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It is the purpose of this study to correlate environmental parameters with the breeding behavior and spermatogenic cycle of the male spring peeper, Hyla crucifer. The dates of calling male H. crucifer along with daily high temperature, low temperature, photoperiod, and rainfall were recorded in 1973-1975. A standardized discriminant function analysis indicated that photoperiod had the most significant effect upon the total number of days that H. crucifer called (N = 248), followed by high temperature, low temperature, and rainfall, respectively.

A multiple regression analysis was used to correlate testicular data, climatological data, and the number of days per month frogs called. The above analysis included sample groups from the wild, an outdoor cage group, and an indoor cage group. Highly significant negative correlations were found between the mean monthly temperature and mean monthly photoperiod to the number of days per month frogs called. Highly significant negative correlations also existed between mean monthly temperature and mean monthly photoperiod to the mean number of spermatogonia. A highly significant positive correlation was found between the number of days per month frogs called and the mean number of sperm, and a high negative correlation between the mean number of sperm and mean monthly temperature.

Gonadal cell counts from the three sample groups were compared by analysis of variance. This analysis and photographs indicated that the spermatogenic cycle of the indoor cage population (March-July) exhibited abnormal lags and accelerations.

These data indicate that the breeding season and reproductive behavior of H. crucifer may be initiated and terminated by photoperiod.

It may also be concluded that once the breeding season has begun, temperature and rainfall become the controlling environmental cues for breeding behavior. This study also supports the hypothesis that temperature has the most significant effect upon spermatogenesis during the breeding season. This study disputes reports from the literature that H. crucifer exhibits a discontinuous spermatogenic cycle, but possibly displays a potentially continuous or continuous spermatogenic cycle.

ENVIRONMENTAL EFFECTORS OF BREEDING CALLS AND SPERMATOGENESIS IN THE MALE SPRING PEEPER, *HYLA CRUCIFER*, IN GUILFORD COUNTY, N.C.

by

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APPROVAL PAGE

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INTRODUCTION

Studies of anuran sexual behavior and sexual cycles date back to the latter part of the 17th century to the Dutch scientist, van Swammerdam (van Oordt, 1960). The earlier experimental investigations of anurans centered primarily upon the genera Rana and Bufo. The study of reproductive physiology in male Hyla crucifer by Rugh (1941) was probably one of the earliest investigations of the anuran genus Hyla.

Hyla crucifer, the spring peeper, is a small tree frog measuring between 2-3 cm in snout-vent length (Rosen and Lemon, 1974). It varies in colors being either brown, gray, or olive, with a characteristic darker cross or "X" across the back.

Hyla crucifer ranges from northern Florida to southern Canada, west to east Manitoba, and as far west as east Texas (Conant, 1975). It is rarely found in the wild until the breeding season, at which time the males can be located by their call.

Calling

Extensive literature indicates that anuran vocalization is related to sex recognition, the facilitation of breeding, and reproductive isolation (Aronson, 1943; Littlejohn, 1958; Blair, 1958a, 1958b, 1961; Crenshaw and Blair, 1959; Martof, 1961; Martof and Thompson, 1958, 1964; Gerhardt, 1973a, 1973b; Loftus-Hills, 1974; Lofts, 1974; Oldham, 1974).

Of the 14 species of hylid frogs in the United States (Blair, 1958b), the mating call of Hyla crucifer is similar to only one other call structure, Pseudacris ornata (Gerhardt, 1973a). Although Hyla crucifer is

sympatric with Pseudacris triseriata in Guilford County, N.C., and breeds at approximately the same time of year, no hybrids have been found in the wild. Reproductive isolation is probably enforced by calling structure and calling sites. Pseudacris triseriata calls in the water while Hyla crucifer calls one cm to six m above the ground or water.

The male H. crucifer emits a shrill peep about once per second with an average dominant frequency of 3200 Hz. and duration of 0.13 seconds (Martof, 1961). The male spring peeper also produces a trill, implicating territorality when another male is near by (Rosen and Lemon, 1974). The trill is also a release call given when a male is grasped by another male or given by a non-gravid female (Aronson and Noble, 1945; Aronson, 1943). The trill is longer in duration and has a smaller amplitude than the peep.

Hyla crucifer is considered a cool weather or winter breeder. Mating calls can be heard in the night from late November to May in the South, and into the latter part of June further north (Conant, 1975). In Guilford County, N.C., the spring peeper mating call can be heard from early January until early May, with peak activity and chorusing occurring from the middle of February to the middle of March.

The number of days that mating calls of H. crucifer were heard in Guilford County were observed to be sporadic. During the breeding season the cessation of calling sometimes followed a severe cold front or dry spell. On several occasions mating calls of H. crucifer were heard two to three months prior to the actual breeding season. Following a weak cold front and 1.3 cm of rain, sporadic calls of H. crucifer were heard in Guilford County from 29 September to 31 October 1974. Also on 14 November 1974 between the hours of 2200 and 2300, several H. crucifer were heard calling at approximately 5°C during 0.18 cm of rainfall.

It is possible that temperature is the most important environmental factor initiating and regulating mating calls and the breeding season in anurans (Rugh, 1941; Moore, 1942; Brattstrom and Warren, 1955; Martof, 1960; Blair, 1961; Bellis, 1957; Gosner and Black, 1957; Jones and Brattstrom, 1961; Salthe and Mecham, 1974). Records of breeding calls of many anuran species show that populations from more northern latitudes and higher altitudes exhibit delayed breeding seasons. Rana sylvatica and Hyla crucifer are winter breeders in the South, but breed in April and May in Alaska and Ontario.

Rugh (1941) postulated that temperature was the major environmental factor determining the breeding season of Hyla crucifer. Jones and Brattstrom (1961) suggested that the initiation, continuation, and cessation of calling H. crucifer were primarily controlled by temperature, and that the rate of calling by H. crucifer is temperature-dependent. Martof and Thompson (1964) reported that an increase in temperature resulted in an increase in the rate of calling by P. triseriata near Athens, Ga. Martof (1960) noted that reproductive activity in H. crucifer was associated with the interactions between seasonal changes (temperature, light, rainfall) and intrinsic metabolic processes. Martof (1960) also reported that calling and spawning in H. crucifer were initiated in September near Athens, Ga., by the passage of a weak cold front and subsequent 6.8°C drop in temperature. Gosner and Black (1957) suggested that while rainfall is the primary factor controlling reproductive activity in Scaphiopus h. holbrooki, temperature was an important cue in determining the beginning of the breeding season. Highly significant positive correlations were found by Bellis (1957) between the frequency of calls and water temperature

in Pseudacris nigrita triseriata and P. clarki and call length and temperature in P. nigrita triseriata and Bufo terrestris charlesmithi. Blair (1961) postulated that the calling periods of Pseudacris streckeri near Austin, Texas, were largely determined by temperature. That temperature is an important controlling factor for the time of breeding in a given locality was suggested by Moore (1942) for five northeastern anuran species. Salthe and Mecham (1974) pointed out that temperature played a dominant role in the regulation of reproductive activity in anurans, and that high temperature was associated with the initiation of the breeding season of Rana aurora, Rana pretiosa, Bufo americanus, and Bufo variegatus. Salthe and Mecham (1974) also suggested that the combination of rainfall and temperature complemented the breeding periods of Bufo valliceps and Bufo bufo. The environmental effects of temperature, rainfall, and photoperiod upon mating calls and breeding behavior will be discussed in detail below.

Schmidt (1966a, 1966b, 1968, 1973) found that intraperitoneal injections of anterior pituitary or human chorionic gonadotrophin into Hyla cinerea and H. versicolor induced mating calling when taped recordings of conspecific calls were played. Testosterone pellet implants by Greenberg (1942) also induced mating calling in Acris gryllus. Castrated individuals in test groups of H. versicolor with anterior pituitary and human chorionic gonadotrophin injections failed to call after ten days but began to call after implantation of two testes per frog (Schmidt, 1966a).

Following a series of lesions and electrical stimulations of the brain, Schmidt (1966a, 1968, 1973) found the ventral magnocellular preoptic nucleus of H. cinerea and H. versicolor to be essential for mating calling

(Fig. 1). Furthermore, stimulation of the trigemino isthmic tegmentum produced a release call in Rana pipiens (Schmidt, 1973). (Fig. 1). Schmidt (1973) suggested that the immediate area surrounding the preoptic nucleus is an androgen receptor site which, when stimulated by testicular hormone production, activated sensory and motor coordination centers of the brain which simultaneously activated calling. This postulate may be supported by the study of Kelly et al (1975). Following the administration of tritiated testosterone in male Xenopus laevis, large numbers of labelled cells were observed by autoradiography in the anterior preoptic area, the ventral infundibular nucleus, the dorsal tegmental area of the medulla, and in the posterior lateral medulla. Kelly et al (1975) suggested that testosterone uptake in the above mentioned areas of the brain in Xenopus laevis may be involved in reproductive behavior.

Spermatogenesis

Testicular histology has revealed that spermatogenesis in anamniotic vertebrates occurs in clusters or groups of cells ensheathed by a germinal capsule or cyst (Reed and Stanley, 1972). Lofts (1968) described this variety of spermatogenesis as cystic. A fibroblastic-like follicle cell with a darkly staining crescent-shaped nucleus surrounded each primary spermatogonium and matured into a germinal capsule as the spermatogonium proliferated (Lofts, 1974).

