AN ABSTRACT OF THE THESIS OF

Jennifer Lee Specker for the degree of Master of Arts

in General Science (Biological Science) presented on December 2, 1977

Title: REPRODUCTIVE CYCLE OF THE MALE NEWT TARICHA GRANULOSA: EXOGENOUS AND ENDOGENOUS REGULATION OF PLASMA ANDROGENS

Abstract approved: Redacted for Privacy
Frank L. Moore

During a 13-month period starting in October of 1976, adult male rough-skinned newts (Taricha granulosa) were collected periodically to study seasonal changes in reproductive status. Plasma androgen concentration, measured by radioimmunoassay, is elevated during autumn and spring (about 50 ng/ml). Winter and summer levels are considerably lower (less than 10 ng/ml). Seasonal changes in testis composition indicate one annual cycle of evacuation (October-March) and spermatogenetic recrudescence (March-October).

Concomitant with decreasing ambient temperature in autumn are the occurrence of second metamorphosis, which includes the assumption of nuptial characteristics such as caudal fins, and the onset of spermiation and sex behaviors. To test the hypothesis that temperature influences plasma androgens through its effects on the pituitary-gonadal axis, newts were maintained at 12° or 18°C for 6 weeks in the laboratory. Androgen levels remained unchanged. Caudal fins regressed in 18°C newts and developed in 12°C newts, suggesting that prolactin, but not luteinizing hormone, was affected by water temperature.

To assess the contribution made by the mature testicular zone to plasma androgen levels, newts were subjected to either bilateral total or bilateral hemicastration. Hemicastration involved extirpation of the mature zone from each lobe of each testis. Each testis consists of several lobes; each lobe is comprised of distinct zones of different maturation stages. The mature zone, containing seminiferous lobules that are already evacuated or still contain sperm, was found to produce a significant portion of the plasma androgens.

Reproductive Cycle of the Male Newt *Taricha granulosa*: Exogenous and Endogenous Regulation of Plasma Androgens

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Arts

Completed December 2, 1977

Commencement June 1978

APPROVED:

Redacted for Privacy

Assistant Professor of Biology
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Date thesis is presented December 2, 1977

Typed by Leona M. Nicholson for Jennifer Lee Specker

ACKNOWLEDGEMENTS

I thank Dr. Frank L. Moore for his good-naturedness and for his continuous help in the design and interpretation of this research.

Appreciation is extended to Dr. Lloyd Swanson, Department of Animal Science, for teaching me the radioimmunoassay techniques. I thank Dr. David L. Willis for his encouragement.

For considerable support and assistance at every stage, I thank Tish Tuttle, Finn Pond, Joel Hagen, Russell Messing, Steve Whitfield, Keith King, Diane Bunting, Steve Trenholm, Ron Riley, Debbie Earley, Randy Schafer, and Leona Nicholson. I am grateful to Cathy Gilbert for help in preparing the graphs and photomicrographs.

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INTRODUCTION

Reproductive cycles of various Amphibia have been described in terms of changes in the testes, secondary sex characteristics, and behavior. Neither the endocrine control of seasonal changes nor the biological action of particular steroids is thoroughly understood. Hormonal activity, behavior, and environmental parameters such as temperature interact to unknown extents.

Steroids secreted by testes are chemically and biosynthetically similar in many of the vertebrates that have been studied (see Ozon, 1972). Consequently, to investigate steroid hormone functions in nonmammalian vertebrates, research has been directed toward target organs and responses known to be influenced by gonadal steroids in mammals. For several reasons results from these investigations have provided confusing and contradictory literature, especially for amphibians. Variability in experimental parameters such as environmental conditions, sexual maturity, species specificity toward drugs, and route of drug or exogenous hormone administration has created discrepancies among the published results. For most nonmammalian vertebrates, researchers are hindered by not knowing the normal range of plasma concentrations, the clearance rate or biological half-life in the vascular system, or the seasonal variations in biological activity of the gonadal steroids. At times excessive or inadequate amounts of steroids have been administered. Accurate

descriptions of seasonal variation in steroid secretion are needed for understanding reproductive cycles and interpreting experiments conducted at different times of the year.

Gonadotropin regulation of testicular activity in both anurans and urodeles has been established (reviewed by Lofts, 1974). Seasonal changes in germinal epithelium have been correlated with pituitary gonadotropin secretion in various species. Ablation of the entire pituitary gland, or just the pars distalis, results in testicular atrophy, a process which can be prevented by injections of pituitary extracts. There are seasonal fluctuations in germinal epithelium sensitivity to purified mammalian gonadotropins in Rana esculenta (Mialhe-Voloss, 1956) and hypophysectomized Rana pipiens (Basu and Nandi, 1965). Also, elevated testosterone levels correspond to the highest density of gonadotropic cells in the pituitary gland of R. esculenta (Rastogi and Chieffi, 1970; D'Istria et al., 1974).

Androgens have had sundry effects on amphibian testes. This topic has been reviewed by van Oordt (1960), Lofts (1968), Chester Jones et al. (1972), and Ozon (1972). Exogenous androgen treatments have reduced, increased, and had no effect on testicular size in various amphibian species. Testosterone, at various doses and in different species, has both stimulated and inhibited mitotic proliferation of secondary spermatogonia. Although development of spermatocytes and spermatids has been unaffected by exogenous testosterone injections, spermiogenesis has been increased (Rastogi et al., 1976).

Many of the inhibitory effects of testosterone on amphibian spermatogenesis may be explained in terms of suppression of gonadotropin secretion, because some animals had intact pituitary glands, but others did not. Basu and Nandi (1965) reported that exogenous gonadotropin did not mitigate the collapse of the germinal epithelium caused by testosterone in R. pipiens. They surmised that androgens might have a direct suppressive action on the amphibian testis. In fact, Moore (1975) reported that in hypophysectomized Ambystoma tigrinum larvae, testosterone either stimulated or inhibited spermatogenesis depending on initial hormone treatment.

The role of androgens in mammalian spermatogenesis has been extensively studied (see reviews by Steinberger, 1971; Franchimont, Chari, and Demoulin, 1975). Testosterone clearly maintains spermatogenesis in hypophysectomized mammals. Although low doses produce testicular damage by suppressing pituitary gonadotropin secretion in intact mammals, high doses compensate for suppressed pituitary secretion by a direct enhancement of spermatogenesis.

Secondary sexual characteristics are clearly modulated by gonadal activity (reviewed by Lofts, 1974). Amplexus, clasping behavior during courtship, is facilitated in Amphibia by toe callosities called nuptial pads, which help the male cling to the female. Nuptial pads regress following castration and grow after testosterone treatments. Testicular transplants induce development of nuptial pads in female toads. Seasonal changes in nuptial pads correspond to the testosterone cycle in R. esculenta (D'Istria et al., 1974). Swollen cloacal glands, indicative of breeding

condition in male urodeles, are also androgen dependent (reviewed by Norris and Moore, 1975). In newts, variation in tail height is a secondary sexual characteristic since it is related to breeding and is confined to male animals. Growth of the tail fin, however, is stimulated by prolactin (Singhas and Dent, 1975) and is part of the process called "second metamorphosis," associated with return to the aquatic environment (reviewed by Bern and Nicoll, 1968).

During the breeding season, reproductive behavior is influenced by gonadal secretions in fishes (see Baggerman, 1968), birds (see Hinde and Steel, 1966), and mammals (Beach and Holz-Tucker, 1949; Money, 1961; Hart, 1967). Evidence is ambiguous for amphibians. Castrated frogs do not display sex behaviors during the breeding season (Shapiro, 1937; Schmidt, 1966; Palka and Gorbman, 1973). Male sex behavior has been restored in castrated Xenopus laevis (Kelley and Pfaff, 1976) and R. pipiens (Wada and Gorbman, 1977) with implants of testosterone. However, researchers have failed to influence amphibian sex behavior with systemic androgen injections (Wolf, 1939; Blair, 1946; Schmidt, 1966; Palka and Gorbman, 1973; Wada and Gorbman, 1977). In male Taricha granulosa sex behavior has not been shown to be correlated with androgen levels, nor induced by exogenous androgen injections (Moore and Muller, 1977; Moore, Specker and Swanson, 1978).

Reproductive patterns in urodele amphibians are generally correlated with climate (for extensive review see Salthe and Mecham, 1974). Oliver and McCurdy (1974) studied the reproductive cycle of the rough-skinned newt on southern Vancouver Island. Their results

showed that females migrate to overwinter on land, while many adult males remained aquatic. In contrast, Efford and Mathias (1969) reported that both males and females in a population of T. granulosa from a permanent pond on the British Columbia mainland leave by mid-October to overwinter on land. In both populations breeding occurred throughout the spring.

Migration patterns of newts in the Benton County, Oregon area were described by Storm (1948) and Pimentel (1960). Females migrate to the ponds in aggregations starting in November and leave the ponds by July. Due to the lack of visible male migration, Storm (1948) surmised that males either gradually migrate to the ponds or return to the water at an immature stage and remain there until mature. Pimentel (1960) described the males as entering the breeding ponds in October and leaving by August. Pimentel (1960) and Chandler (1918) believed that both male and female T. granulosa avoid dessication during the dry summer months by burying themselves in the ground.

Miller and Robbins (1954), in describing the reproductive cycle of Taricha torosa, produced the most extensive work on urodele reproductive cycles. Their results are described below. Migration to ponds in the Palo Alto area occurs with the onset of rains, usually in October. Males enter the ponds first and leave last. Breeding begins in late December and continues until early February. Foraging for terrestrial invertebrates in the forest lasts throughout the spring. Newts then estivate during the dry summer months.

Seasonal changes in the testes were found to correspond to this behavioral cycle (Miller and Robbins, 1954). Each testis consists of several lobes; each lobe is comprised of visually distinct zones of different maturation stages. Maturation occurs in a caudo-cephalic direction in each lobe. As breeding season begins the connective tissue between the mature sperm and the untransformed spermatids thickens. Spermiogenesis is apparently abortive once this separation zone is established, because the spermatids usually degenerate. However, spermatocytes and spermatogonia within the immature zone remain unchanged during the breeding season and serve as a reservoir for the next season. The testis is maximally developed as estivation ends in mid-October. At this time 75% of total testicular weight is mature spermatozoa. The remaining 25% of the testis consists of spermatids, spermatocytes, and spermatogonia. Miller and Robbins (1954) noted a three-month variation in gonadal status, probably associated with the length of the breeding season. Furthermore, approximately one-half of adult newts had undeveloped gonads. As breeding terminates, the glandular region derived from the evacuated area is resorbed. Spermatogenesis then extends from May to July, when the gonadosomatic index is maximum (1.0%). Spermiogenesis apparently occurs during estivation.

Histologically, newt testes more closely resemble piscine rather than anuran structure. Each testis is composed of multiple lobes. These lobes, ensheathed in a fibrous coat, are connected by cords of spermatogonia along the dorsal body wall. Each lobe is composed of seminiferous lobules, analogous to seminiferous tubules

in other vertebrates, which are formed by connective tissue elements. Short efferent tubes drain the contents of the lobules into a large collecting duct that connects to the vasa efferentia.

Spermatogenesis is of the cystic type in all species of urodeles. Lobules, then, contain germinal cysts, each derived from a single primary spermatogonium. Follicle cells, of mesenchymal origin, form the walls of the germinal cysts. In any lobule, many germinal cysts are at similar stages of development, and all germ cells within a germinal cyst are at the same stage of development.

In urodeles each lobe is divided into distinct zones of development. Spermatogenesis occurs in a caudo-cephalic wave. Therefore, when maximally developed, the caudal zone of each lobe is filled with mature spermatozoa and appears white. As spermiation occurs, the evacuated lobules appear yellow and this evacuated region is called the yellow zone (Vellano, 1969) or the glandular tissue (Miller and Robbins, 1954). Lobules that contain spermatozoa along with those that are evacuated comprise the mature zone.

Connective tissue elements forming the lobules are called lobule boundary cells. The perilobular cells originate from fibroblast-like components, as do the interstitial Leydig cells typical of higher vertebrates, and are separated from the germinal cysts by a distinct collagen layer and a thin basement membrane. For most of the testicular cycle they remain inconspicuous. Prior to evacuation, however, they begin to hypertrophy. Once spermatozoa are discharged, these lobule boundary cells and the Sertoli cells compress into the aforementioned glandular tissue. Sertoli cells

are derived from the follicle cells during the late maturation period of spermatids. As breeding terminates, the lobule boundary cells in the glandular tissue degenerate and are resorbed. Spermatogenetic recrudescence occurs along with the resorption of this tissue (see Lofts, 1974).

