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THE ROLE OF PINEAL SECRETIONS IN HIBERNATION OF THE GOLDEN HAMSTER

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the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

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Master of Science in Biology

by

George B. Laroussini

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THE ROLE OF PINEAL SECRETIONS IN HIBERNATION OF THE GOLDEN HAMSTER

by

George B. Laroussini

APPROVED BY:

SUPERVISOR OF THESIS Everett C. Simpson
Dr. Everett C. Simpson

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY
James S. McDaniel
Dr. James S. McDaniel

DEAN OF THE GRADUATE SCHOOL
Joseph G. Boyette
Dr. Joseph G. Boyette

584605

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ABSTRACT

George B. Laroussini. THE ROLE OF PINEAL SECRETIONS IN HIBERNATION OF THE GOLDEN HAMSTER. (Under the direction of Dr. Everett C. Simpson). Department of Biology, December, 1977.

Eighteen male and female golden hamsters (Mesocricetus auratus) were placed in a darkened environmental chamber (DD 24) maintained at a temperature of 6°C. The animals were divided into three, six member, experimental categories. Animals in experimental group I were pinealectomized and implanted with time release capsules containing crystalline melatonin. Those in experimental group II were also pinealectomized, however in this group no melatonin was administered. The animals in group III served as a control, merely undergoing sham pinealectomy and melatonin implantation procedures. All eighteen hamsters were implanted with a Mini-Mitter radio thermometer; their body temperatures were monitored and recorded daily.

On the sixty-second day of the experiment, a chamber malfunction prematurely terminated the investigation. Statistical analyses of the available data (body weights and daily temperature records) showed no significant differences between the three groups.

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INTRODUCTION

The literature is replete with implications of the mammalian pineal organ functioning as a "biological clock" with secretions regulated primarily by photoperiod. Additional investigations indicate a possible thermoregulatory involvement (Stebbins, 1960; Miline, 1971; Binkley *et al.*, 1971); but as yet, no published research has associated this gland with the ability of mammals to hibernate. Popova *et al* (1975) have however indicated a possible reduction in function in the pineal of hibernating sousliks (*Citellus erythrogenys major*).

The present study was designed to establish a functional relationship between the hormonal secretions of the pineal (primarily melatonin) and the ability of the golden hamster (*Mesocricetus auratus*) to hibernate. Experimental manipulations included surgical pinealectomy, selected melatonin replacement, and an environment conducive to hibernation (DD 24; 6°C). The monitoring of body temperature responses, and also the incidence of hibernation, was accomplished using temperature radio-telemetry.

An additional objective of this experiment was an investigation of the ultrastructure of the pineal gland during hibernation. A chamber malfunction prematurely terminated the experiment; consequently this aspect of the investigation was not completed.

REVIEW OF LITERATURE

It has been suspected for many years that the mammalian pineal body secretes biologically active compounds. In 1958, Lerner and his coworkers at Yale University initiated the modern era of pineal research by isolating from bovine pineals the substance, melatonin and identifying its structure as 5-methoxy-n-acetyl tryptamine (Lerner *et al*., 1959). Within a few years, this compound was shown to be physiologically active in mammals; associated with the functioning of numerous organs, including brain, pituitary, thyroid, adrenal, gonad, and smooth muscle (Wendel *et al*., 1974; Chazov *et al*., 1972; Singh and Turner, 1969; Gromova *et al*., 1967; DeBeljuk, 1969; Izumi *et al*., 1973). These observations and the findings that melatonin reverses many of the physiological effects of pinealectomy, seem to justify labeling melatonin a pineal hormone (Minneman and Wurtman, 1974).

Wurtman and Axelrod (1965) have suggested that the mammalian pineal functions as a "biological clock", which secretes more or less of its hormones in response to environmental lighting and time of day. Much data has been published supporting this position particularly in regard to the influence of the pineal on mammalian reproductive structures.

Numerous authors have demonstrated that precocious

sexual maturation, enlarged gonads and accessory sex organs result from the subjection of both mature and immature laboratory animals to a regime of continuous ambient lighting (Browman, 1937; Hemmingsen and Krarup, 1937; Fiske, 1941). During the middle and late fifties Kitay, Altschule and Wurtman, in a series of papers, reported that pinealectomy of immature rats was followed by gonadal stimulation; a stimulation that could be suppressed by administration of pineal extracts (Kitay, 1954; Kitay and Altschule, 1954; Wurtman _et al._, 1959). Fisk _et al._ (1960) found that the pineal glands of rats exposed to continuous light were reduced in weight when compared with pineals from rats housed in continuous darkness. This reduced weight may be correlated with the reduced pineal function, i.e. gonadal enlargement, demonstrated in the pineal intact, continuously lighted animals.

