the adrenal cortex at a time when metabolic changes characteristic of adrenocortical hyperactivity are occurring in the organism. In the absence of the anterior pituitary, adrenal cholesterol remains high under adverse conditions (stress) and usually fixed at a more elevated level than normal. Gould and Cook (17, pp.282) state that the cholesterol in

the adrenal cortex is present in a higher concentration and in a more labile form than in any other tissue. Various types of experimentally induced stress, including hemorrhage, burns, tourniquet shock and fasting, cause a rapid and marked decrease in adrenal cholesterol concentration, due presumably to an accelerated conversion into adrenal cortical hormones (ibid.). For nearly two decades there has been a concentrated effort employed to determine the role cholesterol plays in the biogenesis of adrenocortical steroids. At the present time it has been fairly well established that cholesterol is converted to various sterols in the adrenal cortex, but the fate of that cholesterol which is not converted to steroids is not clear (21, pp.343).

Wet or dry weight of the adrenal is an accurate, if gross, index of adreno-cortical activity in experiments of more than a few days duration. The simplicity of the technique is of considerable advantage. There seems to be a good correlation between adrenal weight and the expected activity of the adrenal cortex, as judged by the severity and duration of the stress to which the animal has been exposed (35, pp.244; 29, pp. 109; 9, pp.258-273). Enlargement of the gland occurs within 6 to 48 hours after the animal has been exposed to "stress" (35, pp.255; 26, pp.53).

METHODS AND PROCEDURES

Webster Strain Swiss male white mice, from six to nine weeks old and weighing between 18 and 25 grams, were obtained from the "Animal Supply Company", Napa, California. Upon arrival the animals were placed in a number of holding pens. As the animals were needed they were isolated in gallon cans with hardware cloth lids. All animals were isolated for 3 weeks before any experiments were performed.

The mice were maintained on Rockland Mouse Diet in pellet form, an abundance of which was provided at all times. Water was supplied ad libitum. Each gallon can was equipped with a water bottle having a long glass tube which was placed through the hardware cloth top. The animals were housed in a room illuminated from 6 A.M. to 6 P.M. and darkened from 6 P.M. to 6 A.M. Temperature was maintained at 78 ± 6° F.

The method used for determining the number of eosinophils was that described by Spiers and Meyer (38, pp.404-405). The technique employed by these authors for holding the animals securely for taking blood from the tail was slightly modified in the present study. A conical chamber was designed with a lucite bottom, and a fine copper screen cone connecting to the base with a moveable gate to restrain the animal from backing out.

This gate also had a small opening at the bottom to allow free extension of the tail when the gate was closed. The body size of the animals to be used determined the size of the chamber needed. Figure 1 gives the design and dimensions of the animal restrainer used in the present study on white mice.

Since previous work has shown a diel eosinophil rhythm in tail blood taken from house mice (18, pp.670; 19, pp.73), and since strain differences in characteristic eosinophil levels apparently exist (19, pp.835), it was important to determine the periodicity of the rhythm before a blood sampling schedule could be planned. To obtain the desired information 24-hour series of eosinophil counts were taken once a week for four weeks. The same 24 animals were used each week to determine the eosinophil levels. Blood samples were taken from the tail of four mice every four hours for a consecutive 24-hour period. The mice were partially randomized every seven days so that the same animal would not necessarily be used during the same four hour period as that of the preceding week. A total of from 14 to 16 counts were thus obtained for each of six time intervals over the four weeks (fig. 2). Each week the study was begun at different, partially randomized, time intervals. From the