Spermatogenesis is a mitotic-meiotic process that results in the growth and maturation of haploid spermatozoa from diploid stem spermatogonia. An ensheathed diploid secondary spermatogonium transforms into a diploid primary spermatocyte; a mitotic division produces a cyst of diploid secondary spermatocytes which undergo a meiotic reduction division producing

Fig. 1. Diagrammatic sagittal section through the brain of Rana pipiens. A. Cerebellum; B. Tectum; C. Cerebral hemisphere; D. Olfactory bulb; E. Ventral magnocellular preoptic nucleus; F. Trigemino isthmic tegmentum; G. Hypothalamus; H. Chiasma; I. Hypophysis (Schmidt, 1966b; Lofts, 1974).

haploid spermatids that metamorphose into spermatozoa (Rugh, 1951). The maturation process of spermatids is called spermiogenesis, spermateleosis, or spermatohistogenesis (Lofts, 1974).

Spermatogenesis in the anuran testis is either a continuous, discontinuous, or potentially continuous cycle (van Oordt, 1960; Basu, 1969; Lofts, 1974). The continuous cycle is usually displayed by anurans inhabiting a tropical or subtropical climate, while a discontinuous spermatogenic cycle occurs in species inhabiting a more temperate and cooler environment. In the continuous cycle, the production of spermatogonia and germinal cysts of spermatocytes persists throughout the entire year. Spermatogenesis is interrupted in the discontinuous cycle and is confined to a specific period of the year. Spermatogonia enter a resting stage or inactive period between October and January during which no mitotic activity occurs (Lofts, 1974). Some species of anurans exhibit a potentially continuous spermatogenic cycle. During unfavorable environmental conditions, spermatogenesis continues at a slower rate with activity returning to normal when environmental factors become optimum.

The annual cycle of spermatogenesis in anurans with either a discontinuous or a potentially continuous cycle is divided into three periods. The interruption of the cycle is described as the resting period (autumn and winter); the breeding season during late winter and early spring is the spermiation period; and the cytospermatogenic period (Hahn, 1964) which occurs during spring and summer (van Oordt et al 1959; van Oordt, 1960; van Oordt and Lofts, 1963; Lofts, 1974). These seasonal periods vary with interspecific adaptations and may contribute to reproductive isolation. For example, the three periods of spermatogenesis in

Rana catesbiana and Hyla versicolor, which are discontinuous summer breeders, are altered significantly from the above mentioned seasons.

Spermatogenesis in anurans is primarily controlled by the secretory activity of the pars distalis in the pituitary gland. The gonadotrophic producing cells in the pars distalis are stimulated by a neurohumoral substance produced by the preoptic nucleus that is sensitive to seasonal environmental changes (van Oordt, 1960). Amphiphils type I or β-cells in the pars distalis secrete FSH-like (follicle-stimulating hormone) substance during the cytospermatogenic period which increases the mitotic activity and number of spermatogonia. ICSH-like (interstitial cell stimulating hormone, also known as LH) substance which has the opposite effect on cytospermatogenesis, is secreted by the amphiphils type II or λ-cells of the pars distalis during the spermiation period and induces the interstitium and Leydig cell system of the testis to produce the androgen testosterone (van Oordt, 1960; van Oordt and Lofts, 1963; Lofts, 1964). Testosterone induces the release of sperm bundles (spermiation) and inhibits the mitotic capacity of spermatogonia; but does not affect the spermatocytes or spermatids (Basu and Nandi, 1965). The inhibitory ability of testosterone on spermatogonia is thought to be a feedback mechanism affecting the FSH-producing amphiphils of the pars distalis (van Oordt, 1960; Lofts, 1974). Basu and Nandi (1965) observed the suppression of spermatogenesis at the spermatogonial level in Rana pipiens after injections of testosterone.

Anuran spermatogenesis is also regulated by the seasonal changes in the sensitivity of the germinal epithelium of the testis to gonadotrophic hormones. It is probable that testosterone inhibits the sensitivity of

the germinal epithelium to gonadotrophins directly, or indirectly via the feedback mechanism to the pars distalis (van Oordt, 1960; Basu and Nandi, 1965; Basu, 1969).

Another regulatory factor in anurans with a truly discontinuous spermatogenic cycle is the inherent mechanism. Witschi (reported in van Oordt, 1960) changed the habitat of Rana temporaria which did not change spermatogenesis at all, and concluded that the annual cycle was regulated by genetic factors.

Environmental Effectors of Spermatogenesis

Rainfall is essential as a trigger mechanism for the breeding behavior in most anurans, but has no significant effect on spermatogenesis (Lofts, 1974). Temperature is also important for initiating the breeding season and has a regulatory effect on spermatogenesis (Lofts, 1974). van Oordt and Lofts (1963) found that when Rana temporaria was exposed to high temperatures the secretory activity of the FSH-producing β-cells in the pars distalis was accelerated while that of the ICSH-producing λ-cells was diminished. The accelerated FSH output caused the breakdown and extrusion of sperm bundles and Sertoli cells, and initiated cytospermatogenesis.

In anurans with a potentially continuous spermatogenic cycle, such as Rana esculenta, spermatogenic activity is increased or decreased by raising or lowering the environmental temperature (van Oordt, 1960). Ifft (1942) found that if the salamander, Triturus viridescens, were continually exposed to elevated temperatures, spermatogenesis was stimulated. Aldridge (1975) postulated that temperature and a temperature threshold were essential for the stimulation of spermatogenic activity in the rattlesnake, Crotalus viridus, while Licht (1971, 1972) indicated that spermatogenesis in reptiles, specifically Anolis carolinensis, was initiated by temperature.

van Oordt (1960) postulated that photoperiod had no direct effect on spermatogenesis in anurans, but may induce spermiation and breeding behavior. Photoperiod also appears to have no effect on the spermatogenic cycle in the rattlesnake Crotalus viridus (Aldridge, 1975). However, Werner (1969) found that increasing the photoperiod advanced the spermatogenic cycle in the salamander Plethodon cinereus; Licht (1971) theorized that the regressive phase of spermatogenesis in the lizard Anolis carolinensis appeared to be maintained by photoperiod.

Feeding behavior in anurans is not a primary factor in the spermatogenic cycle although complete starvation will impair testicular function (van Oordt, 1960; Werner, 1969).

It was the purpose of this study to discuss climatological data affecting the reproductive activity of anurans, specifically Hyla crucifer. While the effects of environmental cues on reproductive behavior is species-specific, the literature indicates that temperature is the most important environmental cue with little or no inference placed on photoperiod. Lack of knowledge of the effects of photoperiod is probably due to experimental design. Rainfall is necessary for stimulation of breeding behavior and migration towards breeding sites in most anurans. The combined influence of these environmental stimuli upon the reproductive cycle of H. crucifer will be discussed in detail below.

MATERIALS AND METHODS

Field Techniques

Specimens of Hyla crucifer crucifer were collected from January to April in 1974 and 1975. Collecting sites were semi-permanent pools located in the Guilford Courthouse National Park approximately 10 km north of Greensboro, Guilford County, N.C.; a power-line cut approximately 1.6 km north of Guilford Courthouse Park; and an open field beside a small creek on Chimney Rock Rd. approximately 16 km west of Greensboro.

All collections were done at night between 2000 and 2400 hrs. The majority of specimens caught were calling males, which are distinguished by an olive-brown throat sac (Oplinger, 1966). Samples taken from the wild were located by their calls. Weather permitting, samples were taken on the 26th, 27th, or the 28th of each month.

The dates of calling male Hyla crucifer were recorded throughout the entire breeding season in 1973, 1974, and 1975. Calling data were recorded in order to discuss environmental parameters affecting breeding and calling.

Climatological data were obtained from the U.S. Weather Bureau located at the Greensboro-Winston-Salem-High Point Regional Airport. Photoperiodic data were obtained from the American Ephemeris and Nautical Almanac; 1972-1975.

No Hyla crucifer were taken from the wild in 1973. In 1974, during January-April, a total of 103 Hyla crucifer were captured. Seventy-six frogs were placed into a wooden-framed, screen-wire cage, approximately

61 cm X 122 cm X 91 cm, and kept outdoors from March-July. The cage contained a glass bowl, sand and leaf litter to a depth of 15 cm, and was doused with water at least three times weekly. Caged frogs were fed cultured Drosophila melanogaster and D. virilis.

Sample numbers from the wild consisted of seven Hyla crucifer from January, none in February (split breeding season), 10 in March, and 10 in April. The breeding season was interrupted for approximately four weeks in January and February due to a severe cold front. May, June, and July samples were taken from animals held in captivity. Samples from captivity consisted of 10 frogs in May, 10 in June, and two in July (only two remaining alive). All samples were preserved in 10% neutral buffered formalin.

In January and February of 1975, a total of 139 Hyla crucifer were caught. Eighty-two were placed into the outdoor cage, and 49 frogs were placed into a 38-liter aquarium indoors. Although there was no control over inside lighting, the aquarium was situated below a large glass window allowing the indoor caged frogs to experience photoperiods similar to natural conditions. The laboratory temperature was maintained at approximately 22.5°C.

Three sample frogs were taken from the wild in January, and five in February, March, and April. Also in March and April five sample frogs were taken from the outdoor cage and five from the indoor cage. Each May, June, and July sample consisted of five Hyla crucifer from the outdoor cage and five from the indoor cage.