In addition to sexual development, the pineal organ has also been linked with the maintenance of certain circadian and also circannual rhythms. In 1968, Gaston and Menaker reported that the circadian rhythm of perching activity was lost after pinealectomy in the house sparrow. McMillan (1972) demonstrated that the pinealectomy of white-throated sparrows, maintained under constant conditions in dim light, abolishes the circadian rhythm of nocturnal spring and fall migratory restlessness.

In rats, however, pinealectomy does not modify the 24-hour rhythm of locomotor activity (Quay, 1968), but may accelerate the entrainment of an activity rhythm to a modified lighting schedule (Quay, 1970a). From these data it seems likely that a major function of the pineal organ is to emit a hormonal signal (melatonin and perhaps other hormones) whose amplitude depends upon time of day and environmental lighting. This photic trigger may not, however, be the sole regulator of this "circulating pineal clock" (Cardinali and Wurtman, 1975). Quay (1970b), in assessing the endocrine activity of this gland, also recognizes the importance of photoperiod, but includes other environmental factors as possible regulating agents.

One such factor being investigated is ambient temperature. The pineal was first linked to thermal stimulation by Stebbins (1960) who demonstrated that in both laboratory and field observations, lizards which have undergone pinealectomy or removal or shielding of the parietal eye are deficient in their thermoregulatory capabilities. Likewise, pinealectomized rats were seen to be more sensitive than control animals to lowered environmental temperature on a variety of physiological measures (Miline, 1971). Binkley, Kluth and Menaker (1971) have shown that normal circadian temperature rhythm was lost in pinealectomized sparrows maintained in darkness and further that the body temperatures of the pineal-

ectomized birds did not drop to the daily minimum. It should be noted that Arutyunan et al. (1964) have shown that in mice a lowering of body temperature may be induced by the exogenous administration of melatonin.

This knowledge of the pineal's involvement with thermoregulation readily lends itself to an investigation of the pineal's influence on hibernation. Furthermore, several hibernation related characteristics have definitely been shown subject to pineal modification. According to Hoffman (1964), reduced reproductive function appears to be characteristic of all organisms preparing for hibernation. Related studies by Hoffman et al. (1965), show diminished testicular weight and function to be induced by short daily photoperiods and further that this testicular atrophy can be prevented by prior removal of the pineal gland.

Lyman (1948) demonstrated that in addition to an inactive reproductive system, preparation for hibernation in the golden hamster is accomplished by a reduced body weight. Hoffman et al. (1965) showed that short photoperiods or low temperature can act alone or additively to induce these processes. In addition, Hoffman (1964) stated that an increase of brown fat was typical of hibernators in the preparatory phase and that short photoperiods or low temperature could likewise produce these increases. It is noteworthy that Heldmaier and Hoffman (1974) induced similar production of this tissue in hamsters by the

administration of exogenous melatonin.

Several additional hibernation related occurances have been attributed to the administration of exogenous melatonin. Rust and Meyer (1969) found that subcutaneous implantation of melatonin in weasels had a striking effect on the animals' change from summer to winter pelage. The normal summer brown and spring white animals changing to brown, grew a white (winter) coat when administered this hormone. Barchas _et al_. (1967) reported a barbituate-like "sleep" in mice and 4-day old chicks, following melatonin injections. Marcznski _et al_. (1964) induced a similar state in cats following the introduction of melatonin directly into their hypothalamic region.

From the evidence outlined above, it is obvious that experimentation regarding the pineal's influence on hibernation is certainly warranted. It is in this light that the following thesis experiment has been developed.

MATERIALS AND METHODS

Animals

Eighteen golden hamsters (*Mesocricetus auratus*) that had never experienced hibernation were used in this experiment. Both males and females were utilized, with ages ranging from six to ten months. Prior to being placed in the experimental chamber, the animals were maintained at a near constant temperature of 22°C in a lighting regime consisting of 14 hours of light and 10 hours of darkness. Throughout both the preparatory stage and the course of the experiment, food (Wayne Laboratory Chow) and water were provided *ad libitum*.

