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A study of the circadian rhythm and pre and post puberty concentrations of serum prolactin in male prairie deermice (*Peromyscus maniculatus bairdii*)

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A STUDY OF THE CIRCADIAN
RHYTHM AND PRE AND POST PUBERTY
CONCENTRATIONS OF SERUM PROLACTIN IN MALE
PRAIRIE DEERMICE (PEROMYSCUS MANICULATUS BAIRDII)

A Thesis

Presented To

The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Arts

by

Michael Kevin Thompson

1978

APPROVAL SHEET

This thesis is submitted in partial fulfillment
of the requirements for the degree of

Master of Arts

Author

Approved, August, 1978

Eric L. Bradley, Ph. D.

C. Richard Terman, Ph. D.

Carl W. Vermeulan, Ph. D.

DEDICATION

This thesis is dedicated to my wife, Caron, and children
Jennifer and Michael Jr.

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ABSTRACT

A radioimmunoassay system for measuring serum prolactin in rats was tested for its ability to measure serum prolactin concentrations in male deermice (Peromyscus maniculatus bairdii). Preliminary assays showed that this system could be used in mice and that it had a sensitivity of 5 to 10 ng/ml. Serum prolactin was then measured at various times throughout the day to determine if a circadian rhythm existed in male mice. The results of this study indicated a single peak in serum prolactin concentrations occurred at 9:00 P.M., one hour prior to the end of the dark cycle. This study suggests that blood samples taken for prolactin analysis should be taken either at the end of the light cycle or the start of the dark cycle. It was found that serum prolactin concentrations were constant during this period.

A study was done to determine pre and post puberty concentrations of serum prolactin and to determine if any correlation existed between these prolactin concentrations and the growth of the reproductive system in male mice. Serum prolactin was measured in groups of males from 10 to 90 days of age and compared with changes in body weight, testis weight, seminal vesicle weight and adrenal gland weight of males during the same age span. A significant positive correlation was found between serum prolactin and testis weight, seminal vesicle weight and adrenal gland weight between the ages of 10 and 90 days.

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INTRODUCTION

Prolactin is one of the nine known hormones produced in the adenohypophysis. It is a polypeptide and has a molecular weight of $23,000 \pm 1,000$. It is the most versatile of all adenohypophysial hormones. Sometimes it is referred to as lactogenic hormone, mammotrophin, galactin, lactogen or luteotrophin (Turner and Bagnara, 1971).

No other hormone has been found to have so many widely different effects and so many target organs. It has been found to have effects in amphibians, reptiles, birds, fish and mammals. In birds, it is responsible for stimulating the feather papillae to produce a new plumage, synergism with estrogen in the formation of the brood patch and the parental actions of sitting on eggs and feeding of young (Turner and Bagnara, 1971). It is also responsible for the production of crop milk in pigeons. This was the basis for a widely employed assay procedure for many years (Bern and Nicoll, 1968). The best known effect in amphibians is the phenomenon of water drive. The red eft stage of the spotted newt metamorphoses and lives a terrestrial life for 3 or 4 years. When sexual maturity is reached, it returns to the water (Turner and Bagnara, 1971). It is also responsible for increased tail length in the tadpoles,

increased body weight and larval growth in amphibians (Bern and Nicoll, 1968). In reptiles, it has been found that prolactin is responsible for skin molting and somatic growth (Turner and Bagnara, 1971). The effects of prolactin in teleost fish include nest building, fin fanning, skin mucous secretion, renal secretion and control of osmoregulation (Turner and Bagnara, 1971). Not much more work has been done with prolactin in these organisms. Most of the effort has been directed at the effects of prolactin in mammals.

The main action of prolactin, from the teleost fish to the mammal, is concerned with reproduction and parental care. Some of its effects found in mammals include mammary gland development, mammary tumor growth, lactation, sebaceous gland activity, hair maturation, maintenance of the corpus luteum in the mouse and rat, increased fertility in the dwarf mouse and stimulation of growth (Bern and Nicoll, 1968). Most of the studies of prolactin in mammals deal with its effects in females. It has been found to be an important factor in reproduction in the female.

Unlike the situation in females, few functions of this hormone have been found in the male mammal. Some investigators think prolactin is necessary for the growth and maintenance of the sex accessory organs. Bartke (1965, 1966, 1970), in his work with genetically sterile dwarf mice, has been able to show that injections of prolactin can restore some degree of fertility. Also, prolactin

acts synergistically with luteinizing hormone (LH) to increase levels of testosterone in hypophysectomized rats (Hafiez et al., 1977) and increase stores of esterified cholesterol in the testes of mice (Bartke, 1971). Other work has shown that prolactin will increase various enzymes in the testes that are needed for spermatogenesis (Hafiez et al., 1970; Musto et al., 1972).

If prolactin has a role in sex accessory organ growth in the male, then changes in circulating levels of the hormone could be expected to occur during the time of sexual maturation. The intention of this study was to determine pre and post puberty serum prolactin concentrations in male prairie deermice, Peromyscus maniculatus bairdii, and then to try to determine if any correlation exists between the serum prolactin concentration and growth of the testes and seminal vesicles.

At the start of this research program, preliminary investigations included research to determine the best time of day to collect samples. Blood sampling should be done at a specific time every day in order to avoid the effects of any circadian rhythm and in addition, the time chosen should be a period during which the hormone level is relatively constant. Many investigators pick a single time period for sampling to avoid any possible circadian rhythm effects. However, in this study, it was decided to determine if there was a circadian rhythm and if there were to sample only during a relatively stable plateau in

that rhythm to avoid the problems of unknowingly sampling during a set period subject to rapidly changing concentrations. Also, no papers could be found dealing with circadian rhythm of prolactin in Peromyscus and there was the possibility that the deermice could be entirely different from the rats.

A review of some of the work done by other investigators showed a great deal of variability in the circadian rhythm found. Several investigators working with male rats found no evidence of circadian rhythm of serum prolactin concentrations (Taya and Igarashi, 1974; Seggie and Brown, 1976). Other investigators found peaks in the middle of the light cycle (Hefco and Lackey, 1974), in the middle of the dark cycle (Dunn et al., 1972), just prior to the change from the dark cycle to the light cycle (Ronnekleiv et al., 1973) and just prior to the change from the light cycle to the dark cycle (Dunn et al., 1972).

