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THE RELATIONSHIP OF WET WEIGHT AND LACTATE CONCENTRATION TO AGE IN
THE CRYSTALLINE LENS OF PEROMYSCUS MANICULATUS BAIRDII

by

ROBERT JAMES KINDERS

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Sciences

June

1975

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Kirt Vener for his constant availability and guidance in the preparation of this thesis. Thanks are also due to Dr. Albert Rotermund and Dr. Jan Savitz for their aid in the respective areas of biochemistry and biometrics. Special thanks are also due to Mark and Suzanne Kinders for their help in preparation of the manuscript and to Colette Burns for moral support.

VITA

The author, Robert James Kinders, is the son of Robert Kinders and Clara (Marek) Kinders. He was born February 12, 1948 in Chicago, Illinois.

His elementary education was accomplished at St. Richard School, Chicago, Illinois, and his secondary education at St. Laurence High School, Burbank, Illinois, where he was graduated in 1966.

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Following graduation he was inducted into military service, and was honorably discharged from the Army in May, 1973. In September, 1974, he was granted an assistantship in biology at Loyola University.

His publications are:

with Benedict Jaskoski: DDT and Methoxychlor in a Frog population in Cook County. Trans. Il. State Aca. Sci., 1974. 67:341-344;

with Kirt Vener: Continuous remote body temperature monitoring. Physiol. Teacher, 1974. 3:1-4.

In May, 1975, he presented two papers before the American Zoological Society, of which he is a student member:

The effects of aging on lactate concentration in the crystalline lens of Peromyscus maniculatus bairdii, with Kirt Vener;

Lactate and LDH isozyme distribution in the crystalline lens of Mus musculus and Peromyscus maniculatus bairdii as a function of age: A preliminary report, with Kirt Vener, Daniel Linert and Colette Burns.

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INTRODUCTION

The intent of this research is to describe an accurate method of age determination in small mammals. This necessitates the defining of parameters against which the age of an unknown mammal can be accurately estimated. The variables that will be examined as possible parameters are the wet weight of the crystalline lens, and the concentration of lactate in the lens, in mammals of known age. If one or both of these variables is to be used for age determination, statistical analysis should demonstrate that any patterns found within the variables are a population wide phenomenon. Therefore, any deviations from an established pattern should be predictable using an equation or standard. If this can be accomplished the variables can be used as parameters for age determination. The present experiments attempt to demonstrate that the concentration of lactate in the crystalline lens of the eye, and wet lens weight, are usable parameters for age determination in small mammals.

The accurate determination of age is important in population ecology because many of the models used for determination of population growth and structure require an age breakdown of the population (Krebs, 1972). In addition to an immediate practical use in population studies and management, the finding of a true aging parameter would be valuable information in the aging field. Finally, the information in this report should be useful in clarifying the biochemistry of the crystalline lens.

LITERATURE REVIEW

THE LENS AS AN AGING PARAMETER

For a number of years, the crystalline lens has been used by researchers as an alternative to dentition and bone fusion studies for age determination. One of the most common techniques is use of the lens growth curve, using either dry or wet lens weight correlated with age. These techniques have met with varying success, and are reviewed by Friend (1967).

The pioneering study of lens growth curves for age determination was performed by Lord (1959) using captive cottontail rabbits, Sylvilagus floridanus mearnsii. His study of dry lens weight (correlated with age to 30 months) showed that individual variability was too great to separate age differences of less than one year. Two other pieces of valuable information were obtained in this experiment: that a poor diet resulted in retarded lens growth rates; and that wet weight variability between lenses of the same rabbit was no more than 3.9%.

Berry and Truslove (1968) studied dry lens weight after alcohol fixation in Microtus arvalis and Mus musculus strains C57BL/Gr, CBA/Gr, and C3H/HRH, and in a wild population of M. musculus. The lens growth curve was usable in aging Microtus when the Log_{10} of lens weight was plotted against an inverse age function. However, in M. musculus, lens weights were so variable as to be unusable. Lens weight to body weight ratios were also too variable for use in age determination. Statistical analysis showed no significant difference between weights of left and right lenses

taken from the same animal.

In studies on a population of Michigan cottontail rabbits, Dudley (1965) concluded that lens growth curves could be used to determine age with the formula

$$\text{Log}_{10} \text{ Age} = .534102 + .0054579 (X)$$

where age is given in weeks and X is the lens weight in milligrams.

However, there is a serious problem with these results. The purpose of Dudley's experiments was to obtain data for a wild population of animals. Consequently, the actual age of the animals was unknown, but only estimated from knowledge of the natural history of the animals and other biometrical techniques. This contradicts one assumption of linear regression analysis theory, that the error of the variables must be known to validly predict the value of one variable from the other. Clearly, the error of the age estimate is not determinable in Dudley's experiment. The alternative to this design is to use animals of known age from a laboratory population. Unfortunately, laboratory populations are not wild, and their gene pools tend to deviate from those of wild populations of the same species. This problem can be overcome by avoiding the use of inbred strains, and by using whose parents were wild, or whose near ancestry was wild, and in which populations inbreeding was avoided. Thus we can see Dudley's dilemma.

Kirkpatrick (1966), studying lens growth curves of the cotton rat, Sigmodon hispidus, found definite contradictions in data obtained from wild versus laboratory populations. He speculated that diet was responsible. One could argue the point that he was dealing with two populations, hence two gene pools. In this case, anomalies in the laboratory popula-

tion due to inbreeding and lack of gene dilution may be more important than dietary differences. However, Berry and Truslove (1968) found no differences between growth curves of wild and laboratory populations of M. musculus. This thoroughly confuses the issue, suggesting that a good experimental design should consider both genetic and dietary factors. The question of applying data obtained from a laboratory population to a wild population is still a valid one, and so there may not be a way out of Dudley's dilemma.

Myers and Gilbert (1968) performed an elaborate study of a wild Australian rabbit population using 924 wild rabbits aged by biometrical techniques and 199 rabbits of known age from a captive population. Their data analysis regressed age on dry lens weight, using age in days and weight in mgm. Their successful equation was

$$\text{Age} = \frac{-b + c}{\text{Log}_e (a/w)}$$

where a is the asymptote of the lens weight growth curve, b is the Y-intercept, c is the growth curve slope and w is the weight of the lens. This equation could be used accurately only to separate young, one year and two year old animals. In addition, their data agreed with Lord (1959), that the growth of the lens is moderately sensitive to environmental changes.

Connolly et. al. (1969a and 1969b) used growth curve transforms of lens weight to devise linear regression formulae to determine age of Black-tailed Jack Rabbits, Black-tailed Deer and Mule Deer. In all cases this method was more accurate than aging methods using bone suture closure techniques, but in no case could 95% confidence limits be attained beyond age two years. A major deficiency of the technique was a decreased

precision with increasing age, although the source of the loss of precision was not traced. In addition, successful determinations were limited to one year intervals. The data and the aging equations for the Jack Rabbits proved significantly different from that obtained from a Kansas population, indicating a variance in the actual numerical data from population to population.

Fisher and Perry (1970), performing studies of lens weight to age curves of 885 gray squirrels, Sciurus carolinensis, were able to distinguish age differences of one year. No statistical differences were found between sexes or between the two lenses of the same animal.

Erickson et. al. (1970) evaluated the three major techniques currently in use for age determination, with Mule Deer as the selected animal. The techniques, molar tooth ratios, visual evaluation of molariform tooth wear and regression of lens weight on age, all proved unacceptable for use with 95% confidence limits for year age-class determinations.

Summarizing the available data on mammals, the following points are apparent:

- 1) 95% confidence limits are not available by use of lens weight growth curves;
- 2) the best available techniques in general usage regress lens weight on age to separate animals into age classes by year;
- 3) no significant statistical difference between growth curves of the two lenses of the same animal are apparent;
- 4) no evidence is available for differences in lens growth rates between sexes;
- 5) some evidence is present for dietary effects on growth of the lens,

although the lens is affected less than other organs;

- 6) the exact growth curves that are obtained are not only species specific, but population specific;
- 7) the accuracy of prediction of age from a regression analysis decreases with age, due to a loss of precision;
- 8) the most rapid and most predictable period of lens growth is during the first year of life.

Use of lens growth curves for age determination is not limited to mammals. Indeed, the most rigorous analysis of this technique in the literature was undertaken by Fink, Hockwin and Weigelin (1968), who used cockerels in the first year of life. By analysis of variance, they showed that variance of lenticular weight between two eyes of one cockerel was significantly less than that between lenticular weight of several cockerels of the same age. However, analysis of variance could not separate variance sources completely, due to a positive skewness of the data. This skewness was traced to the technique of combining lenses of several animals of the same age.

Non-linear regression (multiple regression in this case) was used to attempt age inferences from lens weight. Curves generated from this technique (regression of age on lens weight) differ from those generated by regressing lens weight on age. This curve is also less predictable (90% confidence limits versus 95% confidence of lens weight on age). The loss of precision can be attributed to the reversal of analytical techniques between the observational process and the application of the experimental conclusions. Another conclusion of the study is that the confidence of the predictions can be increased by increasing the number of

parameters used for prediction over prolonged time periods of measurement, provided that the parameters can be shown to be correlated with aging. Finally, the loss of precision in prediction of age from lens weight was statistically traced to increased variability in lens weight of older animals, and the tendency of the curve to become asymptotic.