Histology

Testes from H. crucifer were removed under a dissecting microscope. Fatty tissue surrounding a testis was removed carefully with dissection needles and scalpel. With the use of an ocular micrometer, the lengths and widths of all testes were recorded. Testes color and stomach contents were also noted. Paired testes from each frog were stored in 10% neutral buffered formalin in 12 mm X 35 mm glass vials.

The testes were prepared for histological staining and sectioning by the Erlich's Hemotoxylin Bulk Stain method (Appendix I, Gray, 1964). This method was used in order to locate the small testes while embedding. Paraffin used for embedding was 53°C - 55°C Histowax. Final embedding was accomplished in a vacuum oven at -7.26 kg/2.54 cm^2. Paired testes from each frog were embedded and cut together.

The tissue was placed in salt dishes for the paraffin I and II steps (Appendix I). For final embedding, as the testes were being removed from the vacuum oven, a heated metal instrument was used to line paired testes parallel in the bottom of the salt dish. Salt dishes were then placed in ice to facilitate the extraction of embedded tissue. Upon removal of the paraffin block (embedded tissue), excess paraffin was trimmed in a manner that allowed the parallel testes to be mounted on a 1.91 cm^2 wooden block. The wooden block was screwed tightly into the vice of the microtome, and the tissue was cross-sectioned at three and four micrometers. Depending on the texture and size of the tissue, two to six slides per set of testes were made. A beaker of boiling water was placed near the microtome while sectioning in order to decrease static electricity

in the lab atmosphere which facilitated in decreasing the "clinging and curling effect" of the paraffin tissue-ribbon.

At times the testes appeared harder than normal and indeed were more difficult to embed and cut. When this difficulty occurred, the tissue was washed in distilled water for 24 hours when taken from Bouin's fixative, and the embedding time was increased.

Gonadal Cell Counts

Slides containing the cross section of the median portion of each testis were used for spermatogonium, spermatocyte, spermatid, and sperm counts (Atherton, 1974). Identification of cell types was according to Rugh (1951). Counting was achieved with a Wild microscope at 400 power in bright field. Five seminiferous tubules with approximately the same diameter were chosen randomly as the counting sites. Each tubule was morphologically distinct which disallowed the repetition of any one tubule.

Spermatogonia are located around the periphery of the seminiferous tubule next to the basement membrane. They are somewhat elongated spheres two to four micrometers in diameter. Primary spermatocytes are much larger cells, with a large vesicular nucleus, approximately 10-12 micrometers in diameter. Secondary spermatocytes are half the size of primary spermatocytes, lie closer to the lumen of the tubule with a darkly stained basophylic nucleus, and are approximately six to eight micrometers in diameter. Spermatids are small and darkly stained with a diameter of two to four micrometers. Sperm of H. crucifer are spirally shaped (Rugh, 1941) and are normally arranged in bundles around the periphery of the tubule. An ocular grid, 100 micrometers^2, was used for sperm counts. A cross

section of a sperm bundle revealed that approximately 100 sperm occupied a 10 micrometer^2 grid.

Photography

Cross sections of testes were photographed with a Wild shutter mechanism in a 35 mm Nikon camera at 400 diameters on bright field with 1/8 sec shutter speed while using high speed Ectachrome tungston film.

Statistical Methods

Calling data versus environmental data are described by the standardized discriminant function analysis (Nie et al, 1975). Discriminant analysis statistically analyzes two or more groups (calling frogs versus non-calling frogs) by comparing these groups with discriminating variables, month (photoperiod), rainfall, low daily temperature, and high daily temperature. The importance of each variable is defined in the discriminant function correlation coefficient.

Multiple regression analysis was used to correlate the following variables: date, total monthly rainfall, mean monthly temperature, mean monthly photoperiod, number of days per month frogs called, mean testis length and width, mean number of spermatogonia, mean number of primary and secondary spermatocytes, mean number of spermatids, mean number of sperm, and seminiferous-tubule diameter. The correlation coefficient of the multiple regression analysis predicts a positive or negative correlation among the above mentioned dependent and independent variables (Nie et al, 1975).

Gonadal cell types of frogs from the wild, outdoor cage, and indoor cage were compared by an analysis of variance with the frequencies of types considered significantly different if the F value was less than .05.

RESULTS

Calling

Mating calls of male H. crucifer are generally heard after sunset and continue until sunrise. On 28-30 October 1974, several H. crucifer were heard calling at the Chimney Rock Rd. study area at 0930 hrs and from 1700 to 1800 hrs, but ceased calling at sunset. All individuals observed calling during the breeding season were found out of the water. Specimens in the field were difficult to capture at the beginning and end of the breeding season, but were more easily caught during the peak of the breeding period. During field observations it was noted that sometimes an auditory stimulus was required to initiate calling. Generally, a group of specimens would begin calling following the call of one individual, a leader, or often calling began as an airplane or car passed by (Bellis, 1957; Jones and Brattstrom, 1961; Rosen and Lemon, 1974).

The earliest mating calls of H. crucifer were heard on 29 September 1974 near Lake Jeanette, Guilford County, during hard rains in the night, and the latest calling was heard late at night on 19 May 1975 at the Guilford Courthouse study area. The earliest and latest amplexed pairs of H. crucifer were observed 29 January 1974 and 21 March 1975, respectively. Although the breeding periods of H. crucifer and Pseudacris triseriata feriarum overlap from the latter part of January to early March in Guilford County, no interspecific amplexus was observed.

In 1973, 1974, and 1975, H. crucifer called on a total of 248 days. For each day from July 1972 through July 1975, rainfall, photoperiod, low

and high temperature were recorded. The standardized discriminant function analysis compared the above environmental variables for each day that frogs called. The discriminant function coefficient indicates the impact each environmental variable has on calling, with month or photoperiod being the most significant, followed by high daily temperature, low daily temperature, and rainfall (Table 1). Using the discriminant function coefficient (DFC) for the study years and months and the DFC of the monthly mean environmental variables, the formula given in Table 1 predicted with 79.75% accuracy that H. crucifer would call in a particular month.

The multiple regression analysis correlated environmental data with the number of days that frogs called per month and with testicular data. The multiple regression correlation coefficients indicated high negative correlations of mean monthly temperature (-.75) and mean monthly photoperiod (-.50) to the number of days per month frogs called, and a positive (.55) correlation of the mean number of sperm to the number of days per month frogs called (Table 2).

The R Square value of the multiple regression analysis, as shown in Table 3, represents the variability of each environmental factor as a percentage and increases in significance as each of the environmental factors is considered. As can be seen in Table 3, the effects of mean monthly temperatures on the dependent variable, number of days per month that frogs called, increase from 57% to 64% when the remaining independent environmental variables are combined. The analysis indicated that 64% of the data (number of days per month frogs called) were explained by the mean monthly temperature, mean monthly photoperiod, date, and total monthly rainfall combined.

Table 1. Standardized Discriminant Function Coefficients

Month:	.55618
H Temp:	.31947
L Temp:	.19640
Rainfall:	.09161
Year:	.12232

With Call (Y) = 0 (no call) or 1 (call), the analysis predicted 79.75% correctly for calling within a particular month when Y = Dis. Fun. Coe. (Month) + DFC (Year) + DFC (Rainfall) + DFC (H Temp) + DFC (L Temp).

MULTIPLE REGRESSION CORRELATION COEFFICIENTS

	(02)	(03)	(04)	(05)	(07)	(08)	(09)	(10)	(11)	(12)	(13)	(14)
(02)	1.000											
(03)		1.000										
(04)												
(05)												
(07)			-.7536	-.5019								
(08)						1.000						
(09)							1.000					
(10)			-.6831	-.7081								
(11)									1.000			
(12)										1.000		
(13)			-.6560		.5479			.5173				
(14)												1.000

Table 2. Significant Correlation Coefficients. (02) Date, (03) Rainfall, (04) Mean Temp., (05) Mean Photoperiod, (07) Number of days frogs called, (08) Mean testis length, (09) Mean testis width, (10) Mean number of spermatogonia, (11) Mean number of primary and secondary spermatocytes, (12) Mean number of spermatids, (13) Mean number of sperm, (14) Mean seminiferous tubule diameter.

Table 3. Multiple regression analysis with R Square values. The R Square variability, shown as a percentage, represents the influence of each independent environmental variable upon the dependent variables number of days per month frogs called, mean number of spermatogonia, and mean number of sperm.

# of Days per Month Frogs Called

	R Square
Mean Monthly Temperature:	.5680
Mean Monthly Photoperiod:	.6364
Date:	.6401
Total Monthly Rainfall:	.6403

Mean # of Spermatogonia

	R Square
Mean Monthly Photoperiod:	.5014
Mean Monthly Temperature:	.5254
Date:	.5283
Total Monthly Rainfall:	.5311

Mean # of Sperm

	R Square
Mean Monthly Temperature:	.4303
Total Monthly Rainfall:	.4591
Mean Monthly Photoperiod:	.4784
Date:	.4790

Testicular Data

Of the 129 specimens which were observed, one H. crucifer had three testes and one had only one testis. Twelve pairs of testes were a dull grey while the remaining pairs were solid black. Histological examination revealed very few sperm bundles but active cytospermatogenesis within the seminiferous tubules in all but two pairs of the dull grey testes. Testes from a male specimen taken one day after amplexus had occurred exhibited a greyish coloration and very few sperm bundles within the seminiferous tubules. This confirms Rugh's (1941) report that solid black testes contain sperm bundles while the dull grey testes have no or very few sperm bundles.