In three of the six reports, the pattern of illumination was identical. In the other three reports, the start of the dark cycle was either one hour earlier or one hour later. Because of the small amount of variability in the pattern of illumination, it is not likely that the variability in the results is due to the timing of the light dark cycle.

In all cases, the various investigators cited above refer to the lack of a clear circadian rhythm pattern and state that the difference is probably due to the sampling

method used and or different environmental stimuli present. It is well documented that serum prolactin concentrations are greatly affected by any form of stress. Others have found that serum prolactin concentrations are affected by the blood sampling technique used (Ajika et al., 1972; Wuttke and Meites, 1970; Raud et al., 1971; Terkel et al., 1972; Neill, 1972), sacrifice position (Seggie and Brown, 1976) and exposure to a novel environment (Baldwin et al., 1974). One study deals with parallel tests to determine the circadian rhythm of prolactin in male rats sampled by decapitation and by ether anesthesia (Dunn et al., 1972). These results show a different rhythm in each case even though all other environmental variables were the same.

By comparing these papers, it was found that all the variability could not be explained by differences in blood sampling technique alone. In two papers reporting no significant circadian rhythm (Seggie and Brown, 1976; Taya and Igarashi, 1974), one study obtained the samples by decapitation and the other by ether anesthesia. Comparison of the studies performed by obtaining the blood samples by decapitation show that some found no circadian rhythm (Seggie and Brown, 1976) while others found a peak in the middle of the light cycle (Hefco and Lackey, 1974) and still others found a peak in the middle of the dark cycle (Dunn et al., 1972). Comparison of the papers with the investigators using ether anesthesia in obtaining blood samples show that in some cases no significant

circadian rhythm was found (Taya and Igarashi, 1974) while in other cases, a peak was found just prior to the change from the dark cycle to the light cycle (Ronnekleiv et al., 1973) or just prior to the change from the light cycle to the dark cycle (Dunn et al., 1972).

Because of the lack of any available data on circadian rhythm of serum prolactin in Peromyscus and the great amount of variability found in the work that has been done with rats, a circadian rhythm study was included in this research. This made it possible to sample at a relatively stable plateau for the pre and post puberty study.

In order to study peripubertal concentrations of serum prolactin, it is first necessary to define the criteria that will be used to identify "puberty" per se. Some investigators refer to puberty as an exact period of time when a certain event occurs (McCann, 1974; Ramirez and Sawyer, 1965). Others define puberty as a period in which the animal becomes sexually mature and the sexes become fully differentiated (Marshall and Hammond, 1966; King, 1968). The definition used in this study is that of King (1968). Puberty in male Peromyscus can be identified by the appearance of spermatozoa in the caudal epididymus and an increase in the testicular weight, sperm tubule diameter, epididymal weight and seminal vesicle weight. The male Peromyscus reaches puberty sometime after 40 days of age and is usually sexually mature by 60 days of age (King, 1968).

In order to determine if any correlation exists between serum prolactin and the growth of the testes and seminal vesicles at puberty, this study was conducted on groups of male deermice 10 to 90 days of age. Regardless of how puberty is defined, the mouse is past puberty by 90 days of age. Any developmental changes leading to full maturity should be seen by this time.

MATERIALS AND METHODS

Production Colony

The parent pairs were made by placing 60 to 90 day old female deermice with non-sibling males of approximately the same age. Each pair was placed in an opaque plastic cage measuring 12.7 cm. wide, 15.2 cm. high and 30.5 cm. long with a wire grid top. Food and water were provided ad libitum. The parent pairs were checked daily and any new litters were recorded and standardized in size so that there was at least one of each sex in each litter and the litter had between 3 and 6 pups.

Circadian Rhythm Study Animals

The young were kept with their parents until they reached 21 days of age. Then, they were weaned and the males and females of each litter were placed in separate cages. They were maintained in this manner until they reached 45 days. At this time, the males and females were randomly assigned to the following treatment groups: mice to be killed at 3:00 A.M., 7:00 A.M., 11:00 A.M., 1:00 P.M., 5:00 P.M., 9:00 P.M. and 11:00 P.M. The males in each group were then paired with females in the group that were

the same age ± 5 days. No sibs were paired. Each group consisted of 5 pairs of mice. The mice were maintained as pairs until the males were 60 ± 1 days old. At that time, the males were rapidly etherized and blood collected in less than two minutes. Serum was obtained by centrifugation and frozen at -30°C for the prolactin analysis. The bodies were placed in 10% buffered formalin for later dissection.

Pre And Post Puberty Study Animals

The same production colony was used in this experiment. The parent pairs were checked daily and any new litters were recorded and standardized in size. At day ten, all the young were checked and the males and females of each litter were randomly assigned to treatment groups. The treatment groups are shown in Table 1.

TABLE 1

<u>Treatment Group</u>	<u>Age To Be Killed (Days)</u>	<u>Number Of Pairs</u>
1	10 ± 1	26
2	15 ± 1	26
3	20 ± 1	13
4	30 ± 1	13
5	40 ± 2	13
6	50 ± 2	13
7	60 ± 2	13
8	90 ± 2	13

The animals in the 10 day old treatment group were killed at this time. The rest were returned to their parents. At day 15, the proper animals were taken from the parent cages and killed. When the mice were 20 days old, they were weaned. The animals in the 20 day old group were killed. The remaining males were assigned to females in the same treatment group that were the same age $\pm$ 2 days. These mice were paired and maintained until they reached the proper age. No sibs were paired. In all cases, only males were used. The samples were all taken between 8:00 A.M. and 10:00 A.M. Serum from the 10 and 15 day old animals was pooled in groups of two animals. The bodies were saved in 10% buffered formalin and the reproductive system and adrenal glands removed.

Animal Care

All animals were housed in a room measuring 4.25 x 4.50 meters. The cages were placed on metal racks each of which held 25 pairs of cages. The temperature ranged from 18 to 26°C. Light was provided by four-forty watt white fluorescent tubes. An artificial light program was set up with 14 hours of light and 10 hours of dark with the change to the dark period at 12:00 P.M. A ventilation system completely replaced the air five to ten times per hour. Bedding, which consisted of wood shavings, was changed every 14 days except if the date fell three days or less prior to the scheduled sample date or if

newborn were present. Food and water were provided ad libitum.