A different approach to age determination using lens growth has been introduced by Dapson and his colleagues (1968, 1972). This technique measures aging rather than lens growth. The rationale is measurement of accumulation of soluble protein, which is considered to be growth, and of insoluble protein, which is considered aging. This technique has been used as an aging parameter by gerontologists (Walford et. al., 1969). The insoluble and soluble fractions were measured colorimetrically as tyrosine concentrations, and protein determinations were performed by Lowry's method. The study demonstrated three key advancements over previous methods:

- 1) 95% confidence limits could be attained;
- 2) animals could be separated by age into groups readily identifiable at three month intervals;
- 3) the loss of precision of the method with increasing is overcome by use of the ratio.

A serious disadvantage of this method is the requirement of a cold centrifuge (10,000 - 14,000 G) to obtain fractions, a tool which may not be readily available to the field investigator. The technique is also rather time consuming. Recent work by Harding (1972a, 1972b) indicates that insoluble protein in the lens fractions is an artifact, making Dapson's technique challengeable.

In a short paper, Scuderi et. al. (1973) reported that lactate concentrations in rabbit lenses decreased with age. This finding was peripheral to the intent of their research, and so statistical analysis was not supplied for this data. A more complete study by Hockwin et. al. (1974) demonstrated that lactate concentration does indeed decrease with age in bovines, and that this change is due altered carbohydrate metabolism with age.

THE BASIC ANATOMY AND PHYSIOLOGY OF THE CRYSTALLINE LENS

The crystalline lens of vertebrates is a fascinating tissue and is uniquely suited to research. While it represents a highly evolved and complex tissue, it is at the same time relatively simple. This simplicity resides in the fact that it is composed of only one cell type, which is present at various stages of development (Kronfeld, 1969). The latter attribute makes the lens especially desirable for aging studies, since one may view lens aging in terms of only a single lens, studying the various cell types present, or in terms of many lenses of different ages, all with the same basic pattern of development.

The basic lens cell is the epithelial cell, which is low cuboidal, clear, nucleated, and which contains the characteristic vertebrate sub-cellular particles (van Heyningen, 1969). The lens grows throughout life, never shedding cells. The point of growth is at the equatorial zone of the anterior lens, in the epithelial layer. These cells may divide, spreading outward over the surface of the lens, forming new epithelium. Alternatively, they may become presumptive fiber cells which begin differentiation, losing their nuclei, mitochondria, and other organelles, and

begin laying down the fibrous tissue characteristic of older lens cells (Riley and Devi, 1967; Young and Fulhorst, 1969a, 1969b; Waley, 1969; van Heyningen, 1969a). Using ^3H -thymidine, Riley and Devi (1967) determined the turnover time of the epithelial cells to be 23 days peripherally and 198 days centrally.

The epithelium is known to contain transport enzymes as well as being the point of origin of lens growth. Kinoshita et. al. (1961) demonstrated the presence of an ouabain inhibited Na/K ATPase in the lens epithelium. Larger molecules also have been shown to be actively transported by the lens, such as myo-inositol and various amino acids (van Heyningen, 1969a; Cotlier, 1970). At this time, however, the exact location of this activity is not known.

The cortical region of the lens, that area just beneath the epithelium, is the region of this cellular transformation. Since the epithelium continuously divides without shedding cells, the new cortical fibers are accumulating on top of older ones, and the cortical region in effect moves outward. Centrally, those fiber cells which have completely differentiated assume a rod-like appearance in regular arrays. This characteristic repeating structure is used to delimitate the outermost boundary of the nucleus of the lens, which like the cortex, is always growing outwards (Cohan, 1965; Kobayashi, 1969; Kronfeld, 1969; Leeson, 1971).

The lens is completely enclosed in a capsule which is laid down in two stages, the first of which is characterized by a large increase in surface area due to increased lens volume. The second stage is characterized by increased thickness of the capsule (Fisher and Pettit, 1972). The capsule is a complex acellular matrix composed of collagen, hexose-

amine, sialic acid, hexuronic acid, and various mono- and disaccharides. The percentage composition varies on a species basis, but the representative compounds are remarkably constant. The collagen present is of a non-fibrous type similar to that found in other extra-cellular membranes, such as Descemet's membrane (cornea), Reichert's membrane (yolk-sac), and the glomerular basement membrane. Indeed, the lens capsule is underlaid by its own basement membrane (Fukuguski and Spiro, 1969; Spiro and Fukuguski, 1969). The capsule is permeable to sugars, amino acids and smaller metabolites. Active transport apparently takes place across the lens capsule. Cotlier (1971) demonstrated the activity of an ouabain sensitive Na/K ATPase in the capsule, and that transport of lysine into the lens was dependent on activity of this enzyme. Takeguchi and Nakagaki (1970) demonstrated the presence of membrane potentials across isolated bovine lens capsules in vitro, implying that the capsule is the site of at least some of the enzymes.

The lens has no direct vascular supply after the optic cup splits from the ectoderm of the embryo. Nutrient and gaseous exchange take place via the aqueous humor, which is secreted by the ciliary body and absorbed through the scleral venous sinus (Arey, 1968).

The transparency of the lens has long been attributed to its highly ordered structure (Cohan, 1965; Leeson, 1971). The orderliness of the structure resides in the arrayed, hexagon-shaped fiber cells. The fiber cells of the cortical and shallow nuclear sections are composed of three complex, soluble structural proteins, known as alpha, beta and gamma crystallins. The sclerotic, insoluble protein of the deep nucleus is called "albuminoid" and is insoluble even in 7M urea (Clark et. al., 1969;

Zigman et. al., 1969; Hunter et. al., 1970).

Recently, some of the ultrastructure of these proteins has been elucidated. Maisel and Perry (1972) studied chick lens fiber cells by analytical ultracentrifugation and electron microscopy. They determined that the alpha and beta crystallines combine to form particle chains and filaments, which complexes represent the major structural subunits of the lens. The particles are 12-15 nm in diameter, the filaments 7-9 nm in diameter, and straight segments are 12-14 nm in diameter. Continuing the work with chick lens, Truman et. al. (1971) estimated the molecular weights of beta crystallin at 59,000 daltons (d), composed of eight possible subunits of molecular weight 16,500 d, with any four comprising a polymer. The gamma crystallin was estimated at 158,000 d, composed of any four of six different monomers of molecular weight 25,000 d.

The "albuminoid" protein of lens deep nucleus is an extremely large insoluble protein. Harding (1969) proposed that the albuminoid fraction arose from condensation of alpha, beta and gamma crystallin subunits by disulfide bond formation. The major evidence for this theory is indirect. Young and Fulhorst (1966a and 1966b), chasing ³⁵S-methionine incorporation into structural protein over a 24 hour period, found that 90% of the methionine appeared in crystallin of peripheral fiber cells, and about 2% in albuminoid. Once the labeled methionine became incorporated into lens protein, it never appeared as waste products in the vitreous. Many age related structural changes occur in the lens, but discussion of this topic is deferred until after a review of lens biochemistry.

The lens was for quite a long time thought to be a passive, non-respiring organ (van Heyningen, 1969a). The classical example of this viewpoint is Rabaey's 1964 study of glycogen in bird lenses. Still influenced by the old views of the lens, Rabaey postulated that the purpose of the glycogen was to serve as a transparent structural element of the lens. This point of view is no longer held; the lens is known to be a metabolically active organ.

Studies of the lens over the past 75 years have been motivated primarily by the desire to find the cause and cure of lens cataract. It is not surprising, therefore, that little research exists on lens metabolism per se; most of it is related to various theories of cataractogenesis. A better understanding of this section may be obtained from a succinct but clear and informative review by van Heyningen in Davson's 1969 work, The Eye, which this researcher has found invaluable.

The lens is a somewhat easier organ on which to perform metabolic studies than most, because it contains its own sensitive metering system, its transparency. Kinoshita and Wachtl (1958) showed that any inhibition of carbohydrate metabolism in the lens results in opacification. The isolation of all the enzymes and products of the Embden-Meyerhoff pathway was completed in 1955 (Green et. al., 1955a, b, c). This was the first complete metabolic pathway demonstrated in the lens, and it was followed by identification of enzymes and products of the pentose phosphate shunt, the sorbitol pathway, the Krebs cycle, and the electron transport chain.

