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CHANGES IN THE BIOCHEMICAL COMPOSITION OF TESTES DURING SPERMATOGENESIS IN ASTERIAS VULGARIS, WITH EMPHASIS ON THE ROLE OF POLYAMINES IN REGULATING PROLIFERATION

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BY

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B.A., Cornell University, 1978

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WITH EMPHASIS ON THE ROLE OF POLYAMINES
IN REGULATING PROLIFERATION

by

Frank F. Smith
University of New Hampshire, May, 1985

Testes of Asterias vulgaris are potentially useful for investigating mechanisms regulating spermatogenic events because of their structural simplicity and annual spermatogenic cycle. Examination of major biochemical classes during the spermatogenic cycle provides a definition of the changing chemical microenvironment influencing germinal cells and also suggests temporal relationships among successive spermatogenic events. Testes from seastars collected throughout the year were homogenized and lyophilized and aliquots assayed for DNA, RNA, total protein, free amino acids, total lipids, glycogen, and other carbohydrates; spermatogenic stage was determined by examination of paraffin sections. The resulting data, expressed as 1. mg/g dry mass, 2. mg/mg DNA, and 3. total content, were analyzed by weighted periodic regression and circular-linear correlation with spermatogenic stage and time of year. The observed patterns of change correlate closely with cytological variations observed within the germinal epithelium. Biochemical

data thus complement existing data on the cytology of the germinal epithelium.

Activity of ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine synthesis, increases during the proliferative phase of spermatogenesis. Testicular ODC activity correlates well with DNA synthetic rate. To test the possible role of polyamines in regulating initiation of spermatogonial mitoses, intact testes near the beginning of the proliferative phase were incubated *in vitro* with exogenous polyamines. They subsequently showed a significant increase in incorporation of ^{3}H-thymidine into DNA. No statistically significant difference in thymidine incorporation could be detected among testes exposed to three incubation regimes: polyamines present on first day only, present second day only, and present both days; in all three cases, ^{3}H-thymidine was added for the third day of incubation with the same type of medium as that used on the second day. Thus, the effect on DNA synthesis of extrinsic polyamines appears to be sustained after removal of the extrinsic polyamines. These results suggest a direct role for polyamines in the regulation of spermatogenic proliferation in *A. vulgaris*. Evidence for a regulatory role of polyamines in the initiation of proliferation, together with existing information on the environmental, hormonal, and cytological interactions, facilitates development of a preliminary model for regulation and entrainment of spermatogenesis.

INTRODUCTION

A wide variety of cellular systems have been used for the study of proliferation. These systems can be conveniently divided into three major groups: (1) transformed cells, in which events normally responsible for regulating proliferation are disrupted (e. g., Russell and Snyder 1968; Fillingame et al. 1975; Fong et al. 1976; Fuller et al. 1977; Sweet et al. 1980; Herbst and Branca 1981; Rumsby and Puck 1982; McConlogue et al. 1983); (2) normal (i. e., non-transformed) cells undergoing active proliferation (e. g., Russell and Snyder 1968; Chan et al. 1981); and (3) cells in mitotically quiescent populations which are stimulated to begin proliferation (e. g., Temin 1971; Pardee 1974; Otto et al. 1979). Proliferation in all three of these groups appears to be regulated differently (Cress and Gerner 1980; Haddox et al. 1980; Cameron and Pool 1981; Magun and Gerner 1981); indeed, cells transformed by different chemical or viral agents may differ in the mechanisms by which their growth is regulated (Magun and Gerner 1981). Although thorough understanding of mechanisms regulating cellular proliferation may require synthesis of information gained from all three groups of systems, differences in regulatory mechanisms must be considered when interpreting results.

Many studies of cell cycle regulation, regardless of type of cell system used, have involved three fundamental approaches. In the first, limitation of cellular growth by restriction of some required resource is followed by relaxation of the restrictive condition. This approach includes serum starvation and re-feeding (e. g., Temin 1971; Pardee

1974; Fong et al. 1976; Rumsby and Puck 1982) and dilution of confluent cultures (e. g., Cress and Gerner 1980). In the second, a population of cells is stimulated to proliferate by the addition of a mitogenic or transforming agent not normally present in those cells (e. g., Fillingame et al. 1975; Chen et al. 1976; Cheetham and Bellett 1982). In the third, an agent which may serve some regulatory function in physiologically normal cells is directly manipulated (e. g., Cohen et al. 1970; Dion and Cohen 1972; Herbst and Branca 1981; Schoenmakers et al. 1981b; Pearse and Eernisse 1982).

Spermatogonia in the testes of Asterias vulgaris form a population of mitotically quiescent cells which is annually stimulated by an environmental cue to begin proliferation (Walker 1980; Pearse and Walker in press). Asteroid testes therefore provide a potentially useful system for examining factors involved in regulating proliferation in normally functioning cells. Manipulation of the testicular microenvironment can provide important insights into regulatory events controlling the cell cycle of normal cells stimulated to begin proliferation. In addition, later in the spermatogenic cycle asteroid testes provide a population of normal cells undergoing active proliferation. Thus, it would be possible to compare regulation of actively proliferating spermatogonia to that of mitotically quiescent spermatogonia stimulated to proliferate. The assertion that regulation of actively proliferating cells differs from that of mitotically quiescent cells (Cress and Gerner 1980) can therefore be examined with a system which normally switches from one mode to the other, rather than relying upon laboratory manipulation to force actively proliferating cells to temporarily stop proliferation.

Spermatogenesis in Asterias vulgaris has several additional advantages (Walker 1980). Compared to mammalian spermatogenesis, spermatogenesis in asteroids appears to relatively simple. The testes are structurally simple: essentially double sacs with relatively few tissues (Walker 1974). Although steroids appear to be involved in regulating and coordinating reproductive events and nutrient transport (Voogt et al. 1984), there seem to be no detectable secondary sexual characteristics. For example, I have been unable to detect any sex-related differences via morphometric analysis. Thus, it may be possible to study reproductive functions of hormones in isolation from somatic effects such as production and maintenance of secondary sexual characteristics. Furthermore, some of the major spermatogenic events, such as initiation of proliferation and differentiation, are separated temporally rather than spatially as occurs in mammalian testes. Examination of these events in isolation from other spermatogenic processes is therefore facilitated. Finally, the presence of multiple (10) testes in an animal allows use of randomized block experimental design to account for some variability among animals in a population. However, testes prior to or shortly after initiation of proliferation are often extremely small (occasionally less than 1 mg dry mass per testis); experiments conducted at that time of year may occasionally require biochemical measurements near the limit of sensitivity for the assays used (see Chapter II).

Cytological changes during spermatogenesis in Asterias vulgaris have been described in considerable detail (Walker 1980). The brief description that follows is intended to provide orientation to the timing of major events during the annual spermatogenic cycle. Four

successive phases can be distinguished histologically. The aspermatogenic phase (1) begins after spawning in late May or June and continues until approximately October in populations from the Gulf of Maine. During the early aspermatogenic phase, somatic cells in the testicular lumen phagocytize residual spermatozoa from the preceeding spermatogenic cycle. Other somatic cells surround spermatogonia in the germinal epithelium, enclosing them in follicle-like compartments. In the late aspermatogenic phase (Fig. 1), many of the luminal phagocytic cells apparently convert to "vesicular" secretory cells. These cells contain large secretory vacuoles, extensive smooth endoplasmic reticulum, and well-developed Golgi apparati; in addition, they show weak histochemical evidence for $\Delta 5$-3β-hydroxysteroid dehydrogenase activity (Walker 1980). (2) The beginning of the proliferative phase (Fig. 2) is marked by a significant increase in mitotic rate of previously quiescent spermatogonia. Initiation of spermatogonial proliferation appears to be mediated by steroid hormones (Voogt et al. 1984), probably secreted by "vesicular" somatic cells. Proliferating spermatogonia are released from their follicle-like compartments, and somatic cells begin to organize the germinal epithelium into columns of germinal cells (mostly primary spermatocytes) arranged around a scaffolding formed by a single or a few somatic cells. At the same time, the genital haemal sinus fills with periodic-acid Schiff (PAS) and mercuric bromphenol blue (MPB) positive material, which is presumably nutritive. As the proliferative phase continues, spermatogenic columns lengthen as spermatogonial mitoses continue at their bases (Fig. 3). Primary spermatocytes higher in the columns have passed through pre-meiotic interphase and are arrested in prophase of

the first meiotic division (Walker 1980). Proliferation continues at the bases of columns until approximately early March; cessation of proliferation corresponds with loss of PAS and MPB positivity in the genital haemal sinus. (3) The differentiative phase begins approximately in January, and thus overlaps the latter portion of the proliferative phase by several months. Primary spermatocytes at the tips of thousands of adjacent columns contemporaneously proceed through meiosis and spermiogenesis (Fig. 4). During the latter portion of the differentiative phase, spermatogonial proliferation ceases and the columns begin to degrade. Eventually, the testicular lumen is filled with numerous spermatozoa. (4) Finally, stored spermatozoa are released during the brief evacuative phase in late May or early June. The testicular lumen then contains residual spermatozoa and phagocytic somatic cells as the testis returns to the early aspermatogenic phase.

Although the cytological changes during spermatogenesis have been extensively described (Walker 1980), and information has been obtained on the environmental (Pearse and Walker in press), hormonal (Voogt et al. 1984), and cytological (Walker and Larochelle 1984) interactions involved in entraining and controlling the spermatogenic cycle, little is known about the proximate biochemical events involved in regulating specific spermatogenic events. A reasonable initial step in defining changes in the biochemical microenvironment during spermatogenesis is careful characterization of major biochemical classes in the testes throughout the spermatogenic cycle (see Chapter I). Such a study complements existing cytological and ultrastructural information and provides useful information about fundamental metabolic changes in the testes.

In conjunction with characterization of fundamental changes in biochemical composition of the testes, examination of metabolic pathways which may be involved in regulating specific spermatogenic events further helps to define changes in chemical microenvironment and cellular physiology that are critical to progress through the spermatogenic cycle. Because polyamines have been implicated as serving a regulatory function for proliferation in a variety of cell systems (e. g., Dion and Cohen 1972; Fillingame et al. 1975; Fuller et al. 1977; Kusunoki and Yasumasu 1978; Haddox and Russell 1981; McCann et al. 1981; Sunkara and Rao 1981), the possible role of polyamines in regulation of spermatogonial mitoses in Asterias vulgaris is considered here (Chapters II and III). Preliminary evidence (Chapter II) indicates a good correlation between polyamine synthesis (measured by specific activity of ornithine decarboxylase, the rate-limiting enzyme in the polyamine biosynthetic pathway) and the proliferative phase of spermatogenesis. A regulatory function for polyamines can be indicated by showing that increased polyamine content is sufficient for enhancement of commitment to mitosis (as measured by rate of ^{3}H-thymidine incorporation into DNA).

The research presented here thus provides further definition of the chemical microenvironment influencing germinal cells during spermatogenesis in Asterias vulgaris and provides an indication of proximate biochemical events important in regulating initiation of spermatogonial proliferation.

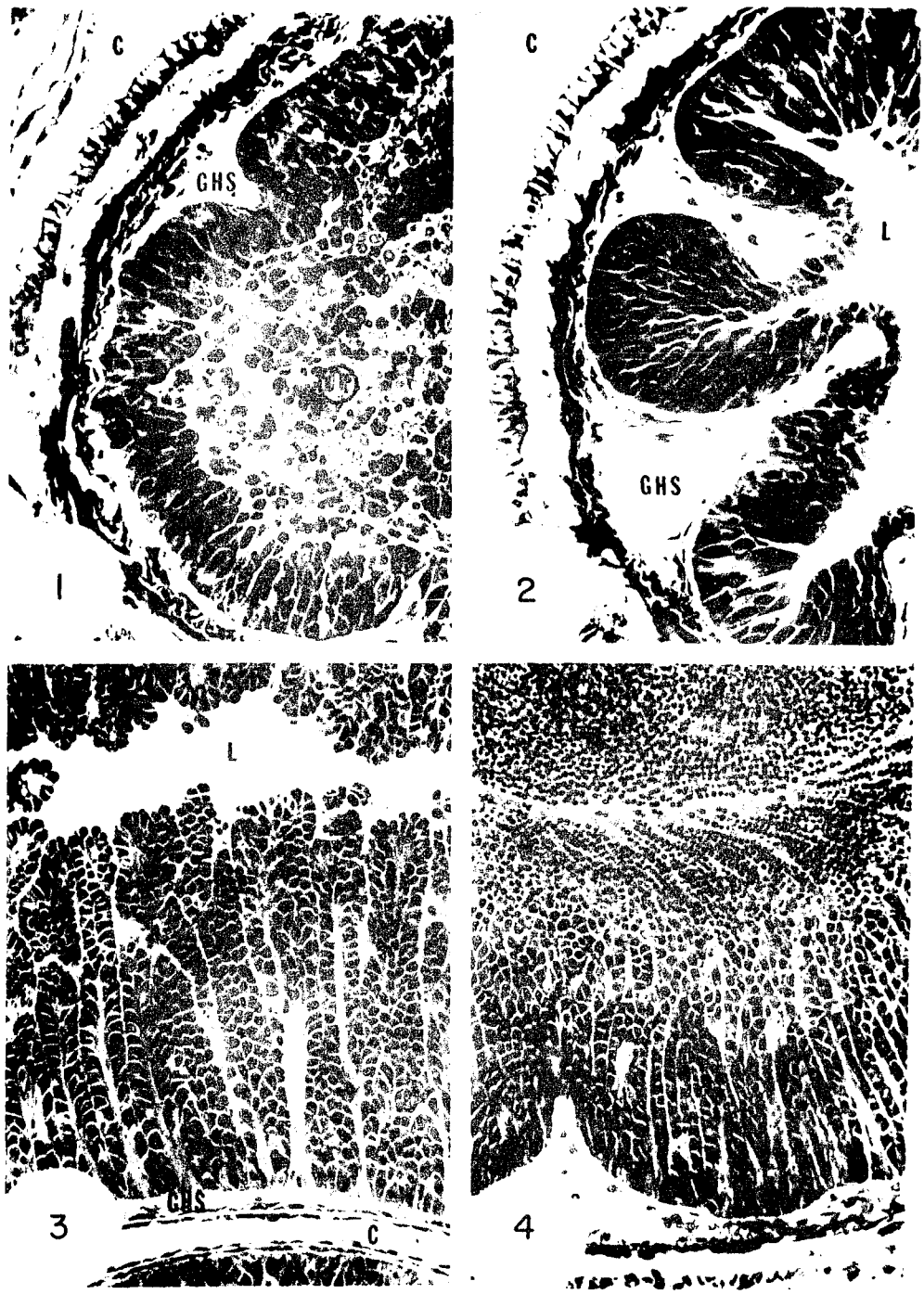

Figure 1. Testis of Asterias vulgaris during the aspermatogenic phase.