Sample Collection

Blood samples were obtained by anesthetizing the animals with diethyl ether. The ascending vena cava was cut at a point directly above the renal vein. The blood pooled in the body cavity and was collected with an unheparinized syringe. The total time interval between the initial contact with the animals and the complete collection of the blood sample was two minutes or less in the 30 to 90 day old animals. In the younger animals, problems with identifying the proper animal to be killed in the litter sometimes made it difficult to stay within this limit. In these cases, identity was made as quickly as possible and the time interval between identification and complete collection of blood was two minutes or less. If possible, enough blood was collected so that the sample could be run in duplicate.

The blood samples were allowed to clot at room temperature, 20°C, for at least 30 minutes and not over one hour. The samples were then centrifuged at 9,000 x g for two minutes at room temperature. The serum was drawn off and immediately frozen at -30°C until the assay.

After the blood was collected, the body was weighed, tagged and placed in 10% buffered formalin. At a later date, the reproductive system and adrenal glands were

removed, cleaned of fat, blotted dry and weighed as paired organs to the nearest 0.1 mg. on a Cahn electrobalance interfaced with a Wang 700 B computer.

Materials

All hormones and antihormones were provided by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases (NIAMD), National Institute of Health. NIAMD-Rat Prolactin I-1 with a biological potency of approximately 30 international units per mg. was used for the radioiodination. The first antibody was NIAMD anti-Rat Prolactin Serum². NIAMD Rat Prolactin RP-1, with a biological potency of approximately 11 international units per mg. served as the reference hormone. Goat anti-rabbit gamma globulin (Antibodies Incorporated, Davis, California) was used as the second antibody. The hormone was labelled with ¹²⁵I (Cambridge Nuclear, Billerica, Massachusetts).

Radioiodination

Two gel columns were prepared prior to radioiodination. In each case, a 1x30 cm. chromatography column was used. Nylon cloth was placed over the porous glass plate on the bottom. The column was filled with the proper gel, previously equilibrated overnight in 0.01 M borate buffer, pH 8.6, at room temperature, to a height of 25 cm. The column was then coated with 2% egg white (EW) in phospho-

saline buffer at pH 7.6 and then thoroughly washed with borate buffer. A sephadex G-50 (Pharmacia, Piscataway, New Jersey) gel column was prepared for the purification process and a sephadex G-75 gel column was prepared for the re-purification process.

Purified rat prolactin (Rat Prolactin I-1) was trace labelled with ^{125}I at room temperature by a modification of the methods of greenwood et al., (1963), and Niswender et al. (1968, 1969). ^{125}I was diluted with 0.5 M phosphate buffer, pH 7.6, to a concentration of 1 MCi/25 ul. Twenty-five ul. were then transferred to a 1.0 ml. serum vial containing 25 ul. NIAMD Rat Prolactin previously diluted to 100 ug. per ml. in 0.01 M phosphate buffer, pH 7.6. The contents of the vial were gently mixed by finger tapping. To this mixture was added 15 ul. chloramine-T (Eastman Chemical, New York) at a concentration of 10 mg. per 10 ml. of 0.05 M phosphate buffer, pH 7.6. The chloramine-T was prepared just prior to use. A stopper was inserted in the vial and the vial gently agitated for 20 seconds. At that time, the reaction was stopped by adding 50 ul. sodium metabisulfate (Fischer Scientific, Richmond, Virginia) at a concentration of 24 mg. per 10 ml. of 0.05 M phosphate buffer, pH 7.6. This was also prepared just prior to use. One hundred ul. of transfer solution containing 4 grams of sucrose and 0.25 grams of potassium iodide in 25 ml. of 0.05 M phosphate buffer at pH 7.6 were then added. The entire contents of the reaction vial were then placed on the top of a sephadex G-50 gel

column and allowed to enter the top layer of gel. The column was eluted with 0.01 M borate buffer, pH 8.6. Fractions of 2 ml. were collected in 12 x 75 mm. borosilicate test tubes containing 500 ul. of 1% egg white in 0.01 M phosphate, 0.15 M NaCl and 0.1% sodium azide, pH 7.5, (PBS). Sixteen tubes were collected. These tubes were placed in a Beckman Biogamma II radioactivity counter (Beckman Instruments, Irvine, California) and counted for one minute in channel A with the discriminating windows set at upper = 10.0 and lower = 0.42. Two peaks of radioactivity were detected (Figure 1). The first peak was usually around tubes 3 to 6 and contained the iodinated rat prolactin. The second peak was usually around tubes 8 to 11 and contained the free iodide. The fraction high on the trailing shoulder of the first peak was stored in the refrigerator at 1°C. All other tubes were discarded.

No later than the following day, the iodinated prolactin was repurified on a sephadex G-75 column. Fourteen tubes were collected with 2ml. each of eluate and 500 ul. of 1% EW-PBS. These were counted and again showed two peaks of radioactivity (Figure 2). The first peak was around tubes 3 to 6 and the second peak was around tubes 8 to 11. In the purification process, both peaks were approximately the same height, but in the repurification process, the second peak was very small. Again, the fraction high on the trailing shoulder of the first peak was saved for the radioimmunoassay (RIA). All other tubes were

discarded.

Preliminary Checks

Preliminary checks were performed on the iodinated rat prolactin to determine its immunoreactivity prior to use. At the same time, first and second antibody titers were also performed. Borosilicate glass test tubes (12 x 75 mm.) were used. To each tube was added 700 ul. of 1% EW in 0.01 M PBS, pH 7.6. The total count, background and iodination check tubes (all run in triplicate) each had 200 ul. of 2% normal rabbit serum (NRS) in 0.01 M PBS, pH 7.6, added. Anti rat prolactin, the first antibody, was initially diluted with 2% NRS-PBS to a 1:2,000 dilution, then further diluted for the titer to 1:15,000, 30,000, 60,000, and 100,000. Two hundred ul. of first antibody in the proper dilution was added to the tubes for the first antibody titer and 200 ul. of first antibody at a dilution of 1:2,000 was added to the tubes for the second antibody titer. To each tube was added 100 ul. of ^{125}I rat prolactin diluted with 0.1% EW in 0.01 M PBS, pH 7.6, so that a known amount, about 20,000 cpm in a Beckman Biogamma II counter with the ^{125}I isoset, was contained in 100 ul. All tubes were incubated 12 to 18 hours at 4°C. After incubation, 32 ul. of second antibody and 68 ul. of 0.01 M PBS, pH 7.6, was added to the iodination check and first antibody titer tubes. For the second antibody titer, the amount of second antibody in each tube was serially doubled five times with the first

containing 2 ul. All tubes were then incubated for 6 hours at 4°C. After incubation, the tubes were centrifuged at 9428 x g (5,000 rpm, Beckman RC2B) for 20 minutes. The supernatant was discarded and 0.2 ml. distilled water added to the precipitate in each tube. Radioactivity in the tubes was counted on the Biogamma II. Calculations were then made to determine the immunoreactivity of the iodinated hormone. The amount of first and second antibody to be used for optimal precipitation was also determined.