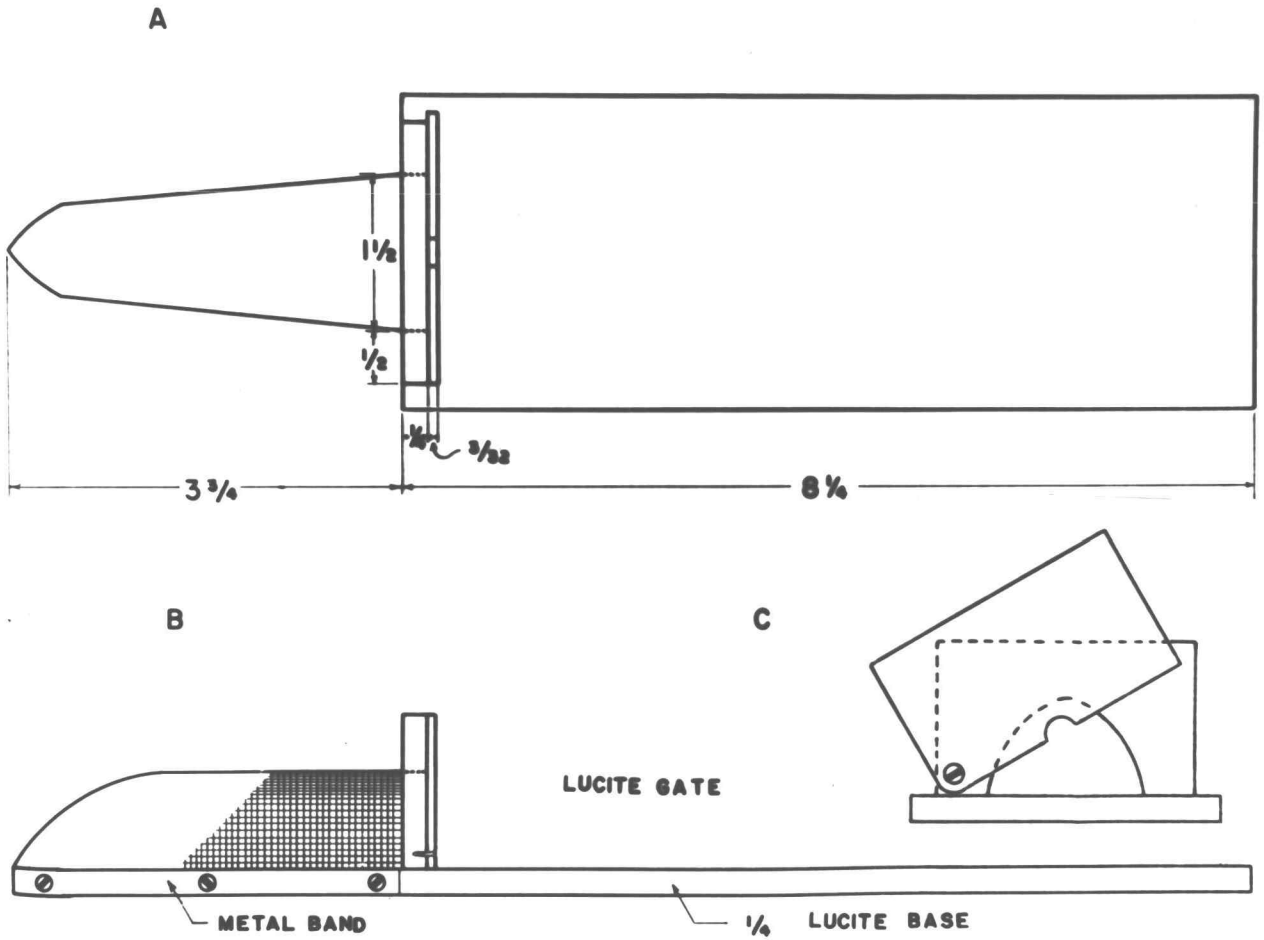

FIGURE 1. APPARATUS FOR HOLDING MICE. (A) TOP, (B) SIDE AND (C) END VIEWS.