Environmental Effects on Gonadal Cell Types

The multiple regression analysis indicated no significant correlations between environmental factors and mean testis length and width, mean seminiferous tubule diameter, mean number of primary and secondary spermatocytes, and spermatids. High negative multiple regression correlation coefficients existed between the mean number of spermatogonia and mean monthly temperature (-.68) and mean number of spermatogonia and mean monthly photoperiod (-.71). There was a positive (.52) correlation coefficient between the mean number of spermatogonia and mean number of sperm. The analysis also indicated a high negative correlation coefficient between mean number of sperm and mean monthly temperature (-.66) as well as a positive correlation between mean number of sperm and days per month frogs called (.55) (Table 2).

The R Square variability in the multiple regression analysis of each environmental factor's effect on gonadal cells indicated that the

mean monthly photoperiod represents 50% of the data for mean number of spermatogonia, with 53% of the data being explained by the combination of mean monthly photoperiod, mean monthly temperature, date, and total monthly rainfall, respectively. Also, 43% of the data for mean number of sperm were explained by mean monthly temperature with 48% of the data being explained by mean monthly temperature, total monthly rainfall, mean monthly photoperiod, and date (Table 3).

Gonadal Cell Comparisons

An analysis of variance compared each gonadal cell type from all sample frogs taken from the wild, outdoor, and indoor cages from January through July. The analysis indicated that the mean number of spermatogonia, spermatids, and sperm from sample testes taken from the wild and the outdoor cage in March were not different, but both were significantly different from the indoor cage population. The analysis also indicates that the mean number of spermatogonia and primary and secondary spermatocytes from all three sample groups during April were similar, while the mean number of spermatids and sperm counts from the wild and outdoor cage population were significantly different from the indoor caged population.

The gonadal cell counts from the indoor cage population exhibited abnormal lags and accelerations in the spermatogenic cycle. Following one month of captivity, the mean number of spermatogonia displayed an abnormal decrease and an abnormal increase after two months of captivity (Fig. 2). In the third month the primary and secondary spermatocytes exhibited an abnormal decrease followed by an extremely abnormal increase during the fourth and fifth months (Fig. 3). The mean number of spermatids also displayed an abnormal decrease after the third month of captivity but

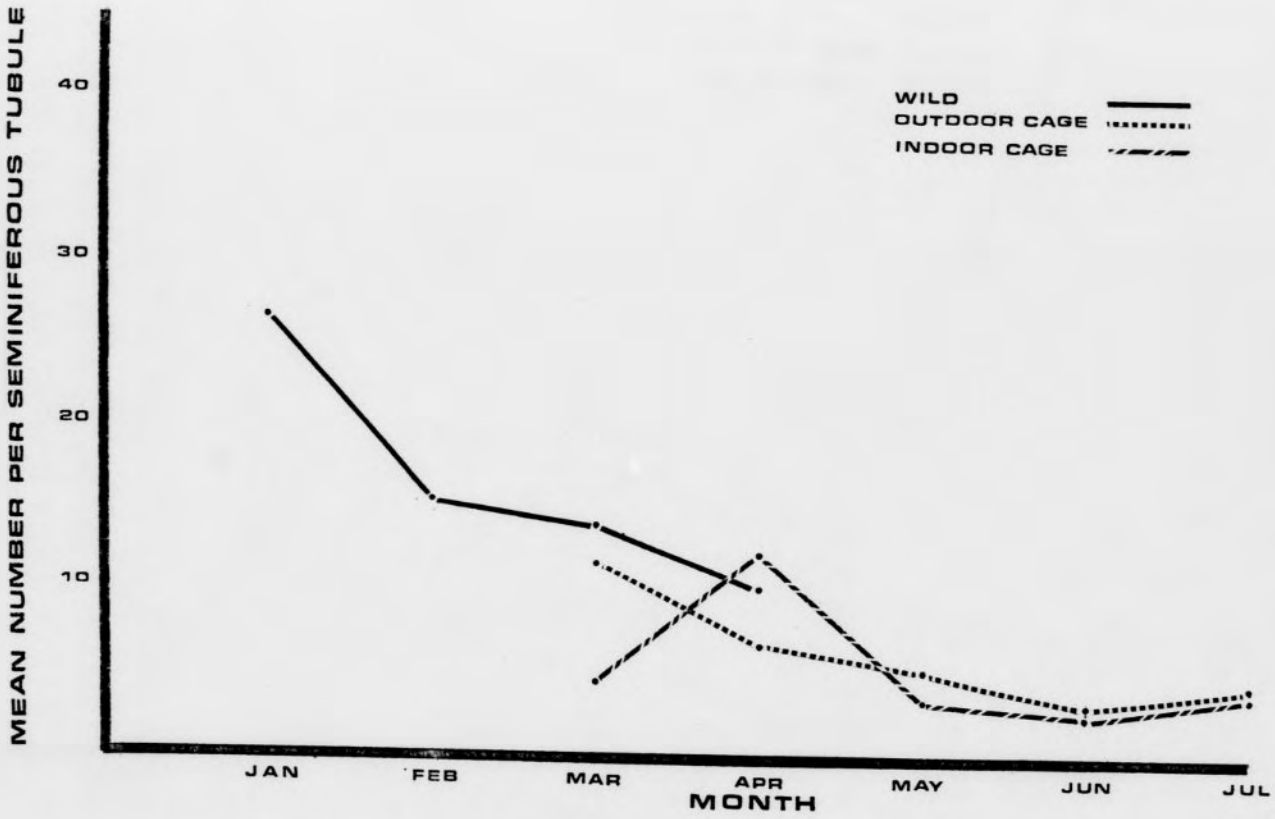

FIG. 2. NUMBER OF SPERMATOGONIA

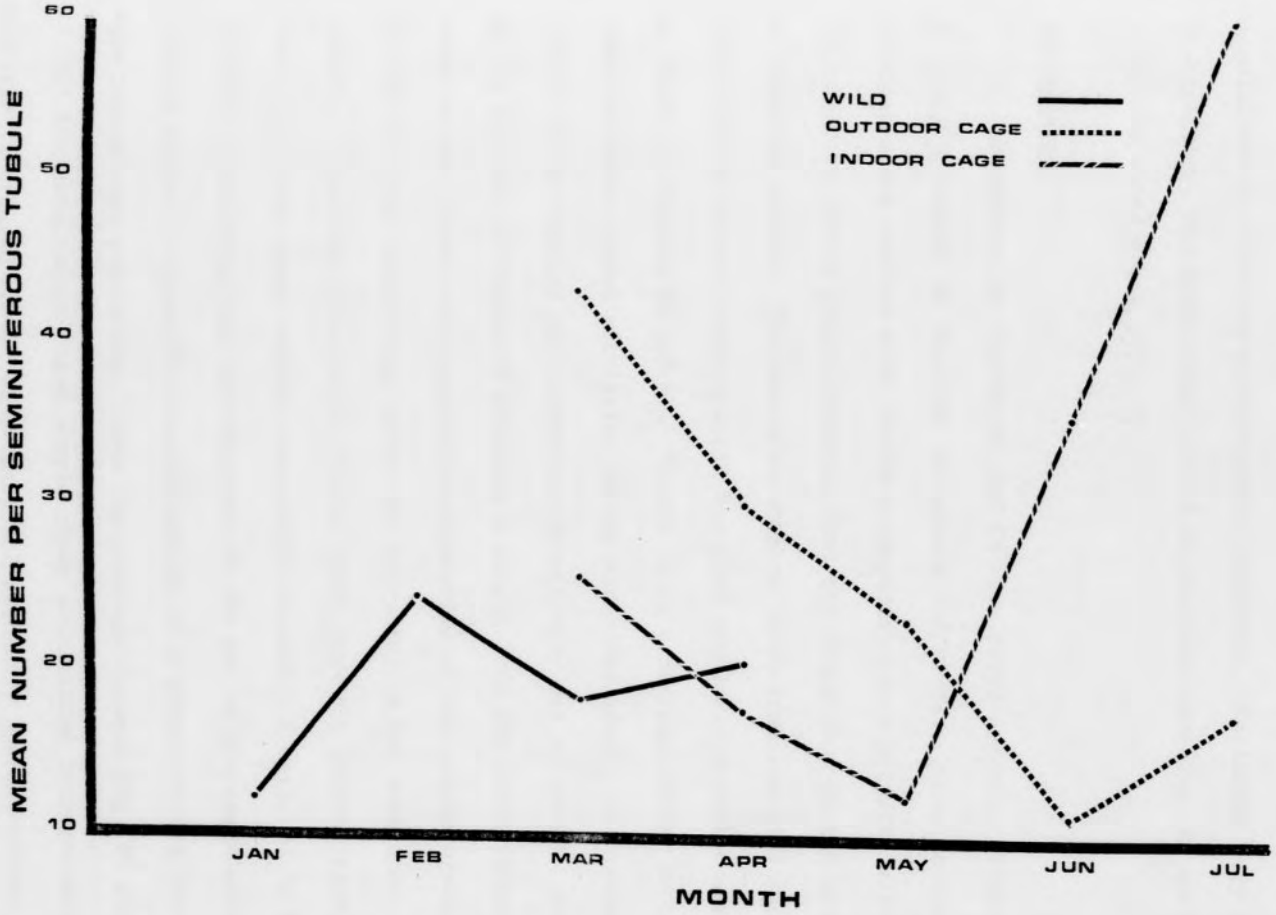

FIG. 3. NUMBER OF PRIMARY AND SECONDARY SPERMATOCYTES

increased to an abnormally high number during the fifth month (Fig. 4). The analysis of variance indicated that the mean number of primary and secondary spermatocytes and spermatids from the outdoor and indoor caged populations in July are significantly different. Following only one month of captivity, the sperm count showed an abnormal decrease and an increase after the third month (Fig. 5).