Radioimmunoassay (RIA)

Serum prolactin was measured by a double antibody radioimmunoassay using modifications of the methods described by Bast et al., (1974), Neill et al., (1971) and Sinha et al., (1972). Standards, standard sera and unknowns were added to tubes (12 x 75 mm. borosilicate) along with the diluent buffer of 1% EW-PBS, pH 7.6, so that all tubes had a common volume of 500 ul. To each tube was added 200 ul. of first antibody at the proper dilution that would bind 30% of the radioiodinated prolactin in an assay tube lacking unlabelled hormone. All the tubes were incubated for 36 hours in a cold room at 4°C. After incubation, 100 ul. of ¹²⁵I prolactin was added with the micromedic automatic pipette (Micromedic Systems Inc., Huntsville, Alabama) using the sample 50 ul. dispense 50 ul. mode so that each tube contained about 20,000 cpm. All tubes were again incubated at 4°C for 24 hours. After incubation, 200 ul. of

second antibody was added at the proper dilution obtained from the second antibody titer. Again, all tubes were incubated at 4°C for 72 hours. After incubation, the tubes were centrifuged at 9428 x g for 20 minutes at 4°C. The supernatant was discarded and the tubes left inverted on a paper towel for five minutes. Two-tenths of one ml. of distilled water was added to each tube and the radioactivity counted in the Biogamma II with the ^{125}I isoset in channel A for five minutes per tube.

Protocol

NIAMD rat prolactin standard (RP-1) previously diluted to 0.1 mg. per ml. with 1% EW-PBS, pH 7.6, was further diluted with the same buffer to 160 ng. per ml. and serially diluted for the standard curve. Standard sera used in the analysis included Peromyscus lactating females (PLF), intact female Sprague Dawley (Charles River) rats (NFR) and hypophysectomized female Sprague Dawley (Charles River) rats (HFR). The protocol followed for the analysis was that described by Duddleson et al., (1972). Total count, non-specific and buffer control tubes were run in triplicate. Standards and standard sera were run in duplicate. Unknowns were run in duplicate where possible. Standards were serial dilutions related to one another by a constant factor. All pipetting of standards and standard sera was done with a Hamilton syringe. Pipetting of the buffer, first antibody, ^{125}I -PRL and second antibody was done with the micromedic

automatic pipette.

Statistics

The counts per five minutes for each sample were calculated and transferred to IBM cards for data processing with an IBM 370 computer and run according to the RIANAL 7 program purchased from R. A. Midgley, University of Michigan, (Duddleson, Midgley and Niswender, 1972). The standard curve data was transformed to log dose and logit response and the hormone concentration was then calculated from the linear regression equation. Hormone concentrations are expressed in terms of NIH-PRL-RP-1. Following the method described by Murr et al., (1971), concentrations lower than the limits of sensitivity were given those values in calculating means and standard errors.

Serum prolactin values obtained in the circadian rhythm assay were grouped according to the times collected and the different groups compared using the F-test and the Student's t test. Values obtained for body weights, paired adrenal gland weights, paired testis weights, paired seminal vesicle weights and prolactin in the pre and post puberty study were grouped according to the different ages at which collected. For each parameter, the different groups were compared with multiple F-tests and Student's t tests (Sokal and Rohlf, 1969). In those cases where the F-test and Student's t test both showed significance, the data was analyzed by the Mann-Whitney U test (Siegel, 1956). Data

was then subjected to linear regression analysis and the correlation coefficient determined (Siegel, 1956). In all cases, the five per cent level of probability was regarded as significant.

RESULTS

Iodination

The elution pattern from the sephadex G-50 column and also from the sephadex G-75 column was similar in all assays performed. The elution patterns obtained in the assay to determine circadian rhythm are shown here. Figure 1 shows the elution pattern of radioiodinated prolactin in the sephadex G-50 column. The first peak, usually between tubes 3 and 6, contained the labelled prolactin. The second peak presumably contained mostly free iodide (Sinha et al., 1972). An aliquot of the first peak was passed through the sephadex G-75 column for repurification within 24 hours. Figure 2 shows the typical elution pattern obtained in this column. Again, the first peak contained the labelled prolactin and the second peak contained the free iodide (Sinha et al., 1972).

Radioimmunoassay

Figure 3 and figure 4 show the standard curves obtained in the circadian rhythm assay and the pre and post puberty assay, respectively. In the circadian rhythm assay, the limit of detection was 1.2 ng. in a 100 ul. sample as given by the standard deviation of the assay. In the pre and post

Legend for Figure 1

Elution profile of prolactin iodination reaction mixture from a 1 x 30 mm. column of sephadex G-50. Each point represents the radioactivity in 2 ml. aliquots. Radioactivity counted for one minute per tube in a Biogamma II counter with the discriminating windows set at upper = 10.0 and lower = 0.42.

FIGURE 1

Legend for Figure 2

Elution profile obtained when an aliquot of the first peak from the sephadex G-50 column was passed thru a 1 x 30 mm. sephadex G-75 column. Each point represents the radioactivity in successive 2 ml. aliquots. Radioactivity counted in a Biogamma II counter with the discriminating windows set at upper = 10.0 and lower = 0.42.

FIGURE 2

puberty assay, the limit of detection was 583.7 pg. in a 100 ul. serum sample as given by the standard deviation of the assay.