Figure 2. Early proliferative phase of spermatogenesis.

Figure 3. Late proliferative phase of spermatogenesis.

Figure 4. Period of overlap of proliferative and differentiative phases.

(Micrographs courtesy of Dr. Charles W. Walker)

CHAPTER I

BIOCHEMICAL CHANGES IN THE COMPOSITION OF THE TESTES DURING SPERMATOGENESIS

Introduction

Asteroid testes are a useful model system for the study of mechanisms regulating spermatogenesis. Because fertilization is external in Asterias vulgaris, the spermatogenic system lacks many of the structural and functional elaborations typical of systems adapted for internal fertilization and continuous production of spermatozoa (Walker and Larochelle 1984). Instead, testes of asteroids are morphologically simple, essentially consisting of a double sac with relatively few tissues (Walker 1974, 1979). Spermatogenesis in Asterias vulgaris occurs in an annual cycle with temporal separation between most major events such as the initiation of proliferation, meiosis, and spermiogenesis. This temporal separation allows study of these processes both in isolation and in combination with other spermatogenic processes. Prior studies have provided information on the environmental (Pearse and Eernisse 1982), hormonal (Schoenmakers et al. 1981b; Takahashi and Kanatani 1981; Voogt et al. 1984), and cytological (Walker 1980; Walker and Larochelle 1984) factors influencing gametogenesis in asteroids. This information will eventually facilitate a detailed description of spermatogenic regulation. Finally, the presence of multiple (ten) testes in a single animal allows reduction of variation among experimental treatments

through randomized block experimental designs, thereby simplifying interpretation of experimental manipulations of the spermatogenic system.

Basic characterization of gross biochemical changes in the testes complements existing cytological and ultrastructural information and is vital in developing a more detailed understanding of spermatogenesis as a whole. Such a study can help to define the nutrients which are necessarily delivered to the germinal epithelium during gamete production and can begin to allow definition of the chemical microenvironment influencing germinal cells during specific stages of the gametogenic cycle.

Cytological and ultrastructural changes in the germinal epithelium during spermatogenesis in Asterias vulgaris have been described in detail by Walker (1980). To summarize briefly, spermatogenesis can be divided into four phases: the aspermatogenic, proliferative, differentiative, and evacuative phases. In Asterias vulgaris, the aspermatogenic phase begins immediately after spawning and in populations from the Gulf of Maine lasts from approximately June to September. During this time, the testes are extremely small (wet masses approximately 0.1-1% of whole animal wet mass). In the early aspermatogenic phase, testes contain spermatogonia, residual spermatozoa, and phagocytic somatic cells. Later in the aspermatogenic phase, as residual spermatozoa are phagocytized, somatic cells in the testicular lumen become "vesicular", synthetic and secretory cells. In October and early November, the proliferative phase begins with an increase in mitotic index of previously quiescent spermatogonia. At this time, the genital haemal sinus fills with presumptive nutrient

material and the spermatogonia and somatic cells begin to organize into spermatogenic columns (Walker 1980). Proliferation continues at the bases of the columns until approximately March, overlapping by several months with the beginning of the differentiative phase. In January, meiotic division and spermiogenesis are initiated contemporaneously at the tips of columns throughout a large portion of the testis. After proliferation ceases, continued meioses and spermiogenesis result in shortening of the spermatogenic columns. Finally, in late April or May, the columns have been degraded entirely, and the testicular lumen is filled with spermatozoa. Stored spermatozoa are normally expelled in late May or early June, when spawning usually occurs, thus returning the gonad to the aspermatogenic phase.

Although structural changes occurring within the germinal epithelium during spermatogenesis are now fairly well understood, relatively few studies have thoroughly examined variations in biochemical constitution of asteroid gonads over the annual reproductive period (Greenfield et al. 1958; Greenfield 1959; Giese 1966; Boolootian 1966; Ferguson 1975a, 1975b; Lowe 1978; Oudejans and Van der Sluis 1979). In general, these previous studies have concentrated primarily on structural and metabolic components of the gonads, such as proteins, lipids, and carbohydrates. An examination of the levels of nucleic acids and a description of the variations in other constituents relative to the nucleic acid level are essential for completely meaningful interpretation (Giese 1967); however, only Greenfield (1959) and van der Plas and Voogt (1982) have assessed changes in biochemical composition during gametogenesis relative to DNA levels. Van der Plas and Voogt (1982) considered only the pyloric

caeca of female Asterias rubens, but their work provides useful insights into the problem of nutrient translocation from the pyloric caeca during vitellogenesis. Although Greenfield (1959) did include estimates of both DNA and RNA concentration, he dealt primarily with the lipid component of the gonad; he did not consider relationships between lipid fractions and nucleic acids, and he did not attempt to correlate his observations with structural changes in the germinal epithelia of his specimens. Prior to the present study, there have been no studies on biochemical composition of asteroid gonads which have adequately related observed biochemical patterns in a variety of biochemical components to cytological changes documented for the germinal epithelium during gametogenesis. Consequently, it is often difficult or impossible to generalize previous findings beyond the specific system studied, even to other populations of the same species (Oudejans et al. 1979; Jangoux and Van Impe 1977).

In this study, major biochemical components of the testes of Asterias vulgaris have been measured at intervals throughout the annual spermatogenic cycle. These measurements are given in terms of: 1. concentration per unit mass, 2. concentration per unit quantity of DNA (designated "level" in this paper), and 3. total content of component present in the testis. Cyclical patterns in these measures are detected and analyzed through periodic regressions. Although regression analysis is powerful, the interpretation of fitted regressions also requires an examination of the data for periods in which the residuals are consistently either positive or negative. Such periods may represent portions of the reproductive cycle during which the regression function does not provide an adequate description of the

actual changes. Generally, these periods can be adequately explained by consideration of structural changes occurring in the gonad at the time. Thus, it is essential to also relate changes in biochemical composition to observed changes in the cytology of the germinal epithelium in the same animals.

Materials and Methods

Animals

Specimens of *Asterias vulgaris* were collected by divers from several populations in the Gulf of Maine. Most specimens were collected at either the Isles of Shoals on the New Hampshire-Maine border or at Nubble Point in York, Maine. The ambient ocean temperature was noted at the time of collection. Animals were maintained in the laboratory at ambient ocean temperatures in Jewel Oceanic 35 aquaria and allowed to feed *ad libitum* on mussels (*Mytilus edulis* and *Modiolus modiolus*). Animals were maintained in the laboratory for no more than three weeks in order to ensure that the animals were still in approximately the same gametogenic stage as the sampled population; because temperature, light cycle, and food availability could not be accurately and consistently matched to environmental conditions, gametogenesis could not be assumed to follow precisely parallel courses over a long period for both laboratory specimens and the field populations.

Several morphometric measurements were taken on each specimen; these measurements were used in morphometric analyses (not considered in detail here). Because the oral surface is generally flatter than the aboral surface, measurements were taken on the oral side to improve precision. Ray length (R) was measured from center of mouth to tip of

ray along the ambulacral groove. Disk radius (r) was measured along an inter-radius from center of mouth to edge of disk. Ray width at the base (B) was measured at the widest point of the ray, generally 0.5 - 1 cm distal to the inter-radial circumference. In order to obtain reasonably consistent wet masses, specimens were drained on paper towels for approximately five minutes (Giese 1967) before being massed on a triple-beam balance.

Animals with a ray length greater than 5.5 cm were massed, narcotized in cold, 8-10% magnesium chloride for approximately five minutes or until the tube feet ceased to respond to mechanical stimulation, and dissected to collect testes and pyloric caeca. Organ indices for both testes and caeca were determined as the ratio of organ mass (both wet and dry) to whole animal wet mass. Gonad indices were used for estimating total content of biochemical components in the testes of a hypothetical 100g animal. This approach was taken to facilitate comparisons of the results presented here with those of previous studies (such as Oudejans and van der Sluis 1979).

Histological procedures

One testis from each animal was fixed in Bouin's fluid, embedded in paraffin, and sectioned on a rotary microtome at 7 µm. Selected sections were stained with Harris's hematoxylin and eosin Y (Thompson 1966). These sections were examined and scored qualitatively for the presence of luminal somatic cells, presence and height of spermatogenic columns, and presence and abundance of spermatids and mature sperm; these observations were used to rank the specimens by degree of progression through the spermatogenic cycle, according to the criteria described by Walker (1980).

Biochemical procedures (described in detail in Appendix B)

The eight or nine testes remaining were pooled and lyophilized to determine dry mass; resulting lyophilized material was stored at -16°C until use for chemical determinations. Proteins and nucleic acids were isolated by a modification of the method of Schmidt and Thannhauser (1945) from aliquots of testes homogenized in sodium phosphate buffer (pH 7.2). RNA was hydrolyzed from the trichloroacetic acid (TCA)-insoluble fraction by incubation in 0.3 N KOH for one hour at 37°C; DNA and protein were precipitated by acidification with concentrated HCl and addition of cold (0-4°C) TCA. DNA was then hydrolyzed from the precipitate by incubation in 0.5 perchloric acid for 15 minutes at 70°C. The remaining precipitate, containing protein, was dissolved in 1 N KOH.

A separate portion of lyophilized testes was used for determination of other components. Lipids were separated from the homogenate by the biphasic chloroform-methanol method of Bligh and Dyer (1959); glycogen, other reducing sugars, and free amino acids were separated essentially according Van Handel (1965) (see Appendix B).

Quantitative estimates of concentration were based on standard spectrophotometric methods. Protein was determined by the method of Lowry (Oyama and Eagle 1956) with a bovine serum albumin standard, DNA by diphenylamine (Burton 1968) with calf thymus DNA standard, RNA by orcinol (Almog and Shirey 1978) with Torula yeast RNA standard, glycogen and other reducing sugars by the anthrone method (Seifter et al. 1950) using a glucose standard, and free amino acids by ninhydrin (Moore 1968) with a glycine standard. Total lipids were estimated gravimetrically. The resulting concentrations were expressed in terms

of unit dry mass and unit quantity of DNA. Total testicular content of each component was then estimated as the product of component concentration and gonad dry mass index, in order to provide an approximation of total content corrected for variation in animal mass. All biochemical assays were performed at least in triplicate.

Statistics

The biochemical data were fitted to the following periodic trigonometric function (Batschelet 1981):

$$y = M + A \cdot \cos\{\omega(t-t_0) + \nu_s \cdot \cos(\omega(t-t_0)) + \nu_p \cdot \sin(\omega(t-t_0))\}$$

where ω, the angular frequency, was set for an annual periodicity and the other parameters were determined by nonlinear regression (BMDPAR: Dixon et al. 1983). Parameters of amplitude (A), skewness (ν_s), and peakedness (ν_p) were selected by forward search with parameters included only if the associated reduction in residual sum of squares was significant at the 0.05 level. Data points were weighted by the inverse of their estimated variances to account for unequal variances (Neter and Wasserman 1974). Variances were estimated for each sample by applying standard formulae for propagation of error (Beers 1957) with the assumption that all spectrophotometer readings for a given component had essentially equal variability. Confidence bands were constructed around the fitted curves by the Working-Hotelling method (Neter and Wasserman 1974), using standard deviations of predicted values as provided by the regression program and fitting a similar trigonometric function through the resulting points. Arcsine transformed organ indices were fitted similarly, using unweighted regression; the resulting regression equations were back-transformed

for plotting. In Figures 6-26, data points are coded by relative weight. The symbols used are summarized in Table 1.

Histological specimens ranked for progress through spermatogenesis were correlated to collection date and biochemical data using nonparametric circular correlation coefficients (Mardia 1975, 1976).

Chemicals

Bovine serum albumen, DNA, RNA, glucose, glycine, diphenylamine, orcinol, anthrone, and ninhydrin reagent were all obtained from Sigma Chemical Company.

Results

Because no differences attributable to year were detected, data for several years were combined in the following analyses.

Correlations

Circular-linear correlation coefficients (Table 2) have been calculated for each of the biochemical components correlated with both date and spermatogenic stage. The actual test statistic is shown together with a statistic normalized to lie in the range [0,1] (Mardia 1975, 1976). Spermatogenic stage correlates strongly with date; the circular rank correlation coefficient of 0.82 shows extremely significant difference from zero ($p<<0.001$). This very good correlation justifies the use of time of year as an approximate and convenient measure of progress through the spermatogenic cycle. Circular-linear correlations of measurements of biochemical component and gonad indices with both stage and date were significant at the 0.05 level at least, except for correlations of RNA concentration with date (parametric correlation) and lipid concentration and percent dry mass

with both date and stage. Percent dry mass of the testis was essentially constant throughout the annual cycle at an average value of approximately 0.18.