Photographs

Photographs in Figures 6a and 6b show cross sections of testes from H. crucifer caught in the wild in January and March. These sections show embedded sperm bundles with little cytospermatogenic activity within the seminiferous tubule which indicated that the frogs were in the spermiation or breeding period. The remaining samples taken from the wild and outdoor cages during January through April revealed similar testicular conditions as shown by Figures 6a and 6b. Figure 7a is a representative photograph from the indoor caged population during March and April. A complete breakdown of sperm bundles and spermatogenic activity can be noted. However, as can be seen in Figure 7b which is a sample from the indoor population taken in May, there was a partial recuperation of the spermatogenic cycle showing that the indoor cage group was adjusting to the laboratory environment. Figure 8a is a sample testis taken from the outdoor cage group in June. Note that sperm bundles were still present but appeared to be breaking down, indicating that the frog was at the end of its spermiation or breeding period. Figure 8b is a photograph of a sample testis from the June indoor cage population. Note the contrast between Fig. 8b and Fig. 8a in that the June indoor caged sample shows accelerated cytospermatogenic activity (increased number of primary and secondary spermatocytes) as

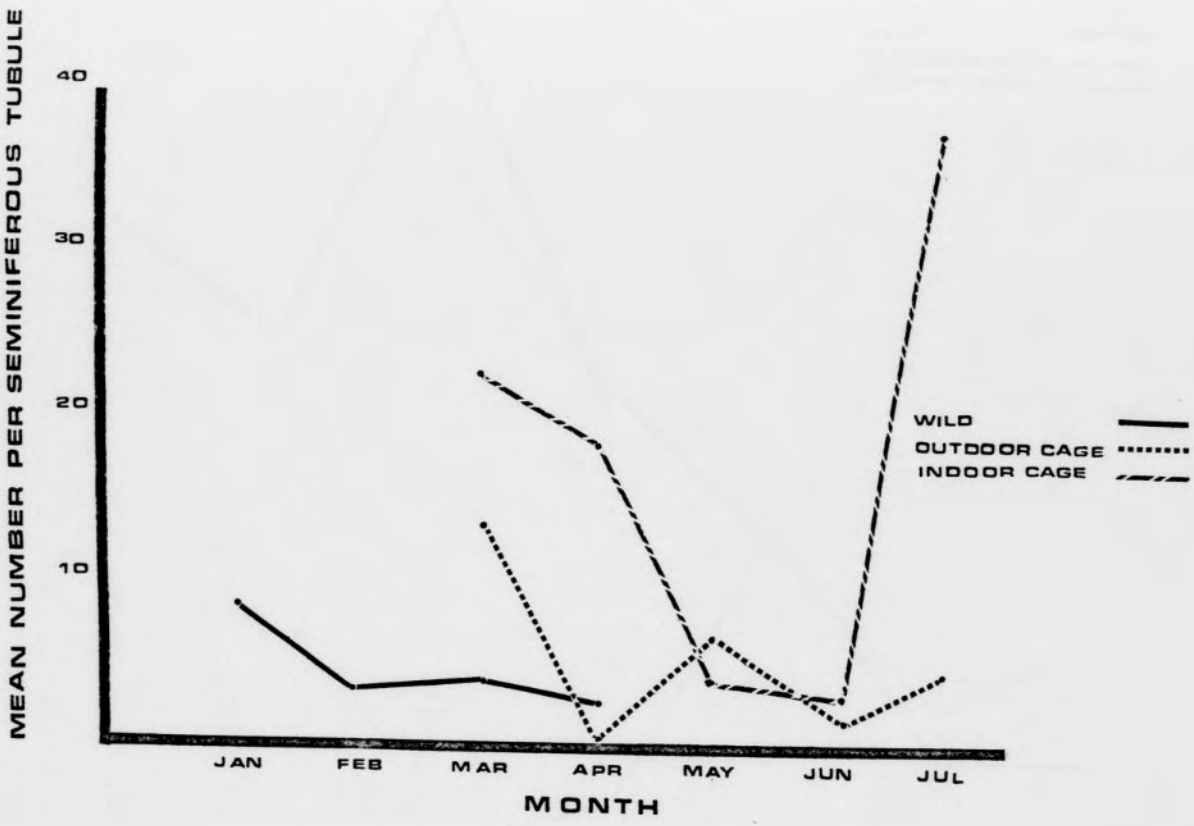

FIG.4. NUMBER OF SPERMATIDS

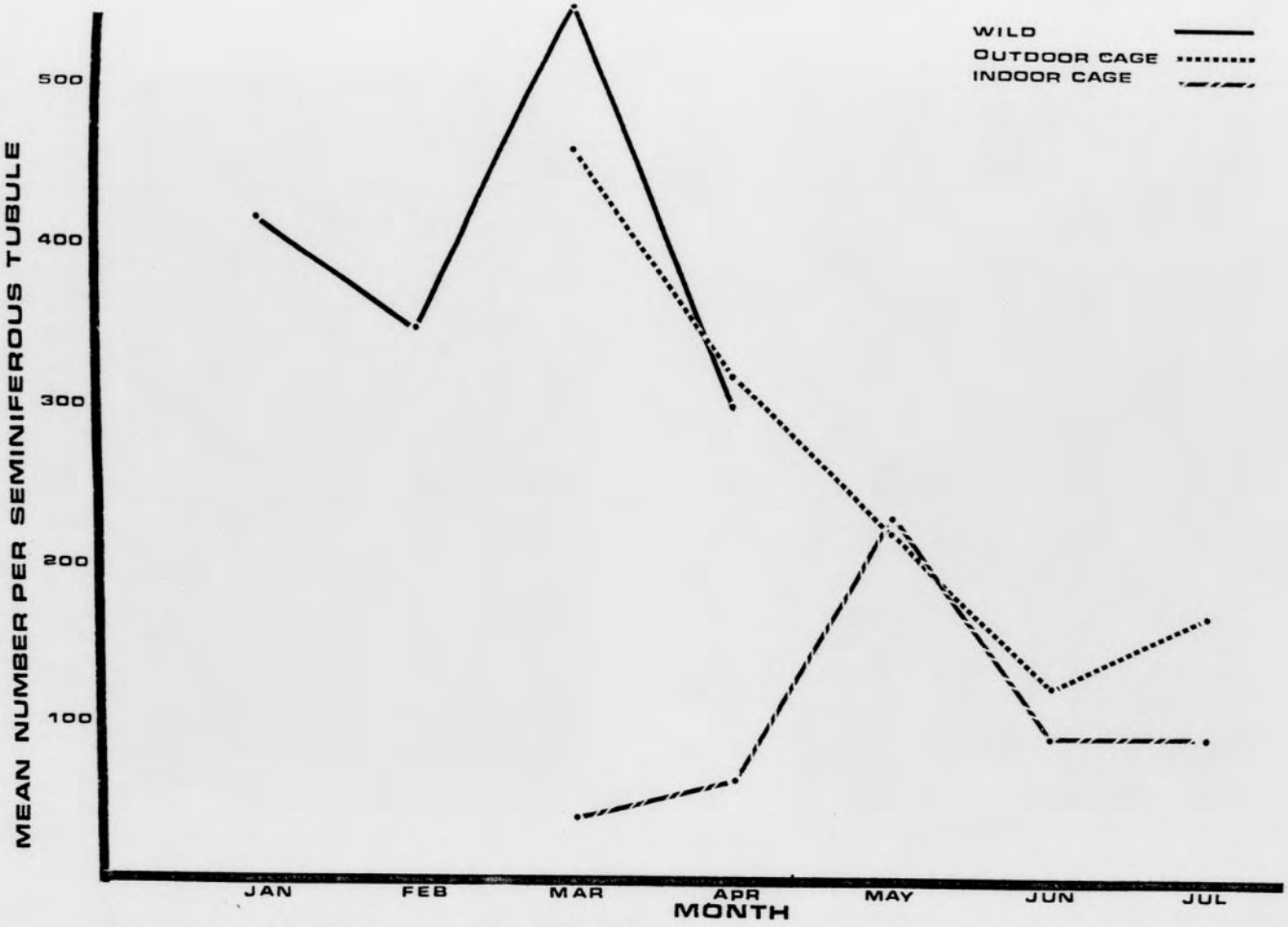

FIG. 5. NUMBER OF SPERM

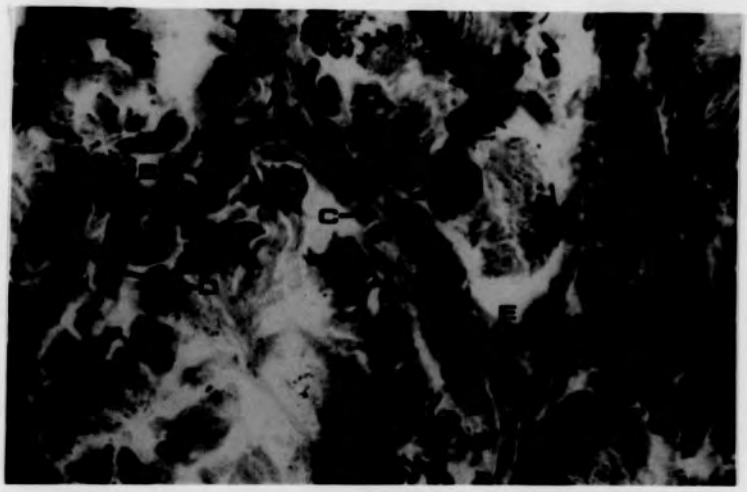

Fig. 6a. Sample testis from the wild in January.
A. Sertoli cell; B. secondary spermatocyte;
C. spermatogonium; D. follicle cell;
E. interstitium. 400X