Design of Experiment

The hamsters were placed into three, six member, experimental categories. Animals in experimental group I were pinealectomized (Hoffman and Reiter, 1965) and subsequently implanted (dorsal subcutaneous) with time release silicone tubes filled with crystalline melatonin (Turek *et al*., 1975). Those in experimental group II were also pinealectomized, however, in this group no exogenous melatonin was administered. The six animals in group III acted as a control, merely undergoing a sham-pinealectomy. The animals in groups II and III were also implanted with silastic tubing, however, in these tubes

no melatonin was included. All eighteen hamsters were implanted (cheek pouch) with a Mini-Mitter radio thermometer for the purpose of nondisruptive, protracted temperature monitoring (Dunham and Herrold, 1962). The transmitters used were shunt-fed Hartley oscillators which incorporated a temperature sensitive thermister. Each oscillator unit thus produced a click signal with a frequency directly proportional to the environmental temperature surrounding the thermister. This signal could then be detected using any commercial AM band radio receiver tuned between stations (Verner and Kinders, 1974).

All eighteen animals were weighed and then placed (19 February, 1977) in a darkened environmental chamber (DD 24) having a temperature of $6^{o}C \pm 1^{o}C$. With the exception of several brief temperature elevations (up to $+7^{o}C$), incurred as a result of opening the chamber during cage maintenance (bi-weekly) and chamber power failures (twice), this constant temperature was maintained.

Temperature monitoring was accomplished through the use of individual antenna-wire circuits to an AM radio receiver external to the environmental chamber. Each animal was maintained within a separate metal walled cage (17 x 26.5 x 12.5 cm), thus allowing individual radio impulses to be isolated. The cages were provided with ample bedding material to accomodate nesting and also a large stockpile of food (within cage) to facilitate the

hoarding behavior characteristic of hamsters preparing for hibernation (Lyman and Chatfield, 1955).

Temperatures of all animals were monitored and recorded daily (mid-morning). Since the prelethargic period (i.e. time in cold environment before hibernation) established for the hamster may extend to ninety days (Smit-Vis and Smit, 1963), an experimental design of this duration was necessitated. Normal body core temperature of the golden hamster is 37°C and temperature drops approaching ambient (plus 1 or 2°C) were considered indicative of hibernation.

Experimental Procedures

Pinealectomy

The surgical removal of the pineal gland was accomplished following the procedure developed by Hoffman and Reiter (1965). The animals were anesthetized (0.15 cc sodium pentobarbital at 50 mg/ml, IP), shaved (posterior margin of eyes to posterior margin of ears, and secured in the head immobilization apparatus of a standard stereotaxic instrument. A disc saw had been constructed using stainless steel stock, turned down and drilled so as to form a thin walled tube, terminating in a small, solid shaft. The completed saw thus resembled an ordinary dental bit (Fig. 3). Fine teeth had been filed in the end of the tube and a movable collar fitted around it. The collar was

secured using an Allen screw, thus allowing rapid adjustment and the selection of an exact depth of cut. The circular saw was then mounted in a standard hand-held dentral drill, to be used in cutting a disc shaped access through the skull to the pineal organ.

An incision was made in the scalp, directed antero-posteriorly along the midline, extending across the shaved area. The skin flaps were reflected and the underlying fasciae and the origins of the temporal and occipital muscles were scraped free. Careful removal of these tissues from the field was necessary to prevent entanglement with the collar and saw during the drilling of the skull.

At this point, the sutures of the cranial bones are plainly visible and were used as guidelines for positioning the point of entry. The drill was centered on the junction of the sutures joining the parietal and interparietal bones, and a hole then cut to the desired depth, as set by the adjustable collar. The optimal depth is not quite equal to the thickness of the skull, as at this depth, the bone disc is movable, but still adherent to the dura. With care, the disc was removed with a minimum of bleeding from the underlying vascular channels. Although the pineal gland was not immediately seen, a little practice permitted the blotting dry of the exposed dura and with careful observation, location of the white body of the gland (0.5 mm) visible in the sinus. A fine pair of watchmaker's forceps were used to

grasp the stalk and pluck the gland from the brain (Fig. 1).

Excessive hemorrhaging was prevented by rapidly returning the bone disc to its original position and by the application of slight pressure using a sterile gauze pad. Bleeding, and possible infection, was further controlled by dusting the wound with powdered sulfur. The skin flaps were then pulled together and secured using standard wound clips.

Transmitter Preparation and Implantation

Prior to implanting the transmitters into the cheek pouches of the hamsters, it was necessary to apply a protective coating to the instruments and also to calibrate them using known temperatures. The following is a procedural outline prepared by Verner and Kinders (1974) paraphrasing the technical sheets provided by the Mini-Mitter Company.

1.) The transmitter capsules were first coated with a 50/50 (beeswax-paraffin) mixture and allowed to dry.

2.) They were then coated with a layer of silicone-rubber aquarium cement and allowed to dry (type A silastic brand medical adhesive was substituted for aquarium cement in this experiment).

After the rubber cement had dried, calibration of the transmitter was as follows:

1.) A 1000 cc beaker was filled with water and placed

on a magnetic stirrer. The initial water temperature was adjusted to the lowest temperature to be used in the calibration curve (5°C).