Coefficients of fitted regression equations and equations for confidence bands are shown in Table 3.

Organ Indices

Periodic regression of arcsine-transformed organ indices (Fig. 5) provides a good description (R^2 = 0.87) of gonad wet mass index; peak values attained approximately in early April began rapidly declining when spawning occurred (ν_s = - 0.35) and remained extremely low throughout the aspermatogenic phase in the summer. Gonad index increased detectably shortly after the proliferative phase began in October and November.

DNA

DNA concentration (Fig. 6) increased at a nearly constant rate throughout the proliferative and differentiative phases, increasing from a minimal value of approximately 5 to 10 mg/g dry mass during the aspermatogenic phase to a maximum of approximately 50 mg/g in late April and May. In May and June, when spawning normally occurs, DNA concentration decreased rapidly, as indicated by the large, negative parameter of skewness in the fitted function. Total DNA content in the testes (Fig. 7) showed an even more dramatic increase from a minimum value of approximately 0.5 mg in the late aspermatogenic phase to amounts in excess of 60 mg and in several cases over 100 mg shortly before spawning.

RNA

Both concentration (mg/g dry mass) (Fig. 8) and level (mg/mg DNA) (Fig. 9) of RNA increased during the late aspermatogenic phase. The increase was sudden, as indicated by a high, positive parameter of skewness. Both measures remained high or declined slightly during proliferation and began to decline greatly near the end of the differentiative phase as spermiogenesis concluded, and subsequently declined smoothly to the low values seen after spawning in the early aspermatogenic phase. The fitted curve for RNA content (Fig. 10) closely parallels that for DNA content, as would be expected from the near constancy of RNA level during much of the proliferative phase. RNA content began to increase in the late aspermatogenic phase, apparently somewhat sooner than the similar increase in DNA content, and began to decline again in the latter part of the differentiative phase.

Protein

Protein concentration (Fig. 11) began to increase during the late aspermatogenic phase and continued to increase throughout the proliferative phase, including the period of overlap with differentiation. It then remained nearly constant or decreased slightly during spermiogenesis. There was no major decrease in protein concentration obviously associated with spawning, although the concentration decreased gradually during the aspermatogenic phase. Protein level (Fig. 12) further showed an increase in amount of protein relative to DNA in the late aspermatogenic phase and much of the proliferative phase.

Free Amino Acids

The concentration of free amino acids (Fig. 14) began to increase in the late aspermatogenic phase, and continued to increase until March; amino acid concentration then declined smoothly to the minimum values attained in the early aspermatogenic phase. Amino acids expressed as concentration per unit mass of water (Fig. 15) show essentially the same pattern as concentration per unit dry mass. In contrast, free amino acid level (Fig. 16) appears to show a sudden increase during the late aspermatogenic phase, with a relatively rapid decrease in the early proliferative phase; the level remained approximately constant through the differentiative phase, with a slight decrease at spawning.

Simple Reducing Sugars

The concentration of simple sugars (Fig. 18) increased dramatically during the aspermatogenic phase, declined as proliferation began, and retained a fairly constant low value throughout proliferation and differentiation. The level of these compounds relative to DNA (Fig. 19) shows a similar pattern, but the acrophase has been shifted later in the aspermatogenic phase. Again, the level declined in November at the beginning of the proliferative phase. Finally, there is a suggestion of a slight increase in total content (Fig. 20) during September and October.

Glycogen

Glycogen (Fig. 21-23) shows a pattern similar to that described for simple sugars, but with several important differences. Maximum values for both concentration and level of glycogen were attained approximately two months later than the maxima for simple sugars. In

addition, glycogen content shows no clear evidence of the slight increase mentioned with regard to sugars.

Total Lipid

Lipid level (Fig. 25) showed no detectable pattern with time: fitting a weighted periodic regression to the amount of lipid per unit amount of DNA did not produce an estimated amplitude significantly different from zero. Lipid concentration (Fig. 24) also showed relatively little fluctuation over the year; the concentration appears marginally higher near the end of the differentiative phase. Because of the statistical constancy of the lipid:DNA ratio, lipid content (Fig. 26) showed a pattern very similar to that of DNA content.

Discussion

Because spermatogenic stage correlates very well with date, the latter can be used as a convenient, readily quantifiable measure of progression through the spermatogenic cycle. The effectiveness of this measure is further shown by the good agreement between coefficients of correlation for biochemical components based on spermatogenic stage and date (see Table 2). In only one case, that of RNA concentration, do the two correlation coefficients provide conflicting information about the significance of the biochemical measure. In this case, the disagreement can be readily explained by recognizing that correlation with date is a parametric statistic, and therefore potentially sensitive to violations of its underlying assumptions about the distributions of the two variables. RNA concentration deviated sufficiently from a simple sinusoidal function of date so that the parametric statistic was less powerful than the non-parametric circular-linear correlation coefficient based on spermatogenic stage.

This conclusion about the source of the disagreement between the two coefficients is confirmed by the observation that the non-parametric correlation coefficient between RNA concentration and date differs significantly from zero ($p<0.01$).

Previous authors (e. g.: Farmanfarmaian et al. 1958; Giese et al. 1959; Greenfield 1959; Boolootian 1966; Lawrence 1973; Jangoux and Vloebergh 1973; Jangoux and van Impe 1977; Oudejans and van der Sluis 1979; van der Plas and Voogt 1982) have frequently used some form of gonad index as a measure of progress through gametogenesis, as well as a means of correcting for effects of animal size; unfortunately, it is not clear that those authors have questioned either the appropriateness of gonad indices for their purposes or the validity of the underlying assumptions and, hence, of the index itself. Although intuitively appealing and mathematically simple, the use of any gonad index entails theoretical difficulties which must be addressed (see Appendix A for additional details and alternatives). First, a linear variable such as gonad index is not entirely appropriate for description or estimation of a circular variable such as gametogenic stage. Even if the correlation between gonad index and gametogenic stage is very good, so that gonad index can be predicted as a function of gametogenic stage, the inverse relationship will not be an unambiguous function. That is, given only gonad index, one cannot unambiguously determine gametogenic stage; testes in both the early aspermatogenic phase and early proliferative phases, for example, have comparable gonad indices, but are extremely different structurally, biochemically, and physiologically. A second and potentially more serious problem is violation of the assumptions underlying any use of organ indices as a

means of accounting for differences in organ size resulting from differences in animal size. As Gonor (1972) indicated, an organ index is a valid measure of relative organ size if and only if organ index is independent of animal size. Thus, use of an organ index requires that organ size be strictly proportional to animal size, other things being equal. There is no *a priori* reason for this assumption to hold; for *Strongylocentrotus purpuratus*, at least, it does not (Gonor 1972). Most investigators do not seem to have verified this basic assumption for their systems, yet if the assumption is violated, an organ index is no better than a coarse approximation of reality. Nevertheless, the computational ease of the organ index approach may justify its use as a crude approximation for some purposes (*e. g.*, the comparison of total content of some biochemical component in an organ for animals of different sizes at different gametogenic stages), provided that the approximate nature of the resulting pattern is recognized. Gonad indices have been used in this paper for estimating total content of biochemical components in the testis for a hypothetical 100g animal, in order to facilitate comparisons of the findings presented here with those of previous studies. However, testis mass does not appear to be strictly proportional to animal mass in *Asterias vulgaris* (see Appendix A); the patterns for total content of biochemical components must therefore be viewed as approximations, dependent on sampling variability of animal mass over the year.

The regression function used to describe changes in biochemical components may not be most appropriate in all cases. In particular, its relatively smooth shape may not be able to adequately describe sudden changes, such as those which occur in total testicular content

during spawning. Careful examination of residuals can often indicate areas in which problems exist, as well as suggesting the actual pattern. It may be possible to modify the regression function in order to allow representation of discontinuity while retaining periodicity. Alternatively, it may be more appropriate in some cases to consider the spermatogenic cycle to have a discrete end and beginning and to perform analyses with non-periodic regression functions. In any case, if emphasis is placed on cytological events and changes in cellular activity, discontinuity is much less of a problem. Spawning is not associated with major discontinuities in metabolically active cells, those which are involved in proliferation and differentiation.

The increase in DNA concentration seen in Figure 6 during the proliferative and differentiative phase results from production of large numbers of very small cells. Because an ever-increasing portion of cell mass is located in the DNA fraction, the overall concentration is higher. At spawning, cells with the highest per cell concentration of DNA, the sperm, are preferentially released from the gonad. Hence, the overall DNA concentration in the testis decreases upon spawning. However, there are several specimens in June, shortly after spawning, whose DNA concentrations are considerably above the fitted curve. The higher DNA concentrations in these specimens result from residual spermatozoa remaining in the testes after spawning is complete; these sperm will be phagocytized by somatic cells in the spermatogenic epithelium during the early aspermatogenic phase (Walker 1980). As residual spermatozoa are digested by phagocytic somatic cells, the DNA concentration and content of the testes decline to the minimal levels seen in mid-summer.

Total content of DNA increases greatly from summer to the end of spermiogenesis (Fig. 7). Total DNA content during the aspermatogenic phase is approximately 0.1 to 0.6 mg, whereas total DNA content reaches a maximum in April of up to nearly 140 mg. The amount of this increase agrees with the observation (Walker 1980) that each diploid spermatogonium produces approximately 1600 haploid spermatozoa. In addition, Figure 7 suggests that DNA synthesis is essentially complete by the end of March: the fitted acrophase occurs at approximately 20 March. Thus, essentially all cells which will undergo meiosis have by that time completed the pre-meiotic S phase and are committed to meiosis and spermiogenesis, although this conclusion does not imply that all of the spermatocytes have proceeded beyond prophase of meiosis I. Presumably, the DNA content remains high through April and May, until spawning. However, estimation of the standard deviations used to assign weights results in necessarily lower weights assigned to specimens with larger gonad indices. Thus, data points late in the spermatogenic season have lower weights and thus less influence on the fitted curve, allowing the fitted curve to decrease sooner than actual DNA content. In this regard, the two data points in late May illustrate the effect of spawning. Histological observations show that one such specimen had not yet spawned, whereas the other had spawned, although residual sperm were present in the testicular lumen. DNA content of these two specimens shows the expected relationship described above: DNA content declines from the high level observed in all specimens beginning in late March to a value appropriate to the early aspermatogenic phase.

DNA content begins to increase in October and November, at the beginning of the proliferative phase, but the initial rate of increase is relatively slight. The actual rate of increase rises as proliferation continues, so that the period from October to February is reminiscent of exponential growth seen in cultured cells. In fact, cells *in vitro* are a reasonable analogy for the microenvironment of the germinal cells in the proliferative and early differentiative phases. The rapid, possibly exponential, increase in DNA content in this period can be ascribed to two principal causes. First, the initial number of spermatogonia undergoing mitosis is relatively small. As the proliferative phase progresses, the number of mitotically active spermatogonia increases as a result of prior mitoses. Later, primary spermatocytes begin to prepare for meiosis by additional DNA synthesis, resulting in further increase in DNA content, particularly during the period of overlap between the proliferative and differentiative phases.

When spermatogonia in the late aspermatogenic phase begin preparations for proliferation, they undergo a variety of metabolic processes, *e. g.*, cellular growth, synthesis of enzymes for cell division, and synthesis of receptor molecules for reception of triggering signals. An early requirement for these preparations is the transcription of the genetic program onto mRNA and manufacture of cellular machinery, such as rRNA, necessary for synthetic activity. RNA concentration thus constitutes a marker for overall metabolic activity. Consequently, the concentration and level of RNA increases during the late aspermatogenic phase, as both spermatogonia and testicular somatic cells begin preparations for proliferation. The fairly rapid increase observed in Figures 8 and 9 is consistent with

the existence of one or more triggering events which activate spermatogenesis; i.e., many cells contemporaneously begin preparations for proliferation over a fairly short period of time. The RNA level would then remain high throughout the proliferative phase and much of the differentiative phase as the spermatocytes and spermatids remain metabolically active. As spermiogenesis proceeds, however, nuclear condensation occurs. In such cells, no new RNA is produced, and existing RNA is gradually degraded. Thus, late in the differentiative phase, an ever-increasing portion of the testis ceases to be synthetically active, and concentration, level, and content of RNA in the testis as a whole begin to decline. Unlike the initiation of proliferation, the completion of spermiogenesis in the entire testis occurs gradually. Hence, average RNA level declines slowly but steadily from approximately March to May, merging smoothly with the RNA level seen in the (relatively) metabolically inactive early aspermatogenic phase.

Because protein synthesis, and therefore DNA synthesis, is dependent on RNA, one would expect RNA synthesis to begin somewhat sooner than either DNA or protein synthesis, as is suggested by the content of those components in the entire testis (Figs. 7, 10, and 13). The increase in protein level in the late aspermatogenic phase probably represents preparation of spermatogonia for mitosis by growth in G1. The subsequent decline seen in the differentiative phase probably has two major contributing factors. By that point in the spermatogenic cycle, a large number of primary spermatocytes have passed through pre-meiotic S phase, thereby doubling the amount of DNA present in that cell without a concommitant doubling of protein. In addition,

spermiogenesis produces a large number of cells in which nuclear material predominates, and the cytoplasmic proteins are relatively less important. Although exhaustion of presumptive nutrient material in the genital haemal sinus by the end of proliferation may also contribute to the observed decline in protein concentration, I suspect that any such contribution is relatively minor. In addition, protein components of that nutrient material are likely to be utilized as protein, so that the amount of protein present in the testes would not be decreased as the nutrient material is exhausted.