The only free monosaccharide present in the aqueous humor which the lens can use as an energy source is glucose. The glucose concentration of the lens closely parallels that of the aqueous. The glucose concen-

tration of the aqueous, in turn, is approximately 90% of that of the blood. Ordinarily, rapid fluctuations of glucose level in the aqueous occur only in cases of diabetes; either extremely low or high concentrations will cause cataractogenesis (Chylack, 1971). Elbrink and Bihler (1972) demonstrated that glucose readily enters the lens by facilitated diffusion and should not be a limiting factor in metabolism. There appears to be no way for the lens to regulate glucose availability to the glycolytic chain, however. In vitro studies of rat lens showed that insulin can increase glucose utilization by only 7 to 20% (Levari et. al., 1964; Patterson and Bunting, 1965) and this effect is neither at the glucose entry point, nor at the glycolytic enzymes. However, it is known that 3',5'-cAMP effects lens glycolytic rates. Glycogen deposits have been measured in lenses of many species, including both mammals and birds. The amounts present are variable from species to species and decrease with age (Hockwin, 1973).

The relative importance of the various pathways in lens metabolism is not as well known as in other tissues. Anatomical and biochemical studies have demonstrated that aerobic respiration in the lens is limited to superficial layers, and is of secondary importance in energy metabolism (Kinoshita and Wachtl, 1957; Virmaux and Mandel, 1963; Cohan, 1965; Chylack, 1971; Leeson, 1971). Under normal concentrations conditions the primary energy source in the lens is anaerobic glycolysis. Estimates of the percentage of energy produced in the lens ranges from 10 to 30% for aerobic processes, and the remainder from anaerobic processes (Chylack, 1971; Trayhurn and van Heyningen, 1971). An early in vitro study (Kinoshita and Wachtl, 1957) of the stoichiometry of lens glycolysis

demonstrated the production of 41 micromoles of lactate from 27 micromoles of glucose. In fact, many of the prominent investigators in the field (Kinoshita, Chylack, van Heyningen) do not consider aerobic metabolism at all important in lens energy production. Trayhurn and van Heyningen (1971) inhibited lens aerobic metabolism with KCN, amytal and nitrogen in the absence of glucose with loss of clarity only in the two former cases. In the case of the nitrogen atmosphere, clarity was restored when glucose was added to the medium. By this criterion, the lens is seen to be strongly anaerobic.

The activity of the sorbitol pathway, hexosemonophosphate shunt and the pentose phosphate shunt are all closely related and dependent on the ratio of NADP to NADPH in the cytoplasm (Chylack, 1971). The K_m of aldose reductase is so high that under physiological glucose concentrations all these pathways are inactive in the lens.

The work of several researchers (Young and Fulhorst, 1966a and b; Riley and Devi, 1967; Elbrink and Bihler, 1972) showed that considerable protein synthesis takes place in the lens, without loss of the usual catabolic pathways for amino acids. In vitro glucose deprivation resulted in increased ammonia concentration, indicating proteolysis to provide energy. Apparently, glycogen concentrations were not adequate to maintain lens clarity.

Regulation of lens metabolism is primarily a problem of regulation of lens glycolysis. It has been argued that glycolysis does not take place in deep fibers of the lens. Enzyme assays indicate that the limiting factor is probably enzyme concentration in the nucleus, but not in the shallower layers, where high levels of enzymes are available

(patterson and Bunting, 1963; Harding, 1969; Hockwin, 1973). Mitochondria have not been found in the nucleus, but only in the epithelium and shallower cortical layers, where oxygen would be available (Cohan, 1965; Leeson, 1971).

Primary regulation of anaerobic glycolysis is accomplished by hexose kinase (Lou and Kinoshita, 1967), and the maximum rate of lens glycolysis is a direct function of hexose kinase concentration (Patterson and Bunting, 1963; Chylack, 1971). A secondary control point is known to be phosphofructose kinase (PFK), in which the amount of ATP, ADP and AMP control the activity of PFK. On inhibition of PFK, glucose-6-phosphate builds up, inhibiting hexose kinase (Lou and Kinoshita, 1967; Chylack, 1971).

A long term regulation of glycolysis may be accomplished by a change in distribution of lactate dehydrogenase isozymes. Stewart and Papaconstantinou (1966) demonstrated a change in LDH isozyme activity with age in bovine lens cells. The cathodal isozymes (M4), which are usually associated with anaerobic tissues, are present in calf epithelial cells. In calf cortical cells, the intermediate forms of isozymes appear. In cortical cells of adult cattle, the more anodal (H4) isozymes appear most active. These isozymes are usually associated with aerobic tissues. The reason for this change is unclear, but may be regulatory (Markert and Moller, 1959; Everse and Kaplan, 1973).

A great deal of ambiguity and variability regarding lens metabolism can be traced to the different organisms used in the various studies. These range from chickens to mice and rats, cattle and man; many of the important studies have been performed on cattle. For example, glucose dehydrogenase is present in man and cow, but not in rat and rabbit; this

eliminates the pentose phosphate shunt as a metabolic pathway in the latter two (van Heyningen, 1969).

AGE RELATED CHANGES IN THE LENS

There are numerous studies demonstrating age-related changes in the structure and metabolism of the crystalline lens. Most of the studies are concerned with one of two problems: that of age determination in animals, or the cause and cure of senile cataract.

The structural changes in the lens are striking. In addition to the continually increasing growth cited above, there is a change in the structural protein with aging. The distribution of the proteins of the lens shift with age, alpha crystallin increasing, while beta crystallin remains about constant and gamma crystallin decreases. The sum of the three increases with age. Also, there is a steady increase in the amount of insoluble protein ("albuminoid") with aging, which is quite different from the increase in structural proteins that is usually termed growth (Dapson et. al., 1968; Waley, 1969; Dapson and Irland, 1972).

During the aging process, the nucleus of the lens not only becomes larger, but denser, losing elasticity and transparency, and light scattering increases (Zigman et. al., 1969). Concurrent with this, the nucleus undergoes sclerosis. The deep lenticular fibers become irregular in shape and array. The limiting membranes of the fiber cells become indistinct, with increasing interdigitation between adjacent fibers. Many other degenerative changes are observed with the electron microscope (Cohan, 1965; Leeson, 1971). This process closely accompanies the increase in the amount of albuminoid present in the lens. Interestingly,

analysis indicates that this albuminoid is nearly 100% gamma crystallin, insolubilized by crosslinkages, primarily disulfide bond formation. By the time this albuminoid has increased to large concentrations in the nucleus, the soluble protein has changed so that almost 50% of it is gamma crystalline (Dapson et. al., 1968; Harding, 1969 and 1972; Zigman et. al., 1969; Leeson, 1971; Satoh, 1972). Broekhuysen (1969) has shown that there is a shift in the phospholipid constitution of the lens with aging. Apparently, sphingomyelin is synthesized throughout life, while other phospholipids are not. Thus the concentration of sphingomyelin increases while that of all other measured phospholipids decreases with age.

Age related changes in the lens capsule have also been documented. After the initial increase in area due to lens growth, the capsule begins to thicken (Fisher and Pettit, 1972). There appears to be a change in the capsule protein with age, since ratios of various amino acid constituents change markedly with age, such as hydroxylysine:lysine and hydroxyproline:proline, which increase with age (Fukuguski and Spiro, 1969). Evidence is accumulating that the metabolic changes accompanying the obvious physical changes are quite extensive.

The rate of anaerobic glycolysis may be age dependent. There is a fair amount of evidence to support this position. Chylack et. al. (1970) showed that lens hexose kinase activity decreases with age. There is also a transition in LDH isozymes with age: the younger cells contain the more cathodal forms (M4) which are active in the presence of high levels of lactate (K_m of 6.6×10^{-3} M in chick), while the older cells contain more anodal forms (H4), which are inhibited by high levels of lactate

(K_m of 3.3×10^{-4} M in chick). Scuderi et. al., (1973) and Hockwin et. al. (1974) found decreases in lactate concentrations with age, while a decrease in ATP levels and ATP:ADP ratios indicate that there is probably something more happening here than a switchover in metabolism.

Hockwin (1965, 1974) has shown age related increases in the specific activity of fructose diphosphatase with age while activity of glucose-3-phosphate dehydrogenase, glucose dehydrogenase, phosphoglyceric mutase, enolase and pyruvate kinase decrease with age. However, PFK activity increased and concomitantly there is a decrease in ATP levels with age (in calves, rats, chick) which may relieve inhibition of PFK. Work by Sippel (1965) confirms Hockwin's findings that metabolic activity of the lens in general decreases with age.

Accompanying the decrease in anaerobic processes, a decrease in aerobic metabolism seems to be associated with age, at least on a per cent basis. As the lens ages, it increases its surface to volume ratio without increasing the amount of mitochondria proportionately. In addition, the changed surface to volume ratio makes oxygen supply to the deep elements of the lens questionable (Trayhurn and van Heyningen, 1971). This is very strong contradictory evidence to the theory that lens metabolism switches from anaerobic to aerobic with age. Hockwin (1965) also found an increased concentration of HMP intermediates with aging, possibly indicating a partial block of glycolysis.

Probably there is a decrease in all forms of energy metabolism with age. These changes are significant in the observed structural changes and the tendency towards senile cataract observed with lens aging.

MATERIALS AND METHODS

The animals used for this research were Peromyscus maniculatus bairdii trapped wild specifically for this project. The mice were live-trapped in the following forest locations, all in Illinois: Swallow Cliff Woods, Saganashkee Slough, Spears Woods, all in Cook County, and The Mississippi Palisades State Park, in Jo Daviess County. Animals were identified using the criteria of Hall and Kelson (1959), King (1968), and Burt (1972). For the purposes of this experiment, all mice of the strain P. m. bairdii in the state of Illinois are considered to be members of the same population.