Fig. 6b. Sample testis from the wild in March.
A. secondary spermatocyte; B. spermatozoa;
C. interstitium. 400X

Fig. 7a. Representative photograph of testes from the indoor cage population in March and April. A. spermatid; B. spermatozoa; C. interstitium. 400X

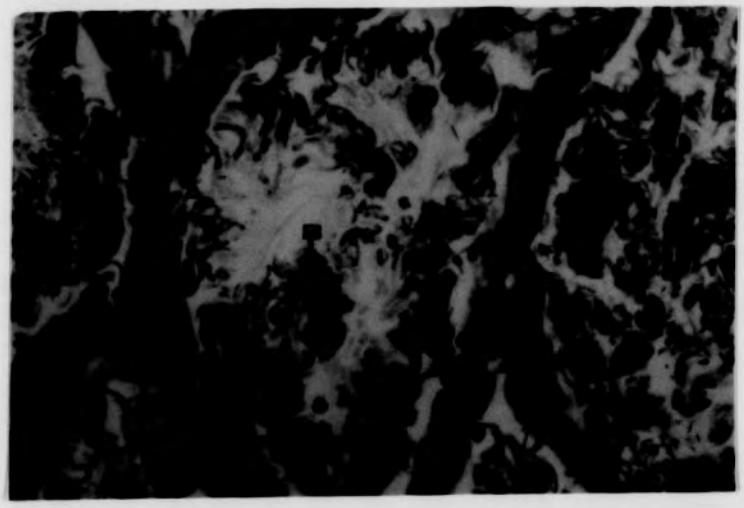

Fig. 7b. Sample testis from the indoor population in May. A. interstitium; B. spermatozoa. 400X

Fig. 8a. Sample testis from the outdoor cage population in June. A. spermatid; B. interstitium; C. spermatozoa. 400X

Fig. 8b. Sample testis from the indoor cage population in June. A. secondary spermatocyte; B. spermatozoa. 400X

compared to the June outdoor cage sample. If Hyla crucifer has a potentially continuous spermatogenic cycle, the cytospermatogenic period would begin sometime in July. Fig. 9a clearly shows that the July outdoor cage sample has entered the cytospermatogenic period of the spermatogenic cycle exhibiting very few sperm bundles with large numbers of primary and secondary spermatocytes and spermatids. Note that the July outdoor cage sample appears to have caught-up with the June indoor cage sample. Fig. 9b, a photograph of an indoor cage sample testis taken from July, shows extremely active cytospermatogenesis and several sperm bundles. Note that the cytospermatogenic activity in the July indoor cage sample appeared to be accelerated approximately one month from the July outdoor sample.

Gastrointestinal Content (Table 4)

The gastrointestinal contents of 125 H. crucifer were noted. Sixty per cent of all stomachs contained food, and 74% of specimens taken from the wild while calling had food in the gastrointestinal tract. This disputes Oplinger's (1967) and Whitaker's (1971) reports that H. crucifer does not feed while in breeding pools in Vigo County, Indiana and near Ithica, N.Y., respectively. Fifty-eight per cent and 38% of specimens taken from the outdoor and indoor cages, respectively, had empty gastrointestinal tracts.

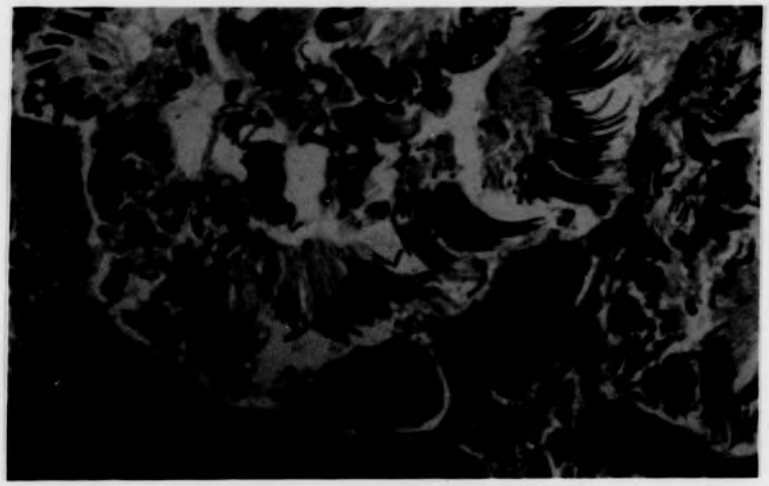

Fig. 9a. Sample testis from the outdoor cage population in July. A. secondary spermatocyte; B. spermatid; C. spermatid undergoing spermiogenesis; D. interstitium. 400X

Fig. 9b. Sample testis from the indoor population in July. A. secondary spermatocyte; B. interstitium; C. spermatozoa.

Table 4. Gastrointestinal Content

Samples	Food Content	Empty
Wild	74%	26%
Outdoor Cage	42%	58%
Indoor Cage	62%	38%
All Samples	60%	40%

DISCUSSIONS AND CONCLUSIONS

Calling

Hyla crucifer has the most extended breeding season of all hylid frogs in Guilford County, N.C. In Guilford County, H. crucifer breeding calls can be heard during the five-month period from January through May. Although the breeding season is long and the environmental ranges are wide (Table 5), critical thermal maxima and minima and a critical amount of rainfall do exist for the breeding season (Bellis, 1957; Jones and Brattstrom, 1961; Dunlap, 1968). A lack of rainfall or a sudden cold wave or hot spell during the breeding season can interrupt breeding behavior. A classical example of an interrupted breeding season due to decreasing temperatures was observed during January and February, 1974. A strong chorus and amplexed pairs of H. crucifer were observed 29 January at the Guilford Courthouse study area. When a severe cold wave moved in the next day, breeding behavior of H. crucifer ceased until 1 March. However, Pseudacris triseriata feriarum called intermittantly during this period indicating that this particular species has a lower thermal tolerance for breeding behavior than does H. crucifer. This confirms Martof's (1960) report that P. triseriata has a lower thermal requirement for reproductive activity which usually begins and ends two weeks prior to H. crucifer.

The standardized discriminant function coefficients in Table 1 indicated that month or photoperiod was the most significant factor contributing to the number of days H. crucifer called. Since the breeding activity of Hyla crucifer and other anurans with a potentially continuous

Table 5. Environmental factors for number of days Hyla crucifer called. N = 248 days.

	Mean	Std. Dev.	Var.	Range
Rainfall	.1692	.3453	.1192	0-2.41 in.
Photoperiod	12.92	1.134	1.286	9.7-14.05 hrs
Low Temp.	43.52	9.270	85.93	$26^{o}F$-$85^{o}F$
High Temp.	65.20	9.160	83.91	

and discontinuous spermatogenic cycle is seasonal, the reproductive season should begin and end with similar photoperiods year after year. This would tend to confirm van Oordt's (1960) report that photoperiod initiates the breeding season in anurans.

Although H. crucifer is considered to be a winter breeder, it is interesting to note that the discriminant function coefficient for high temperature rather than low temperature follows photoperiod (Table 1). This is probably due to the tolerance in the breeding behavior of H. crucifer to wide ranges of temperatures extending into the warmer months of May and sometimes into June (personal observation, 1976). It was observed that a subsequent rise in temperature during the cool winter days of the breeding season appeared to have a qualitative and quantitative effect upon calling. Note that in Table 1 the DFC for the study years was larger than the DFC for rainfall. A possible explanation for this is the interruption of the breeding season in January and February, 1974, by a sudden cold period. The analysis indicated that calling within a particular year was significant in the overall three-year observation of breeding calling.

Although H. crucifer mating calls can be heard April through May in Guilford County, high negative multiple regression correlation coefficients (Table 2) of mean monthly temperature (-.75) and mean monthly photoperiod (-.50) to the number of days per month frogs called indicate that H. crucifer prefers the cooler months for reproductive activity. Multiple regression analysis also indicated a positive (.55) correlation coefficient between the mean number of sperm and the number of days per month frogs called, thus confirming reports from the literature that reproductive gametes must be present for reproductive activity to occur.

The R Square variability of the multiple regression analysis, shown in Table 3, indicates the significance of each environmental factor upon the dependent variable, number of days per month frogs called. Fifty-seven per cent of the data for number of days per month frogs called were related to the mean monthly temperature, while 63.6% can be explained by the combined effects of mean monthly temperature and mean monthly photoperiod.

van Oordt and Lofts (1963) found that FSH and ICSH production by the pituitary in Rana temporaria could be accelerated or diminished by increasing or decreasing temperatures, respectively. Low temperatures stimulated the ICSH producing cells of the pars distalis which subsequently stimulated the production of testosterone by the Leydig cells of the testis. Schmidt (1966a) suggested that testosterone initiated mating calling in Hyla cinerea and H. versicolor by indirect stimulation of pituitary injections. The high negative multiple regression correlation coefficient (-.75) between mean monthly temperature and number of days per month frogs called, and the high R Square value (57%), which indicates the influence of mean monthly temperature upon the number of days per month frogs called, support the above endocrinological explanations.