Histochemical, ultrastructural, and biochemical evidence suggest that this yellow zone is the major source of steroids in the amphibian testis (Ozon, 1967; Picheral, 1970; Della Corte and Cosenza, 1965; Lofts and Bern, 1972). However, manifestations of steroid biosynthesis appear in lobule boundary cells and a significant development of sexual characteristics occurs before spermatozoa are evacuated from lobules (reviewed by Lofts, 1974).

D'Istria et al. (1974), in the only study to date describing seasonal changes in testosterone levels in an amphibian, found that maximum levels occur from December to March (11 to 17 ng/ml) and minimal levels from August to September (about 1 ng/ml) in R. esculenta. High testosterone levels correlate with thumbpad or nuptial pad development, as well as with the highest density of gonadotropic cells in the pars distalis. Curiously, breeding occurs in R. esculenta in the spring when plasma testosterone titers have dropped below 5 ng/ml. D'Istria et al. (1974) failed to investigate the temporal relationships between testosterone levels and testis condition. In fact, no study on amphibians has documented the annual cycles of both plasma androgens and testicular development.

It becomes apparent that the role of androgens in spermatogenesis and reproductive behavior in amphibians remains to be

elucidated. The source of androgens in urodeles is also unclear. The interrelationships among androgen levels, season, temperature, testicular condition, and behavior are not known.

The purpose of this research, then, was to provide needed data concerning seasonal variation in androgen secretion in male urodeles, and thus gain some understanding of their reproductive physiology. Concurrently, somatic and testicular conditions, as well as behavior, were noted. The possibility of extra-testicular androgen sources was investigated by a series of surgical extirpations. The influence of ambient temperature on androgen production and second metamorphosis was also investigated. Such information will hopefully aid in design and interpretation of further research in this field. T. granulosa was chosen for study primarily because it is the locally abundant urodele. Collecting males should have had no effect on the reproductive output of the population, because they are polygamous. Also, the thorough study of the reproductive cycle of T. torosa by Miller and Robbins (1954) provides a strong foundation upon which to build.

METHODS AND MATERIALS

Seasonal Changes in Male Reproductive Status

During a 13-month period starting in October of 1976, adult male rough-skinned newts, T. granulosa, were collected periodically to study seasonal changes in plasma androgen concentrations associated with changes in primary and secondary sexual characteristics. All newts were collected, using a long-handled dip net, from Fathead Lake which is located 3 miles west of Hoskins in Benton County, Oregon.

Animals were brought to the laboratory where live weight, snout-vent length, and tail length were measured within 24 hours. Tail height, taken about 2 cm behind the cloaca, was also measured. All newts were killed by decapitation within 24 hours to collect blood for radioimmunoassay of androgens. Several methods of collecting blood for the assay were tried: placing heparinized capillary tubes on the carotids; injecting 0.5 ml of 1.0% heparin intramuscularly 30 minutes before decapitation and then collecting the blood in a centrifuge tube; or using heparinized centrifuge tubes. The latter method was used because of its expediency. The possible effect of these three methods on plasma testosterone measurements was evaluated in Rana catesbeiana; no effect was found (Muller, personal communication). Blood was centrifuged for 10 minutes and the plasma stored at - 20°C until assayed for androgens.

In order to investigate the relationship between plasma androgen levels and testicular condition, the testes were removed, weighed, fixed in 10% neutral buffered formalin and stored in 70% ethanol. Testes were prepared for microscopic analyses using standard histological procedures (see Appendix I for details). Serial sections of 7-8 μm were mounted on glass slides and stained with hematoxylin-eosin (Appendix II). To evaluate testis development, seminiferous lobules containing mature spermatozoa, immature spermatogenic stages, and lobules that were already evacuated were counted using the method of van Oordt (1960). Four to six sections, evenly spaced over the entire testis, were counted; the counts were converted into a percentage of the total counts made.

Radioimmunoassay of Plasma Androgens

Plasma androgen concentrations were measured using the procedure of Louis, Hafs, and Seguin (1973) for progesterone. No purification was involved. The antiserum used, anti-testosterone-3-ovine-bovine serum albumin (from Gordon Niswender and described by Ismail, Niswender, and Midgley, 1972), reacts appreciably with dihydrotestosterone (17β-hydroxy-5α-androstan-3-one). Since both testosterone and dihydrotestosterone are present in this species (Moore and Muller, 1977), measurements are referred to as "androgen concentration." Moore, Specker, and Swanson (1978) have reported the validation of this assay using T. granulosa plasma.

Samples were extracted in triplicate (30 μl aliquots) with benzene-hexane (1:2) and the extracts air-dried at 40°C. To

calculate extraction efficiency, ^{3}H-testosterone tracer (6000 cpm in 10 µl ethanol) was added to every third tube 30 minutes before extraction. These extraction efficiency tubes were then counted in 10 ml toluene-based scintillation fluid. The results for each sample were corrected for extraction efficiency. Average extraction efficiency for these assays was 87.5 ± 1.0% (mean ± SE).

Antiserum (200 µl) diluted 1:4000 with 0.1% gelatin in phosphate-buffered saline (GPBS, pH = 7) was added to the dried extract. Following a 20 minute incubation at room temperature, competitor ^{3}H-testosterone (12,500 cpm in 100 µl GPBS) was added to each tube. After a 16-22 hour incubation at 5°C, 1 ml dextran-charcoal was added to separate bound from free androgen. After 10 minutes at 1°C, tubes were centrifuged (2500 x g). A fraction of the supernatant (0.6 ml) was counted in 7 ml of toluene:Triton X-100 scintillation fluid for 10 minutes or 10,000 counts. The average binding for this antiserum was 39.2 ± 1.4% (mean ± SE), of which 1.2 ± 0.1% was non-specific.

A calibration curve was established for known amounts of testosterone: 25, 50, 100, 250, 500, 1000, and 2000 pg in 100 µl ethanol. The interassay precision and accuracy of this method were evaluated by adding 0.25 (n = 5), 0.50 (n = 5), 1.00 (n = 4), 10.00 (n = 5), and 20.00 (n = 5) ng testosterone to samples of pooled Taricha plasma (diluted 1:100 dH_2O). Plasma for the pool was collected from nuptial males in February. These standards were assayed and the androgen concentration of the diluted pool subtracted. Resulting androgen concentrations were 0.24 ± 0.17

(mean ± SE), 0.53 ± 0.05, 1.07 ± 0.08, 9.63 ± 0.35, and 19.11 ± 0.63 ng, respectively. Within-assay variability was determined from replicates (n = 10) of the plasma pool (1:10 dH_2O) and the androgen concentration was found to be 3.21 ± 0.25 ng/ml (coefficient of variation = 7.63%). Androgen concentrations in 25 µl and 50 µl aliquots (10 of each) of the pooled Taricha plasma (1:10 dH_2O) were measured in 5 different assays to check for volume effect: no volume effect was found ($t = 0.92$, n.s.). The sensitivity of the assay, the smallest amount significantly different from zero ($t = 5.52$, $df = 19$, $p < 0.001$), was 10 pg/tube or 0.33 ng/ml.

Effect of Castration and Hemicastration on Plasma Androgens

Eighty-two adult male newts were collected from Hamer Lake, 2 miles west of Nashville in the Oregon Coastal Range on May 9, 1977, at which time water temperature was 13° to 15°C. Newts were maintained in the laboratory in clear plastic boxes (about 10 males/4 l of aged tapwater). Temperature was maintained at 14.5°C by Westinghouse cooling units attached to water baths. The photoperiod was not controlled and the newts were not fed.

Newts were toe clipped for identification and randomly assigned to one of four experimental groups: total bilateral castration (n = 21), bilateral hemicastration (n = 21), sham-operated controls (n = 20), or intact controls (n = 20). Prior to surgery each newt was placed in anesthetic (tricaine methanesulfonate) for about 5 minutes or until it could no longer right itself.

All newts except the intact controls received a single lateral incision about 2 cm in length. Total castrates had both the right and left testes entirely removed. Hemicastrates had the mature zone of each testicular lobe carefully removed. The extirpated mature zone consisted of the white-appearing zone of mature sperm and the adjacent yellow-appearing evacuated region. In half the sham-operated animals the zones were cut apart to disrupt blood circulation, as happened in the hemicastrates. Incisions were closed with 9 mm Autoclips (Clay Adams). The next day and twice weekly thereafter, the incised newts were immersed in a solution of antibiotics (Benson, 1965) for 2 hours.

On day 1 and day 7 post-operation (PO), six animals from each group were decapitated and blood was collected in heparinized centrifuge tubes for determination of androgen concentrations. On day 14 PO the remaining newts were decapitated. Hematocrit levels were determined for some newts on day 7 PO and for all the newts killed on day 14 PO.

Temperature Effects on Plasma Androgens and Tail Height

Forty-four adult males were collected from Fathead Lake on September 9, 1977. Temperatures at collection points were between 18° and 19°C. Newts were maintained in the laboratory in plastic containers filled with 14 l of lake water. Half the animals were maintained at 18° ± 1°C (range). The other 21 newts were acclimated to a colder temperature during a 3-day period and then were maintained at 12 ± 1°C (range) for the duration of the experiment.

Large water baths and Westinghouse cooling units regulated the temperatures. Newts were fed worms ad libitum. Water was changed weekly.

The day after capture all newts were weighed; their tail heights, snout-vent lengths, and tail lengths were measured. On termination days the same measurements were made. After 2 ($n = 7$), 4 ($n = 7$), and 6 ($n = 8$) weeks of laboratory maintenance, newts from each ambient temperature were decapitated and blood was collected for determination of plasma androgen concentrations. On these termination days newts were also collected from the pond and killed for blood collection. To maintain approximately the same density of newts during the experiment, the water volume was decreased by a third each time newts were removed. Testes were removed from killed newts, weighed, and prepared for histological examination, as described earlier.

Statistical Analysis

When appropriate data were analyzed with a one-way analysis of variance (ANOVA), followed by appropriate t-tests. Significance was taken at the $\alpha = 0.05$ probability level, although unless otherwise stated all results were significant at the $\alpha < 0.01$ probability level.

RESULTS

Seasonal Changes in Male Reproductive Status

The assumption of nuptial characteristics occurred during September and October in adult male T. granulosa from Fathead Lake. These changes, some of which are referred to as second metamorphosis, involved development of nuptial pads, swollen glands around the cloaca, smooth skin, and a caudal fin. By April most newts had lost these nuptial characteristics, although a few nuptial males could be found in May. Generally, males from May to September are "rough-skinned" and lack developed caudal fins.