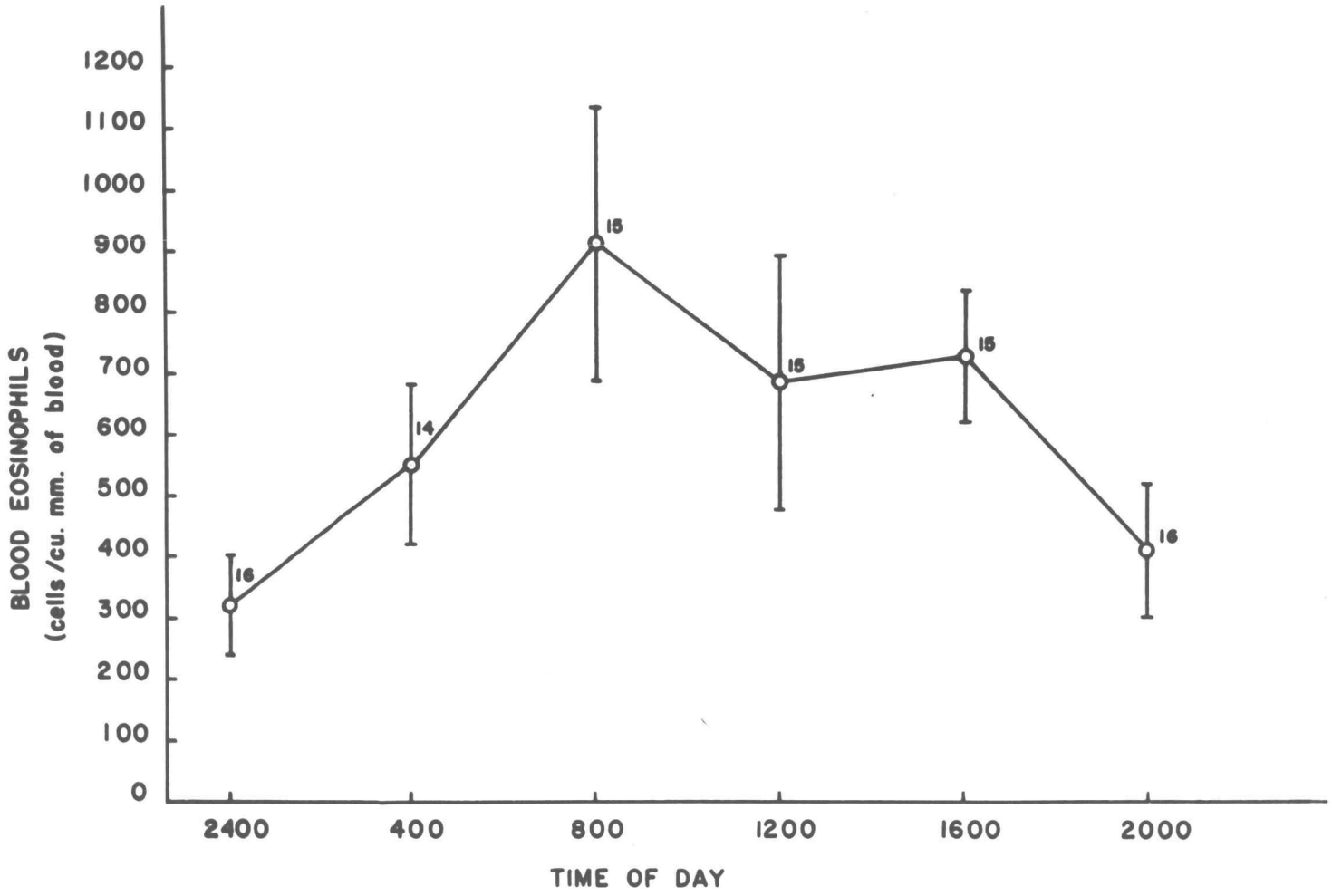

FIGURE 2. DIURNAL FLUCTUATION OF BLOOD EOSINOPHILS IN 10 TO 13 WEEKS OLD MALE WHITE MICE. NUMBER OF MICE USED IS GIVEN ABOVE EACH POINT.

first to fourth week, respectively, the following schedule of starting times was followed: 8:00 A.M., 8:00 P.M., 12:00 noon, and 4:00 A.M.

As mentioned previously each animal was isolated individually three weeks prior to the crowding experiments. They were then placed in groups of 1, 4, 6, 8, 16, and 24 animals per test cage. The test cages were constructed of sheet metal on the bottom and sides with wooden ends. Each cage measured 5-1/2 x 12-1/2 x 14-1/2 inches. Only one group was run per week so the mice crowded in groups of 24 per cage were six weeks older than the animals grouped one per cage. A second series of determinations on isolated mice was run to check the values obtained from the first run controls. These animals were about 75 days older than the first run controls.