It should be noted that the behavioral aspects of reproductive activity in anurans is associated with interactions between temperature, rainfall, and photoperiod and the genetic variation within the species. The species as a population and single organisms of the species react differently to the seasonal climatological variations. Anurans which prefer semi-permanent water for reproductive activity may not breed as a population when the temperature becomes optimum, but will begin to breed

when sufficient amounts of rainfall occur along with the optimum temperature. Anurans confined to permanent waters for reproductive behavior are probably more sensitive to temperature and photoperiodic cues than species which prefer semi-permanent aquatic areas. Reproductive activity in species inhabiting more permanent aquatic areas may even be controlled by olfactory cues such as the odors of algal metabolites (Salthe and Mecham, 1974).

Summary of Calling Data

Eakin (1961) studied the processes extending into the lumen of the pineal gland in tadpoles of the Pacific treefrog, Hyla regilla. He found the processes to be similar to rods and cones of the vertebrate lateral eye and concluded that cells within the processes are photoreceptors. Adler (1970) also found structures in the amphibian pineal gland resembling the rods and cones of the eye, while Dodt and Heerd (1962) and Dodt and Jacobson (1963) found the pineal body of Rana temporaria and R. esculenta to be sensitive to light intensities.

Recent extensive literature indicates that the pineal gland may contribute to the regulation of the vertebrate reproductive system. Unfortunately, for this study, the majority of the pineal investigations have been performed using mammals and birds.

It is well known that melatonin is a pineal secretory product (Quay, 1969). Juszkiewicz and Rakalska (1965) found that melatonin did not affect the spermatogenic response of male Rana esculenta to chorionic gonadotrophin injections, but spermatogenic response to chorionic gonadotrophin injections was diminished by injected homogenized tissues from cow pineal glands. Melatonin and extracts of bovine pineal glands (peptides) decrease

the pituitary gonadotrophin levels in rats (Orst et al., 1973). These peptidic pineal antigonadotrophins (PAG) have been isolated in mammals and birds (Ebels, 1976; Benson et al., 1976; Vaughn et al., 1976), with one having been found to reduce serum and pituitary LH (ICSH) levels in rats (Orts et al., 1974). It is also known that enzymatic PAG formation is class specific in vertebrates, and is increased by the presence of light (Ebels, 1976). Furthermore, Ralph (1976) found that rhythmic changes in melatonin content of rats and Leghorn chickens occurred in phase with photoperiod. Menaker and Zimmerman (1976) reported that the pineal organ plays an important role in the circadian rhythm of the sparrow, Passer domesticus.

Anurans are the only amphibians that possess a pineal end organ (Porter, 1972). Since the pineal is a functional organ in anurans (Adler, 1970) it is possible that pineal antigonadotrophins exist similar to those mentioned above. The standardized discriminant function analysis indicated that month or photoperiod was the most significant environmental factor correlating with the total number of days that H. crucifer called for three years. These data support the hypothesis that the pineal gland may respond to photoperiod by releasing a pineal antigonadotrophin. The PAG is turned off by the appropriate photoperiod at the beginning of the breeding season. With decreasing PAG production and low temperatures, the pars distalis secretes ICSH (LH) which is followed by the breeding period. Once the photoperiodic response of reproductive readiness occurs, temperature and rainfall become the regulatory cues of behavior. As the breeding season and photoperiod progress, the threshold potential for photoperiod in the species reaches a critical maximum. The photoperiod stimulates PAG

production, and as temperatures increase, ICSH production is decreased as FSH output from the anterior lobe of the pituitary is increased. This mechanism along with the feedback mechanism of testosterone aids in the cessation of the breeding season.

The physiological response of reproductive behavior in anurans to environmental cues probably involves several complex pathways. Porter (1972) reported light sensitive photoreceptors in the skin of Rana pipiens and the salamander Ambystoma mexicanum. Jørgensen (1974) postulated that osmoreceptors are located in the area of the preoptic nucleus in the brain of anurans which are responsible for maintaining fluid levels. It is possible that osmoreceptors in the skin and/or brain of anurans comprise one of the complex regulatory environmental-hormonal pathways for reproductive behavior.

TESTICULAR DATA

Spermatogonia and Spermatozoa

The multiple regression analysis indicated highly significant correlations between the environmental factors and the mean number of spermatogonia and sperm. These data support reports from the literature that these two cell types are under the influence of FSH-like and ICSH-like hormone production which appears to be controlled by environmental factors, in particular, temperature. The analysis indicated a high negative correlation (-.68) between the mean number of spermatogonia and mean monthly temperature (Table 2). van Oordt and Lofts (1963) ascribed high temperatures as being responsible for raised FSH output by the pars distalis. FSH has been found to increase the number of spermatogonia in Rana pipiens

(van Oordt, 1960). Although the number of spermatogonia should be greater with higher temperatures, the -.68 correlation in this particular study does not verify this assumption. The -.68 correlation coefficient indicated an increased number of spermatogonia at low temperatures. Increased spermatogonia were noted during the cooler months of the breeding season and decreased continuously until July (Fig. 2). It is known that testosterone is produced during the breeding season and produces an effect opposite to that of FSH on spermatogonia proliferation (Basu and Nandi, 1965). Since spermatozoa and sperm bundles must be present during the breeding season, it seems probable that most FSH produced in that period was being used to maintain the Sertoli cell-sperm bundle complexes (van Oordt and Lofts, 1963). Cytospermatogenesis occurs throughout the entire breeding season to replenish spent spermatozoa (Hahn, 1964). The slight increase in spermatogonia during the breeding season may possibly be accounted for by the replenishment of spent spermatozoa. In addition to maintaining the Sertoli cell-sperm bundle complexes, any excess FSH produced during this period could also stimulate the spermatogonia. This postulate is supported by the high multiple regression correlation coefficient (.52) between the mean number of sperm and the mean number of spermatogonia.

The analysis also indicated a very high negative correlation (-.70) between the mean number of spermatogonia and photoperiod. Spermatogonia were in more demand during the cooler months of the breeding period which corresponds to a decreased photoperiod. The R Square value in the multiple regression analysis indicated that mean monthly photoperiod represents 50% of the data for the mean number of spermatogonia. If Hyla crucifer has a

potentially continuous spermatogenic cycle, these data dispute van Oordt's (1960) report that the spermatogenic cycle in Amphibia with a potentially continuous spermatogenic cycle is completely controlled by environmental temperatures. According to the analysis, 52.5% of data for mean number of spermatogonia can be explained by the combined effects of mean monthly photoperiod and mean monthly temperature. Fifty-three per cent of the data represent the effects of rainfall and the two previously mentioned environmental factors combined (Table 3). While temperature and rainfall are significant, these data indicate that mean monthly photoperiod has the most significant effect upon the mean number of spermatogonia in Hyla crucifer. Since spermatogonia are the predecessors of all testicular reproductive cell types and photoperiod is the most consistent environmental cue year after year, it would be favorable for the proliferation of the species if spermatogonia were regulated by photoperiod.

The multiple regression correlation coefficient indicated a high negative correlation (-.66) between the mean number of sperm and mean monthly temperature. These data confirm the observations that Hyla crucifer prefers the cooler winter and spring days for reproduction. The data also support van Oordt's and Lofts' (1963) report that decreasing temperature stimulates ICSH-like production in the pituitary of anurans which is followed by the manufacture of testosterone by the Leydig cells in the testis and the initiation of the breeding period. Increased numbers of spermatozoa were observed in H. crucifer from January through March (Fig. 5). These data also support the hypothesis that reproductive gametes must be present for reproductive behavior to occur.

Summary

It is interesting to note that the multiple regression analysis computed high correlations and R Square values only between environmental factors and the mean number of spermatogonia and sperm. These two cell types are the first and final products of spermatogenesis, respectively, and they are target cells of FSH and testosterone. Since temperature seems to control FSH and ICSH secretion in anurans (van Oordt and Lofts, 1963), and using these data for support, it may be concluded that temperature is the most significant cue for regulating cytospermatogenesis in Hyla crucifer.

Effects of Captivity

The gonadal cell counts from the indoor caged population exhibited abnormal lags and accelerations while the counts from the outdoor caged group did not. Since the analysis of variance indicated that the gonadal cell counts from the wild and outdoor cage populations are similar (with exception of primary and secondary spermatocytes in March), Alexander and Bellerby's (1935) contention that anurans can be maintained in captivity for research purposes is supported.

Samples from the indoor caged group were consistently exposed to a controlled temperature of 22.5°C and to photoperiods similar to natural conditions (uncontrolled) for 1 to 5 months. The literature suggests that the lags and acceleration of the gonadal cell counts in the indoor caged group were caused by the consistently elevated temperature.

Figure 7a is a representative photograph of a testis from an indoor sample frog from March and April. Note that following one and two months

of captivity indoors, the spermatogenic cycle has completely broken down. The sample frogs appear to be experiencing a form of gonadal shock. However, Fig. 7b shows that after three months of captivity spermatogenic activity had adjusted to the indoor environment. Figures 3 and 4 also indicate that the indoor caged group acclimatized to the laboratory environment. A tremendous increase in primary and secondary spermatocytes and spermatids followed the fourth and fifth months of captivity in Figures 3 and 4. These data indicate that the indoor caged samples recuperated from their gonadal shock and cytospermatogenesis was stimulated.