2.) The transmitter was then suspended in approximately the center of the beaker on one end of a string which had been attached to a clamp secured to a ring stand. A thermometer was suspended in a similar fashion so that it lay within 2 cm of the transmitter.

3.) An AM radio, tuned between stations, was placed near the beaker and the frequency of the oscillator was counted for 2 minutes to determine a click/minute frequency.

4.) The beaker was then warmed through successive temperature steps with the temperature being stable for 2 minutes prior to beginning the click frequency determinations (5°C, 15°C, 25°C, 35°C).

5.) A calibration curve was constructed of clicks/minute versus temperature for each transmitter utilized (Fig. 2).

The surgical implantation of the transmitter followed, in general, the procedure described by Dunham and Herrold (1962). The animals were anesthetized (as above) and a 7-8 mm incision made in their cheek approximately 3 mm from the angle of the mouth. Using careful blunt dissection,

the neck of the underlying cheek pouch was then totally freed from the surrounding tissues. At this point, the mouth of the unconscious animal was forced open and the transmitter passed through it and into the adjacent pouch. By entering through the incision, it was now possible to loop and loosely tie a heavy cotton thread (sterile) around the neck of the partially exposed pouch, thus preventing the expulsion of the transmitter. The cheek skin flaps were then pulled together and secured, again using standard wound clips.

Melatonin Capsule Preparation and Implantation

Time release tubular capsules containing melatonin were prepared as described by Turek *et al.*, (1975). The tubing utilized was medical grade silastic (polydimethylsiloxane) tubing having a 1.47 mm inner diameter and a 1.96 mm outer diameter. Each of the implanted capsules was 200 mm in length; a design intended to provide a 101± 1.6 mg/day administration of the drug (Turek *et al.*, 1975). This daily dosage was selected in accordance with the positive responses reported by Turek *et al.* regarding experimentation with melatonin implants in hamster reproduction.

Twenty-four hours after sealing one end (type A silastic adhesive), the tubes were packed with crystalline melatonin and the remaining end then sealed. All capsules were dried for 24 hours at 60°C and then weighed.

Implantation of the capsules (subcutaneous) was done concurrently with that of the transmitters, under general anesthesia. A minimum of ten days recovery time was always allowed between each of the pinealectomy operations (actual and sham) and the transmitter and tubing implants. A 1 cm incision was made through the skin at a point along the dorsal midline in the sacral region. A blunt probe was inserted beneath the skin and manipulated anteriorly to the base of the neck, separating the connective tissue and thus providing a channel in which to slide the folded length of tubing. Upon completion of the implant, the incision was closed using a single wound clip.

Antennae Design and Construction

A simple coil antenna, contained within a 300 ml glass beaker, was centered in each of the 18 cages. The antenna consisted of approximately 3 meters of 20 gauge insulated copper wire wrapped around a 3 cm x 9 cm cardboard tube. The two ends of the wrapped wire then led out of the beaker through a glass tube in its rubber stopper. This tube penetrated the metal cage lid through its alternate drinking bottle port, and in doing so provided stability to the entire antenna apparatus. The wires led from the glass tube, along the chamber wall, and out of the unit through the wire aperture provided. They then terminated in exposed, numbered, leads taped to the exterior

of the chamber (Fig. 4). This design not only allowed total individual cage monitoring, but also provided the necessary protection against destruction of the antennae by the hamsters.

Located adjacent to the antennae terminals was a standard AM radio receiver having alligator clip leads extending from the coils of its own antenna. By sequentially attaching these clips on to each of the cage antenna leads, the separate circuits were completed and the monitoring of the individual signals from within the chamber was accomplished. Through the use of the pre-established calibration curves (Fig. 2) each animal's clicks per minute signal could then be translated into body temperature and recorded.

Analysis of Data

Data collection was to include daily temperature records, initial and terminal body weights, the incidence of hibernation, and a post-torpor pineal ultrastructural investigation. A chamber malfunction, however, prematurely terminated the experiment, consequently eliminating actual torpor and post-torpor analyses.

Time series analyses (i.e., autocorrelation) were used to establish temperature response patterns for between group comparisons. Temperature records were further analyzed using regression statistics with a comparing of

the resultant slopes.

Analysis of covariance was used to investigate the significance of altered body weights resulting from the experiment. The relative incidence of hibernation for the three groups was to be compared using analysis of variance.