After one week in the test cages eosinophil counts of the tail blood were taken between 8:00 A.M. and 12:00 noon. This time was chosen because the circulating eosinophil level was highest during this period (fig. 2). After the blood samples had been taken the mice were returned to their respective cages. Two days later the animals were weighed to the nearest gram and killed by decapitation. Immediately after decapitation, the abdominal cavity of the animal was completely exposed. A piece of liver from the base of the left lateral lobe

was removed and weighed to the nearest two tenths of a milligram using a torsion balance. After the liver sample had been weighed, it was placed in 1 cc. of 30% potassium hydroxide for digestion. Subsequent to the removal of the liver both adrenal glands were removed. Any excess fat encapsulating the structures was cut away, and the paired adrenal weight was obtained to the nearest two tenths of a milligram with a torsion balance. The glands were then placed into 0.25 cc. of tetramethyl ammonium hydroxide for digestion. Tissue digestion began only after the adrenals and liver sample had been removed from all of the animals to be used in that experimental group.

Liver glycogen concentration and adrenal cholesterol content were both determined colorimetrically. Liver glycogen was determined by the method of Bowman (2, pp.157-161) which is a modification of the procedure developed by Seifter et al. (36, pp.191). The cholesterol content of the adrenal glands was determined using the method described by Herrmann (22, pp.503-505). This method combines the procedures described by MacIntyre et al. (31, pp.XLIII) and Abell et al. (1, pp.357). The liver glycogen was read in the colorimeter the same day the animals were killed, but the adrenal cholesterol samples were stored in a refrigerator and read three days

later. All determinations were made with a Bausch and Lomb Spectronic 20 colorimeter.

RESULTS

The results of the 24-hour study of fluctuations in blood eosinophil level are illustrated in figure 2. A unimodal cycle with an increase in the eosinophil level from 12:00 midnight to 8:00 A.M. is evident. Other workers (32, pp.537; 5, pp.368; 20, pp.73) reported finding maximum depletion of blood eosinophils at 12:00 midnight with a steady increase after that time till a peak is reached from between 9:00 A.M. and 12:00 noon.

The results of the crowding experiments are tabulated (table 1) and summarized graphically in figure 3. A brief report of each series of tests is presented below:

Adrenal Glands

There is a steady increase in adrenal gland weight from the controls (isolated individuals) up to a density of 16 mice per cage. The maximum increase in weight is approximately 15% over the isolated mice. The increase in adrenal weight found here is similar to that which has been reported elsewhere (7, pp.478; 8, pp.292) and is believed to be due primarily to an increase in adrenocortical tissue. Christian (7, pp.479), who used the same densities as those used in the present study with the exception of the highest density, believes that the

decrease in adrenal weight in populations of above 16 mice per cage is due to deterioration of the "social structure" within each cage. Christian (7, pp.479) used groups of 32 instead of 24 mice per cage as his highest cage population density.

Eosinophils

The highest mean level of eosinophils was shown by the controls (isolated individuals). The eosinophil level shows a steady downward trend with increasing crowding, with the exception of an unexplainable slight rise at a density of six mice per cage. A second series of determinations on isolated individuals gave an almost identical mean value (fig. 3).

Adrenal Cholesterol

Values for adrenal cholesterol concentration (expressed as mg./gm. of adrenal) showed no consistent trend, with considerable variation as indicated by the large standard errors. Nevertheless, there appears to be a drop in cholesterol level at population densities greater than four per cage (fig. 3). Between densities of six and 24 per cage, however, the concentration of cholesterol seems to have reached a more or less constant level. The slight increase shown by the animals grouped

four per cage over the isolated mice is probably not significant. The mean cholesterol level from the second run on isolated mice was very close to that from the first run (fig. 3).