Both tail height and body weight peak during autumn and spring and are low in winter and summer. Tail height variation is shown in Figure 1. Caudal fins were most developed in March and April, averaging 20 ± 1 mm (mean ± SE). By August they regressed to 11 ± 1 mm (mean ± SE). Total body weight changes, as shown in Figure 2, paralleled the seasonal changes in tail height. Males weighed the most as spring breeding began in February (21.5 ± 0.6 g, mean ± SE), but then decreased to the lowest weight by May ($F = 12.26$; $df = 3, 33$). Body weight increased between May and November ($F = 4.40$; $df = 6, 54$), but decreased during winter from 17.8 ± 0.8 g to 15.4 ± 0.6 g (mean ± SE). Between January and February there was again an increase ($F = 26.64$; $df = 2, 35$). No seasonal changes in snout-vent length were found in this population of newts (Table 1). Development and regression of the caudal fin

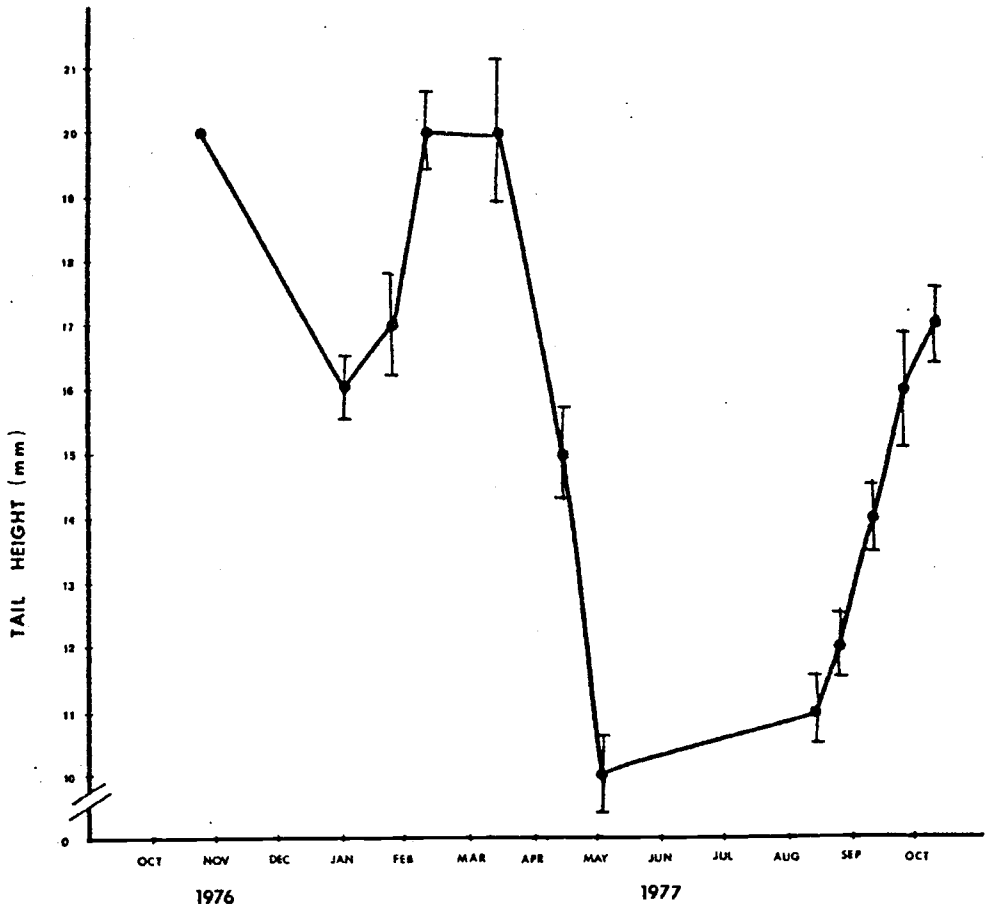

Figure 1. Seasonal changes in tail height of adult male newts (Taricha granulosa). Vertical lines represent ± SE.

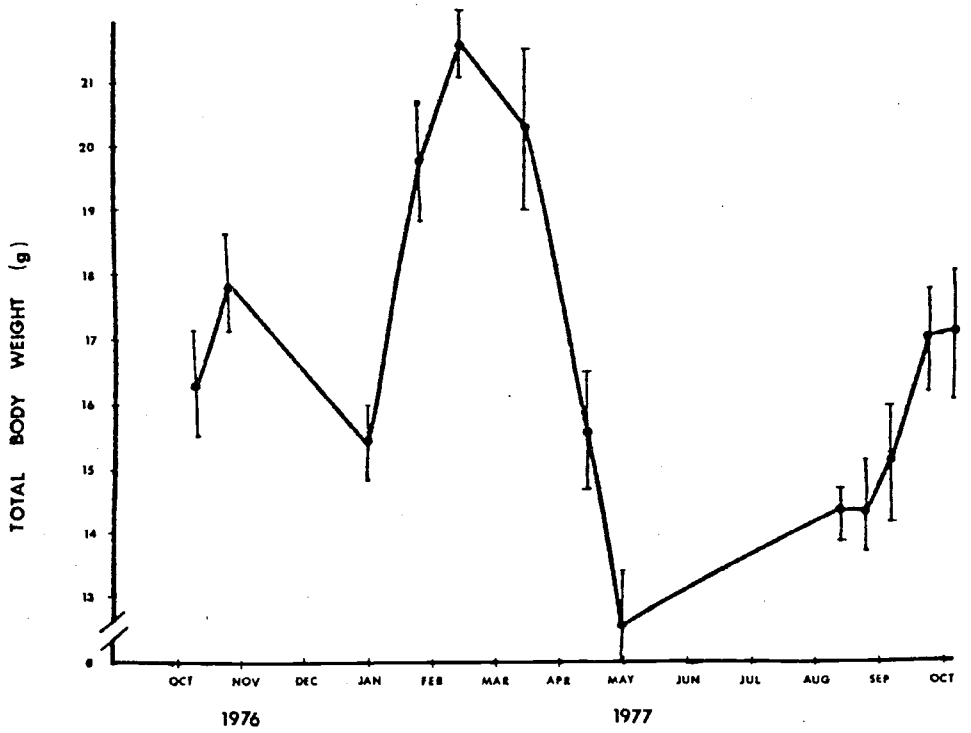

Figure 2. Seasonal changes in total body weight of adult male newts (Taricha granulosa). Vertical lines represent ± SE.

Table 1. Seasonal variation in snout-vent length and tail length in adult male newts (Taricha granulosa).

Collection date	N[1]	Snout-vent length (cm) Mean	SE	Tail length (cm) Mean	SE
10-22-76	11	6.62	± 0.14	10.91	± 0.28
11-8-76	15	6.99	± 0.08	10.75	± 0.16
1-16-77	15	6.53	± 0.09	10.71	± 0.15
2-7-77	8	6.84	± 0.10	10.45	± 0.28
2-27-77	12	6.78	± 0.05	10.42	± 0.19
3-31-77	12	6.60	± 0.15	10.40	± 0.21
4-28-77	5	6.72	± 0.21	10.02	± 0.40
5-16-77	5	6.20	± 0.19	9.98	± 0.13
8-31-77	6	6.90	± 0.12	10.13	± 0.28
9-7-77	6	6.98	± 0.09	10.70	± 0.35
9-23-77	6	6.83	± 0.11	10.90	± 0.26
10-7-77	6	6.85	± 0.08	10.72	± 0.20
10-21-77	9	6.84	± 0.10	11.12	± 0.32

[1]N refers to the total number of newts collected. These sample sizes are applicable to the other parameters presented in other figures.

are directly reflected in tail length changes (Table 1).

Seasonal changes in testicular development and regression are presented in Figure 3 and given in further detail in Table 2. Spermatogenesis resumed around March, as indicated by the increasing percentage of the testis occupied by seminiferous lobules containing immature spermatogenetic stages, concomitant with increasing testis weights. Seminiferous lobules in March contained many secondary spermatogonia (Figure 4). In fact, testes weights increased dramatically between March and August ($F = 37.19$; $df = 3, 26$), as shown in Figure 5. By May the glandular tissue is almost entirely resorbed, the unevacuated sperm are degenerating, and there are numerous primary spermatocytes (Figure 6).

Spermiogenesis was completed by late August, as is evidenced by the large percentage of testis comprised of mature spermatozoa. In September the seminiferous lobules that contained sperm bundles at early stages and that were near the immature zone typically appeared more hydrated, as shown in Figure 7. Spermiation began in early October and continued through March (see Figure 3). In late October the lobule boundary cells appear to have pulled away from the basement membrane, as shown in Figure 8. Testes weights during this period of evacuation dropped from 0.31 ± 0.04 g to 0.06 ± 0.01 g (mean $\pm$ SE), ($F = 28.63$; $df = 8, 88$). In March, when evacuation is completed, the lobule boundary cells and the Sertoli cells are the most hypertrophied, filling the entire seminiferous lobule, as shown in Figure 9. Changes in the percent ratio of testes in total body weight, the gonadosomatic index, is depicted in Figure 10.

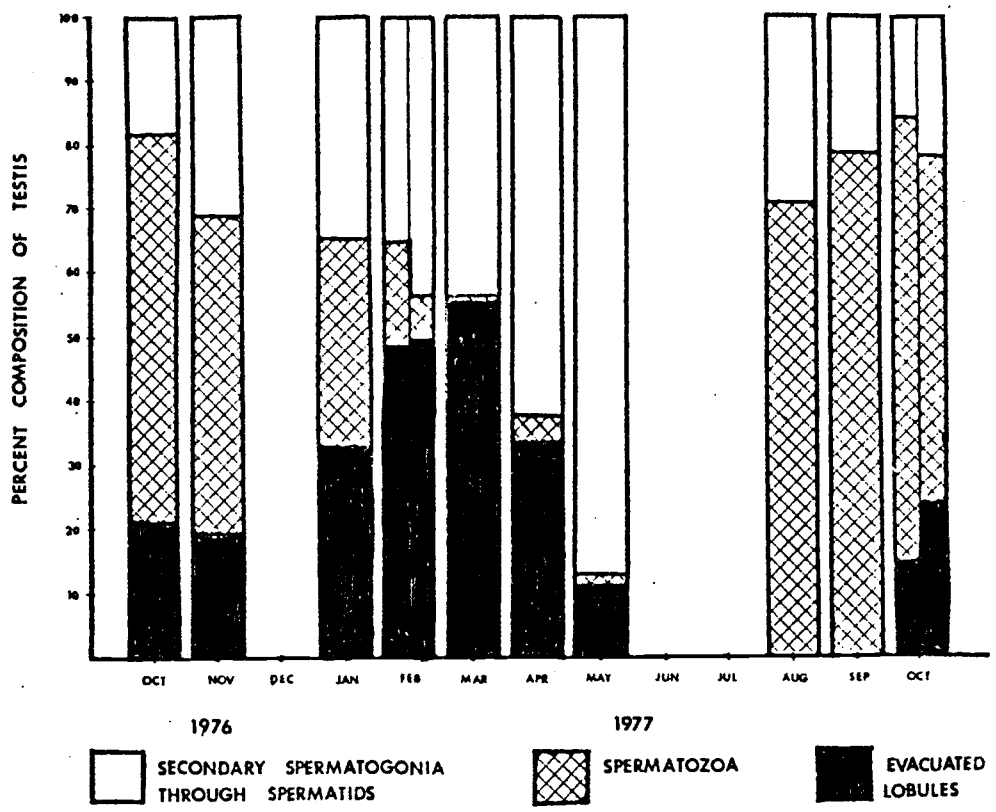

Figure 3. Seasonal variation in testis composition in adult male newts (Taricha granulosa). Four to six sections, evenly spaced over the entire testis, were counted. The number of seminiferous lobules counted for each category was converted into a percentage of the total number counted.

Table 2. Seasonal variation in testis composition in adult male newts (Taricha granulosa). Four to six sections, evenly spaced over the entire testis, were counted. The number of seminiferous lobules counted for each category was converted into a percentage of the total number counted.

Collection date	N[1]	Percent of lobules evacuated		Percent of lobules containing mature spermatozoa		Percent of lobules containing secondary spermatogonia through spermatids	
		Mean	SE	Mean	SE	Mean	SE
10-22-77	7	21.5	± 7.5	60.1	± 6.2	18.3	± 5.1
11-8-77	9	19.6	± 2.8	49.6	± 3.5	30.8	± 5.6
1-16-77	5	33.0	± 4.7	31.7	± 2.6	35.3	± 3.2
2-7-77	5	48.3	± 6.0	15.8	± 8.5	35.8	± 4.9
2-27-77	5	49.5	± 4.1	6.6	± 2.3	44.0	± 4.5
3-31-77	5	55.4	± 3.2	0.5	± 0.3	44.1	± 3.3
4-28-77	5	33.4	± 3.9	4.5	± 2.9	62.3	± 3.0
5-16-77	4	10.9	± 2.2	1.3	± 0.9	87.8	± 1.7
8-31-77	3	0		70.3	± 6.5	29.7	± 6.5
9-23-77	5	1.0	± 0.7	78.4	± 2.4	20.6	± 2.3
10-7-77	5	14.5	± 2.9	69.5	± 2.6	16.0	± 3.3
10-21-77	5	23.7	± 3.3	54.1	± 4.2	22.3	± 1.7

[1]N refers only to the number of animals for which testis composition was calculated.