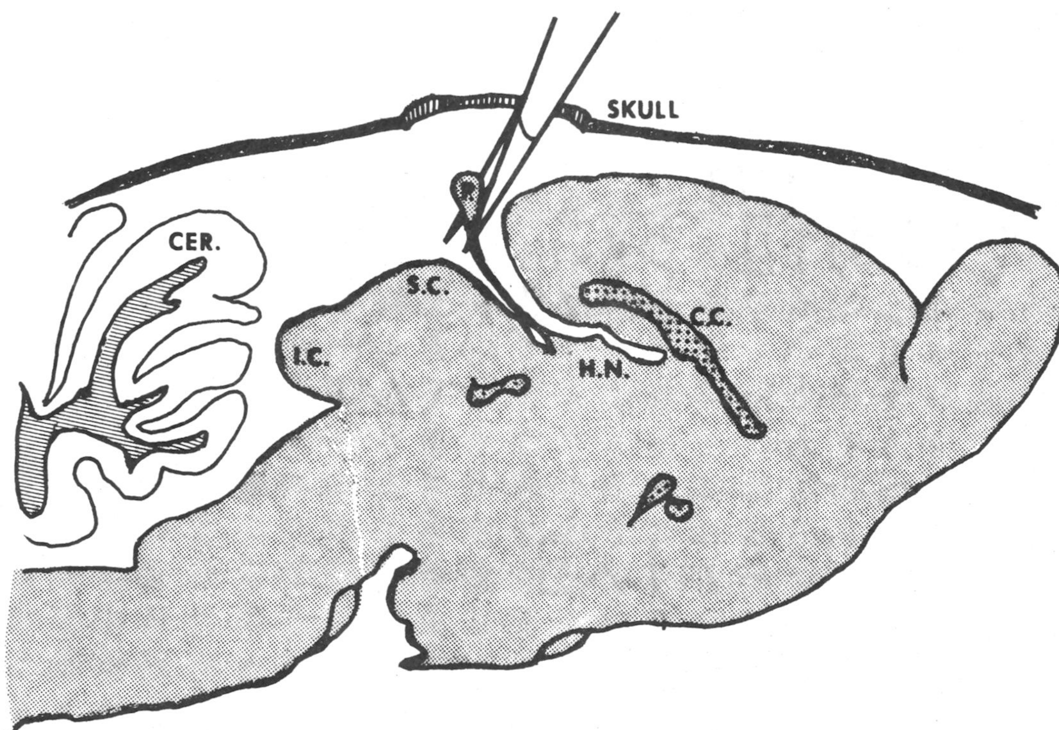

Figure 1. Diagram of a mid-sagittal section of an adult hamster brain showing the relationships of the pineal organ, P, with the surrounding structures and the direction of approach for pinealectomy. C. C., corpus callosum; CER., cerebellum; H. N., habenular nucleus; I. C., inferior colliculus; S. C., superior colliculus. (Hoffman and Reiter, 1965)