Liver Glycogen

The concentration of liver glycogen (expressed in mg. glucose per gm. liver) appears to be exceedingly high in the isolated control individuals of the initial run. In the check determinations on isolated mice, made with animals about 75 days older, the glycogen level is considerably lower (fig. 3). Nothing could be found in the literature concerning the effects of age on liver glycogen levels of white mice. Thus, it is difficult to decide which of these mean values represents the true liver glycogen level for the isolated mice. Between densities of four to 16 per cage, however, there is a more or less consistent rise in concentration of liver glycogen. At a population density of 16 per cage the glycogen level is approximately 100 percent greater than at a density of four per cage. At a density of 24 per cage, the glycogen level decreases about 15 percent from the maximum. This decrease may not be significant. Farner (16) has found that liver glycogen in birds may fluctuate this much

due to the blood glucose level in the liver at the time the animals were killed.

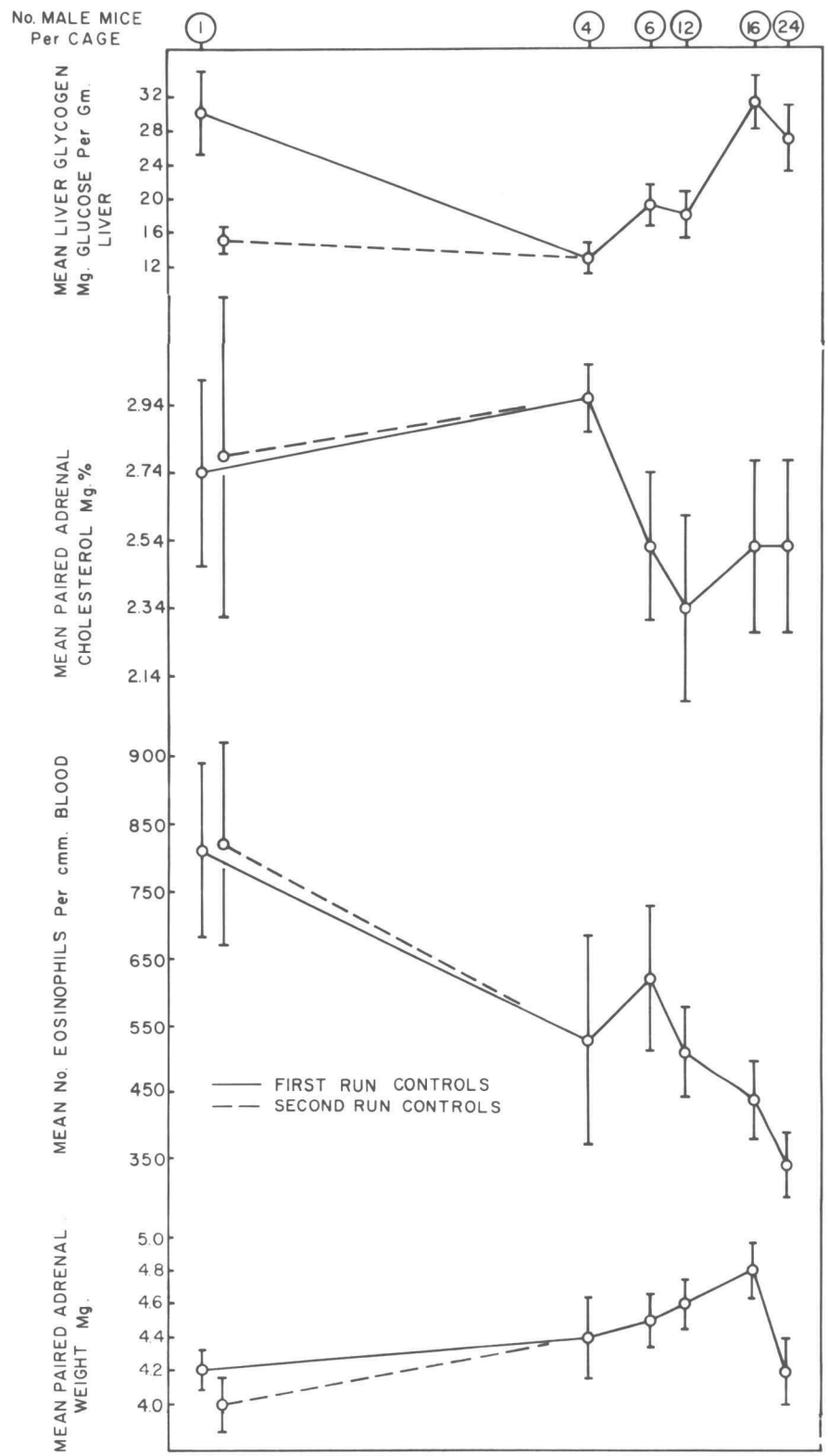

FIGURE 3. MEAN VALUES FOR PAIRED ADRENAL WEIGHT, BLOOD EOSINOPHILS, ADRENAL CHOLESTEROL, AND LIVER GLYCOGEN OF MALE WHITE MICE PLOTTED AGAINST THE LOG OF THE POPULATION SIZE. VERTICLE LINES REPRESENT THE STANDARD ERROR.

DISCUSSION

The findings of the present study are in general similar to those of other workers investigating the effects of crowding on adrenal gland activity. However, some of the physiological parameters employed in this investigation, i.e., adrenal cholesterol and liver glycogen concentrations, have not, to the author's knowledge, been previously employed in analogous work. Christian (11, pp.7) states that biochemical measurements are not a desirable means for measuring adrenal activity in population density studies, because the added stress of handling the animals might produce responses overshadowing the response to crowding. Southwick (37, pp.157) found that handling his control mice per se for a few seconds did not produce significant eosinopenia, but these animals were not exposed to any type of population stress previously. Although the results obtained in the present investigation do not necessarily prove that handling does not produce responses overshadowing the response to crowding, it is interesting to note that the paired adrenal weights obtained here are quite similar to those obtained by Christian (7, pp.477-480; 8, pp.292-300), even though the animals had blood taken from their tails only two days prior to adrenal gland weighing. Although Christian's values are slightly higher, a point which

should not be overlooked is that strain differences between the mice used in the present study and those used by Christian could explain the slight weight differences.