Free amino acids have been widely thought to serve a predominantly osmoregulatory role in asteroids, although Ferguson (1975a) provides evidence for other functions, especially protein and purine synthesis and nitrogen storage. If the free amino acids were serving primarily for osmoregulation, one would expect the concentration of amino acids per unit mass of water to be essentially constant. However, examination of that concentration (shown in Figure 15) suggests that although a constant osmoregulatory concentration of free amino acids may be contributory to the observed quantity, it does not adequately explain the pattern of changes seen. Two additional processes help explain the observed increase from the minimum concentration and level attained shortly after spawning. First, amino acids, perhaps bound temporarily into peptides, must be mobilized from nutrient storage sites to support protein and nucleotide synthesis. This mobilization of nutrients can account for the observed increase in amino acid concentration and level during the proliferative phase. Second, the breakdown of protein components in residual spermatozoa during the aspermatogenic phase may account for the highly variable free amino

acid increase observed during the aspermatogenic phase, especially in July and early August.

As with free amino acids, the increase in simple reducing carbohydrates seen in the aspermatogenic phase (Figs. 18 and 19) may represent, at least in part, the degradation of residual spermatozoa by phagocytic somatic cells. Some of this carbohydrate appears to be subsequently converted to glycogen or a glycogen-like substance, possibly constituting temporary intra-gonadal storage for nutritive and structural material. The relatively slight increase in simple carbohydrates observed in September and October may represent the arrival of periodic-acid Schiff positive material in the genital haemal sinus. This material, presumed to be nutritive in character (Walker 1980), is then either directly utilized or converted into glycogen. If the latter occurs, the increase in glycogen is not apparent, as it would disappear into the larger peak represented by increasing gonad mass with a fairly constant glycogen concentration. Although the increase in simple carbohydrates which I am here ascribing to the arrival of nutritive material in the genital haemal sinus is quite small, this should not be taken to imply a minor role for that nutritive material; it is by no means certain that all of the nutritive material present in the genital haemal sinus throughout the proliferative phase arrives simultaneously (see Beijnink et al. 1984). Thus, additional material could be arriving at a continuous rate and simply not be detectable with the techniques employed here.

Although the weighted periodic regression of lipid level against time gives a value for amplitude which is not significantly different from zero, the large number of points in Figure 25 with relatively low

weights lying above the fitted regression line in the late aspermatogenic phase and early proliferative phase require consideration. Although they do not provide satisfactory statistical evidence, these points suggest that lipid level may be higher during the aspermatogenic phase, with a subsequent decrease during the proliferative phase to the low and constant level seen from January to May. If this pattern is real, it may be explained by several phenomena. The testicular cell population during the late aspermatogenic phase consists predominantly of diploid somatic cells, with a ratio of approximately 200 somatic cells per germinal cell (Walker and Larochelle 1984). These somatic cells take the form of "vesiculated" cells involved in synthetic activities and contain extensive quantities of smooth endoplasmic reticulum, vacuoles, and well-developed Golgi apparati (Walker 1980, Schoenmakers et al. 1977). Consequently, one might expect a somewhat elevated lipid level due simply to the large amount of internal membrane present. As proliferation proceeds, an increasing number of germinal cells pass through pre-meiotic S phase. The doubling of DNA in those cells would act to reduce the lipid:DNA ratio, even if the high rate of protein and membrane synthesis in actively proliferating cells keeps the amount of lipid per cell fairly high. Finally, during the differentiative phase, a large number of very small haploid cells are produced. Although the amount of DNA per cell decreases as a result of meiosis, the amount of cytoplasm, nuclear membrane, and plasmalemma is also reduced during spermiogenesis. Thus, there would also be a decrease in lipid per cell, offsetting the decrease in DNA.

The patterns described here for principal biochemical components of the testes are generally compatible with those reported for other species of asteroids. The values and overall pattern of changes shown for nucleic acids and total lipids agrees well with those observed for Pisaster ochraceus (Greenfield 1959). Total carbohydrate and free amino acid components are very similar to those of Echinaster (Ferguson 1975a, 1975b), although annual variation in free amino acids in A. vulgaris appears to be less than that in Echinaster. Comparison of the data in this paper with those for female Asterias rubens (Oudejans and van der Sluis 1979) reinforces the expected differences in gross biochemical composition between testes and ovaries. Although the concentration of total lipid in the pre-vitellogenic ovary is comparable to that in the pre-proliferative testis, the testis shows no increase comparable to that seen in the ovary during vitellogenesis. In addition, protein concentration follows a similar pattern in the gonads of both males and females, but the annual variation reported for ovaries is greater than that shown here in the testis. This difference is probably attributable to the deposition of proteins in yolk.

In conclusion, changes observed over the spermatogenic cycle for major biochemical components show clear and illuminating correlations to structural changes in the germinal epithelium. The appropriate use of regression analysis greatly aids the quantitative interpretation of these patterns, but full appreciation of the observed biochemical patterns requires examination of cytological and histological changes in the testes of the same animals. Just as examination of the histological state of the germinal epithelium elucidates the contemporaneous changes in biochemical composition, the biochemical

data enhance our understanding of events at the cellular level leading to observed histological changes. Combining these two distinct approaches thus begins to unify our understanding of the biochemical and cytological events in spermatogenesis.

Table 1. Symbol codes. Figures 6-26 all show weighted regressions with 95% confidence bands; parenthesized values are given for extreme data points (probable outliers). All data are coded on a logarithmic scale by assigned weights relative to maximum weight for that graph, as shown below.

Symbol	Range of Weights
■	$1 \geq$ Relative weight > 0.1
✖	$0.1 \geq$ Relative weight > 0.01
▲	$0.01 \geq$ Relative weight > 0.001
▣	$10^{-3} \geq$ Relative weight $> 10^{-4}$
◍	$10^{-4} \geq$ Relative weight

Table 2. Circular-linear correlations of biochemical components with date and spermatogenic stage. Levels of significance: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; N.S.: not significant at the 0.05 level.

		DATE			STAGE		
		R^2	nR^2		D	U	
DNA	concentration	0.673	28.9	***	0.337	11.6	**
	content	0.664	28.6	***	0.584	20.2	***
RNA	concentration	0.103	4.4	N.S.	0.450	15.5	***
	level	0.410	17.6	***	0.470	16.2	***
	content	0.752	32.3	***	0.571	19.7	***
Protein	concentration	0.273	11.7	**	0.335	11.6	**
	level	0.319	13.7	***	0.449	15.5	***
	content	0.722	31.1	***	0.601	20.7	***
Sugars	concentration	0.294	12.1	**	0.380	12.4	**
	level	0.266	10.7	**	0.492	16.0	***
	content	0.700	28.7	***	0.690	22.4	***
Glycogen	concentration	0.298	12.2	**	0.614	20.0	***
	level	0.313	12.5	**	0.475	15.5	***
	content	0.676	27.7	***	0.656	21.4	***
Amino acids	concentration	0.227	9.3	**	0.370	12.0	**
	aqueous conc.	0.177	7.3	*	0.254	8.3	*
	level	0.412	16.5	***	0.440	14.3	***
	content	0.745	30.5	***	0.653	21.3	***
Lipid	concentration	0.023	1.0	N.S.	0.148	4.8	N.S.
	level	0.358	14.3	***	0.360	11.7	**
	content	0.669	27.4	***	0.675	22.0	***
Gonad wet mass index		0.731	32.1	***	0.656	22.6	***
Gonad dry mass index		0.722	31.8	***	0.668	23.0	***
Percent dry mass		0.053	2.3	N.S.	0.094	3.3	N.S.

Table 3. Periodic regression equations. For each component, the fitted periodic regression equation is given, along with equations for lower and upper limits of the 95% confidence band, the coefficient of determination (R^2) for the fitted curve, and true values for mean and acrophase after accounting for the effects of skewness and peakedness.

Component	Measure	Mesor (M)	Amplitude (A)	Acrophase (t_0)	Skewness (ν_s)	Peakedness (ν_p)	R^2	Mean Level	True Acrophase
Testes	wet mass index	0.1845	0.1534	76.7403	-0.351564	0.509901	0.873	0.1845	96.0458
	lower limit	0.1325	0.1474	73.1658	-0.436624	0.495032			
	upper limit	0.2355	0.1602	79.9790	-0.273847	0.507082			
DNA	concentration	27.0023	24.1173	68.4229	-0.606037	0	0.843	27.0023	98.8951
	lower limit	18.1586	23.7390	65.3871	-0.507339	-0.060508			
	upper limit	35.8506	24.6263	71.3319	-0.688106	0.060345			
	content	25.8026	26.2898	73.438	-0.300147	1.047198	0.599	25.8026	90.1569
	lower limit	12.0293	13.9785	64.0206	-0.529458	1.047198			
	upper limit	39.1915	38.8624	76.132	-0.247327	1.03721			
RNA	concentration	25.7261	13.3203	311.598	1.31748	0	0.547	25.7261	261.6516
	lower limit	17.0844	11.4267	318.876	1.42794	-0.371204			
	upper limit	34.3785	15.3030	306.162	1.18735	0.254551			
	level	1.19166	1.0383	293.595	0.84936	0.583271	0.499	0.9273	265.5883
	lower limit	0.18400	0.4330	343.117	1.15847	-0.347662			
	upper limit	2.1532	1.8276	283.77	0.72185	0.675226			
	content	22.5986	22.3293	75.9631	-0.287454	1.047198	0.667	22.5986	92.0273
	lower limit	11.8803	12.7396	70.7478	-0.50000	1.047198			
	upper limit	32.8037	32.0120	77.0106	-0.240922	0.998597			
Protein	concentration	323.991	52.8891	61.5368	0	0	0.485	323.9911	61.5368
	lower limit	291.782	58.0487	67.2142	0.063457	0.028561			
	upper limit	355.530	48.4963	54.8186	-0.054748	-0.062160			
	level	13.4375	8.668	289.95	0.767371	0.574010	0.558	11.2246	264.082
	lower limit	7.1554	4.5659	316.014	0.990165	0.163669			
	upper limit	19.6244	13.3729	281.386	0.659836	0.66954			
	content	266.301	258.228	76.7037	0	1.047198	0.652	148.7992	76.7037
	lower limit	155.284	158.472	81.0390	0.248076	1.047198			
	upper limit	369.756	356.718	76.4673	-0.032543	0.986661			
Amino acid	concentration	100.558	39.6647	43.8387	-0.728473	0	0.815	100.558	78.7444
	lower limit	83.0536	42.5029	44.4970	-0.515621	-0.075516			
	upper limit	117.275	37.8678	42.7807	-0.939451	0.046709			

	fluid conc.	23.3575	9.2593	46.5231	-1.13105	0.555345	0.781	21.2640	81.9292
	lower limit	19.1975	9.5267	51.1741	-0.888318	0.612285			
	upper limit	27.3577	9.1446	41.5869	-1.3588	0.43283			
	level	4.53643	3.95732	299.872	1.01507	0.445118	0.742	3.7842	265.4234
	lower limit	0.829345	1.65881	47.162	-0.189543	-0.226227			
	upper limit	9.2023	8.1169	287.181	0.782161	0.759582			
	content	72.2925	70.6913	82.1160	0	1.047198	0.584	40.1258	82.116
	lower limit	36.4245	39.3005	82.6557	0.174523	1.047198			
	upper limit	107.212	102.046	82.1145	-0.058597	1.01857			
Simple sugars concent.		6.2709	3.6267	231.865	0.728636	1.047198	0.666	4.7330	212.1738
	lower limit	3.8915	2.1051	230.672	1.2697	1.047198			
	upper limit	8.7625	5.4286	231.730	0.451752	1.047198			
	level	0.2538	0.2113	261.858	0.589982	0.878656	0.414	0.1734	244.316
	lower limit	-0.0348	-0.0953	266.896	-0.469102	1.047198			
	upper limit	0.5454	0.4839	262.082	0.372572	0.957668			
	content	3.0864	3.1012	102.891	0.502065	1.047198	0.795	1.7214	88.9829
	lower limit	1.9871	2.2961	104.106	0.516342	1.01268			
	upper limit	4.1611	3.9154	102.782	0.514019	1.047198			
Glycogen	concentration	6.6056	4.4540	275.447	0	0	0.515	6.6056	275.447
	lower limit	4.3723	2.9048	279.097	-0.017560	-0.042264			
	upper limit	8.8418	6.0132	273.692	0.004608	0.021339			
	level	0.1321	0.0885	291.218	0	0	0.328	0.1321	291.218
	lower limit	0.0561	0.0336	331.827	-0.089610	0.166244			
	upper limit	0.2127	0.1532	283.019	0.035229	0.022941			
	content	2.8561	2.5125	76.7526	0	0.874533	0.750	1.8592	76.7526
	lower limit	1.9009	1.8162	79.1461	0.146297	1.047198			
	upper limit	3.7977	3.236	76.1461	-0.055261	0.772531			
Lipid	concentration	101.45	21.0289	113.356	0	0	0.195	101.45	113.356
	lower limit	71.9706	40.3797	96.4280	-0.038133	-0.054133			
	upper limit	128.755	12.2309	188.170	-0.121822	-0.176714			
	level	2.2285	0.3307	78.8312	0	0	0.044	2.2285	78.8312
	lower limit	1.0654	1.2173	81.3375	-0.036728	-0.114672			
	upper limit	3.3873	-0.5612	84.583	-0.107458	-0.233585			
	content	85.6374	85.6934	86.7529	0	1.047198	0.689	46.6442	86.7529
	lower limit	48.2259	54.8574	84.6132	0.142448	1.047198			
	upper limit	122.892	117.108	87.4573	-0.081334	1.03721			