The dose response curves for Peromyscus lactating females (PLF), normal female rats (NFR) and hypophysectomized female rats (HFR) are shown with the standard curves in Figures 3 and 4. In the circadian rhythm assay, figure 3, the slope of the regression line for the prolactin reference hormone was not significantly different from the slope of the line for the NFR, but it was significantly different from the slope of the line for PLF and HFR. This necessitated the use of the dose response curve for PLF to determine the amount of prolactin in the samples. In the pre and post puberty assay, figure 4, the slope of the regression line for the prolactin reference hormone was not significantly different from the slope of the line for NFR or PLF, but it was significantly different from the slope of the line for HFR.

Circadian Rhythm

Serum prolactin levels were observed at 1:00 P.M., 5:00 P.M., 9:00 P.M., 11:00 P.M., 3:00 A.M., 7:00 A.M. and 11:00 A.M. to determine if a circadian rhythm existed in adult male Peromyscus (Table 2). Serum prolactin levels were found to be constant from 7:00 A.M. to 5:00 P.M. At 5:00 P.M., prolactin levels began to rise sharply and a peak was reached at 9:00 P.M. After 9:00 P.M., the

Legend for Figure 3

Standard curve for NIAMD rat prolactin reference hormone preparation (NIAMD-Rat PRL-RP-1) along with the dose response curves for Peromyscus lactating females (PLF), normal female rats (NFR) and hypophysectomized female rats (HFR) obtained in the radioimmunoassay to determine circadian rhythm of prolactin in adult male mice.

FIGURE 3

Legend for Figure 4

Standard curve for NIAMD rat prolactin reference hormone preparation (NIAMD-Rat PRL-RP-1) along with the dose response curves for PLF, NFR and HFR obtained in the radioimmunoassay to determine pre and post puberty levels of serum prolactin in male Peromyscus.

FIGURE 4

prolactin level fell gradually until 3:00 A.M. when it reached its lowest point (Figure 5). When multiple Student's t tests were performed on the data involved, it was shown that the rise from 5:00 P.M. to 9:00 P.M. was significant ($P < 0.02$) and the corresponding fall from 9:00 P.M. to 11:00 P.M. was significant ($P < 0.02$). The fall from 11:00 P.M. to 3:00 A.M. was found to be different ($P < 0.05$) (Table 2).

On those days when two or more animals were sampled consecutively at a specific time interval, comparisons of prolactin concentrations versus sacrifice order were made to determine if any relationship could be found (Seggie and Brown, 1976). These comparisons show that in 5 out of 7 cases, as the order of sacrifice increased, the prolactin values also increased. In the other two cases, as the order of sacrifice increased, the prolactin values decreased. In both of these cases, the prolactin levels were already elevated (Table 3).

Pre and Post Puberty Concentrations

Serum Prolactin Levels

Serum prolactin was measured in male Peromyscus from 10 to 90 days of age. Data obtained showed that serum prolactin levels were low on day 10, rose to a slight peak on day 15 and fell back to a low level on day 20. Prolactin levels then rose gradually from 20 days of age to 50 days. At age 50 days, a sharp increase was noted to 60 days of age

Legend for Figure 5

Mean Values of serum prolactin concentration (ng/ml)
at various times throughout a twenty-four hour period.

TABLE 2

Comparison of Serum Prolactin Concentrations (ng/ml) at
Various Times Throughout a Twenty-Four Hour Period

<u>Time</u>	<u>N</u>	<u>Mean $\pm$ SE</u>	<u>Student's t test</u>
1:00 P.M.	5	38.08 $\pm$ 1.91	
			P<0.90
5:00 P.M.	5	35.82 $\pm$ 3.56	
			P<0.02
9:00 P.M.	5	48.58 $\pm$ 1.66	
			P<0.02
11:00 P.M.	4	42.23 $\pm$ 0.85	
			P<0.05
3:00 A.M.	5	33.50 $\pm$ 2.59	
			P<0.40
7:00 A.M.	5	38.64 $\pm$ 2.85	
			P<0.90
11:00 A.M.	4	38.33 $\pm$ 4.14	
			P<0.90
1:00 P.M.	5	38.08 $\pm$ 1.91	

Comparison of 9:00 P.M. (highest value) with 3:00 A.M.
(lowest value) P<0.01

TABLE 3

Comparison of Order of Sacrifice and Serum Prolactin Values

<u>Animal Number</u>	<u>Time Of Sample</u>	<u>Prolactin ng/ml</u>
1	7:02 A.M.	30.50
8	7:11 "	41.75
3	4:57 P.M.	26.35
9	5:12 "	35.15
20	1:05 P.M.	38.50
28	1:10 "	40.90
18	11:03 A.M.	27.50
23	11:10 "	44.10
32	5:04 P.M.	41.59
24	5:11 "	45.85
25	9:05 P.M.	53.20
31	9:15 "	47.45
38	9:20 "	43.05
29	11:00 P.M.	44.20
36	11:05 "	41.50
19	6:55 A.M.	33.20
27	7:03 "	42.75

when a peak was reached. From 60 days of age until 90 days prolactin levels fell slightly (Table 4, Figure 6).

Statistical analysis of this data showed that the increase found in the 15 day mice was not significantly higher than the level found in the 10 day old mice ($P < 0.07$). However, the decrease from 15 day mice to 20 day mice was found to be significant ($P < 0.03$). Analysis of the gradual increase from 20 to 50 days of age showed that the different age groups were not significantly different from each other but that the 20 day old mice were significantly lower than the 50 day old mice ($P < 0.001$). The sharp rise from 50 day mice to 60 day was found to be significant ($P < 0.05$). Comparison of the serum prolactin in the 60 day old and the 90 day old showed they were not significantly different ($P < 0.90$) (Appendix B).

Body Weight

The body weight of male Peromyscus 10 to 90 days of age was compared. Data collected showed that the body weight increased sharply from 10 to 30 days of age. From 30 to 90 days, body weight continued to increase but at a much slower rate (Table 4, Figure 6). Statistical analysis of this data showed a significant difference between the body weight of the 10 and 15 day old ($P < 0.00001$), the 15 and 20 day old ($P < 0.00001$) and between the 20 and 30 day old animals ($P < 0.00001$). No significant difference was found between the 30 and 40 day old ($P < 0.20$). A slight increase

was noted between 40 and 50 days and this increase was found to be significant ($P < 0.05$). Body weight did not change significantly between the ages of 50 and 60 days ($P < 0.50$) but did show a significant increase from 60 to 90 days of age ($P < 0.02$) (Appendix C).