Five pairs of wild mice were mated and the first three laboratory generations were used in data collection. The purpose of this approach was to have animals of exactly known age, with genotypes as close as possible to that of the original wild population. Animals were not inbred, to avoid possible deviations from the wild gene pool. It is believed that the resulting statistical analysis will be mathematically valid, and equally important, may be validly applied to members of the Illinois population of P. m. bairdii.

The mouse colony was maintained in a controlled environment. Temperature (68° F.), humidity (50%) and photoperiod (12L/12D) were constant for all animals. All mice were fed a diet of Purina Lab Chow and watered with tap water (chlorinated) ad libitum. Mated animals were kept in plastic cages on alfalfa bedding with cotton added for nesting material.

Unmated animals were kept in cages singly. Mice were selected as they reached the desired age (1, 4, 8, 12, 15 or 20 months), with equal numbers of males and females being used where possible. There was no other criterion for selection of mice.

Animals were killed by etherization and immediately weighed to the nearest .1 mgm on a piece of tared filter paper, using a Sauter type 424/11 analytical balance. A small incision was made in the cornea, both lenses were excised and rolled on bibulous paper to remove aqueous and vitreous humors, and weighed to the nearest .1 mgm. The lenses were then transferred to a Potter-Elvehjem tissue grinder and homogenized in 250 microliters of cold physiological saline in an ice bath. Composition of the saline is listed in Table I of the Appendix. After homogenization, 750 microliters of 8% perchloric acid was added to precipitate all protein, and the sample was transferred to a centrifuge tube and spun down for 10 minutes at 5000 rpm on an International Clinical centrifuge. Pipetting was done with Sigma micro pipettes.

Enzymatic analysis of supernatant for lactate was performed at 340 nm on a Beckman DB-GT spectrophotometer. The protocol for the assay was that of the Sigma Chemical Company (1968). Figure 1 summarizes the reaction sequence. Purified bovine LDH, beta-NAD and glycine buffer (pH 9.2) containing hydrazine were all obtained from Sigma for the assay.

The data analysis can be grouped into three sections: sex differences testing, construction of a prediction curve and equation for animals of unknown age, and analysis of the relationship of metabolic activity (milligrams lactate) to lens growth (milligrams wet lens weight). Testing was designed prior to the experiment to provide data in optimal form

Lens Homogenate + HClO_4 \longrightarrow Protein ppt↓

Centrifuge

Supernatant + NAD + LDH \longrightarrow LDH + Pyruvate + NADH
($\Delta\text{OD} @ 340 \text{ nm}$)

Pyruvate + Hydrazine \longrightarrow Pyruvate complex ppt↓

Figure 1. Lactate assay reaction scheme, Sigma^R technique. This reaction is specific for L(+) lactic acid.

for statistical analysis. The exact experimental procedure was followed in analysis of a laboratory population of Mus musculus C57BL/6. The resulting data proved most usable when grouped, and graphs were made of linear transforms of the variables.

Peromyscus were grouped by age at 1, 4, 8, 12, 15, and 20 months. Each animal was assayed individually, with both lenses of the mouse constituting a sample. This caused some problems in obtaining high enough concentrations for chemical analysis and weighing, but this was solved by manipulations of analytical techniques (for example, dilutions) to avoid problems of the type described by Fink et. al. (1968) and Young and Rickert (1973) (See Literature Review).

Analysis of variance (ANOVA) (Sokal and Rohlf, 1969) and multiple regression analysis were used, regressing age on wet lens weight and lens lactate concentration. The use of regression analysis as a method of estimation of population parameters is both valid and precedented (Goldberger, 1968). The selection of dependent and independent variables was done so that the predictions could be made using the known variables (lactate and lens weight) as independent variables, thus abiding by the assumptions of regression theory. Correlation coefficients are provided for the graphs. Sex differences were tested by analysis of variance (Model II, one-way) for lactate concentration and lens weight.

AGE (in months)	LENS WEIGHT (mgm)				
	<u>SUM</u>	<u>MEAN</u>	<u>STD. DEV.</u>	<u>SUM OF SQ.</u>	<u>N</u>
1.00	64.400	6.440	0.880	6.964	10
4.00	212.600	17.717	2.315	58.976	12
8.00	262.900	21.908	2.835	88.383	12
12.00	139.200	23.200	0.849	3.600	6
15.00	109.600	21.920	2.044	16.708	5
20.00	<u>95.100</u>	<u>23.775</u>	<u>2.012</u>	<u>12.148</u>	<u>4</u>
TOTAL	883.779	18.037	6.582	186.780	49

ANOVA TABLE

	<u>SUM OF SQUARES</u>	<u>DF</u>	<u>MEAN SQUARE</u>
Between groups	1893.0134	5	378.6025
Within groups	<u>186.7795</u>	<u>43</u>	4.3437
TOTAL	2079.7930	48	

$$F = 87.1611$$

$$F_{.05}(5, 43) = 2.45$$

TABLE 1. Relationship of lens weight (mgm) to age (in months). One-way analysis of variance. The weight differences between groups are significant with 95% confidence.

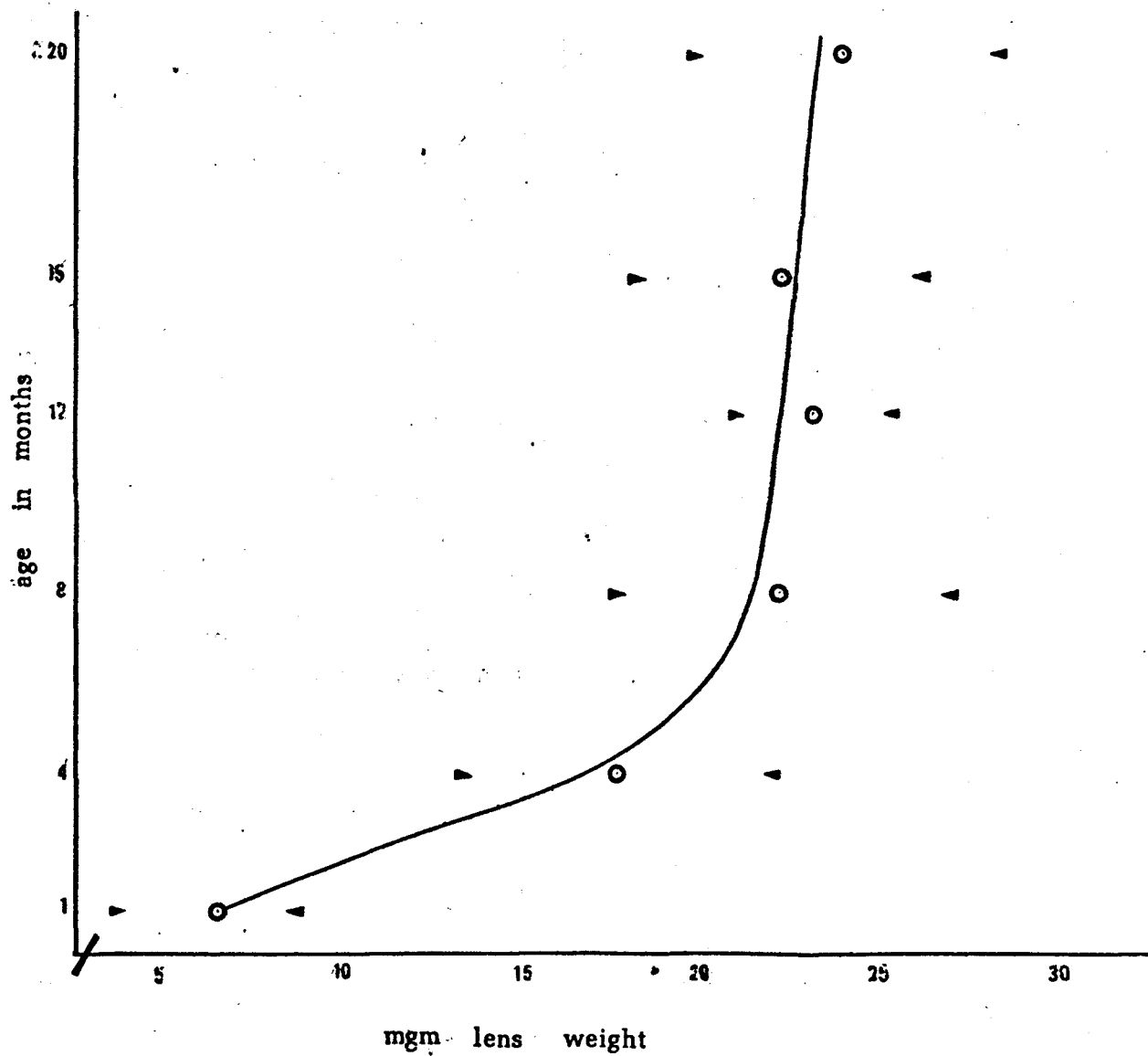


Figure 2. Relationship of wet lens weight to age in Peromyscus. Each circle represents a group mean. Arrows inclose the 95% confidence limits of the group means.

protein fraction is an artifact caused by oxidation of sulfhydryl groups in preparation of the fractions. Since the oxidizing capability of the atmosphere is not constant in all geographical locations, there is no reason to believe that the artifact would be constant from laboratory to laboratory.