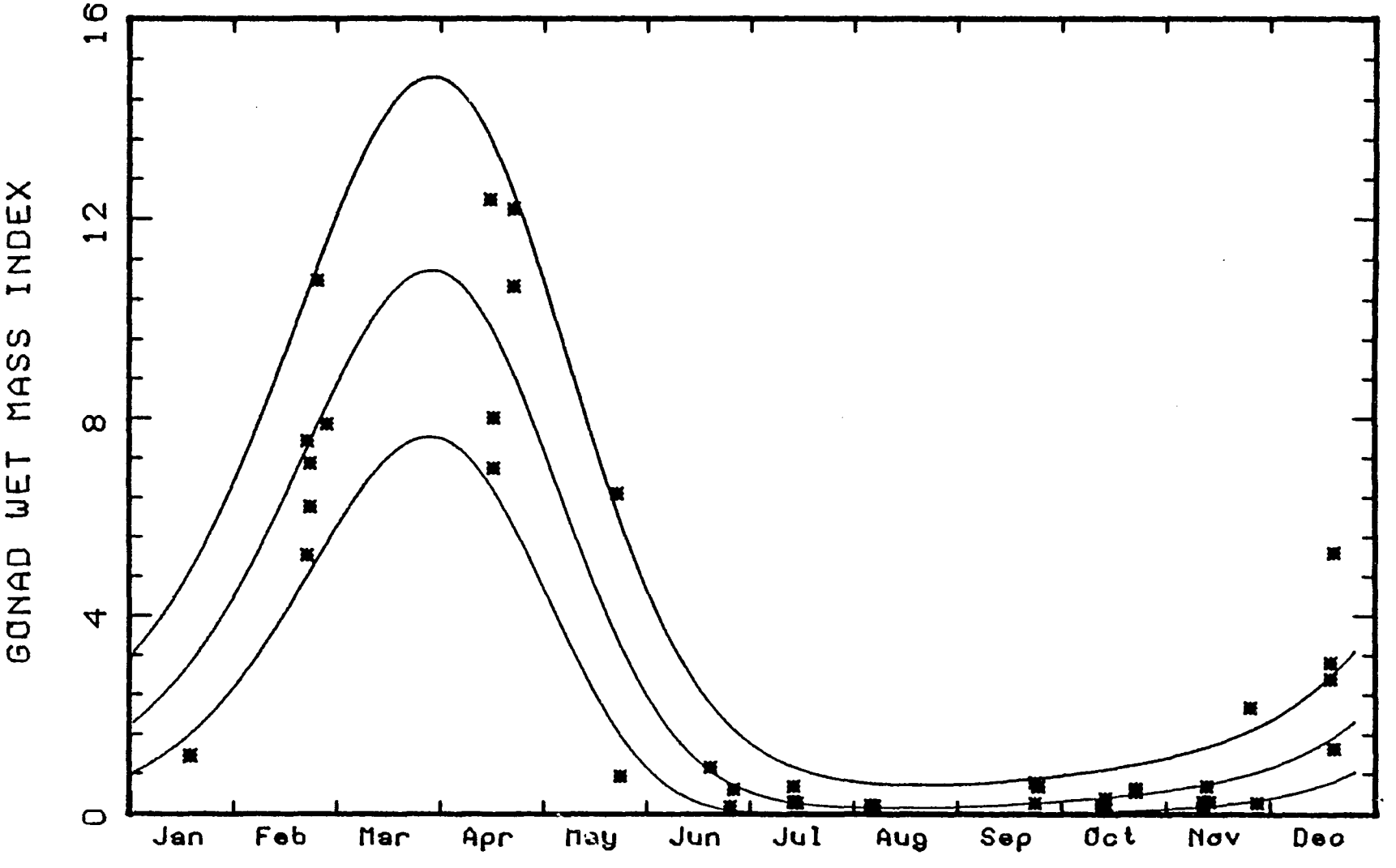

Figure 5. Changes in gonad index during the spermatogenic cycle. Periodic regression with 95% confidence band.

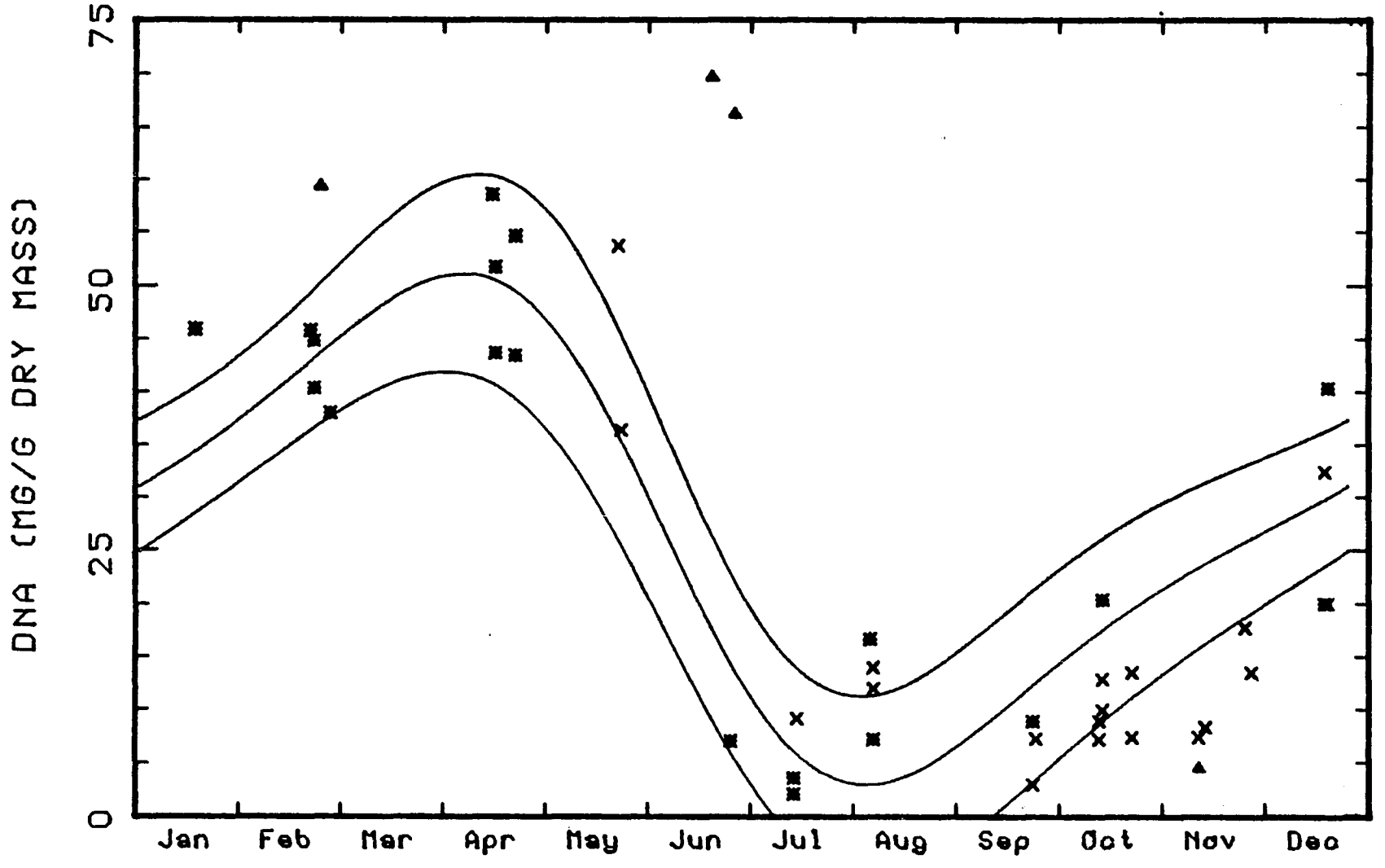

Figure 6. Annual changes in testicular DNA concentration.

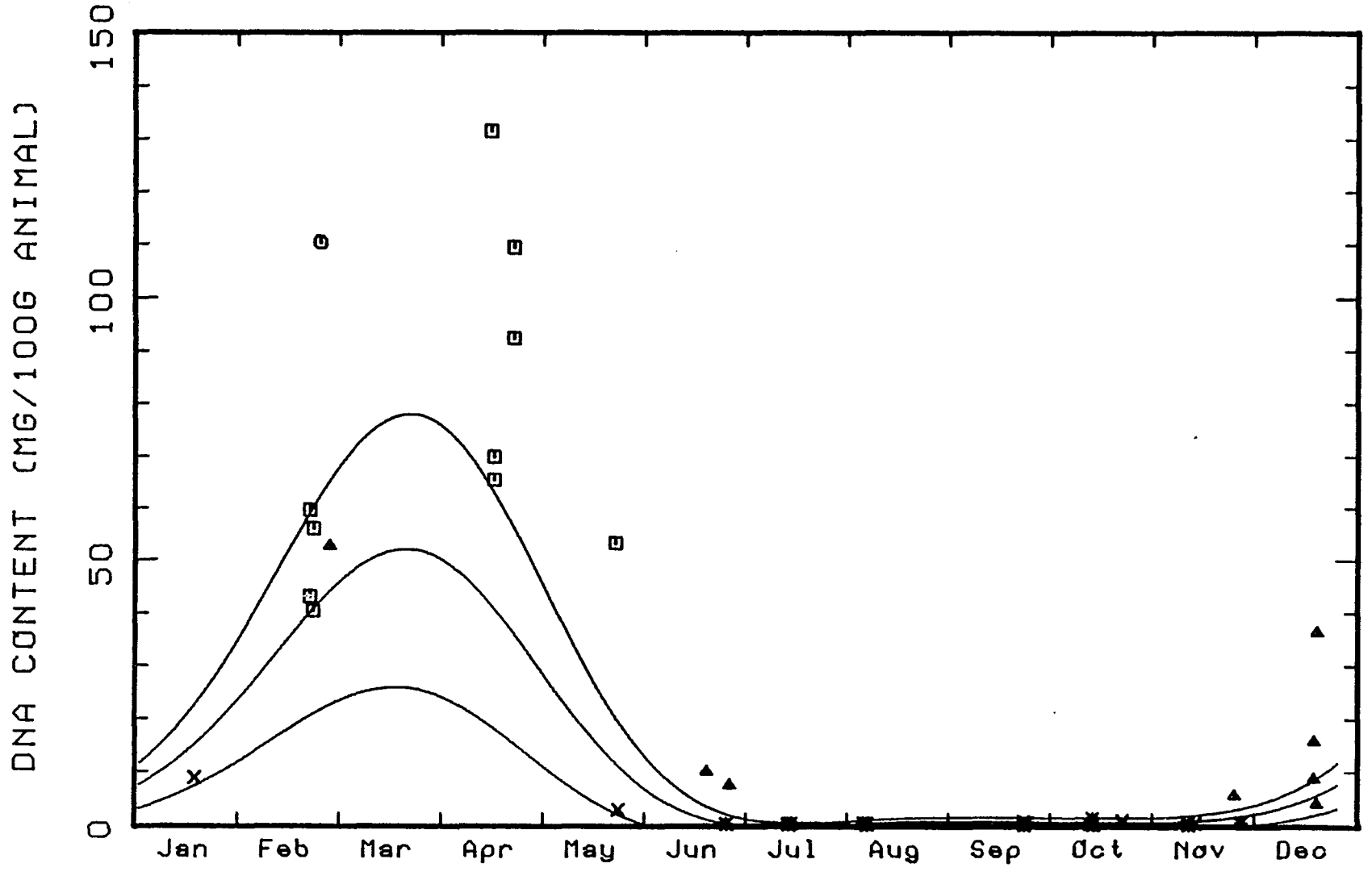

Figure 7. Annual changes in DNA content. Calculated for the testes of a 100g animal as product of DNA concentration and gonad dry mass index.