Paired Testis Weights

The testis weight was determined for mice 10 to 90 days old. The graph of the weights collected showed that testis weights increased the entire time with the greatest increase occurring from 40 to 60 days of age (Table 4, Figure 6). Statistical analysis of this data showed that each age level was significantly different from the previous age group (Appendix D).

Paired Seminal Vesicle Weight

The weights of paired seminal vesicles from mice 10 to 90 days old were collected. Data collected showed little change in seminal vesicle weight from 10 to 30 days. At age 30, a slight increase was noted to age 40 days. At age 40 days, seminal vesicle weight increased rapidly to 60 days of age, at which time weight continued to increase but at a much slower rate until 90 days (Table 4, Figure 6).

Statistical analysis of the 10 and 15 day old animals showed no significant difference ($P < 0.50$). A significant drop was noted in seminal vesicle weight from 15 to 20 days ($P < 0.00006$). After 20 days of age, seminal vesicle

weight increased and all groups measured from 20 to 90 days were significantly different (Appendix E).

Paired Adrenal Gland Weight

The weight of the paired adrenal glands in male mice 10 to 90 days of age was measured. Weights collected showed that adrenal gland weight increased rapidly from 10 to 40 days of age. From 40 to 60 days, adrenal gland weight increased but at a slower rate. After 60 days, the adrenal gland weight leveled off and remained constant till 90 days (Table 4, Figure 6).

Statistical analysis of this data showed a significant difference between the 10 and 15 day old ($P < 0.002$), the 15 and 20 day old ($P < 0.0082$) and the 20 and 30 day old ($P < 0.0089$). No significant difference was found between 30 and 40 day old ($P < 0.10$) or between 40 and 50 day old ($P < 0.50$). A significant difference was found from 50 to 60 day old ($P < 0.05$). The 60 and 90 day old were not significantly different ($P < 0.90$) (Appendix A).

Linear Regression Analysis

Linear regression analysis was performed on prolactin concentration versus age, paired testis weight, paired adrenal gland weight and paired seminal vesicle weight. The correlation coefficients were also determined. No correlation could be found between prolactin concentration and any of these variables if the analysis was done using only

the 10, 15 and 20 day old mice. However, in all cases, a significant positive correlation was found if the analysis was performed using the mice 20 to 90 days old or 10 to 90 days old (Appendix F).

TABLE 4

Summary of Body Weights, Organ Weights and Prolactin Concentrations

Age (Days)	Body Weight (Gm.)	Paired Testis Wgt. (mg)	Paired S.V. Wgt. (mg)	Paired Adrenals (mg)	Prolactin (ng/ml)
10	5.24 $\pm$ 0.08 (25)	7.47 $\pm$ 0.32 (23)	1.03 $\pm$ 0.04 (24)	0.89 $\pm$ 0.05 (25)	14.47 $\pm$ 2.29 (12)
15	6.38 $\pm$ 0.17 (26)	10.55 $\pm$ 0.73 (26)	1.07 $\pm$ 0.03 (24)	1.16 $\pm$ 0.06 (21)	21.09 $\pm$ 2.74 (17)
20	8.47 $\pm$ 0.25 (26)	22.33 $\pm$ 2.05 (24)	0.86 $\pm$ 0.04 (24)	1.45 $\pm$ 0.07 (25)	14.73 $\pm$ 2.20 (23)
30	12.39 $\pm$ 0.43 (12)	56.00 $\pm$ 5.95 (11)	1.27 $\pm$ 0.19 (11)	1.95 $\pm$ 0.20 (11)	21.44 $\pm$ 3.20 (16)
40	13.45 $\pm$ 0.44 (13)	83.57 $\pm$ 8.82 (13)	11.33 $\pm$ 2.52 (12)	2.43 $\pm$ 0.16 (12)	27.61 $\pm$ 4.62 (14)
50	14.99 $\pm$ 0.49 (13)	171.50 $\pm$ 11.47 (13)	52.66 $\pm$ 8.56 (12)	2.59 $\pm$ 0.16 (11)	31.10 $\pm$ 4.14 (15)
60	15.45 $\pm$ 0.30 (13)	253.96 $\pm$ 12.12 (13)	116.76 $\pm$ 13.00 (13)	3.22 $\pm$ 0.25 (11)	50.57 $\pm$ 5.58 (10)
90	17.48 $\pm$ 0.51 (13)	324.59 $\pm$ 17.46 (11)	166.38 $\pm$ 19.28 (13)	3.13 $\pm$ 0.34 (11)	49.31 $\pm$ 3.10 (8)

Legend for Figure 6

Mean serum prolactin concentrations, mean body weights, mean paired seminal vesicle weights, mean paired testis weights and mean paired adrenal gland weights of male Peromyscus 10 to 90 days of age.

DISCUSSION

Circadian Rhythm

The data obtained in this circadian rhythm study indicates relatively constant serum prolactin concentrations from 6:00 A.M. to 5:00 P.M., a peak at 9:00 P.M., one hour prior to the change from the dark cycle to the light cycle, and the lowest level at 3:00 A.M. (Table 2, Figure 5). Comparison of the results of this study to others with rats (Taya and Igarashi, 1974; Seggie and Brown, 1976; Hefco and Lackey, 1974; Dunn et al., 1972; Ronnekleiv et al., 1973) showed the greatest similarity to those obtained by Ronnekleiv et al., (1973). In both cases, the blood samples were obtained under ether anesthesia and the peak was found one hour prior to the start of the light period.

A review of the other papers on circadian rhythm of serum prolactin shows some with no pattern and others with peaks at various times in the light or dark cycle. The differences between the results referred to above and those obtained in this study could possibly be explained by different environmental stimuli or stress from different blood sampling techniques. It is a well documented fact that serum prolactin is affected by stress. Different studies have shown serum prolactin to be affected by method

of obtaining blood samples (Ajika et al., 1972; Wuttke and Meites, 1970; Raud et al., 1971; Terkel et al., 1972; Neill, 1972), sacrifice position (Seggie and Brown, 1976) and exposure to a novel environment (Baldwin et al., 1974).

In all cases cited above, it was determined that the serum prolactin concentration could best be measured at resting levels if the animal was disturbed as little as possible prior to killing, then the blood sample was obtained by rapid decapitation. In this study, blood samples had to be taken by anesthetizing the animal with ether. This was necessary because in the deermice, decapitation does not provide the amount of blood necessary for the assay.