Histological studies show that the cortex of the adrenal gland undergoes hypertrophy and hyperplasia during exposure to increasing population densities. According to Christian (9, pp.270), of the three zones present in the cortex, the zona fasciculata increases to the greatest extent and receives primary credit for the increase in adrenal weight. Jones (25, pp.514-536) found that the zona fasciculata width in mice of both sexes is directly dependent on ACTH, and it is interesting to note that injections of ACTH in mice have also been reported to produce eosiopenia which is almost identical to that produced by mild stress produced by injections of slightly toxic material (39, pp.309). Stachenke and Giroud (40, pp.743) demonstrated the site of ACTH action in beef adrenal slices to be localized in the fasciculata-reticularis cells and found an increased production of total corticosteroids and hydrocortisone. Both eosinopenia and high liver glycogen levels have been reported in animals receiving injections of gluco-corticoids similar to those reportedly found in the adrenal cortex (13, pp.105; 42, pp.299). Other investigators (30, pp.384) claim that

adrenal enlargement is due chiefly to an increased water content and, to some extent, to protein and phospholipids.

No histological observations were made in the present study. However, the physiological data, in particular the eosinophil and glycogen measurements, point indirectly to an increase in the activity of the adrenal cortex, and perhaps more specifically to the zona fasciculata-reticularis with increased crowding "stress" (40, pp.743). Thus the progressive decline in eosinophil count (fig. 3) may be correlated with the steady rise in adrenal weight up to a population density of 16 mice per cage. However, the marked drop in adrenal weight at a density of 24 per cage is unexpectedly associated with a still further decline in eosinophil count. No explanation can be given for this. The data on liver glycogen is more difficult to interpret because of the great difference in the mean values of the two sets of determinations on isolated individuals. Nevertheless, there is a marked upward trend in liver glycogen concentration from densities of four to 16 mice per cage (fig. 3), the total increase amounting to more than 100%. At a density of 24 animals per cage there is a decline of about 15% which may be correlated with the drop in adrenal gland weight at this density. If it may be assumed that the increase in adrenal gland weight is

reflected mainly by increase in the cortex width, then the increased deposition of liver glycogen might be due to increased secretion of gluco-corticoids from this region.