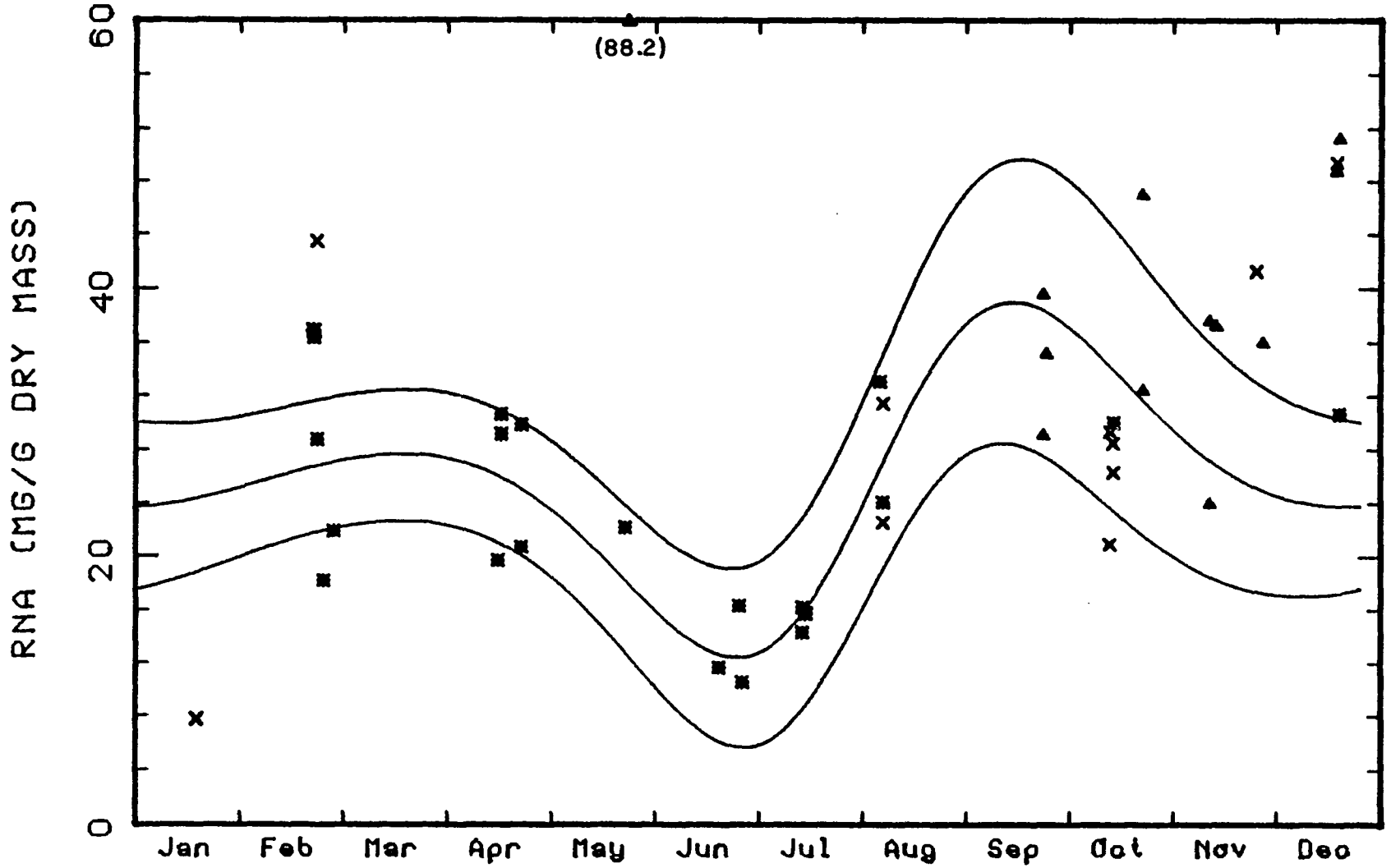

Figure 8. Annual changes in testicular RNA concentration.

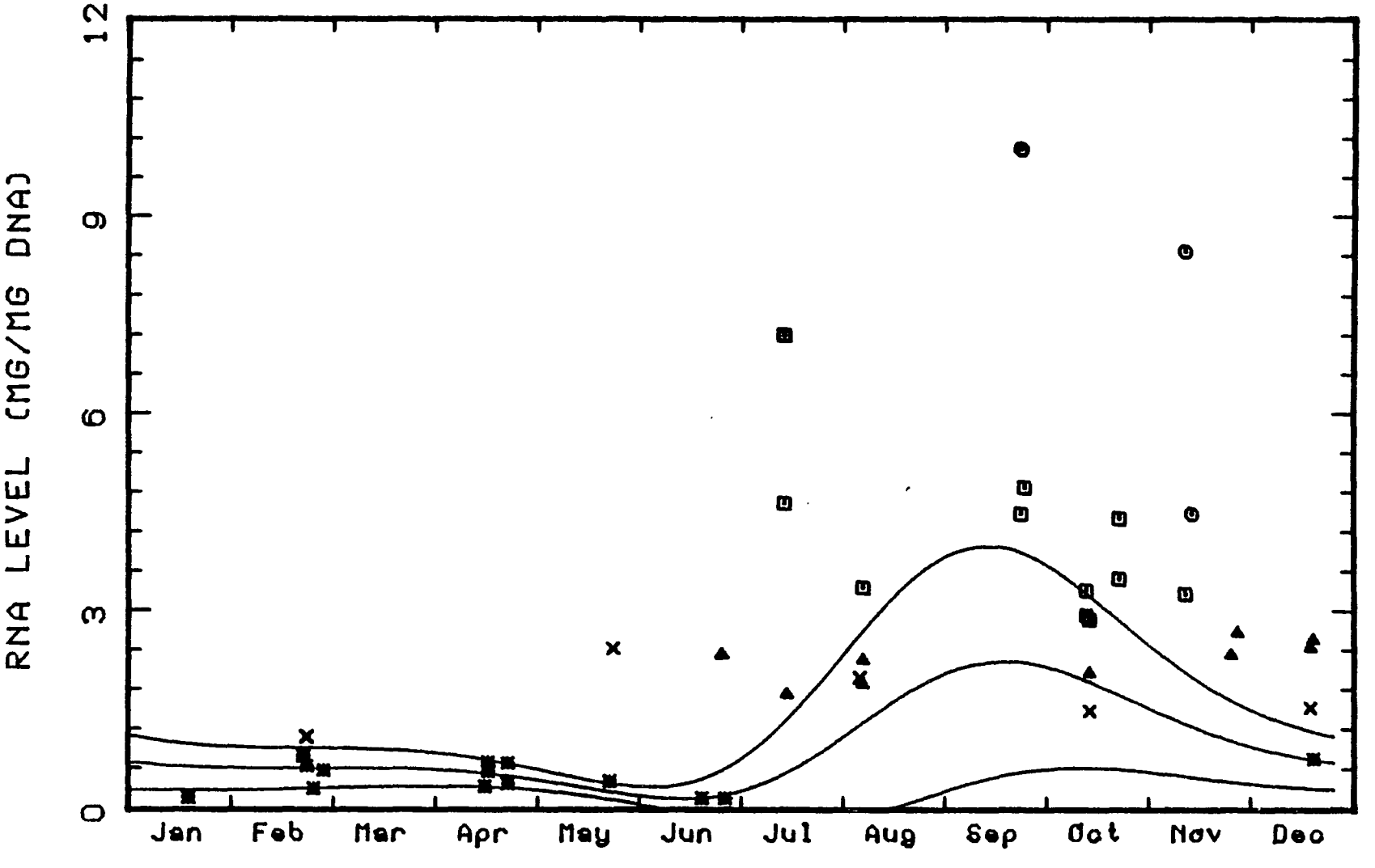

Figure 9. Annual changes in testicular RNA level.

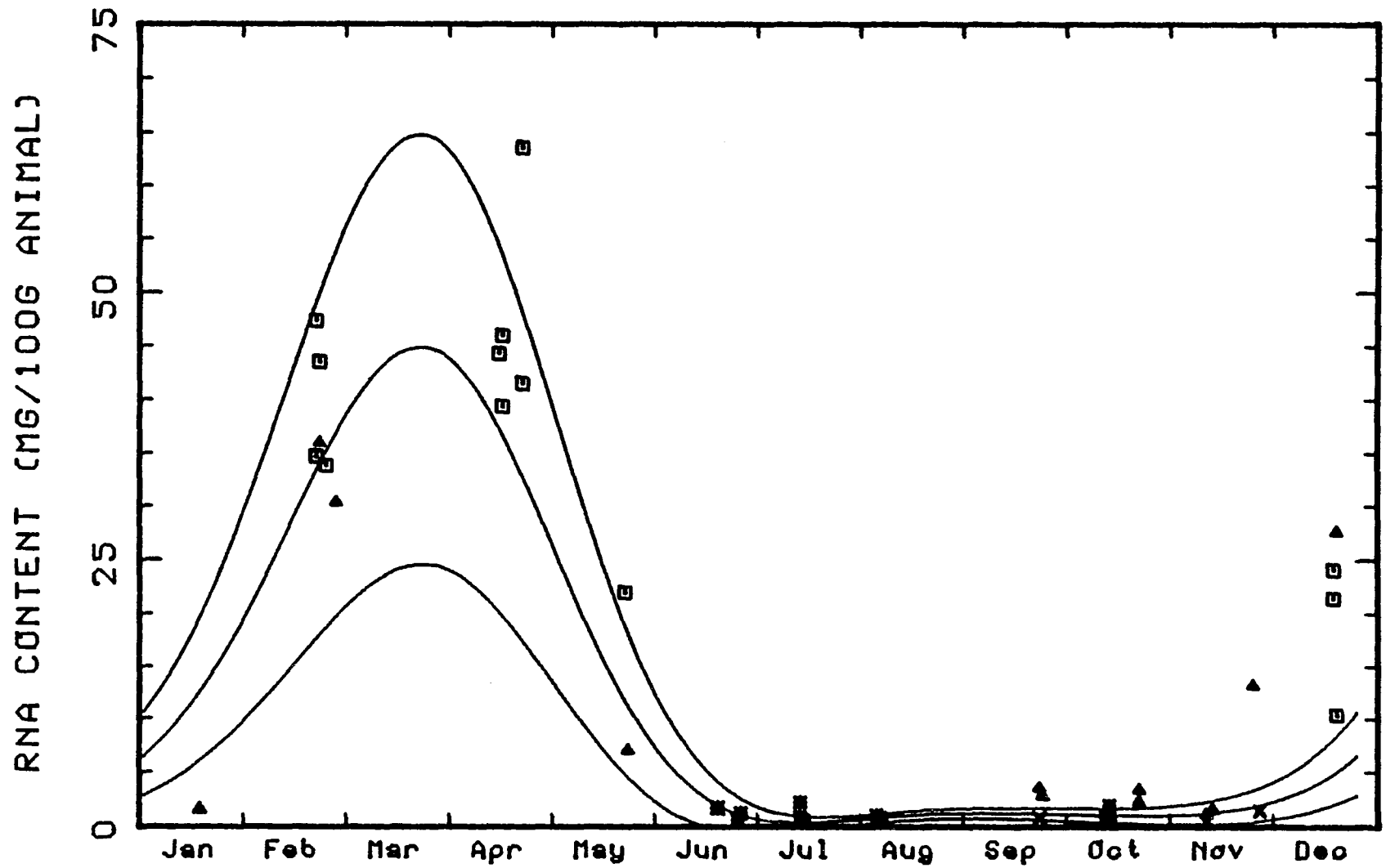

Figure 10. Annual changes in RNA content in the testes.

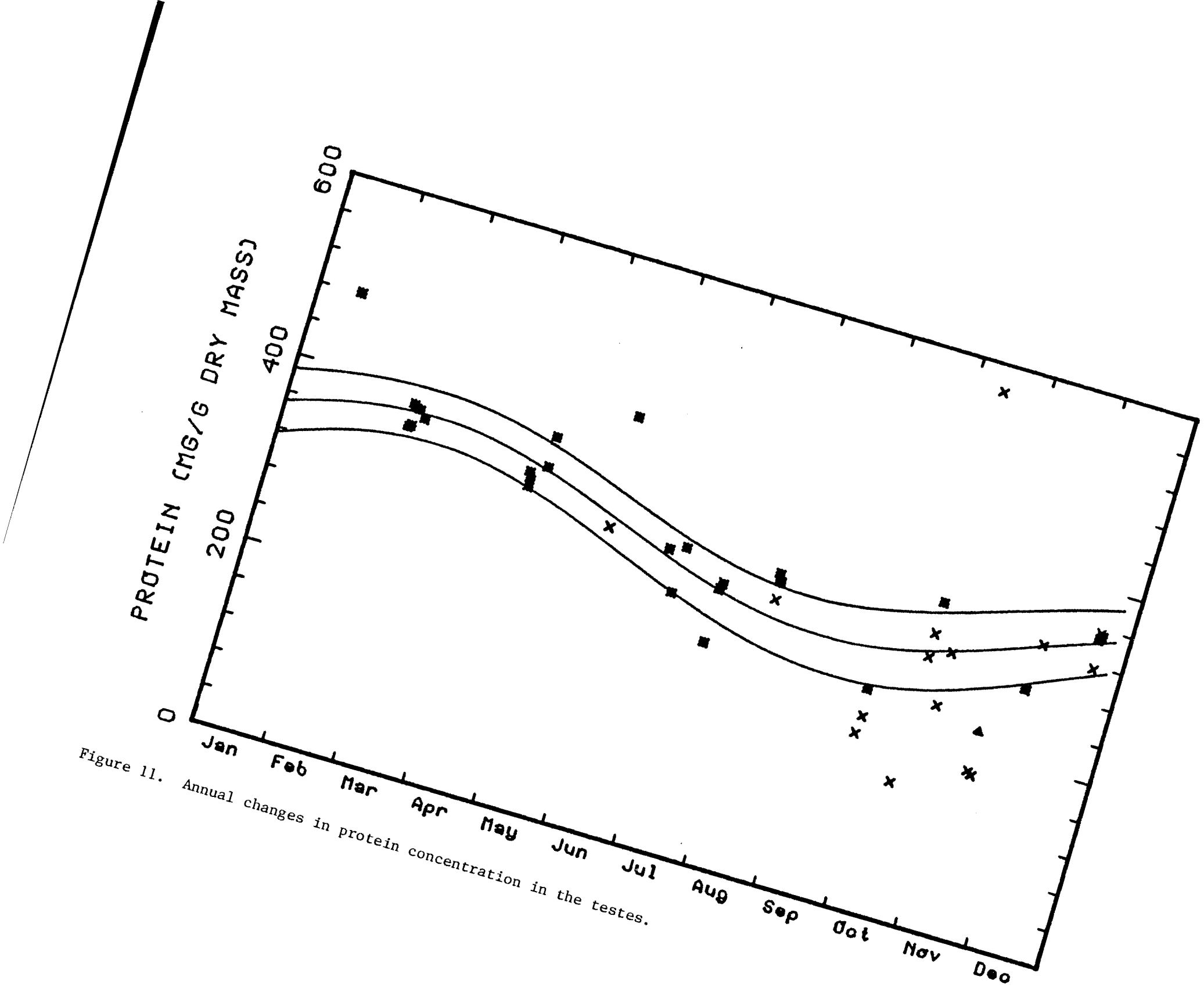

Figure 11. Annual changes in protein concentration in the testes.