The standard deviations of the lens weight as presented in Table 1 indicate that the variability of lens weight with age groups in Peromyscus is fairly constant using the wet lens weight technique, so that this method of reporting data seems justifiable. Analysis of variance demonstrates that there is a strong correlation of these variables, with the lens weight increasing until age eight months and levelling off thereafter. Although the sample population was divided into six groups, a posteriori testing (Table II, Appendix) shows that there are only three lens weight groups: 1 month, 4 months, and 8 through 20 months. Sex differences were also tested using ANOVA and there were no significant differences (Table 2). Errors of measurement of age were due to age grouping only, and all ages should be considered as plus and minus one week.

Regression analysis of age on mgm lens weight as the dependent variable are asymptotic (Figure 2). The curve characteristics do not lend themselves to application in predicting age. The relative constancy of wet lens weight and of the standard deviations of the last three age groups (12, 15 and 20 months) indicate that this technique does not suffer from the loss of precision reported by Fink, Hockwin, or others (1969) (see Literature Review).

The relationship of lactate concentration (in mgm lactate per lens

<u>SEX</u>	<u>LENS WEIGHT (mgm)</u>				
	<u>SUM</u>	<u>MEAN</u>	<u>STD. DEV.</u>	<u>SUM OF SQUARES</u>	<u>N</u>
Male	451.299	18.052	6.701	1077.762	25
Female	<u>432.499</u>	<u>18.021</u>	<u>6.601</u>	<u>1002.059</u>	<u>24</u>
TOTAL	883.798	18.037	6.583	2079.820	49

ANOVA TABLE

	<u>SUM OF SQUARES</u>	<u>DF</u>	<u>MEAN SQUARE</u>
Between groups	0.0195	1	0.0195
Within groups	<u>2079.8203</u>	<u>47</u>	44.2515
TOTAL	2079.8398	48	

$$F = 0.0004$$

$$F_{.05}(1,47) = 4.05$$

TABLE 2. Relationship of lens weight (mgm) to sex. One-way analysis of variance. The weight differences between groups are not significant with 95% confidence.

pair) is presented in Table 3. As with the lens weight data, this table shows that there are only three groups in terms of lactate concentration: 1 month, 4 through 8 months, and 8 through 20 months. The variances in these groups are significantly greater than those of the lens weight groups. This variation may be due to a change in lactate metabolism in these age groups, one which does not necessarily occur at the same rate in all members of the groups. At any rate, it is clear that by age eight months the lactate concentrations in the lens have about levelled off.

The samples were tested for sex differences using ANOVA, and no significant differences were found (Table 4). Regression analysis of the lactate data (Figure 3) shows that here, too, the relationship to age is asymptotic, and that the curve generated is not useful in predicting age.

In keeping with classical methods of reporting data, lactate to lens weight ratios were calculated and tested. The results are presented in Table 5. Analysis of variance demonstrates an age correlation with only three groups: 1, 4, and 8 through 20 months. Sex differences (Table 6) were not present. The graph of the ratio against age (Figure 4) is asymptotic, and regression analysis shows that this curve is not usable in predicting age.

The data of lens weight and lactate concentration versus age suggested that further statistical investigations could be valuable. The first operation performed was a regression of lactate (mgm) on wet lens weight (mgm). A strong correlation ($r = -0.745$) was found, as shown in Figure 5. The equation of the regression line is:

$$\text{mgm lactate} = 0.471 - 0.015 (\text{mgm lens weight}).$$

AGE (in months)	LENS LACTATE CONCENTRATION (mgm)				
	<u>SUM</u>	<u>MEAN</u>	<u>STD. DEV.</u>	<u>SUM OF SQ.</u>	<u>N</u>
1.00	4.019	0.402	0.153	0.209	10
4.00	1.962	0.163	0.079	0.068	12
8.00	1.717	0.143	0.044	0.021	12
12.00	0.821	0.137	0.029	0.004	6
15.00	0.540	0.108	0.052	0.011	5
20.00	<u>0.570</u>	<u>0.142</u>	<u>0.068</u>	<u>0.014</u>	<u>4</u>
TOTAL	9.629	0.197	0.135	0.328	49

ANOVA TABLE

	<u>SUM OF SQUARES</u>	<u>DF</u>	<u>MEAN SQUARE</u>
Between groups	0.5411	5	0.1082
Within groups	<u>0.3281</u>	<u>43</u>	<u>0.0076</u>
TOTAL	0.8693	48	

$$F = 14.1826$$

$$F_{.05}(5,43) = 2.45$$

TABLE 3. Relationship of lens lactate concentration (mgm per lens pair) to age (in months). One-way analysis of variance. The lactate differences between groups are significant with 95% confidence.

SEX	LENS LACTATE CONCENTRATION (mgm)				
	<u>SUM</u>	<u>MEAN</u>	<u>STD. DEV.</u>	<u>SUM OF SQ.</u>	<u>N</u>
Male	5.176	0.207	0.154	0.566	25
Female	<u>4.453</u>	<u>0.186</u>	<u>0.114</u>	<u>0.298</u>	<u>24</u>
TOTAL	9.629	0.197	0.135	0.864	49

ANOVA TABLE

	<u>SUM OF SQUARES</u>	<u>DF</u>	<u>MEAN SQUARE</u>
Between groups	0.0057	1	0.0057
Within groups	<u>0.8636</u>	<u>47</u>	0.0184
TOTAL	0.8693	48	

$$F = 0.3078$$

$$F_{.05}(1,47) = 4.05$$

TABLE 4. Relationship of lens lactate concentration (mgm per lens pair) to sex. One-way analysis of variance. The sex differences are not significant with 95% confidence.

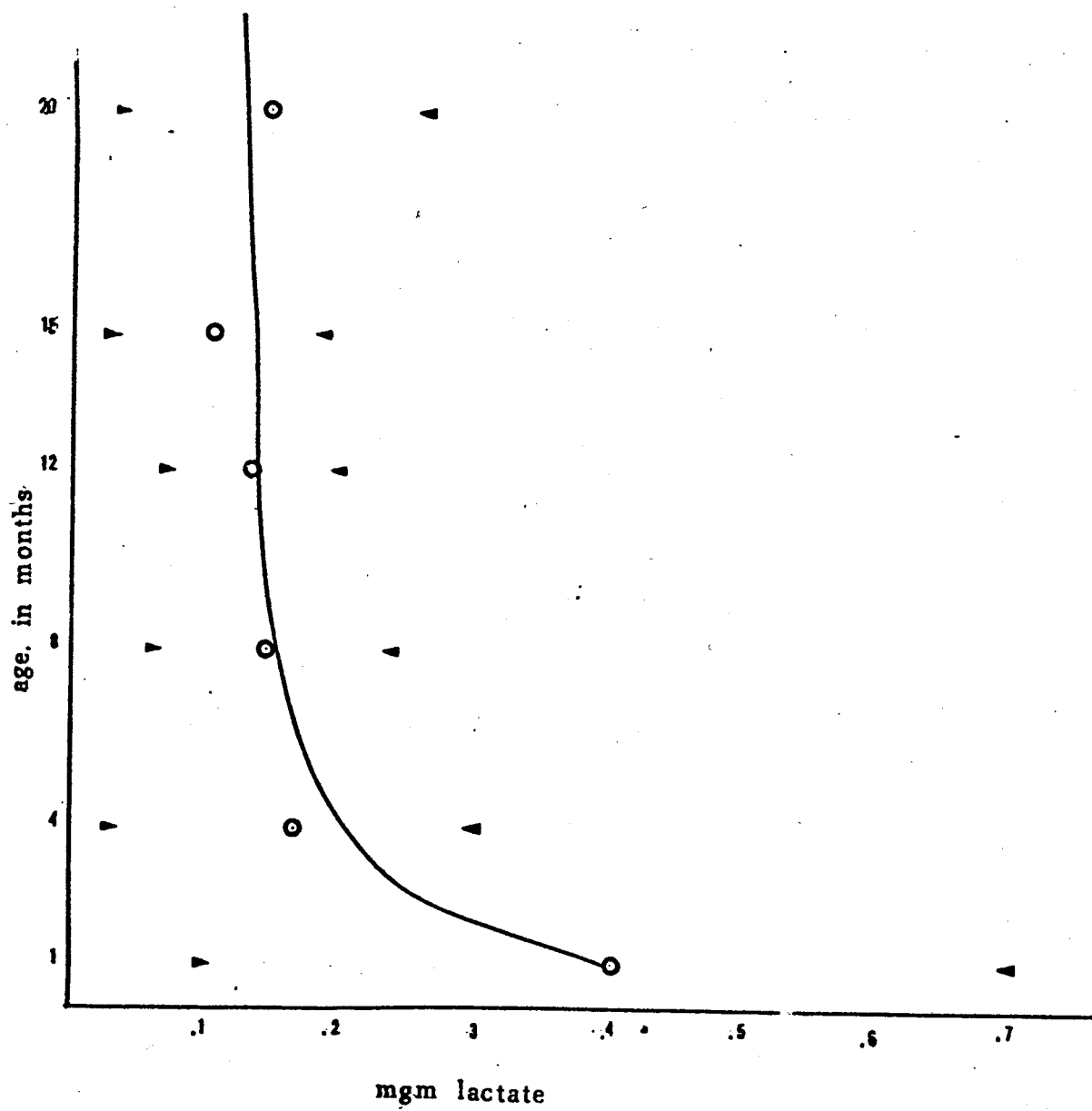


Figure 3. Relationship of lens lactate concentration to age in Pero-
myscus. Each circle represents a group mean. Arrows inclose
the 95% confidence limits of the group means.