Temp. °C.	Clicks Per Minute
5	37
15	52
25	70
35	102

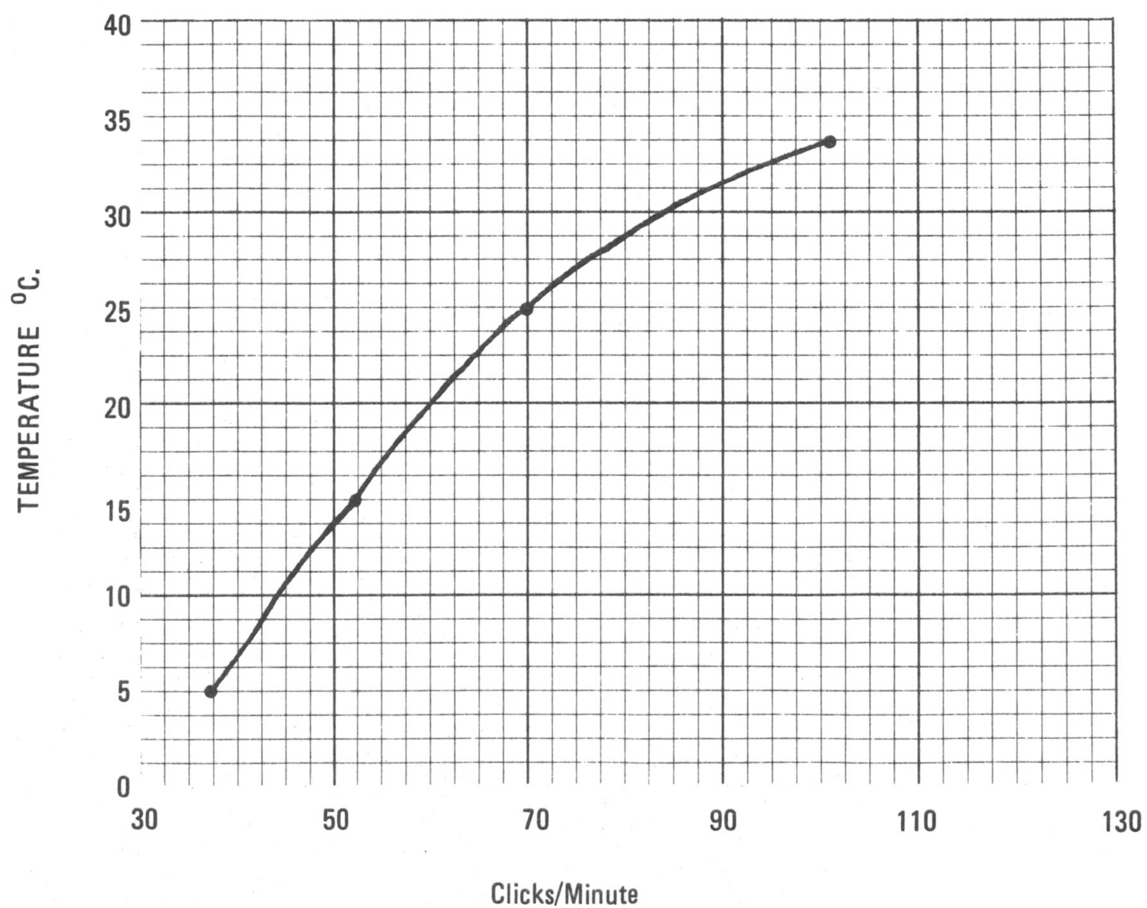

Figure 2. Example of a typical calibration curve for the Mini-Mitter radio thermometer.

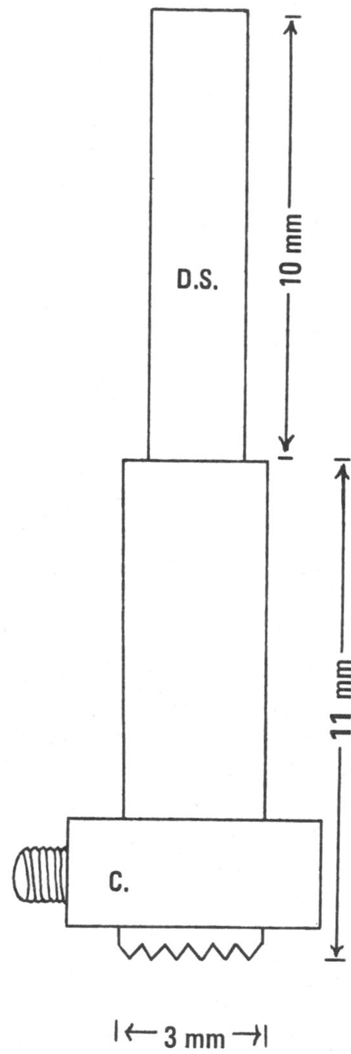

Figure 3. Diagram of the drill complex. C., collar (outer diameter, 6 mm; inner diameter, 3.5 mm); D. S., drill shaft.