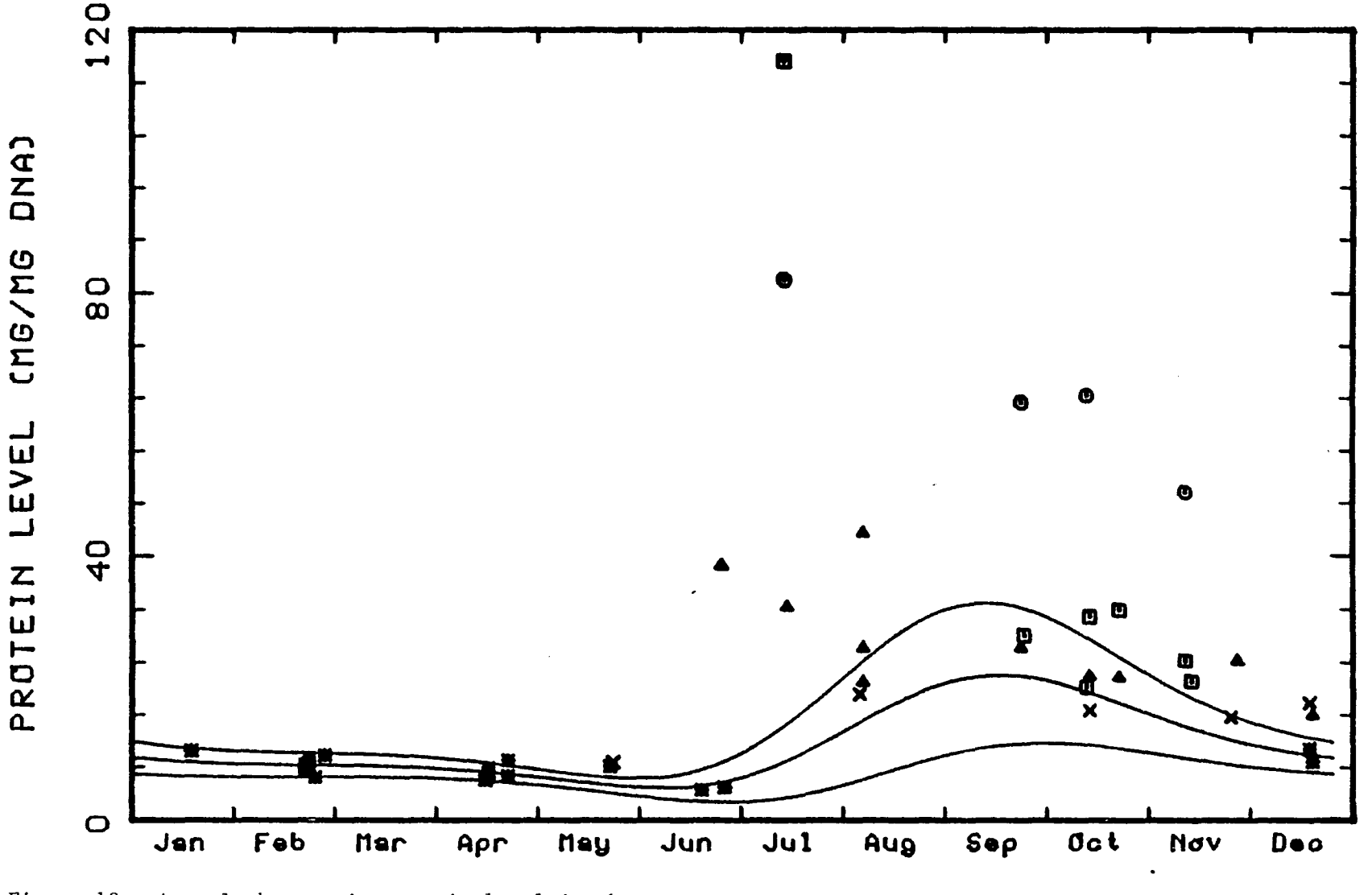

Figure 12. Annual changes in protein level in the testes.

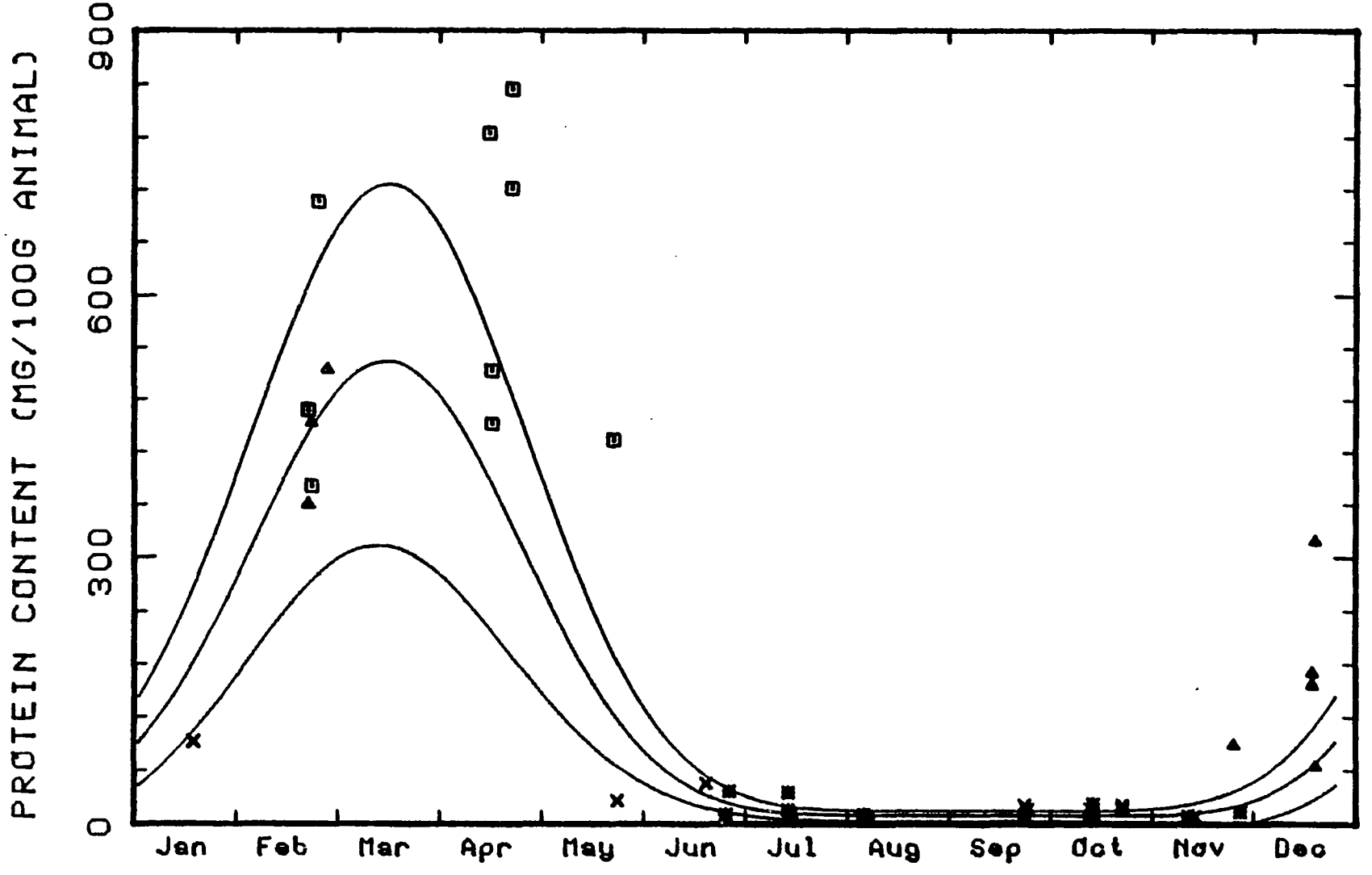

Figure 13. Annual changes in protein content in the testes.

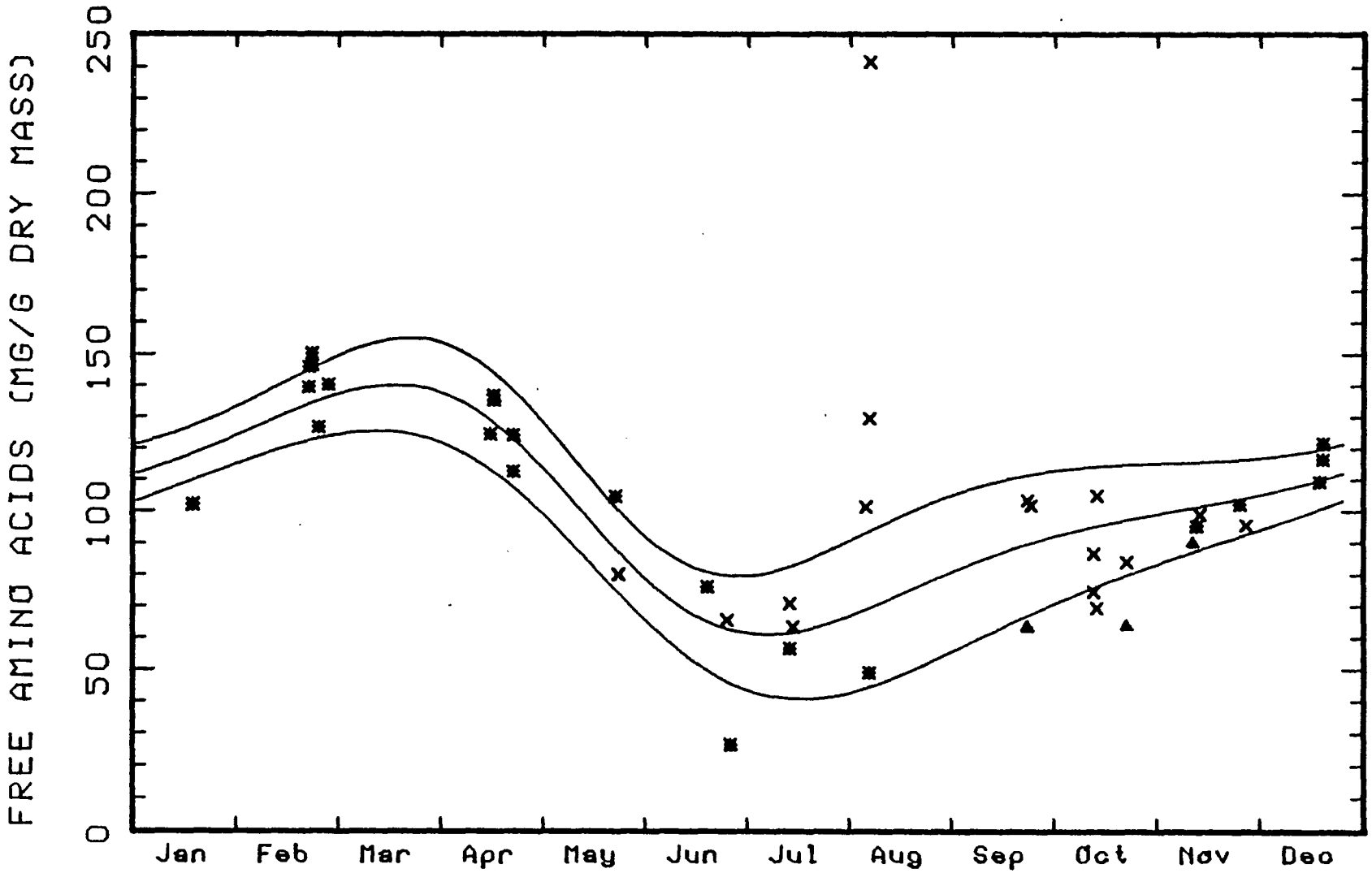

Figure 14. Annual changes in free amino acids in the testes, relative to dry mass.