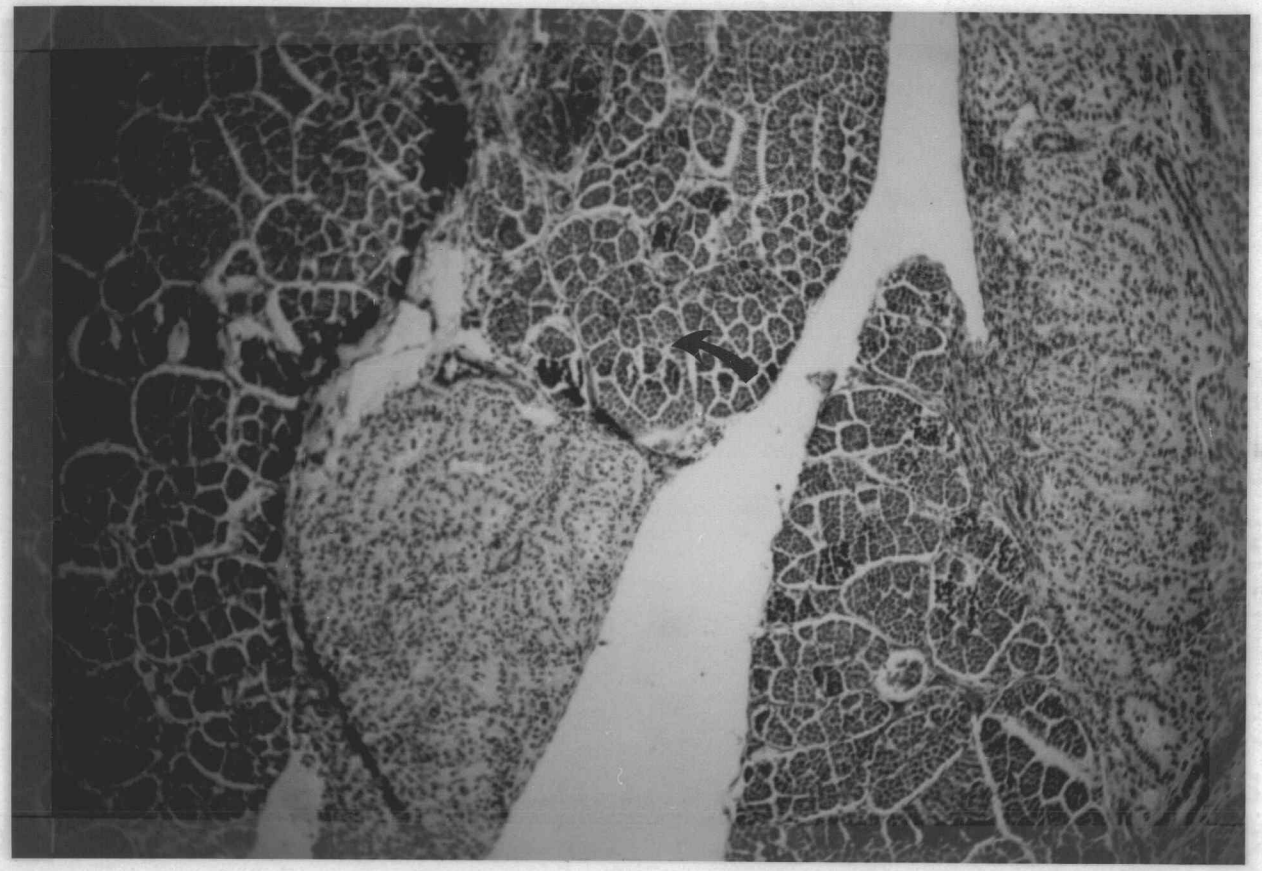

Figure 4. Testis of adult Taricha granulosa collected on March 31, 1977. Note abundance of darkly staining secondary spermatogonia (arrow). (40 X)

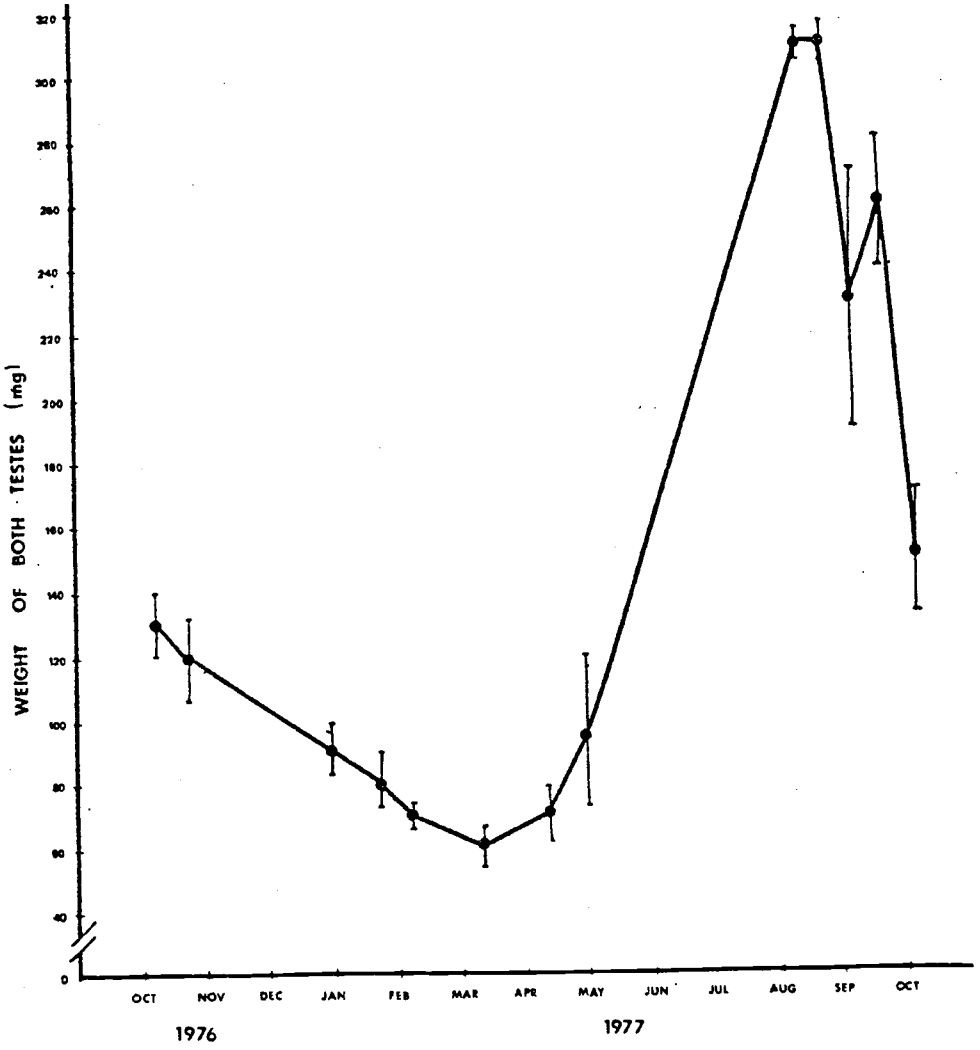

Figure 5. Seasonal variation in paired testis weight in adult male newts (Taricha granulosa). Vertical lines represent ± SE.

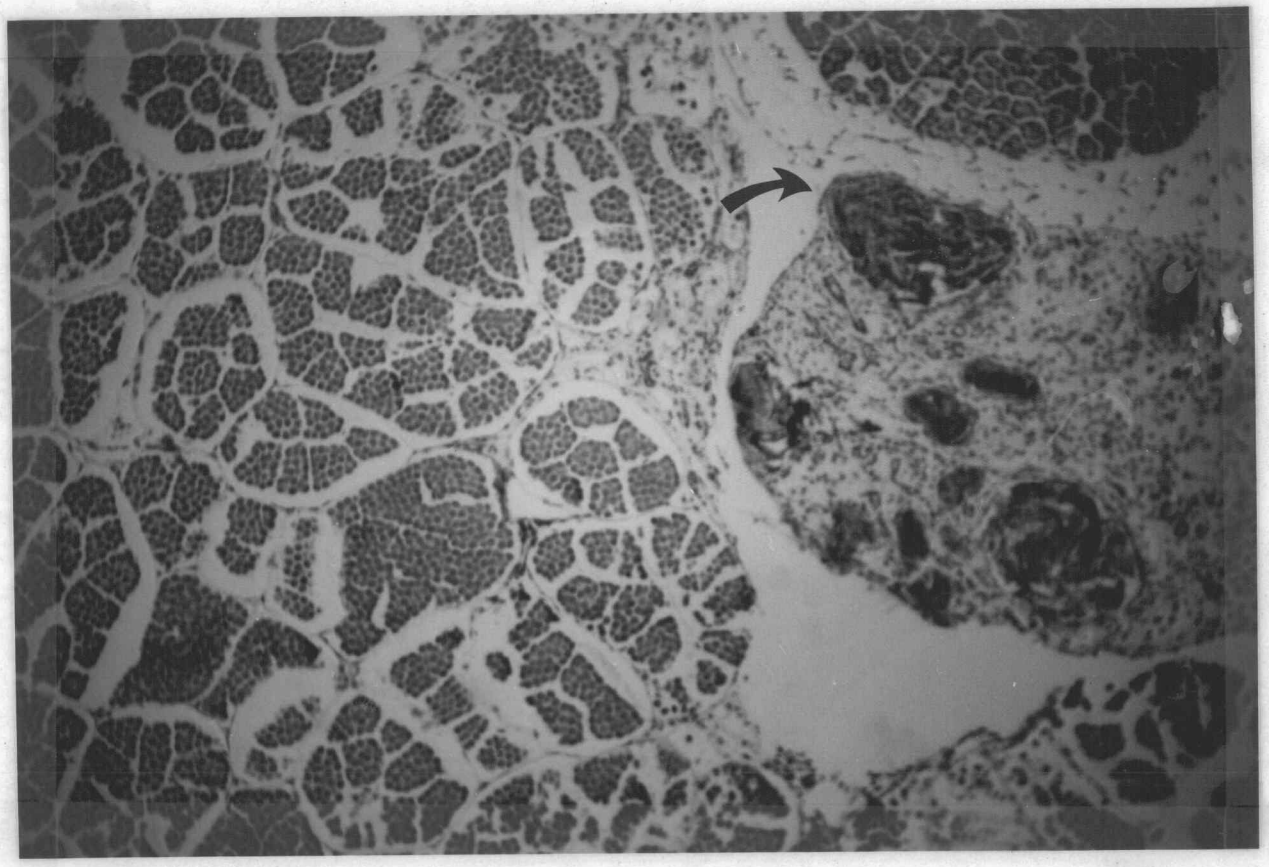

Figure 6. Testis of adult Taricha granulosa collected from Fathead Lake on May 17, 1977. Note numerous primary spermatocytes (left) and degenerating sperm (arrow) amidst resorbing grandular tissue. (40 X)

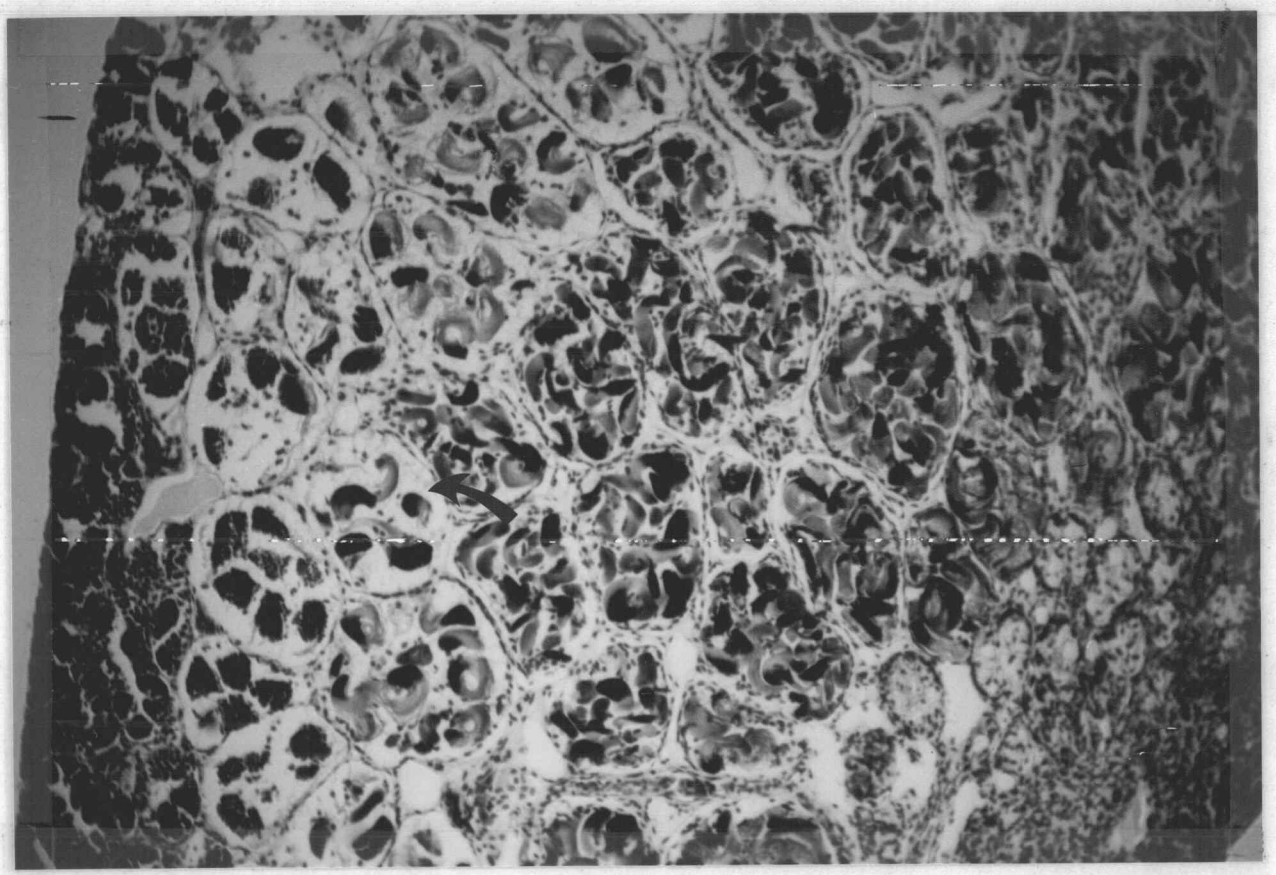

Figure 7. Testis of adult Taricha granulosa collected from Fathead Lake on September 23, 1977. Note appearance of hydration in seminiferous lobules near immature zone (arrow). (60 X)