On the basis of the cited circadian rhythm studies and the above mentioned studies on stress, it can be said that the prolactin concentration is influenced by blood sampling technique, but many other factors must also be considered and must certainly affect the results. It may be that the circadian rhythm of serum prolactin is extremely sensitive to environmental stimuli and the daily activity of the animal. Such things as time of day at which the investigator changes bedding and checks for new litters could possibly be enough to alter the rhythm in each case.

As a result of this study, the samples for the pre and post puberty serum prolactin analysis were obtained between 8:00 A.M. and 10:00 A.M. Prolactin concentrations were found to be relatively stable during this period.

Pre and Post Puberty Serum Prolactin

The data in this study show the serum prolactin concentrations having two major peaks, one at 15 days and the other at 60 days (Table 4, Figure 6). The peak at 15 days is not significantly higher than the ten day old animals ($P < 0.07$) but a trend is established. This peak could be due to the difference in blood sampling technique, the 10 and 15 day old, still being in the litter were hard to identify by toe clipping and usually took longer than the two minute refractory period for an increase of serum prolactin concentrations following the initiation of stress (Terkel, 1972). However, the data obtained are similar to that found by other investigators working with male rats where the first peak in serum prolactin is associated with the growth of the sex accessory organs (Negro-Vilar *et al.*, 1973; McCann, 1974; Yamamoto *et al.*, 1970). A second peak was postulated by these investigators to synergize with testosterone and androgens to bring about a further growth of the sex accessory organs.

The data obtained in this study show testicular weights increase from 10 to 90 days of age. The largest increase is seen between the ages of 40 and 60 days (Table 4, Figure 6). Data on seminal vesicle weights show the weight begins to increase at 30 days of age and experiences the biggest increase from 40 to 60 days of age (Table 4, Figure 6). If the small peak in serum prolactin at 15 days is real, the prolactin increase might be at least partly responsible for

the increase first in testicular weight and then later prolactin and the testicular hormones cause a corresponding increase in seminal vesicle weight at a slightly later time (Table 4, Figure 6). This would be supported by many investigators who believe that serum prolactin is indeed responsible for the growth of these sex accessory organs (Swerdloff et al., 1971; Bartke and Lloyd, 1970; Bartke et al., 1977; Meites and Nicoll, 1966; Antloff et al., 1960).

In this study, no significant correlation could be found between the serum prolactin levels of animals 10 to 20 days of age and the testicular and seminal vesicle weights at this time. This was expected since the prolactin concentrations were rising and falling and the testicular and seminal vesicle weights were just beginning to rise. However, even with no correlation during this period, the increase in the prolactin, or a surge in prolactin in young animals could possibly be a prerequisite for the start of growth of testis development and then the testes and sex accessory organs. Further studies with greater control of stress on the younger animals should shed more light on this relationship.

The large increase in testicular weight and seminal vesicle weight from 40 to 60 days of age correlates well with our definition of puberty. However, the largest increase in prolactin concentration occurs from 50 to 60 days of age. This indicates that prolactin is not

responsible for the increase in organ weight at this age but could be synergizing with other hormones and acting on these organs to bring about sexual maturity and fertility.

Several studies have shown that genetically sterile dwarf mice are prolactin deficient. By using pituitary grafts or prolactin injections, some degree of fertility has been restored. This indicates that prolactin is necessary for reproduction (Cheever et al., 1969; Bartke, 1965; Bartke, 1966; Bartke and Lloyd, 1970). However, by using hypophysectomized dwarf mice, it can be shown that other hormones must synergize with prolactin to produce this effect (Musto et al., 1972). Some investigators believe that prolactin acts on the testes and causes an increased sensitivity to LH. This in turn causes increased activity of the Leydig cells and results in spermatogenesis (Bartke, 1971). Studies show that LH does not increase at puberty, but if the sensitivity to LH increases, then an increase in the hormone concentration would not be necessary (Odell and Swerdloff, 1975; Odell et al., 1974; Swerdloff et al., 1971). Various studies have shown synergism between LH and prolactin. Prolactin and LH administered together have been shown to increase plasma levels of testosterone and androstenedione in hypophysectomized rats (Hafiez et al., 1972), increase esterified cholesterol in the testis of mice (Bartke, 1971), increase the activity of 3 β -Hydroxysteroid Dehydrogenase in the testes (Hafiez et al., 1970)

and increase the activity of 17 β -Hydroxysteroid Dehydrogenase in the testes (Musto et al., 1972).

In contrast, others believe that FSH is responsible for increasing the sensitivity of the testes to LH (Odell and Swerdloff, 1975; Odell et al., 1974; Swerdloff et al., 1971). Serum FSH is high prior to puberty then falls at the time of spermatogenesis. It is thought that the high levels prior to spermatogenesis increase the LH sensitivity.

The data obtained in this study suggests that prolactin may play some role in the initiation of fertility. A significant positive correlation was found between prolactin and testicular and seminal vesicle weight over the entire 90 day period. The rise in prolactin from 20 to 40 days of age may be necessary for preparing the sex accessory organs for puberty. Its rapid increase at puberty signifies that it must play some role in either bringing the organs to maturity or bringing about fertility. The exact effect of the increasing levels of prolactin and the relationship between FSH, LH and prolactin in puberty and sexual maturation can only be known through further research.

The adrenal gland weight of the deermouse 10 to 90 days of age was studied. At this time, no known relationship exists between the adrenal glands and prolactin. However, recent studies show that some relationship may be present. It has been found that adrenalectomy causes increases in serum prolactin (Ogle, 1977), prolactin injections can overcome the effects of adrenalectomy on

puberty (Gelato et al., 1976) and injections of prolactin cause an increase in progesterone in ovariectomized rats (Piva et al., 1973). Various workers have found that adrenalectomy can cause a delay in puberty (Gorski and Lawton, 1973; Gelato et al., 1976; Ramaley and Campbell, 1977; Ramaley, 1974). This delay in puberty is accompanied by a delay in the serum prolactin peak (Ramaley and Campbell, 1977).