AGE (in months)	<u>LACTATE CONCENTRATION</u> (<u>mgm</u>)		<u>LENS WEIGHT</u> (<u>mgm</u>)		<u>N</u>
	<u>SUM</u>	<u>MEAN</u>	<u>STD. DEV.</u>	<u>SUM OF SQ.</u>	
1.00	0.646	0.065	0.028	0.007	10
4.00	0.114	0.009	0.005	0.000	12
8.00	0.079	0.007	0.002	0.000	12
12.00	0.035	0.006	0.001	0.000	6
15.00	0.024	0.005	0.002	0.000	5
20.00	<u>0.025</u>	<u>0.006</u>	<u>0.004</u>	<u>0.000</u>	<u>4</u>
TOTAL	0.923	0.019	0.026	0.007	49

ANOVA TABLE

	<u>SUM OF SQUARES</u>	<u>DF</u>	<u>MEAN SQUARE</u>
Between groups	0.0264	5	0.0053
Within groups	<u>0.0073</u>	<u>43</u>	<u>0.0002</u>
TOTAL	0.0337	48	

$$F = 31.1556$$

$$F_{.05}(5,43) = 2.45$$

TABLE 5. Relationship of the lactate to lens weight ratio (mgm) to age (in months). One-way analysis of variance. The differences in ratios between groups are significant with 95% confidence.

SEX	<u>LACTATE CONCENTRATION</u> (<u>mgm</u>)				
	<u>LENS WEIGHT</u> (<u>mgm</u>)				
	<u>SUM</u>	<u>MEAN</u>	<u>STD. DEV.</u>	<u>SUM OF SQ.</u>	<u>N</u>
Male	0.494	0.020	0.027	0.018	25
Female	<u>0.430</u>	<u>0.018</u>	<u>0.026</u>	<u>0.016</u>	<u>24</u>
TOTAL	0.923	0.019	0.026	0.034	49

ANOVA TABLE			
	<u>SUM OF SQUARES</u>	<u>DF</u>	<u>MEAN SQUARE</u>
Between groups	0.0000	1	0.0000
Within groups	<u>0.0336</u>	<u>47</u>	0.0007
TOTAL	0.0337	48	

$$F = 0.0579$$

$$F_{.05}(1,47) = 4.05$$

TABLE 6. Relationship of the lactate to lens weight ratio to sex.
One-way analysis of variance. The sex differences are not
significant with 95% confidence.



Figure 4. Relationship of the mgm lactate to mgm lens weight ratio versus age in Peromyscus. The circles represent group means. Arrows inclose the 95% confidence limits of the group means. In those groups not inclosed by two arrows, the 95% confidence limits are off the scale of the graph.

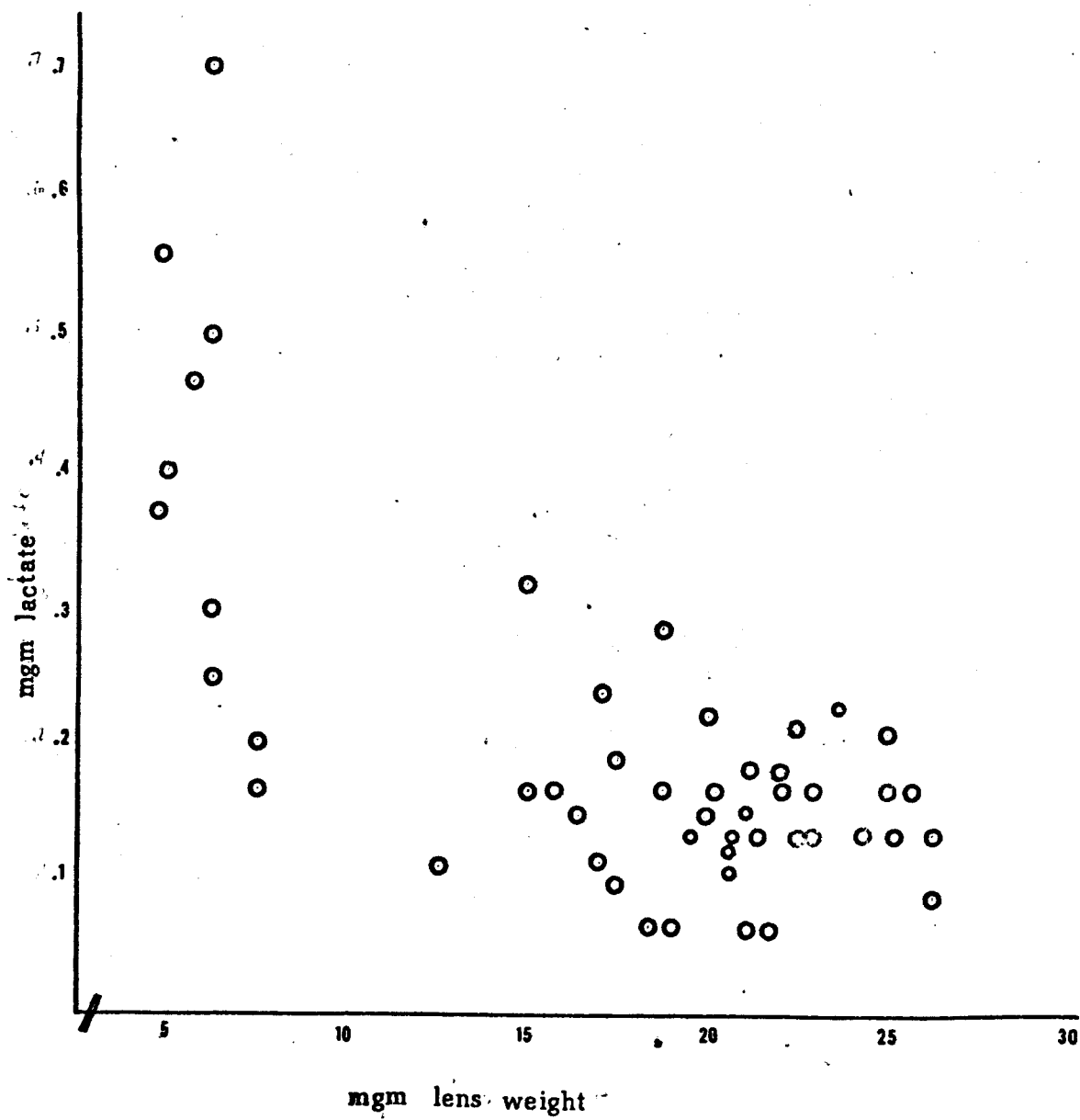


Figure 5. Relationship of lens lactate concentration to wet lens weight in Peromyscus. Each circle represents an individual value, N=49.

The decrease in lactate concentration as lens weight increases is consistent with a decreasing rate of metabolism as growth slows, since the lens is strongly anaerobic (see Literature Review). However, it may be indicative of a change in metabolic pathways used with the cessation of growth (Hockwin, 1965, 1974; van Heyningen, 1969; Kronfeld, 1969). This data, however, does not address the problem of metabolism.

DISCUSSION

Since none of the data obtained was usable in predicting age due to the sigmoidicity of the generated curves, the technique of linear transformation of data was applied and regression analysis was performed again. Regression of lens weight on age was most successful using $1/\text{age}$ (Months) and Log_{10} lens weight (mgm). This gives a curve of the equation:

$$1/\text{age (months)} = 1.4024 - 0.5985 \text{Log}_{10} (\text{wet lens weight in mgm}).$$

This curve is shown in Figure 6. The line thus obtained has a correlation coefficient of -0.972 , which is very good. However, even after transformation, the curve is sigmoidal, making predictions of age beyond eight months a shady proposition. To avoid this problem, and to obtain closer confidence limits on predictions, regression analysis was applied to only four age groups: 1, 4, 8, and 12 months. This enables predictions with 95% confidence limits of the first three age groups without extrapolation, and limits prediction to the ages at which it can validly be applied. The most successful equation obtained was:

$$\text{age (months)} = -2.386 + 0.473 (\text{wet lens weight in mgm}).$$

The correlation coefficient of this line is only 0.851 , but the 95% confidence limits of the mean prediction are plus and minus 2.01 months. These are the smallest confidence limits obtained of any method of data analysis applied. The curve of this function is depicted in Figure 7.