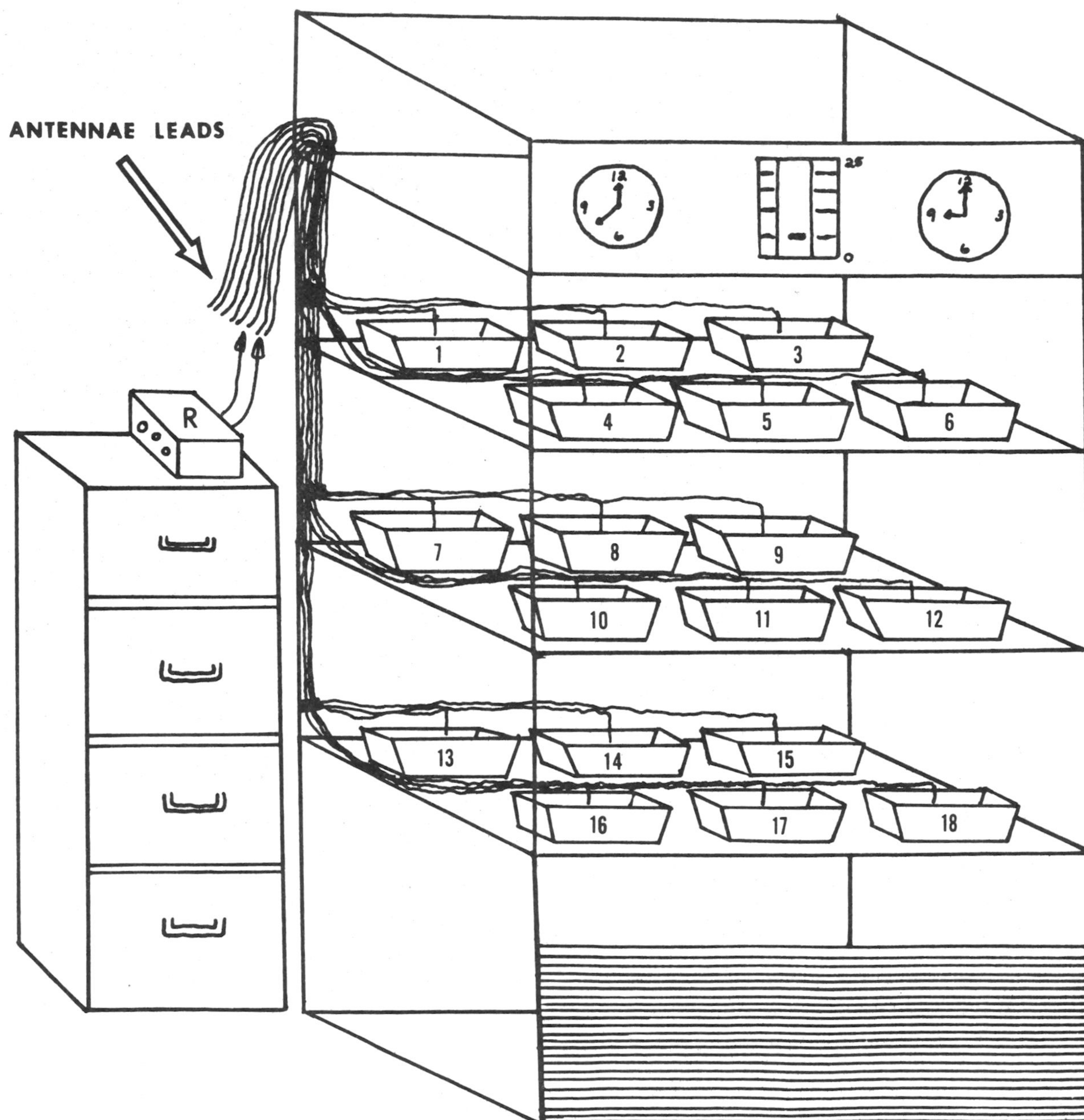

Figure 4. Diagram of the eighteen cages within the environmental chamber, showing the antennae circuitry and positioning of the numbered external leads. R., AM radio receiver.

RESULTS

On the sixty-second day of the experiment a chamber malfunction prematurely terminated the investigation. Statistical analyses were therefore limited to daily temperature records and changes in body weight which preceeded that day (February 19 through April 21).

Autocorrelation analyses were employed to determine patterning within individual temperature response records. These patterns could then be utilized for between group comparisons. The autocovariance values which were derived however, do not indicate a rhythmicity within individual response records and as such, no between group time series comparisons were possible.

Temperature records were further analyzed using regression statistics. A "line of best fit" was established for the complement of temperature responses, from day one through day sixty-two, for each of the three groups. Temperatures considered to be indicative of ongoing hibernation ($< 25^{\circ}C$) were excluded from these analyses. It is evident from the slopes of these regression lines (group I $b = 0.0$; group II $b = -0.02218$; group III $b = -0.03038$) that a similar trend of decreasing temperature exists in the sham-operated (group III) and the pinealectomized-no melatonin replaced (group II) animals. The pinealectomized-melatonin replaced (group I) animals show no such negative trend. Hoffman (1968), in summarizing

the available data on hamster preparation and entry into hibernation, states that cheek pouch temperatures remain essentially constant prior to the initiation of torpor. The discrepancy between these findings and the negative slopes established for groups II and III is not readily explainable; nor is the apparent incongruity of the group II and group III similarity in response.

Both the initial and terminal body weights of the animals were recorded and an analysis of covariance used to investigate the significance of the overall weight losses noted (Table I). Weight analysis did not include the data for three of the eighteen animals due to their abbreviated stay in the chamber (group II, number 6 died April 11; group III, numbers 5 and 6 entered chamber on March 3). Numerous authors have reported body weight losses of up to 40% in hamsters preparing for hibernation (Lyman, 1948; Farrand, 1959; Panuska and Wade, 1958) and those findings are herein corroborated. The effect of differing treatments upon the weight changes noted was shown, however, to be insignificant ($P > 0.50$).

Table I. Analysis of relative weight (g) loss between experimental groups.