Data from this study show an increase in the adrenal gland weight between 50 and 60 days of age. A significant positive correlation was also found over the entire 90 day period. This suggests that the adrenal gland could possibly have some role in puberty and it could either affect the prolactin concentration or be affected by the increasing serum prolactin concentration. At this time, it is unclear what the role of the adrenal gland is in puberty and how it effects prolactin concentration. It could possibly be merely a coincidence that the adrenal gland weight was increasing at the same time the prolactin concentrations were increasing. If the prolactin concentrations are responsible for organ weight changes and puberty, then anything that shifts puberty will automatically shift prolactin. Further research directed at this relationship needs to be undertaken.

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APPENDIX A

Results of Statistics Performed on Paired Adrenal Gland Weights (mg.)

<u>Age (Days)</u>	<u>N</u>	<u>Mean $\pm$ SE</u>	<u>F-test</u>	<u>Student's t</u>	<u>Mann-Whitney U</u>
10	25	0.89 $\pm$ 0.05	P<0.50	P<0.01	P<0.002
15	21	1.16 $\pm$ 0.09	P<0.25	P<0.01	P<0.0082
20	25	1.45 $\pm$ 0.07	P<0.01	P<0.01	P<0.0089
30	11	1.95 $\pm$ 0.20	P<0.50	P<0.10	P<0.1000
40	12	2.43 $\pm$ 0.16	P<0.50	P<0.50	
50	11	2.59 $\pm$ 0.16	P<0.10	P<0.05	P<0.10
60	11	3.22 $\pm$ 0.25	P<0.25	P<0.90	
90	11	3.13 $\pm$ 0.34

APPENDIX B

Results of Statistics Performed on Prolactin Concentrations (ng/ml)

<u>Age (Days)</u>	<u>N</u>	<u>Mean $\pm$ SE</u>	<u>F-test</u>	<u>Student's t</u>	<u>Mann-Whitney U</u>
10	12	14.47 $\pm$ 2.29	P<0.25	P<0.10	P<0.10
15	17	21.09 $\pm$ 2.74	P<0.50	P<0.10	P<0.0446
20	23	14.73 $\pm$ 2.20	P<0.25	P<0.10	P<0.0446
30	16	21.44 $\pm$ 3.20	P<0.25	P<0.40	
40	14	27.61 $\pm$ 4.62	P<0.25	P<0.90	
50	15	31.10 $\pm$ 4.14	P<0.50	P<0.01	P<0.05
60	10	50.57 $\pm$ 5.58	P<0.05	P<0.90	
90	8	49.31 $\pm$ 3.10			

Compare 20 day with 50 day old P<0.001

Exact probability of 10 to 15 day old comparison P<0.076176

Exact probability of 15 to 20 day old comparison P<0.020371

APPENDIX C

Results of Statistics Performed on Body Weights (grams)

<u>Age (Days)</u>	<u>N</u>	<u>Mean $\pm$ SE</u>	<u>F-test</u>	<u>Student's t</u>	<u>Mann-Whitney U</u>
10	25	5.24 $\pm$ 0.08	P<0.001	P<0.001	P<0.00001
15	26	6.38 $\pm$ 0.17	P<0.05	P<0.001	P<0.00001
20	26	8.47 $\pm$ 0.25	P<0.50	P<0.001	P<0.00001
30	12	12.39 $\pm$ 0.43	P<0.50	P<0.20	
40	13	13.45 $\pm$ 0.44	P<0.50	P<0.05	P<0.1000
50	13	14.99 $\pm$ 0.49	P<0.05	P<0.50	
60	13	15.45 $\pm$ 0.30	P<0.05	P<0.01	P<0.02
90	13	17.48 $\pm$ 0.51			

Compare 30 day old with 50 day old P<0.001

Compare 30 day old with 60 day old P<0.001

APPENDIX D

Results of Statistics Performed on Paired Testis Weights (mg.)

<u>Age (Days)</u>	<u>N</u>	<u>Mean $\pm$ SE</u>	<u>F-test</u>	<u>Student's t</u>	<u>Mann-Whitney U</u>
10	23	7.47 $\pm$ 0.32	P<0.001	P<0.001	P<0.0013
15	26	10.55 $\pm$ 0.73	P<0.001	P<0.001	P<0.00003
20	24	22.33 $\pm$ 2.05	P<0.005	P<0.001	P<0.00003
30	11	56.00 $\pm$ 5.95	P<0.10	P<0.05	P<0.05
40	13	83.57 $\pm$ 8.82	P<0.25	P<0.001	P<0.002
50	13	171.59 $\pm$ 11.47	P<0.50	P<0.001	P<0.002
60	13	253.96 $\pm$ 12.12	P<0.25	P<0.01	P<0.02
90	11	324.59 $\pm$ 17.46			

APPENDIX E

Results of Statistics Performed on Paired Seminal Vesicle Weights (mg.)

<u>Age (Days)</u>	<u>N</u>	<u>Mean $\pm$ SE</u>	<u>F-test</u>	<u>Student's t</u>	<u>Mann-Whitney U</u>
10	24	1.03 $\pm$ 0.04	P<0.50	P<0.50	
15	24	1.07 $\pm$ 0.03	P<0.50	P<0.001	P<0.00006
20	24	0.86 $\pm$ 0.04	P<0.001	P<0.01	P<0.00280
30	11	1.27 $\pm$ 0.19	P<0.001	P<0.01	P<0.002
40	12	11.33 $\pm$ 2.52	P<0.001	P<0.001	P<0.002
50	12	52.66 $\pm$ 8.56	P<0.10	P<0.001	P<0.002
60	13	116.76 $\pm$ 13.00	P<0.10	P<0.05	
90	13	166.38 $\pm$ 19.28			

APPENDIX F

Summary of Results of Linear Regression Analysis

	<u>Age Groups</u>	<u>N</u>	<u>r</u>	<u>P</u>
Prolactin versus Paired Testis Weight	10 - 20	51	0.086451	n.s.
	20 - 90	83	0.595707	P<0.01
	10 - 90	111	0.594119	P<0.01
Prolactin versus Paired Adrenal Weights	10 - 20	50	0.176757	n.s.
	20 - 90	79	0.512447	P<0.01
	10 - 90	106	0.532204	P<0.01
Prolactin versus Paired S. V. Weight	10 - 20	49	0.027211	n.s.
	20 - 90	80	0.596058	P<0.01
	10 - 90	108	0.604192	P<0.01
Age versus Prolactin	10 - 20	52	-0.079633	n.s.
	20 - 90	86	0.633012	P<0.01
	10 - 90	115	0.623719	P<0.01

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