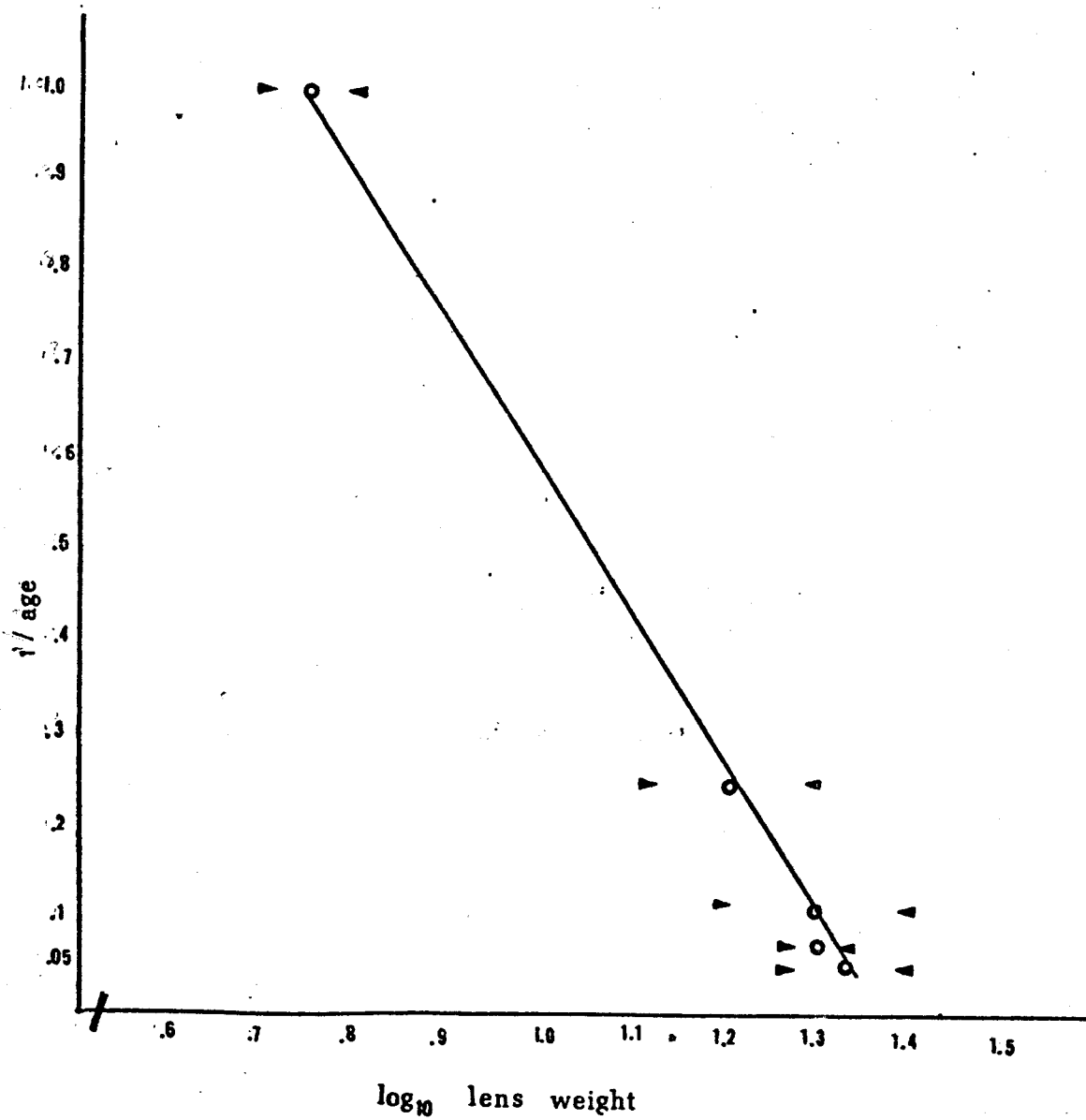


Figure 6. Relationship of \log_{10} lens weight to the inverse of age in Peromyscus. Circles represent group means, and arrows inclose the 95% confidence limits of the group means. The line equation is $1/\text{age (months)} = 1.4024 - .5985 \log_{10} (\text{wet lens weight in mgm})$. The correlation coefficient (r) of the line is -0.972.

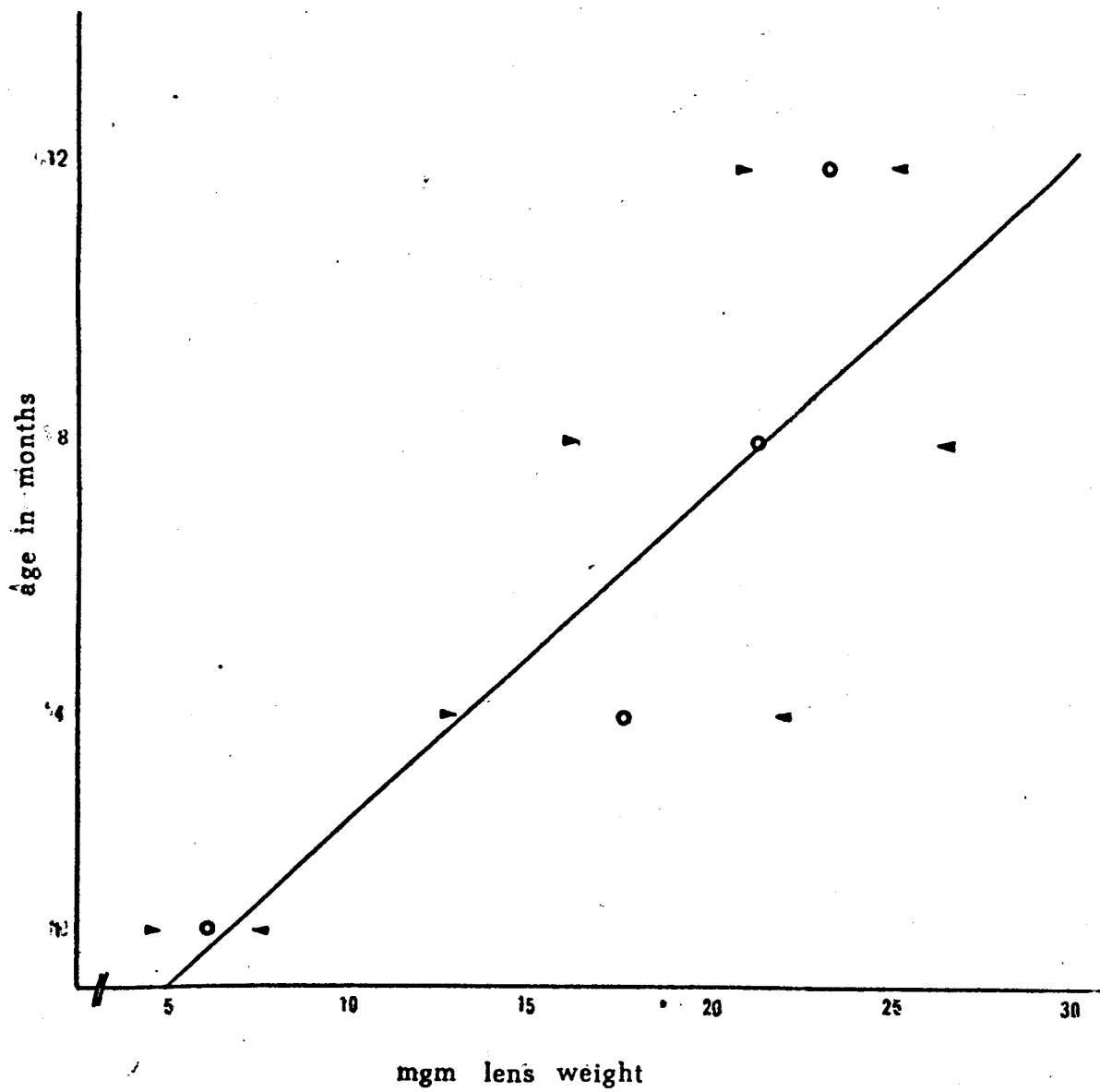


Figure 7. Relationship of wet lens weight to age in the first year of life in Peromyscus. The circles represent the group means, and arrows inclose the group 95% confidence limits. The line equation is $\text{age (months)} = -2.386 + 0.473 (\text{wet lens weight in mgm})$. The correlation coefficient (r) of the line is 0.851.

Similar treatment of the lactate data produced a curve (Figure 8) of the equation:

$$\text{age (months)} = 9.098 - 16.187 (\text{mgm lactate}).$$

The 95% confidence limits on the mean prediction made with this equation are plus and minus 3.04 months. These are fairly good, considering that the correlation coefficient of this function is only -0.607.