Animal No.	Group I Pinealectomized with Melatonin Replacement		Group II Pinealectomized		Group III Sham operated	
	Init. wt.	Term. wt.	Init. wt.	Term. wt.	Init. wt.	Term. wt.
1	130.5	107.0	110.5	99.0	115.0	91.0
2	120.0	102.0	128.5	107.0	137.0	96.5
3	105.5	110.0	113.5	104.5	140.5	108.0
4	105.4	97.0	111.5	99.0	132.5	113.5
5	111.5	98.0	94.5	81.0	133.0	nd
6	138.0	119.0	121.5	nd	119.2	nd
	$\bar{X}_A = 118.5$	$\bar{Y}_A = 105.5$	$\bar{X}_B = 111.7$	$\bar{Y}_B = 98.1$	$\bar{X}_C = 131.2$	$\bar{Y}_C = 102.2$

Accept H_o

$P > 0.50$

DISCUSSION

Several aspects of this investigation warrant further consideration. Foremost of these is the basic design of the experiment. The use of three experimental categories (gland removed, gland removed with hormone replaced and sham-operated) is a standard design used in endocrine studies. It is felt however, that a fourth, unoperated control group would have been of considerable value, in that this group would have allowed an analysis of the impact of the surgical procedures. This additional parameter was however, not included in the present study due to both financial and spacial limitations.

The choice of hormone replacement technique was also of particular concern. The administration of exogenous melatonin through the implantation of time release silastic capsules is a recent approach (Turek *et al*., 1975) to the ongoing problem (Marczynski *et al*., 1964; Barchas *et al*., 1967; Binkley, 1974) of administering physiological rather than pharmacological dosages. This mode of administration seems superior to previous methods (injection or implanted beeswax pellets) in that it allows the introduction of the hormone in determinable dosage at a relatively constant rate for protracted time periods (Turek *et al*., 1975). This chronic administration does not however, entirely mimic the apparently fluctuating secretions of the intact pineal, but it does eliminate the trauma of repeated animal

disturbance which was critical to the success of the present study.

In order to reduce animal disturbance during the bi-weekly cage maintenance sessions a photographic safelight (Kodak filter designation Dark Red - No. 2) was used. Entry into the chamber was done at night and all other lighting was turned off prior to the opening of the chamber door. The safelight used transmits only those wavelengths of light greater than 650 Nanometers (Kodak, 1970), thus excluding all but the red range of the visible spectrum. This lighting was used as it has been shown that deep red light has little or no effect on the rhodopsin visual pigments characteristic of retinal rod cells (Morton and Pitt, 1969). These visual pigments predominate in the retinas of nocturnal animals (Romer, 1971) and it is probable that this is the case in the golden hamster.

Relative humidity as an environmental aspect affecting hibernation is seldom, if ever, considered. During the first two weeks of April a recording humidity gauge monitored the air within the environmental chamber used in this experiment. It was found that the relative humidity within the chamber was rather high (90%) and generally stable throughout the two weeks monitored. This elevated humidity was to be expected considering the number of animals and the confinement of the chamber. Its effect on the incidence of hibernation is however, unknown. It should be noted

that during this period two of the experimental animals did successfully go into hibernation.

Prior to the malfunction of the environmental chamber, three of the eighteen animals had entered hibernation (one group I-melatonin replaced entered on day 27 and two group III-controls on days 38 and 39) and one died (group II-pinealectomized-no melatonin replaced on day 41). With the exception of brief periods of arousal (every two to three days) the hibernating animals were still in torpor when the experiment was prematurely terminated. As to whether or not others of the animals would have eventually gone into torpor cannot be determined. The one animal which died warrants further mention in that prior to its death it exhibited an unusual series of temperature responses. During the five days preceeding its death its temperature was stable at approximately 27 to 29°C ($\simeq$ 5°C drop). This considerable drop in body temperature may represent an attempted entry into hibernation which was unattained and which eventually proved lethal. As a result of the above mentioned chamber malfunction, sixteen of the remaining seventeen animals were also to ultimately die in the chamber.

An interesting occurance regarding the silastic implants was discovered upon removing them from the hamsters following the termination of the experiment. Of the eighteen implanted capsules, sixteen were retrieved in a

seemingly unmodified condition, but two of them were found to be extremely deteriorated (one containing melatonin and one empty). This deterioration is of particular concern as Dow Corning Corporation manufactures this material for use in human surgical implants and describes it as "essentially nonreactive to body tissue or fluid ... will not support bacterial or common fungal growth" (Dow Corning Bulletin, 1973). It is assumed that some aspects of the dead animals "body tissues or fluids" were somehow different than those in the living state and that these modifications were responsible for the capsules degradation.

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