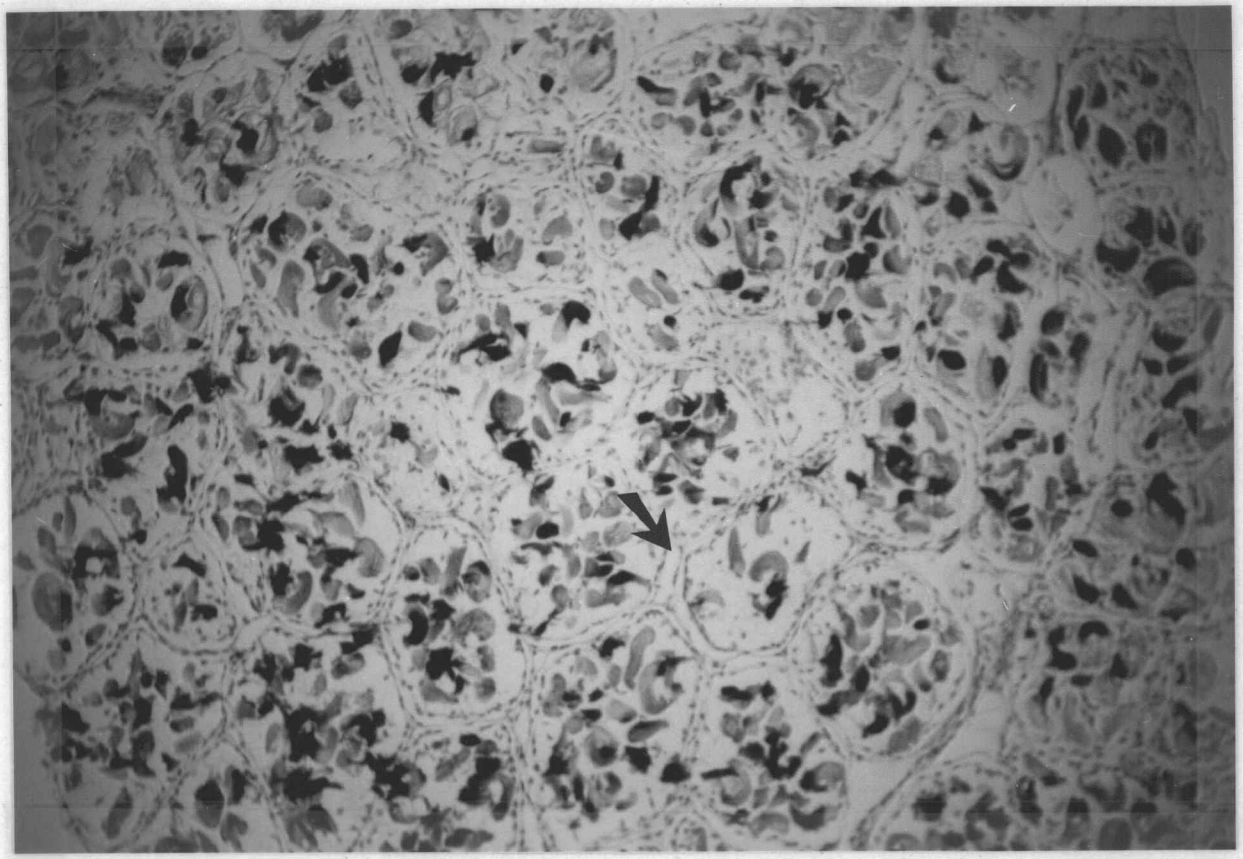

Figure 8. Testis of adult Taricha granulosa collected from Fathead Lake on October 20, 1977. Note that the lobule boundary cells appear to have pulled away from the basement membrane (arrow). (60 X)

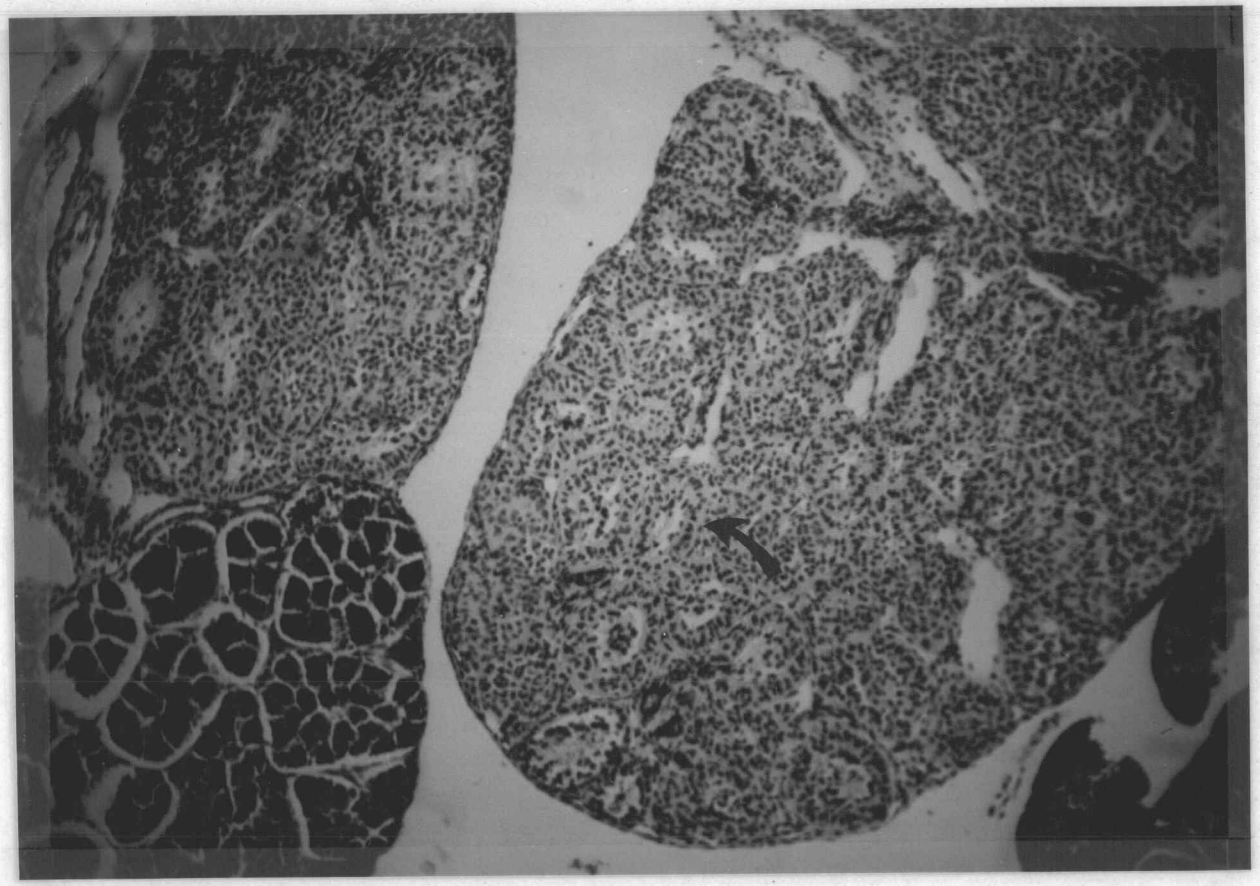

Figure 9. Testis of adult Taricha granulosa collected from Fathead Lake on March 31, 1977. Note that the lobule boundary cells and the Sertoli cells are extremely hypertrophied, filling the entire seminiferous lobule (arrow). (40 X)

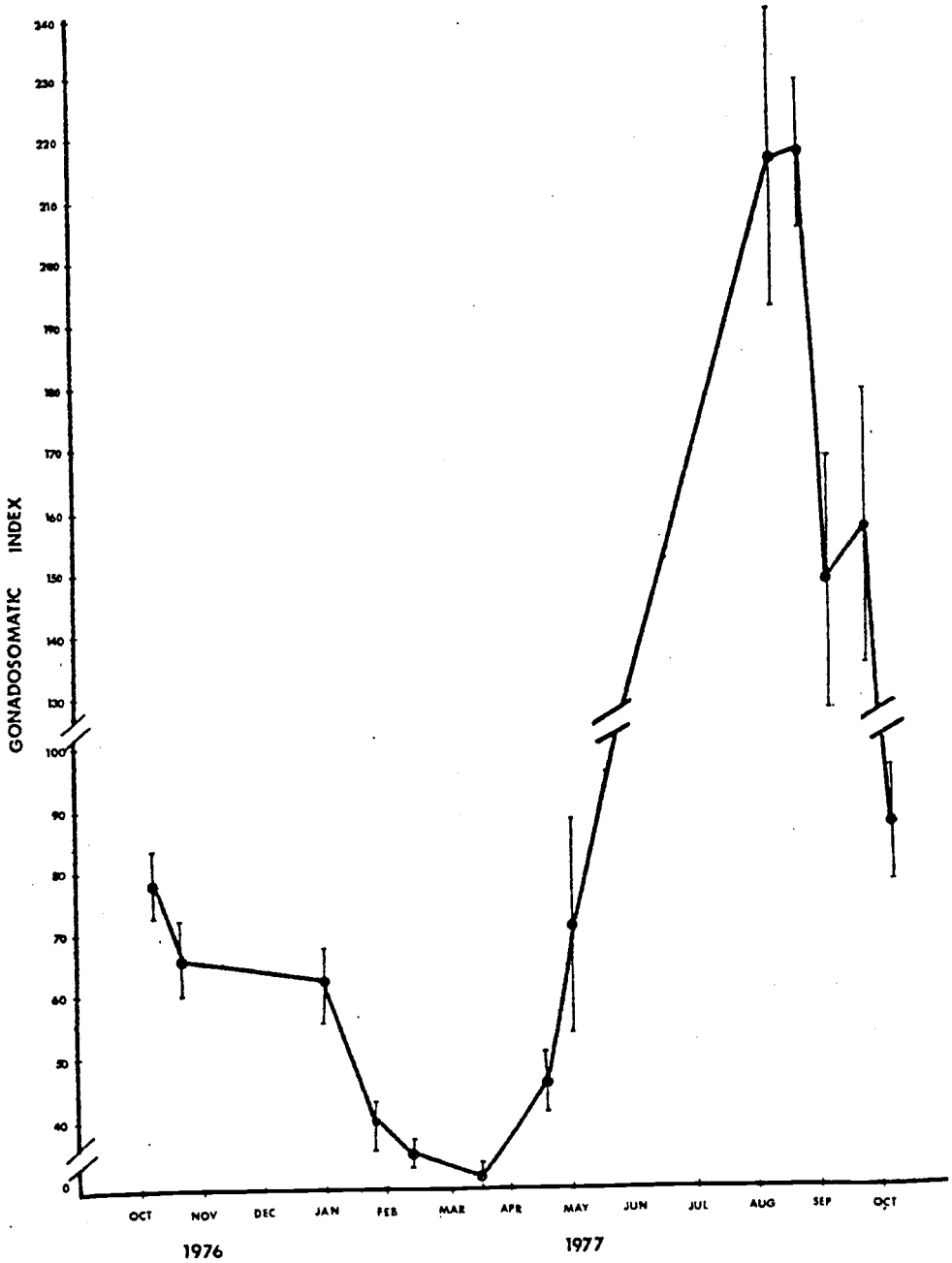

Figure 10. Seasonal changes in the gonadosomatic index (testes/total body weight % ratio) in adult male newts (Taricha granulosa). Vertical lines represent ± SE.