The large standard errors for the adrenal cholesterol values make it difficult to draw anything but tentative conclusions. There appears to be a slight rise in concentrations at a density of 4 per cage, although the standard error for the isolated control individuals is so large that the difference is undoubtedly not significant. At a density of 6 per cage the cholesterol concentration has declined somewhat and appears to remain at this level at the higher population densities (fig. 3). The slightly lower adrenal cholesterol concentrations at higher population densities may be indicative of increased adrenal secretion of corticoids. However, the very important question, still not completely answered, is whether cholesterol in these tissues primarily plays a precursor role wherein it would function as a storage of preformed steroid nuclei to supply trace amounts of hormones as needed. According to Hechter (21, pp.343) the content of adrenal cholesterol in rat adrenals is about 4 percent, and following maximal doses of ACTH, this value declines 50 percent more in three hours. When beef adrenal slices are incubated in

a media containing ACTH, Stachenke and Giroud (40, pp.743) found that cholesterol was converted to hydro-cortisone by the cells of the zona fasciculata and reticularis. Hechter (21, pp.343) states that about one-quarter of the cholesterol which disappears in the rat adrenal as a result of ACTH action is accounted for in terms of conversion to corticoids. The fate of the other three-quarters of adrenal cholesterol still remains in question.

The variability of the results could be due at least in part to genetical differences between the individual mice. Downs, Pennybaker, and Bowling (12, pp.5) have reported in many cases, even where mice have been "inbred" for several generations, that there were marked differences in the normal blood picture of littermates of the same sex. Not infrequently, these differences were as great as between control and experimental results. They found only when breeding animals are selected for close similarity in blood-picture at similar diurnal periods and are bred brother-to-sister for a minimum of three generations to a point of being homozygous for blood-count, are control stocks of value for such studies. This information lends support to the fact that some of the variability found here could be due to genetical differences even though all of the mice were presumably of the same strain.

SUMMARY

The effects of crowding on male Swiss white mice was studied by placing varying number of mice for a period of one week in pens having the same areas. At the end of one week the following physiological parameters were measured: paired adrenal gland weight, adrenal cholesterol concentration, blood eosinophil level, and liver glycogen concentration. Adrenal glands showed an increase in weight up to a density of 16 animals per cage. At a density of 24 per cage the adrenal weight dropped to the control value. The counts of blood eosinophils, though variable, showed a steady decline with increased crowding. Over the range of population densities tested, liver glycogen showed a consistent rising trend. The adrenal cholesterol values were highly variable and showed no consistent trend. The results are discussed with regard to the physiological activity of the adrenal cortex under conditions of crowding.

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APPENDIX

TABLE I. Mean Cage Values, with standard error, for Paired Adrenal Weights, Blood Eosinophil Counts, Paired Adrenal Cholesterol, and Liver Glycogen.

Population Size	Number of Cages	Number of Animals Used	Animal Age in Days When Killed ± 11 days	Mean Body Weight in gm.	Mean Paired Adrenal Weight in mg.	Mean No. Eosinophils per cmm. Blood	Mean Pr. Adrenal Cholesterol in mg. %	Mean Liver Glycogen mg. glucose/gm. liver
1*	12	10	79	25.3 ± 0.54	4.2 ± 0.13	810 ± 130	2.74 ± 0.29	30.44 ± 5.10
1**	14	11	189	30.9 ± 0.88	4.0 ± 0.17	832 ± 150	2.79 ± 0.49	14.95 ± 1.50
4	3	12	86	26.3 ± 0.57	4.4 ± 0.23	526 ± 160	2.96 ± 0.12	12.42 ± 2.80
6	2	12	93	27.7 ± 0.67	4.5 ± 0.17	618 ± 109	2.52 ± 0.23	19.42 ± 3.53
8	2	16	100	26.8 ± 0.88	4.6 ± 0.18	509 ± 69	2.34 ± 0.28	17.82 ± 2.85
16	2	16	107	27.9 ± 0.76	4.8 ± 0.16	436 ± 69	2.42 ± 0.27	31.44 ± 3.45
24	2	16	114	25.3 ± 0.62	4.2 ± 0.19	361 ± 56	2.42 ± 0.27	26.69 ± 4.39

* First run controls

** Second run controls