Apparently, little benefit can be obtained by use of both lens weight and lactate concentration equations in age predictions, since lens weight is much less variable than the latter. However, use of wet lens weight as a method of age prediction in Peromyscus seems justified from the data, for the following reasons:

- 1) the lens weight variances of the six age groups is fairly constant;
- 2) the correlation of lens weight to age by regression analysis, using untransformed data is 0.851;
- 3) 95% confidence limits on mean predictions are small - only plus or minus two months.

This is subject to the limitation that predictions be limited to animals no more than one year of age.

Some of the data obtained in this investigation merit further inquiry, notably the strongly negative correlation concentration of lactate to lens weight. This fits nicely with the concept of slowing metabolism as growth slows, but gives no indication as to the mechanism involved. One area that would seem worthy of experimentation is the possible change in distribution of lactate dehydrogenase isozymes, since it is widely held that lens metabolism remains anaerobic throughout life, and it is known that

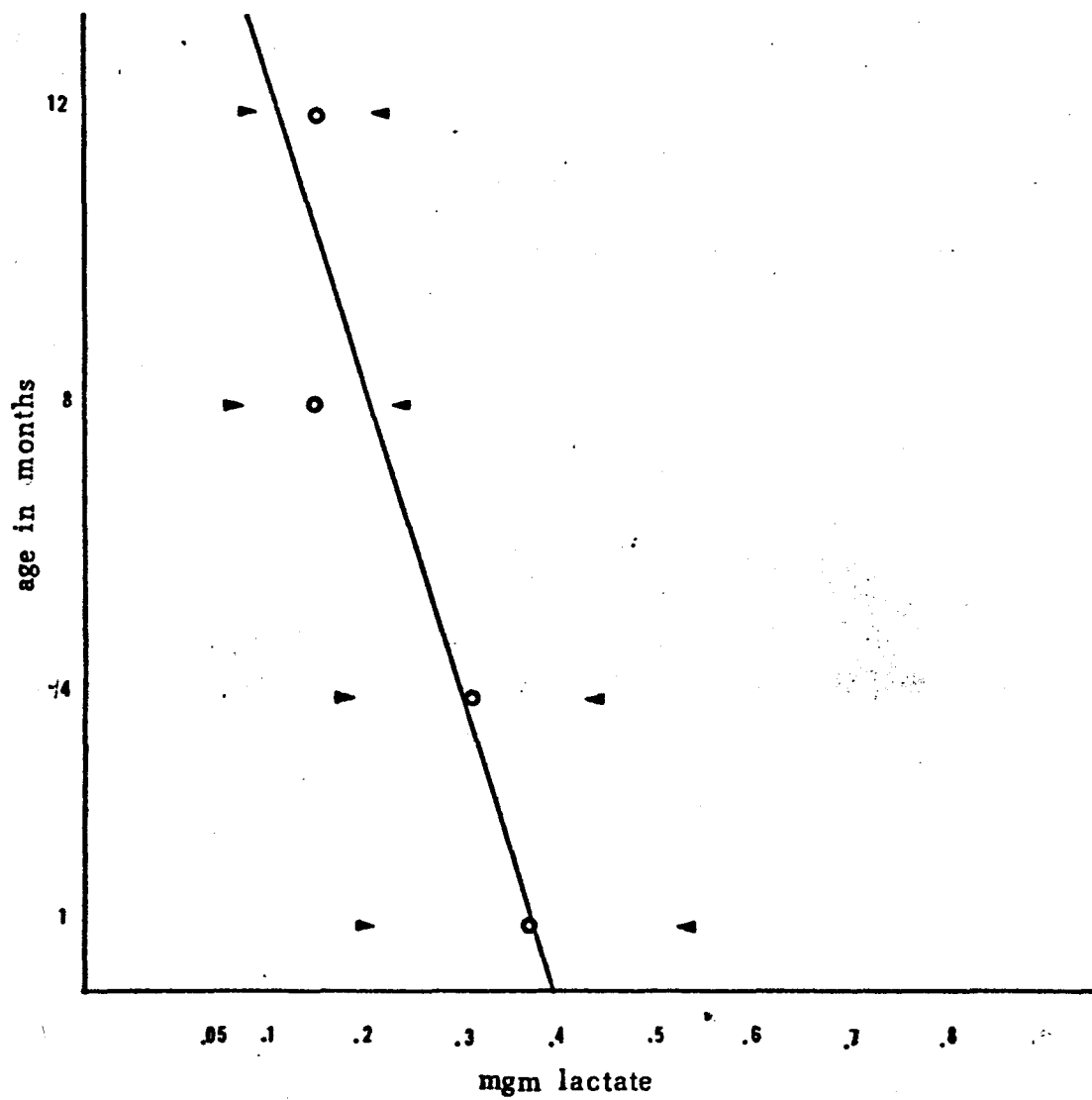


Figure 8. Relationship of lens lactate concentration to age in the first year of life in Peromyscus. The circles represent the group means, and arrows inclose the group 95% confidence limits. The line equation is $\text{age (months)} = 9.098 - 16.187 (\text{mgm lactate})$. The correlation coefficient (r) of the line is -0.607.

there is a change of LDH isozymes in embryogenesis of many tissues, including the lenses of some species (See Literature Review).

CONCLUSION

This experiment has shown that it is possible to predict age of Peromyscus maniculatus bairdii from the wet lens weight of the animal. It is possible to obtain 95% confidence limits of two months on the mean prediction in the one through eight month age groups, and prediction should be limited to this period.

This experiment serves as a model that may be applied to other populations of Peromyscus with the understanding that the data are from a laboratory population, and that a specific standard curve must be obtained for any wild population to which it is applied. Use in wild Illinois populations should be tempered with the realization that it is not possible to duplicate wild conditions in a laboratory situation, and that consequently there is a likelihood of some error in applying the equation supplied above to these wild populations directly, without at least some prior testing for differences in curve characteristics such as the slope and Y-intercept.

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APPENDIX

TABLE I. Physiological saline solution, modified from Bretag, 1969.

No buffers were used in order to prevent possible interference with the assay reaction by lowering the pH of the buffered medium (See Materials and Methods).

	<u>Bretag</u>	<u>Mammalian Tyrode</u>
<u>SALT</u>	<u>mM</u>	<u>mM</u>
NaCl	108.5	138.0
KCl	3.5	5.0
CaCl ₂	1.5	2.7
MgSO ₄	0.69	1.2

pH = 6.2

TABLE II. T-Test of age group differences. M = group mean, () inclose months age of group, S = standard deviation.

Table 1. Age in months versus wet lens weight (mgm).

$$\begin{aligned}
 M(4) - M(1)/S(1) &= 17.717-6.440/.880 = 12.815 & t_{.05}(9) &= 2.262 \\
 M(8) - M(4)/S(4) &= 21.908-17.717/2.315=1.810 & t_{.10}(11) &= 1.796 \\
 M(12)-M(8)/S(8) &= 23.200-21.908/2.835=0.541 & t_{.10}(11) & \\
 M(15)-M(12)/S(12) &= 21.920-23.200/.849 =-1.508 & t_{.20}(5) &= 1.476 \\
 M(20)-M(15)/S(15) &= 23.775-21.920/2.044=0.908 & t_{.10}(4) &= 2/132
 \end{aligned}$$

Table 3. Age in months versus lens lactate concentration (mgm).

$$\begin{aligned}
 M(4)-M(1) / S(1) &= 0.163-0.402/.153 = -1.562 & t_{.20}(9) &= 1.383 \\
 M(8)-M(4) / S(4) &= 0.143-0.163/.079 = 0.253 & t_{.05}(11) &= 2.201 \\
 M(12)-M(8)/S(8) &= 0.137-0.143/.044 = -0.136 & t_{.05}(11) &= 2.201 \\
 M(15)-M(12)/S(12) &= 0.108-0.137/.029 = -4.615 & t_{.05}(5) &= 2.571 \\
 M(20)-M(15)/S(15) &= 0.142-0.108/.052 = 0.654 & t_{.20}(4) &= 1.533
 \end{aligned}$$

Table 5. Age in months versus lactate to lens weight ratio.

$$\begin{aligned}
 M(4) - M(1)/ S(1) &= 0.009-0.065/.028 = 2.000 & t_{.10}(9) &= 1.833 \\
 M(8) - M(4)/ S(4) &= 0.007-0.009/.005 = 0.400 & t_{.10}(11) &= 1.796 \\
 M(12)- M(8)/ S(8) &= 0.006-0.007/.001 = -1.00 & t_{.10}(11) &= 1.796
 \end{aligned}$$

APPROVAL SHEET

The thesis submitted by Robert James Kinders has been read and approved by the following committee:

Dr. Kirt J. Vener, Chairman
Assistant Professor, Biology, Loyola

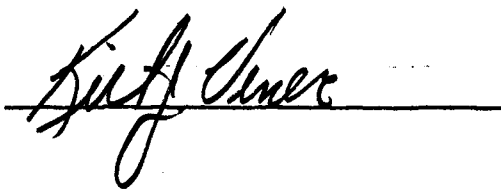
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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Sciences.

June 5, 1975

A handwritten signature in cursive script, reading "Kirt J. Vener", is written over a horizontal line.