The adult male rough-skinned newt, T. granulosa, exhibited two pronounced seasonal peaks in plasma androgen concentrations, as shown in Figure 11. In October, titers reached 54.7 ± 7.9 ng/ml and in March the concentration was 43.9 ± 3.2 ng/ml (mean ± SE). Between early October and November, when the lake temperature dropped from 15° to 7°C, androgen titers fell to 7.2 ± 1.5 ng/ml ($F = 36.69$; $df = 2, 25$). Then, during the period between January and March, androgen concentration increased six-fold ($F = 52.44$; $df = 3, 46$). By May androgen concentration averaged only 3.3 ± 0.02 ng/ml ($F = 23.29$; $df = 2, 21$).

Effect of Castration and Hemicastration on Plasma Androgens

The androgens measured by this assay are apparently secreted by the mature zone of the testis, as is evident upon examination of Figure 12. Data were analyzed by a 3 x 4 ANOVA. Although main effects of time ($F = 65.86$, $df = 2, 18$) and group ($F = 14.51$; $df = 3, 54$) were highly significant, there was some interaction ($F = 2.96$; $df = 6, 54$; $P < 0.05$). On day 1 PO, androgen concentrations in the castrated and hemicastrated newts tended to be lower than in the two control groups, though not significantly ($F = 2.99$; $df = 3, 23$; $P < 0.10$). By day 7 PO hemicastrated newts exhibited the lowest plasma androgen level of the four experimental groups ($F = 8.68$; $df = 3, 23$). Interestingly, on day 7 the bilaterally castrated newts had plasma androgen concentrations very similar to the control newts. By day 14 PO the plasma androgen concentrations in the castrated and hemicastrated newts were significantly lower than the androgen

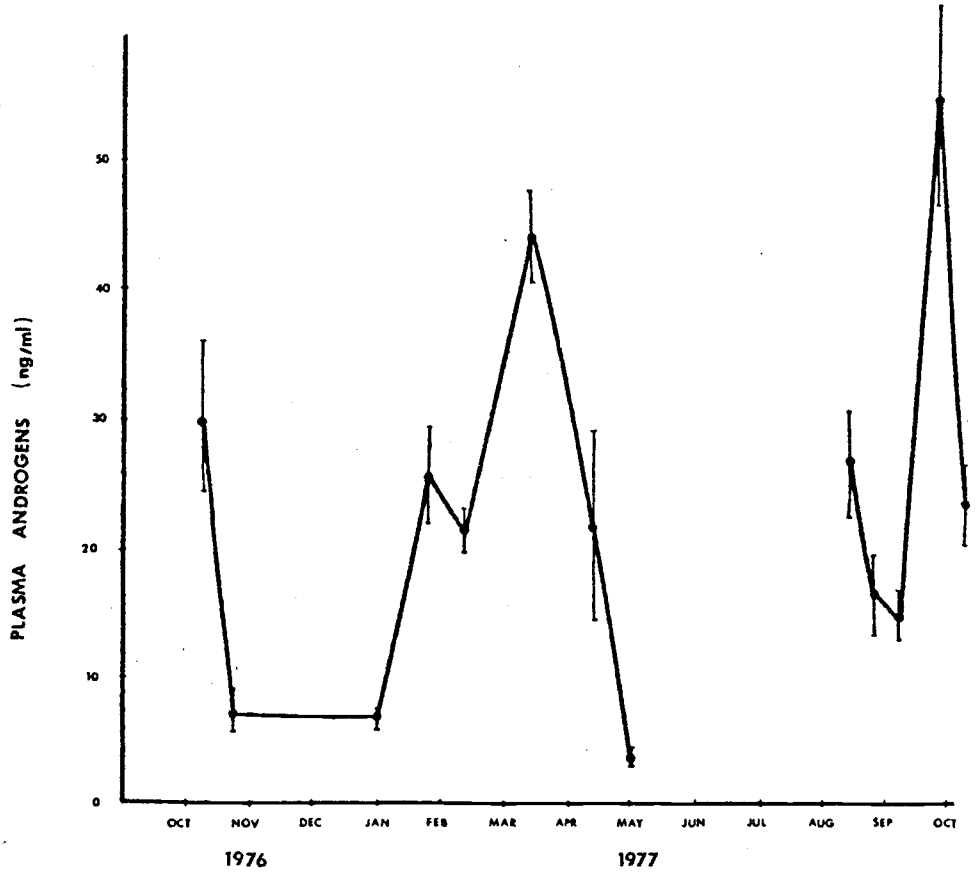

Figure 11. Seasonal variation in plasma androgen concentration in adult male newts (Taricha granulosa). Vertical lines represent ± SE.

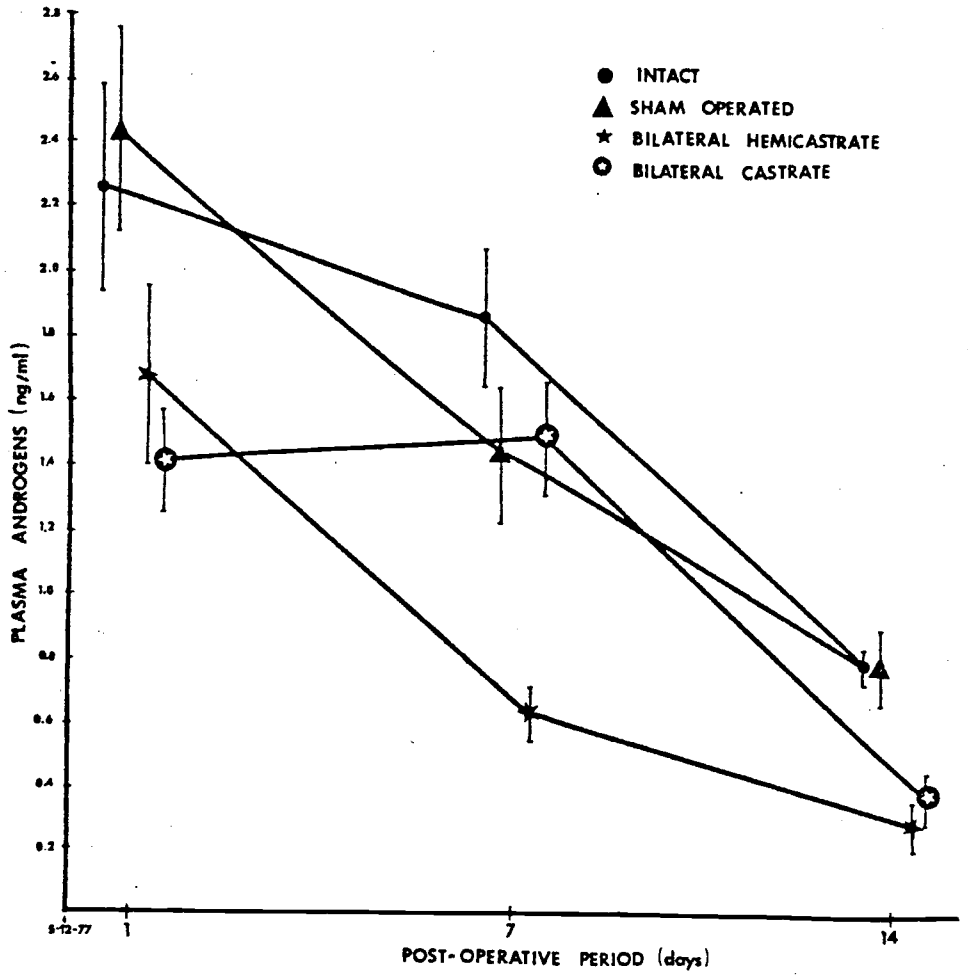

Figure 12. Effect of bilateral hemicastration or castration on plasma androgen concentration in adult male newts (Taricha granulosa). Vertical lines represent ± SE.

concentrations in newts from the two control groups ($F = 10.10$; $df = 3, 28$). At this time, however, no difference existed between the castrated or hemicastrated animals or between the sham-operated and control animals. Therefore, androgens from the testes are secreted by the mature zone and the surgery itself had no appreciable effect on androgen concentration. A significant decrease in androgen levels occurred across time in the intact control group ($F = 15.68$; $df = 2, 19$) and the sham-operated control group ($F = 13.83$; $df = 2, 18$).

Hematocrit levels of newts killed on days 7 and 14 PO are given in Table 3. After 2 weeks hematocrit levels were similar for all four groups ($F = 0.61$, n.s.). However, on day 7 PO the total castrates had lower hematocrit levels than did the other three groups ($F = 5.51$; $df = 3, 14$). Among the control groups and the hemicastrated group there was no difference ($F = 1.44$, n.s.).

Temperature Effects on Plasma Androgens and Tail Height

Newts maintained in the laboratory for 6 weeks at either 12° or 18°C had similar levels of plasma androgens, as shown in Figure 13; whereas newts collected freshly from the lake during the experimental period had levels that increased significantly from 14.9 ± 1.8 to 54.7 ± 7.9 ng/ml (mean ± SE), ($F = 16.72$; $df = 3, 26$). The plasma androgen levels did not change significantly during the experimental period in either the 18°C laboratory newts ($F = 1.43$, n.s.) or the 12°C laboratory newts ($F = 0.28$, n.s.). The significant difference in androgen concentrations among the three experimental groups after 4 weeks can be attributed to the dramatic increase in plasma androgen

Table 3. Hematocrit levels for newts (Taricha granulosa) subjected to surgery or used as controls.

Group	Day 7 PO		Day 14 PO	
	N[1]	Hematocrit Mean SE	N	Hematocrit Mean SE
Control	2	26.5 ± 4.5	8	26.5 ± 1.9
Sham-operated	5	29.4 ± 1.7	7	24.4 ± 3.0
Bilateral hemicastrate	3	33.3 ± 2.7	7	28.4 ± 3.7
Bilateral castrate	5	21.4 ± 1.7	7	23.0 ± 2.0

[1]N refers to the number of newts for which a hematocrit was taken; hematocrits were taken on only some of the newts on day 7 PO and for all of the newts on day 14 PO.

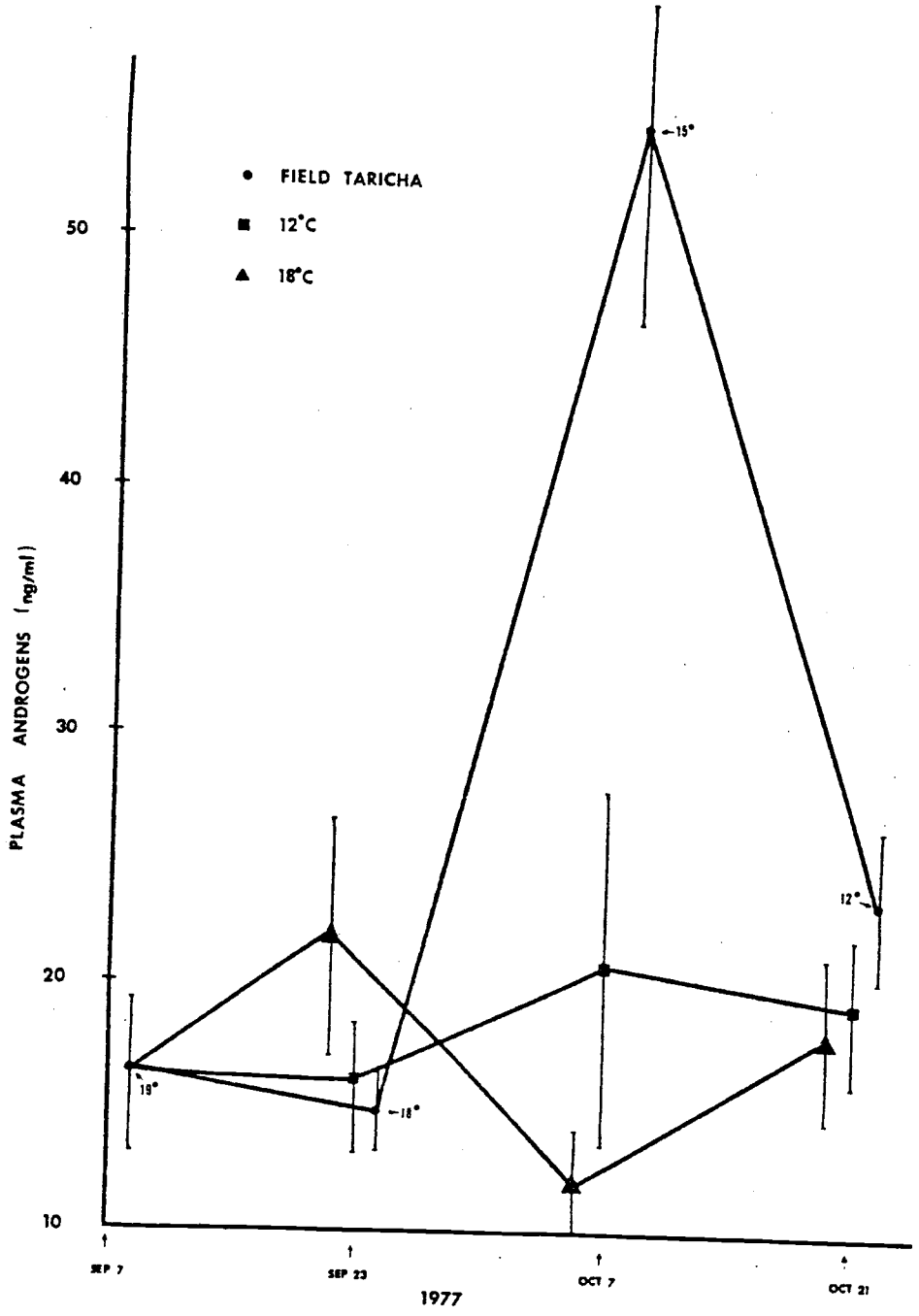

Figure 13. Effect of temperature on plasma androgen concentration in adult male newts (Taricha granulosa) compared to those from Fathead Lake. Vertical lines represent ± SE.