Figure 8a is a photograph of a testis from an outdoor caged sample frog taken in June, probably having experienced conditions similar to the wild population. Note the contrast in Figure 8a and 8b. Figure 8b is a photograph of an indoor caged sample testis from June showing very active cytospermatogenesis. These photographs, along with Figures 3 and 4, indicate that the cytospermatogenic period of the spermatogenic cycle in the indoor population has been accelerated and is approximately advanced by one month from the outdoor cage population. The photograph in Fig. 9a, an outdoor cage sample testis from July, shows the probable beginning of the cytospermatogenic period in H. crucifer. Note the contrast in Figures 9a and 9b. Figure 9b, an indoor caged sample testis from July, shows increased numbers of primary and secondary spermatocytes and sperm bundles indicating again that the cycle appears to be accelerated and advanced.

The above data support van Oordt's and Lofts' (1963) report that high temperatures increase cytospermatogenic activity. The data do not support Rugh's (1941) and Basu's (1969) reports that H. crucifer displays a discontinuous spermatogenic cycle. In anurans with a discontinuous

spermatogenic cycle the initiation of the spermiation and cytospermatogenic periods is regulated by an inherent mechanism (Witschi in van Oordt, 1960). In anurans with potentially continuous and/or continuous spermatogenic cycles, the periods mentioned above are regulated by environmental cues. These data support the hypothesis that H. crucifer displays a potentially continuous or continuous spermatogenic cycle. If a discontinuous spermatogenic cycle exists in H. crucifer the advanced and accelerated cytospermatogenic period, as displayed by the indoor caged population, could not occur. The inherent mechanism would not allow the increased laboratory temperature to initiate cytospermatogenesis.

Conclusions

It is possible that the breeding season and reproductive behavior of H. crucifer are initiated and terminated by photoperiod. It may also be concluded that once the breeding season has begun, temperature and rainfall become the controlling environmental cues for breeding behavior. This study also supports the hypothesis that temperature has the most significant effect upon spermatogenesis during the breeding season. This study disputes reports from the literature that H. crucifer exhibits a discontinuous spermatogenic cycle. It may be concluded that Hyla crucifer probably displays a potentially continuous and possibly a continuous spermatogenic cycle.

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APPENDIX I. Ehrlich's Hematoxylin - Bulk Stain

1. Fix in Bouin's fixative - overnight
2. Wash in 70% alcohol - (days) - until yellow disappears
3. Acidify tissue in (0.1% HCL in) 70% acid alcohol - 30 min. or less
4. Stain from 1 to 6 hours in Ehrlich's hematoxylin
5. Rinse quickly in 70% alcohol
6. Fix stain in wash of alkaline 70% alcohol (Add a pinch of sodium bicarbonate to 100 cc 70% alcohol) - 2 hours
7. Wash in 80% alcohol - 1/2 hr - 1 hr
8. Wash in 95% alcohol - 1 hr
9. Wash in absolute alcohol - 1 hr
10. Wash in absolute alcohol:xylol, 1:1 - 1 hr
11. Wash in xylol - 1 hr
12. Wash in paraffin:xylol, 1:1 - 1/2 hr
13. Wash in paraffin I - 1/2 hr
14. Wash in paraffin II - 1 hr
15. Imbed in paraffin - vacuum oven
16. Block tissue on microtome stage
17. Section tissue and mount on slides - albumen
18. Place slides in coplin jar of xylol - 1/2 hr
19. Place slides in coplin jar of absolute:xylol - 1/2 hr
20. Place slides in coplin jar of absolute alcohol - 15 min - 1/2 hr
21. Place slides in coplin jar of 95% alcohol - 15 min - 1/2 hr
22. Place slides in coplin jar of Eosin or light green - few sec
23. Place slides in coplin jar of absolute alcohol - 1/2 hr
24. Place slides in coplin jar of absolute alcohol:xylol, 50:50 - 1/2 hr
25. Xylol I - 1/2 hr
26. Xylol II - 1/2 hr
27. Mount in clear mount and cover
28. Place on slide warmer

APPENDIX II. Mean, Standard Deviation, Variance, Number and Range for Spermatogonia Counts

Month and Year	Type	Mean	Standard Deviation	Variance	N	Range
1/75	wild	27.000	3.000	9.000	3	20-35
2/75	wild	15.800	3.421	11.700	5	5-24
3/74	wild	9.667	5.220	27.250	9	2-24
3/75	wild	18.60	0.895	0.800	5	15-23
3/75	outdoor	12.000	2.449	6.000	5	6-22
3/75	indoor	4.8000	1.789	3.200	5	2-11
4/74	wild	10.300	2.163	4.678	10	4-18
4/75	wild	10.400	2.608	6.800	5	5-18
4/75	outdoor	7.000	2.646	7.000	5	2-12
4/75	indoor	12.600	13.278	176.300	5	2-10
5/74	outdoor	4.300	4.956	21.122	10	0-19
5/75	outdoor	6.4	1.673	2.800	5	2-12
5/75	indoor	3.6	1.342	1.800	5	1-7
6/74	outdoor	2.600	1.265	1.600	10	0-6
6/75	outdoor	3.8	1.643	2.700	5	1-10
6/75	indoor	2.8	0.837	0.700	5	1-8
7/74	outdoor	6.000	4.243	18.000	2	1-10
7/75	outdoor	3.000	1.000	1.000	5	1-6
7/75	indoor	4.000	0.707	0.500	5	1-6

APPENDIX III. Mean, Standard Deviation, Variance, Number and Range for Primary and Secondary Spermatocyte Counts

Month and Year	Type	Mean	Standard Deviation	Variance	N	Range
1/75	wild	12.000	6.245	39.000	3	2-31
2/75	wild	24.400	6.693	44.800	5	8-38
3/74	wild	16.000	10.805	116.750	9	2-49
3/75	wild	20.400	11.632	135.300	5	4-38
3/75	outdoor	42.600	6.878	47.301	5	31-57
3/75	indoor	25.600	18.676	348.800	5	4-55
4/74	wild	14.600	9.143	83.600	10	0-34
4/75	wild	27.200	5.975	35.700	5	12-42
4/75	outdoor	30.000	17.635	311.000	5	6-63
4/75	indoor	17.400	6.229	38.800	5	8-32
5/74	outdoor	14.100	13.844	191.656	10	1-65
5/75	outdoor	32.600	22.512	506.801	5	4-78
5/75	indoor	12.600	13.259	174.800	5	4-39
6/74	outdoor	16.000	23.314	543.555	10	0-88
6/75	outdoor	6.800	2.280	5.200	5	2-16
6/75	indoor	35.200	35.464	1257.700	5	2-126
7/74	outdoor	11.500	9.192	84.500	2	1-28
7/75	outdoor	24.000	23.206	538.500	5	9-77
7/75	indoor	59.600	22.919	525.301	5	22-102

APPENDIX IV. Mean, Standard Deviation, Variance, Number and Range for Spermatid Counts

Month and Year	Type	Mean	Standard Deviation	Variance	N	Range
1/75	wild	8.333	11.930	142.333	3	0-48
2/75	wild	3.400	3.847	14.800	5	0-37
3/74	wild	4.000	6.764	45.750	9	0-37
3/75	wild	0	0	0	5	0
3/75	outdoor	13.600	16.832	283.300	5	0-57
3/75	indoor	22.600	30.997	960.800	5	0-77
4/74	wild	2.700	8.538	72.900	10	0-39
4/74	wild	0	0	0	5	0
4/75	outdoor	0	0	0	5	0
4/75	indoor	18.200	11.498	132.200	5	0-46
5/74	outdoor	3.700	9.238	85.344	10	0-30
5/75	outdoor	10.000	16.492	272.000	5	0-50
5/75	indoor	4.000	8.944	80.000	5	0-38
6/74	outdoor	1.100	2.601	6.767	10	0-15
6/75	outdoor	2.80	6.261	39.200	5	0-23
6/75	indoor	3.000	6.708	45.000	5	0-41
7/74	outdoor	0	0	0	2	0
7/75	outdoor	4.800	10.733	115.200	5	0-37
7/75	indoor	37.600	24.876	618.801	5	0-124

APPENDIX V. Mean, Standard Deviation, Variance, Number and Range for Sperm Counts

Month and Year	Type	Mean	Standard Deviation	Variance	N	Range
1/75	wild	420.000	52.915	2800.000	3	300-700
2/75	wild	350.800	234.869	55163.298	5	100-1200
3/75	wild	367.333	65.484	4288.125	9	280-500
3/75	wild	760.000	227.156	51600.000	5	400-1200
3/75	outdoor	464.000	293.394	86086.000	5	100-1000
3/75	indoor	42.4	16.087	258.801	5	20-100
4/74	wild	254.800	51.322	2633.965	10	120-400
4/75	wild	347.200	198.945	39579.234	5	10-600
4/75	outdoor	325.200	258.409	66775.187	5	20-700
4/75	indoor	68.000	41.012	1682.00	5	20-200
5/74	outdoor	258.900	116.081	13474.797	10	30-500
5/75	outdoor	190.000	181.758	33036.000	5	20-500
5/75	indoor	237.60	152.049	23118.812	5	30-500
6/74	outdoor	140.800	81.154	6585.957	10	20-400
6/75	outdoor	112.400	100.054	10010.801	5	20-400
6/75	indoor	94.800	70.521	4973.199	5	10-240
7/74	outdoor	269.000	264.458	69938.000	2	40-600
7/75	outdoor	75.600	24.552	602.802	5	20-120
7/75	indoor	95-200	94.948	9015.199	5	10-400