concentrations in the field-collected newts compared to the newts maintained in the laboratory ($F = 13.06$; $df = 2, 19$). No difference in plasma androgen titers was found among the three groups after 2 weeks ($F = 1.27$, n.s.) or after 6 weeks ($F = 0.89$, n.s.).

Tail heights for the three groups tended to diverge during the experimental period, as indicated in Figure 14. Data were analyzed by a 2 x 4 ANOVA. Because of the significant interaction value between treatment and time ($F = 7.86$; $df = 3, 63$), regression analyses were utilized. Tail height changes differed between 12° and 18°C laboratory animals as indicated by the significant differences between the slopes ($t = 3.59$, $df = 54$). Caudal fins regressed significantly in 18°C newts ($t = 2.68$ when $\beta_2 = 0$, $df = 27$) and increased in 12°C newts ($t = 2.37$ when $\beta_2 = 0$, $df = 27$, $P < 0.025$). Caudal fins in field-collected newts grew more rapidly during the experimental period than in either the 12° ($t = 4.44$, $df = 53$) or 18°C laboratory newts ($t = 6.62$, $df = 53$). Live body weight tended to decrease in the 18°C laboratory newts and remain the same in 12°C newts (Table 4). Paired testis weight decreased equally in the laboratory newts but not as much as in the field-collected newts during this time (Table 4).

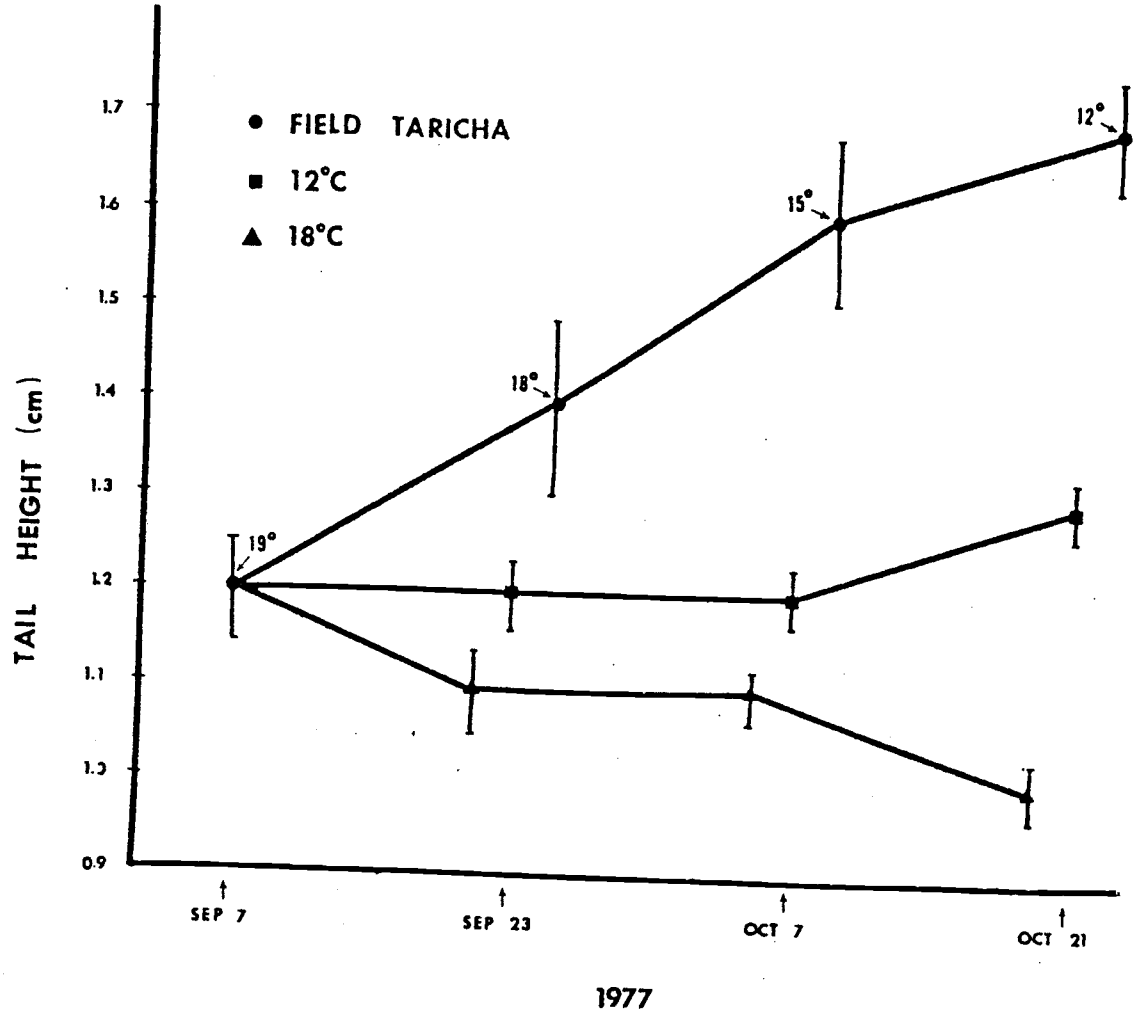

Figure 14. Effect of temperature on tail height in adult male newts (Taricha granulosa) compared to tail heights from Fathead Lake newts. Vertical lines represent ± SE.

Table 4. Effect of temperature on several parameters in the adult male newt (Taricha granulosa).[1]

Collection or termination date	Water temperature		N	Paired testes weight (g)	Live body weight (g)	Tail height	Plasma androgen concentration (ng/ml)
9-7-77	(Lake)	19°	6	0.31 ± 0.03	14.3 ± 0.8	1.2 ± 0.05	16.3 ± 3.1
9-23-77	(Lake)	18°	6	0.23 ± 0.04	15.1 ± 0.9	1.4 ± 0.09	14.9 ± 1.8
		18°	7	0.29 ± 0.02	13.5 ± 0.7	1.1 ± 0.04	22.0 ± 4.8
		12°	7	0.29 ± 0.04	14.2 ± 0.6	1.2 ± 0.04	16.1 ± 2.5
10-7-77	(Lake)	15°	6	0.26 ± 0.02	17.0 ± 0.8	1.6 ± 0.09	54.7 ± 7.9
		18°	7	0.19 ± 0.01	11.4 ± 0.6	1.1 ± 0.03	12.0 ± 2.2
		12°	7	0.21 ± 0.01	12.8 ± 0.5	1.2 ± 0.03	20.8 ± 7.1
10-21-77	(Lake)	12°	9	0.15 ± 0.02	17.1 ± 1.0	1.7 ± 0.06	23.4 ± 2.9
		18°	8	0.19 ± 0.01	11.9 ± 0.7	1.0 ± 0.04	18.1 ± 3.3
		12°	8	0.21 ± 0.01	13.2 ± 0.5	1.3 ± 0.03	19.1 ± 3.0

[1]Values presented as mean ± SE.

DISCUSSION

The adult male rough-skinned newt, T. granulosa, exhibits two pronounced seasonal peaks in plasma androgen concentrations: in October titers reach 54.7 ± 7.9 ng/ml and in March androgen levels average 43.9 ± 3.2 ng/ml (mean ± SE). During winter, levels drop to 6.7 ± 0.7 ng/ml (mean ± SE). After the spring peak, androgens decrease to 3.3 ± 0.02 ng/ml (mean ± SE) in May. Moore and Muller (1977) found androgen concentrations to be 0.54 ± 0.15 ng testosterone/ml and 0.23 ± 0.02 ng dihydrotestosterone/ml (mean ± SE) in June of 1976. In the anuran R. esculenta, the only other amphibian for which seasonal changes in androgens have been studied, elevated titers occur annually: from December to March plasma testosterone concentrations average 11 to 17 ng/ml, while minimum levels of less than 1 ng/ml occur from August to September (D'Istria et al., 1974).

Plasma androgen peaks occur in the newt at both the onset (October) and termination (March) of evacuation of mature spermatozoa from the seminiferous lobules. Seasonal changes in androgen concentration related to testicular conditions will be discussed first, followed by changes related to secondary sexual characteristics. The final stages of spermiogenesis occur in August when androgen levels are 2.6 ± 3.8 ng/ml (mean ± SE). Androgens may be necessary for the later stages of spermatogenesis. Transformation of spermatids into spermatozoa has been recorded in the plaice Pleuronectes platessa (Barr, 1963), during a period of high androgen concentration

(Wingfield and Grimm, 1977). Maximum testicular development, in terms of mature spermatozoa, is attained by September, at which time 78.4 ± 2.4% (mean ± SE) of the seminiferous lobules contain mature spermatozoa. The testis appears hydrated and its weight is maximum from August to early October when evacuation begins.

In Rana temporaria Lofts, Wellen, and Benraad (1972) reported a large increase in testicular water content during spermiation. The water uptake may be caused by the gonadotropin luteinizing hormone (Burgos and Vitale-Calpe, 1967; Burgos, Vitale-Calpe, and Russo, 1968), which has been associated with spermiation in anuran amphibians (Burgos and Ladman, 1957; Lofts, 1961). Luteinizing hormone has been shown to stimulate androgen production by anuran testes (Muller, 1977) and T. granulosa testes (Moore, Muller, and Specker, unpublished). Perhaps a surge of luteinizing hormone is responsible for increased weight of the testes and the large increase in plasma androgen concentration at the onset of spermiation in October.

Androgen levels are low during the coldest winter months (water temperature less than 7°C). Low ambient temperature may insensitize testicular tissue to gonadotropin (van Oordt, 1960). Roth (1974) found that temperature interacted with circulating gonadotropin to control rates of ovarian growth and the pattern of follicular growth within the ovary of the lizard, Anolis carolinensis.

Throughout the spring androgen levels are again elevated. Evacuation of spermatozoa is complete by late March and the testes weigh the least at this time. Curiously, the lobule boundary cells

appear most hypertrophied in late March, when the plasma androgen concentration averages 40 to 50 ng/ml. This is in contradistinction to the interstitial cell cycle in anurans (Lofts, 1964) in which the maximum lipid and cholesterol content occurs in these cells after mating, when plasma androgen concentration declines (D'Istria et al., 1974). Since cholesterol is a precursor to androgens (Ying, Chang, and Gaylor, 1965), it accumulates in the interstitial cells upon reduced androgen biosynthesis (Lofts, 1974).

In T. granulosa spermatogenesis appears to resume in March as indicated by the increased percentage of the testis composed of immature spermatogenetic stages, concomitant with increasing testicular weight. These annual testicular changes correspond to those reported for T. torosa by Miller and Robbins (1954) in which spermatogenetic recrudescence is known to occur around March. Although the effect of androgens on amphibian spermatogenesis is not clear, some research has shown high androgen levels to have an inhibitory effect on spermatogenesis. However, the elevated androgen concentration found in T. granulosa through April did not seem to be inhibitory, since spermatogenesis appears to have resumed in late winter. Furthermore, spermatogenesis continued even when androgens decreased.

Second metamorphosis occurs during September and October in adult male T. granulosa. Nuptial characteristics such as swollen glands of the cloaca, nuptial pads, increased body weight, and caudal fins appear at this time. This condition is retained until April, although a few nuptial males could be found in May.

In urodeles, many of the events of second metamorphosis are induced by prolactin and antagonized by thyroxin (reviewed by Bern and Nicoll, 1968). Prolactin has known stimulatory effects on tail height in this species (Moore, Seide, and Specker, unpublished) as well as in other urodeles (Chadwick, 1941; Tuchmann-Duplessis, 1949; Gona and Etkin, 1970; Vellano, Mazzi, and Sacerdote, 1970; Swanberg and Norris, 1972; Signhas and Dent, 1975; Platt and Christopher, 1977). Prolactin levels may increase with decreasing temperature since many characteristics of second metamorphosis are prolactin-induced and second metamorphosis occurs as ambient temperatures drop in autumn. Increased body weight associated with the prolactin effect of water uptake has been reported in this species (Moore, Seide, and Specker, unpublished) and in Ambystoma tigrinum (Platt and Christopher, 1977). Decreased tail height and body weight in winter and summer may be due to decreased androgen production since some events of second metamorphosis are dependent on a certain level of androgens being present (Singhas and Dent, 1975). These characteristics could also be decreased during winter and summer by increased thyroxin levels, since thyroxin is known to antagonize prolactin-induced effects (see Bern and Nicoll, 1968). Changes in tail height and body weight might also reflect the temperature effect on the biological activity of pituitary hormones.

Male newts were present in Fathead Lake throughout the year. During the coldest part of winter and in the heat of the summer, newts were observed only in deeper parts of the lake. Only in October and March did there seem to be as many females as there

were males in the water. Amplexus was observed from late September through April. The largest number of newts in amplexus was observed in early October and late March. During the periods when females were scarce, it was not uncommon to see a male clasping another male. Curiously, breeding activity was most intense when androgen levels were the highest. Yet researchers have been unable to implicate androgens in the expression of sex behavior in this species (Moore and Muller, 1977; Moore, Specker, and Swanson, 1978). Breeding occurs in R. esculenta after androgen titers drop significantly (D'Istria et al., 1974).

Although this study encompasses only one 13-month period, it represents normal seasonal changes. This is indicated by the close correspondence between seasonal changes in testis weight and composition and that found for T. torosa over a 2-year period (Miller and Robbins, 1954).

To assess the contribution the mature testicular zone makes to plasma androgen levels newts were subjected to either bilateral total or bilateral hemicastration. Hemicastration involved extirpation of the mature zone from each lobe of both testes. The mature zone contained lobules that were either already evacuated or still contained sperm.

The mature zone of spermatozoa and resorbing glandular tissue appears to produce a significant portion of plasma androgens. On day 1 PO the hemicastrated and castrated newts tended to have lower plasma androgen concentrations than did the sham-operated and intact control newts. By day 14 PO androgen concentrations in the two

experimental groups were significantly lower than the androgen titers in the two control groups.

Curiously, on day 7 the hemicastrated newts exhibited the lowest plasma androgen concentrations of all four groups, while the totally castrated newts had levels very similar to the two control groups and to the total castration group on day 1 PO. The decrease in androgen concentration in the hemicastrated newts cannot be accounted for by increased blood loss, since only the totally castrated newts had reduced hematocrit levels on day 7 PO. Decreased hematocrit levels in the castrated animals on day 7 PO suggest that their plasma androgen concentrations might be decreased due to dilution of the plasma, assuming blood volume loss is compensated. Perhaps, instead, the relatively unchanged plasma androgen concentrations found in the totally castrated newts on day 7 PO are indicative of an extended circulating half-life due to a lowered clearance rate of the androgens. Lowered clearance rate could reflect the absence of tissue which binds or metabolizes androgens. In the hemicastrates, on the other hand, androgens could be bound by the remaining testicular tissue. Other possibilities are that once the newt is totally castrated, some chemical message from the testis to the pituitary is terminated and/or other steroidogenic tissue is stimulated by pituitary secretions. The interrenal gland in Pleurodeles waltlii has the potential to produce steroids (Picheral, 1970). In mammals high circulating levels of adrenocorticotropin hormone cause the adrenal to secrete testosterone.

Plasma androgen concentration decreased significantly during the experiment in both the intact control group and the sham-operated control group. Androgen titers were concurrently decreasing in the pond newts, typical of this terminus of the breeding season. Decreasing androgens might also reflect the stress of laboratory conditions, such as interrupted photoperiod and lack of food.

Early fall marks the occurrence of second metamorphosis and the onset of spermiation and sex behaviors. Concurrently decreasing pond temperatures may affect these events through changes in pituitary and gonadal secretions. In fact, temperature, more than photoperiod, seems to play a dominant role in amphibian reproductive cycles (Ifft, 1942; reviewed by van Oordt, 1960; Werner, 1969; see Lofts, 1974). Therefore, it was hypothesized that newts brought into laboratory conditions in early fall and maintained at 12° or 18°C could be expected to diverge with respect to several parameters, relative to each other and to freshly captured newts.

Temperature, per se, did not affect plasma androgen levels in newts maintained in the laboratory at 12° or 18°C. Plasma androgen concentrations did not change over the 6 weeks in either group. Androgen levels in field-collected newts increased three-fold after 4 weeks. However, after 2 and 6 weeks the three groups had comparable androgen levels (about 20 ng/ml).

Plasma androgen measurements, however, reflect several physiological events. For instance, changes in concentration may indicate either a different secretion rate or a different clearance rate. That is to say, even though the newts maintained in the

laboratory had similar plasma androgen concentrations, they might still have had different secretion and clearance rates. Secondly, changes in secretion rate and clearance rate might reflect changes in the biological activity of pituitary hormones, which is a temperature-influenced phenomenon.

Perhaps the unchanged plasma androgens in the laboratory groups--when lake newt titers increased dramatically--reflect the absence of exogenous cues that occurred in the lake and not the laboratory. For example, the influx of females into the pond might, through olfactory cues, trigger a surge of luteinizing hormone from the pituitary. Another possibility is that although all the newts had potential for high androgen production and secretion at this time, stress related to laboratory maintenance inhibited luteinizing hormone release, perhaps through negative feedback of circulating corticosteroids.

Although plasma androgen concentrations did not seem to be influenced by ambient temperature, tail heights in the three groups diverged significantly. Caudal fins regressed in 18°C newts and increased in 12°C laboratory newts. Fin development was even greater in pond Taricha. Considering that tail height is stimulated by prolactin (Bern and Nicoll, 1968) and that this nuptial characteristic is assumed as ambient temperature is dropping, it may be that prolactin levels increase with decreasing temperatures. This would account for differences in tail height between 12° and 18°C laboratory newts. Since thyroxin antagonizes prolactin effects, it could also be that thyroxin levels increased in the 18°C newts.

Plasma androgen concentrations in amphibians do not seem to be influenced by prolactin levels (Muller, 1977). A certain level of androgens are required for prolactin effects to occur, however, as discussed earlier. Tail height almost always decreases in the laboratory and is usually only ameliorated by prolactin injections (Singhas and Dent, 1975; Moore, Seide, and Specker, unpublished).

In summary, seasonal changes in plasma androgen concentration and testicular development were measured for the first time in a urodele. Plasma androgen concentrations in adult male T. granulosa, measured by radioimmunoassay, are elevated in autumn and spring (averaging about 50 ng/ml) and are considerably less in the winter and summer (less than 1 ng/ml). Seasonal changes in testis composition indicate one annual cycle of evacuation of spermatozoa (October-March) and spermatogenetic recrudescence (March-October). Androgen levels peak at both the onset and termination of spermiation. Bilateral castration lowered plasma androgen concentrations indicating that the testis accounts for a significant portion of plasma androgens. Furthermore, extirpation of the mature zone from each testicular lobe decreased plasma androgens equally to bilateral castration. Therefore, the seminiferous lobules either containing mature sperm or already evacuated are the principal source of androgens from the testes. The possibility of an extratesticular androgen source remains. Previous research on amphibian spermatogenetic cycles has indicated that temperature is a principal controlling factor. Therefore, it was hypothesized that temperature

would influence changes in plasma androgen concentration through its effects on the pituitary-gonadal axis. However, newts maintained at either 12° or 18°C for 6 weeks in the laboratory had unchanged plasma androgen levels. Caudal fins regressed in the 18°C newts and developed in the 12°C newts, suggesting that prolactin, but not luteinizing hormone, was affected by water temperature.

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APPENDICES

APPENDIX I

TISSUE PREPARATION

60 min. 85% Ethanol

30 min. 95% Ethanol

30 min. 95% Ethanol

30 min. 100% Ethanol

30 min. 100% Ethanol

30 min. 100% Ethanol

30 min. ½ 100% Ethanol ½ Xylene

15 min. Xylene

15 min. Xylene

15 min. Paraplast

60 min. Paraplast

15 min. vacuum infiltration

Mount in tissue ring

APPENDIX II

HEMATOXYLIN-EOSIN STAINING TECHNIQUE

5 min. Xylene

3 min. Xylene

3 min. ½ Xylene ½ Ethanol

3 min. 100% Ethanol

3 min. 95% Ethanol

3 min. 80% Ethanol

3 min. Distilled H_2O

6 min. Hemotoxylin

Rinse in running water to remove excess stain.

3 dips in 1% Acid Alcohol

Rinse in running water to remove excess Acid Alcohol.

Dip in 1% Ammonia water until tissue turns blue.

10 min. running water

3 min. Eosin

2 min. 95% Ethanol

2 min. 100% Ethanol

3 min. Xylene

3 min. Xylene

Acid Alcohol: 1 ml HCl in enough 70% EtOH to make 100 ml

APPENDIX III

SEASONAL CHANGES IN MALE TARICHA GRANULOSA REPRODUCTIVE STATUS

Collection date	N	Tail height (mm) Mean SE	Total weight (g)	Paired testis weight (g)	Gonadosomatic index (%)	Plasma androgen concentration (ng/ml) Mean ± SE	Range
10-22-76	11		16.3 ± 0.8	0.13 ± 0.01	0.78 ± 0.05	29.9 ± 5.8	70.0- 4.8
11-8-76	15	20	17.8 ± 0.8	0.12 ± 0.01	0.66 ± 0.06	7.2 ± 1.5	18.7- 1.8
1-16-77	15	16 ± 0.5	15.4 ± 0.6	0.09 ± 0.01	0.62 ± 0.06	6.7 ± 0.7	13.2- 3.6
2-7-77	8	17 ± 0.8	19.8 ± 0.9	0.08 ± 0.01	0.39 ± 0.04	25.5 ± 3.6	43.9-13.7
2-27-77	12	20 ± 0.6	21.5 ± 0.6	0.07 ± 0	0.34 ± 0.02	21.2 ± 1.6	27.9-13.5
3-31-77	12	20 ± 1.1	20.3 ± 1.2	0.06 ± 0.01	0.30 ± 0.02	43.9 ± 3.2	59.7-29.3
4-28-77	5	15 ± 0.7	15.5 ± 1.0	0.07 ± 0.01	0.45 ± 0.04	21.6 ± 7.4	47.4- 3.9
5-16-77	5	10 ± 0.6	12.5 ± 0.9	0.09 ± 0.02	0.71 ± 0.17	3.3 ± 0	3.4- 3.2
8-31-77	6	11 ± 0.5	14.3 ± 0.4	0.31 ± 0.04	2.16 ± 0.24	26.6 ± 3.8	37.2-17.9
9-7-77	6	12 ± 0.5	14.3 ± 0.8	0.31 ± 0.03	2.17 ± 0.12	16.3 ± 3.1	25.1- 4.6
9-23-77	6	14 ± 0.9	15.1 ± 0.9	0.23 ± 0.04	1.48 ± 0.20	14.9 ± 1.8	21.3- 8.2
10-7-77	6	16 ± 0.9	17.0 ± 0.8	0.26 ± 0.02	1.57 ± 0.22	54.7 ± 7.9	79.0-36.5
10-21-77	9	17 ± 0.6	17.1 ± 1.0	0.15 ± 0.02	0.87 ± 0.09	23.4 ± 2.9	41.1-13.8