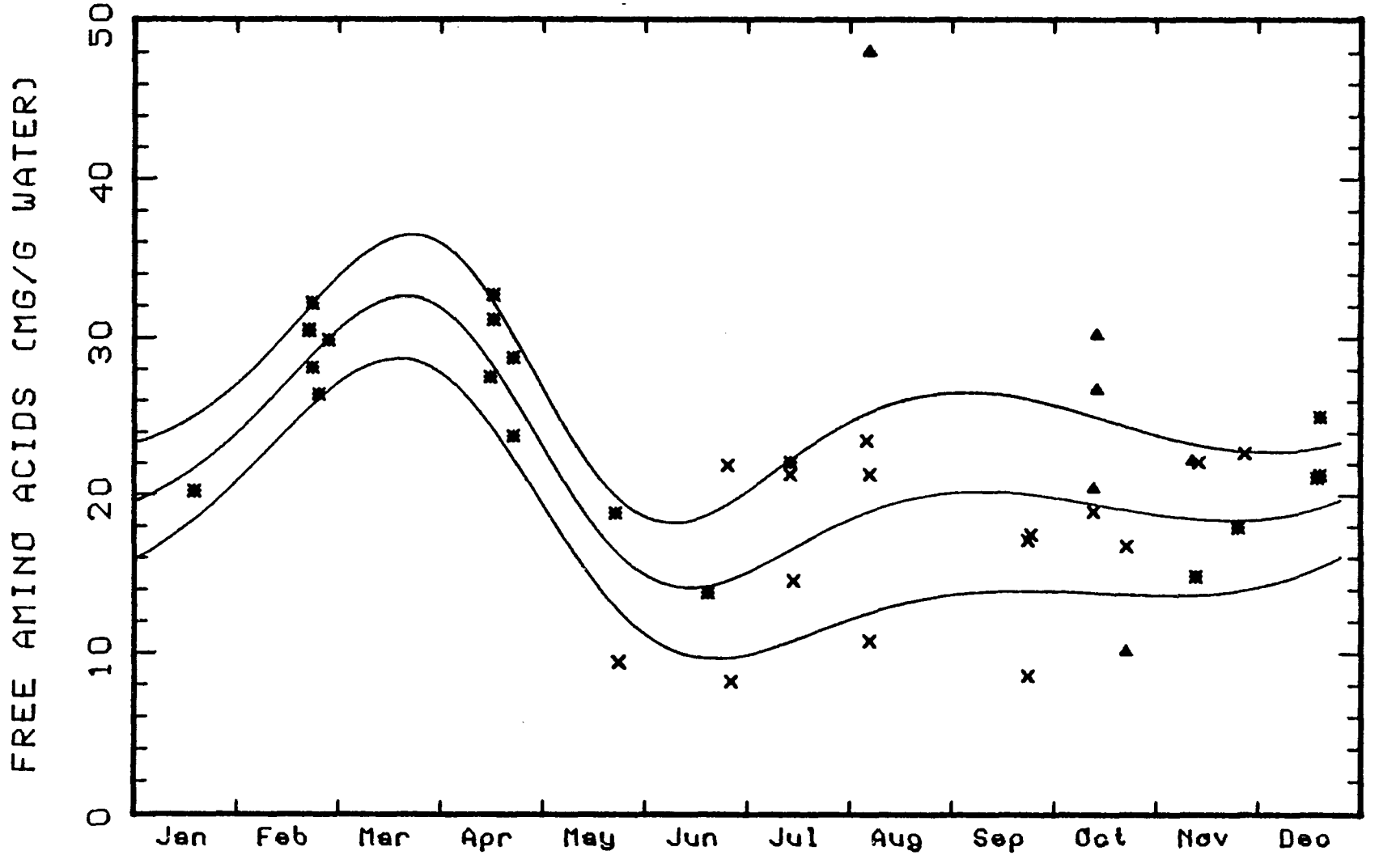

Figure 15. Annual changes in free amino acids in the testes, relative to water content.

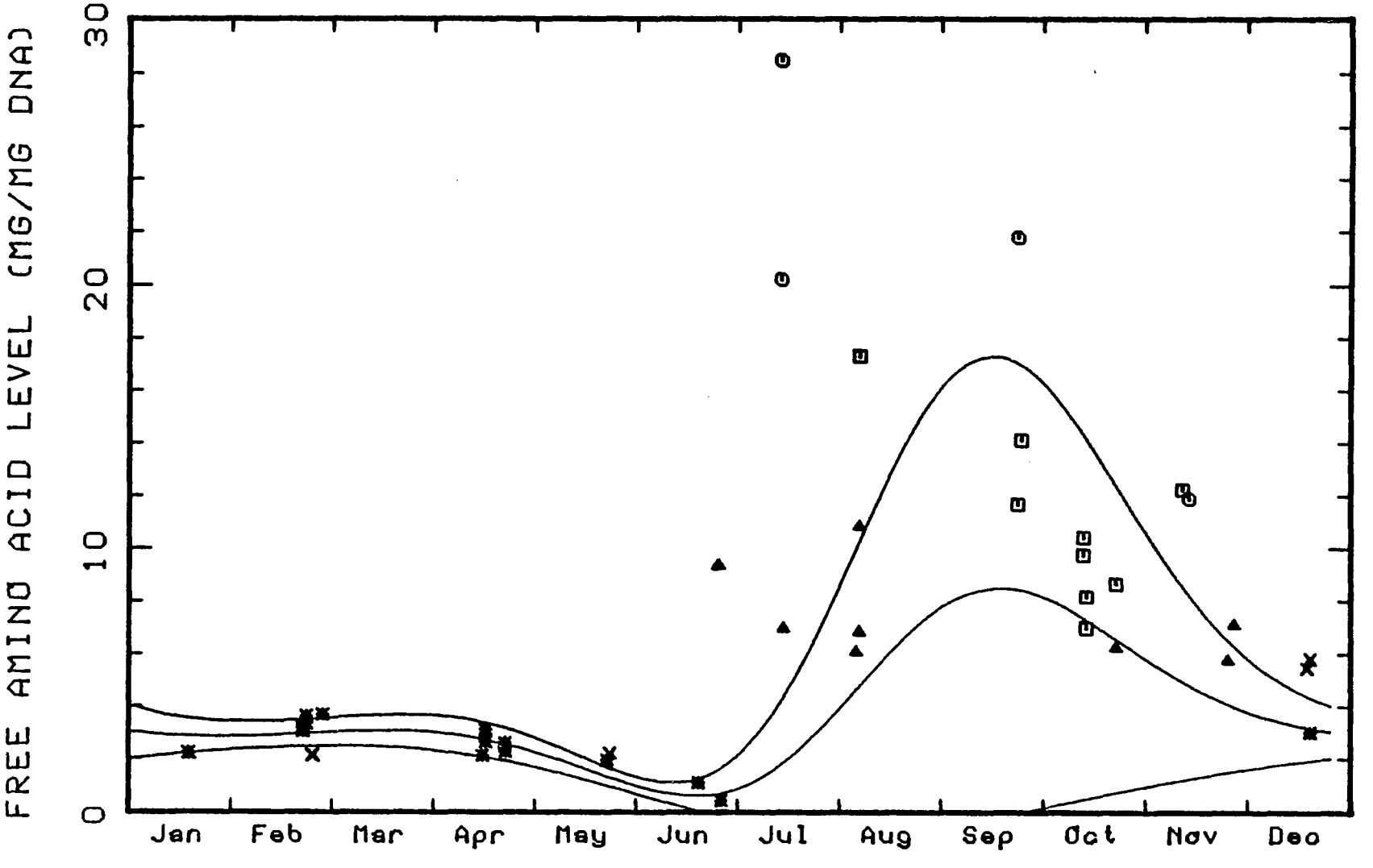

Figure 16. Annual changes in free amino acids in the testes, relative to DNA.

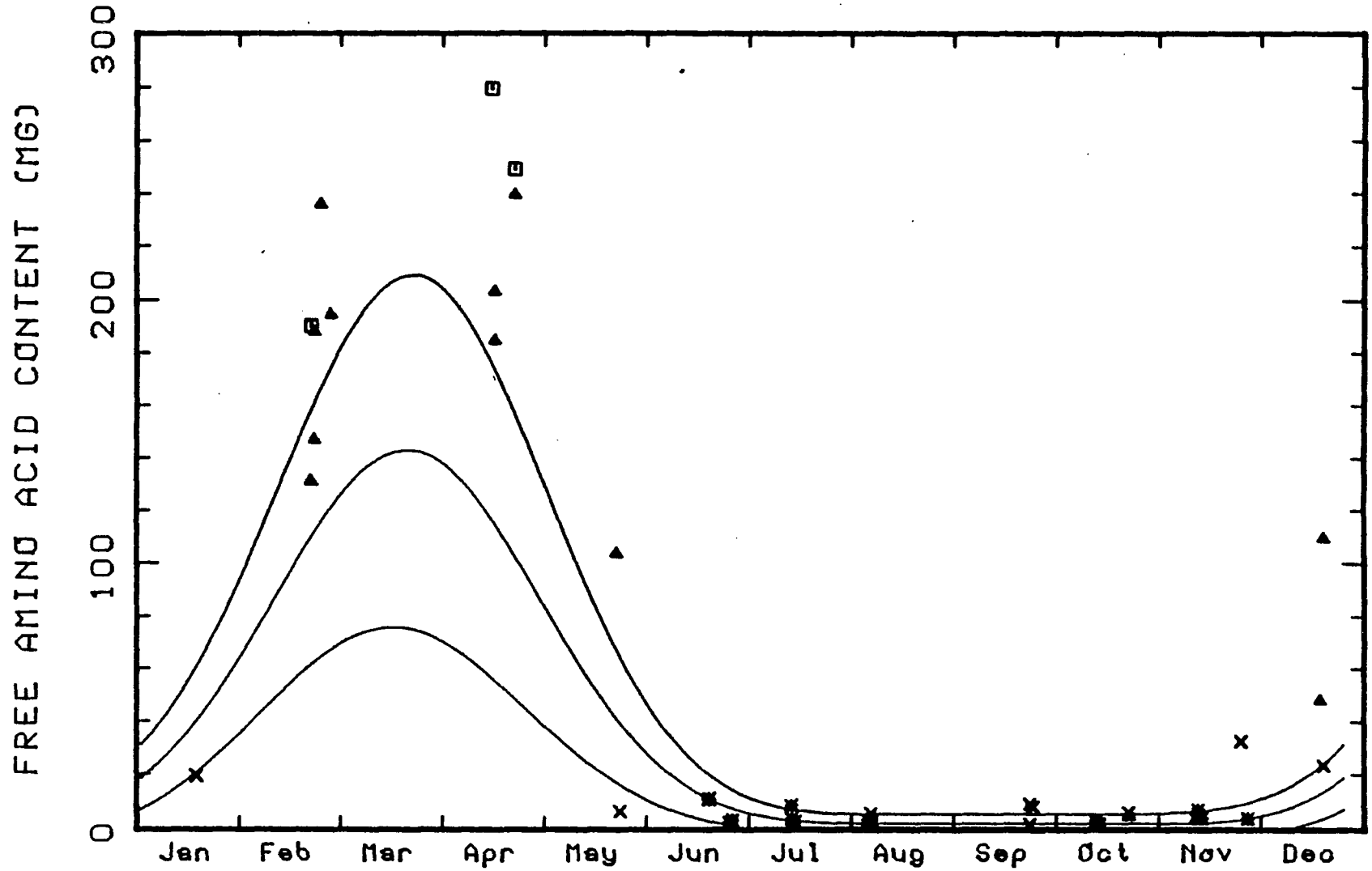

Figure 17. Annual changes in total free amino acid content in the testes.

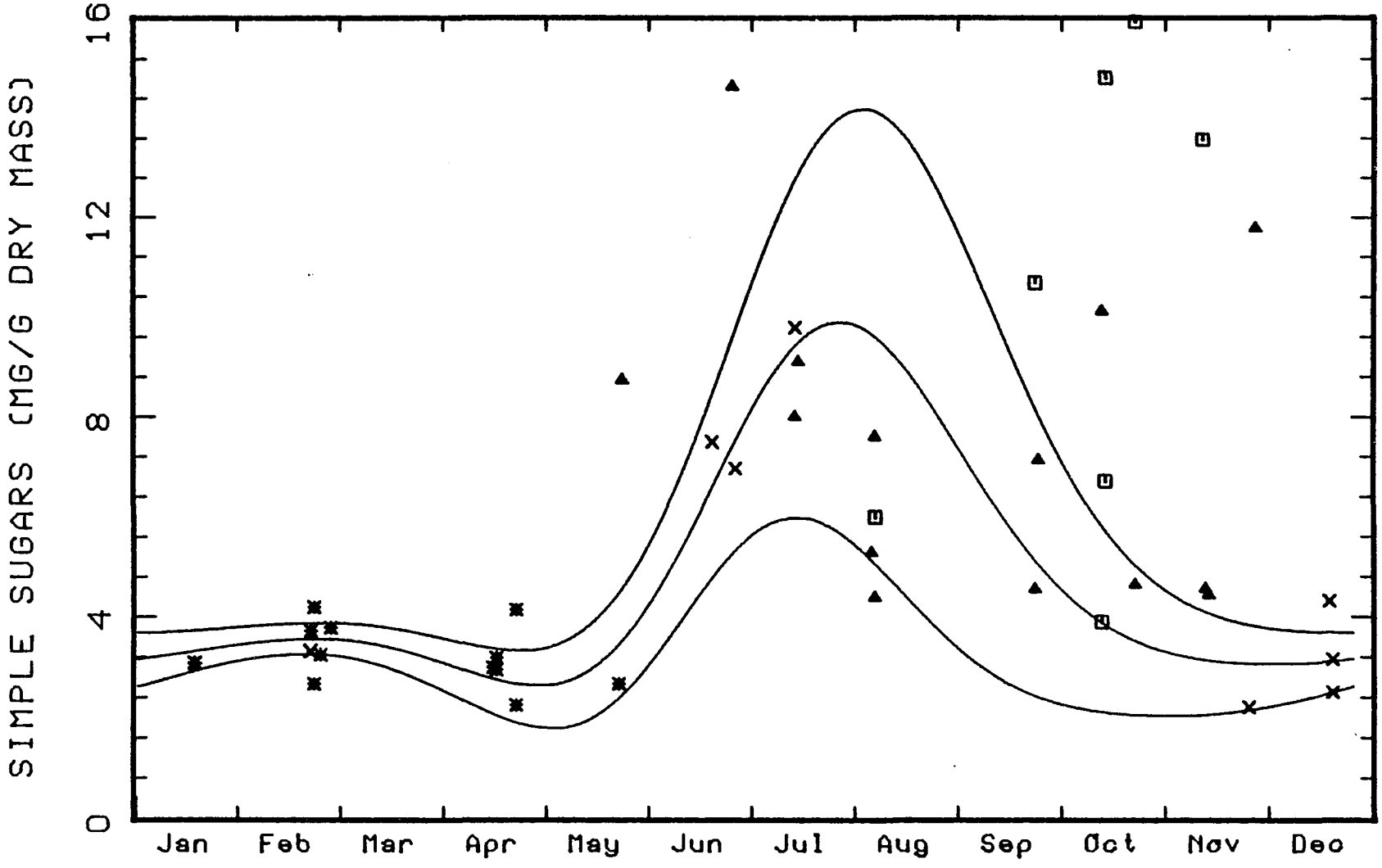

Figure 18. Annual changes in concentration of simple reducing sugars in the testes.

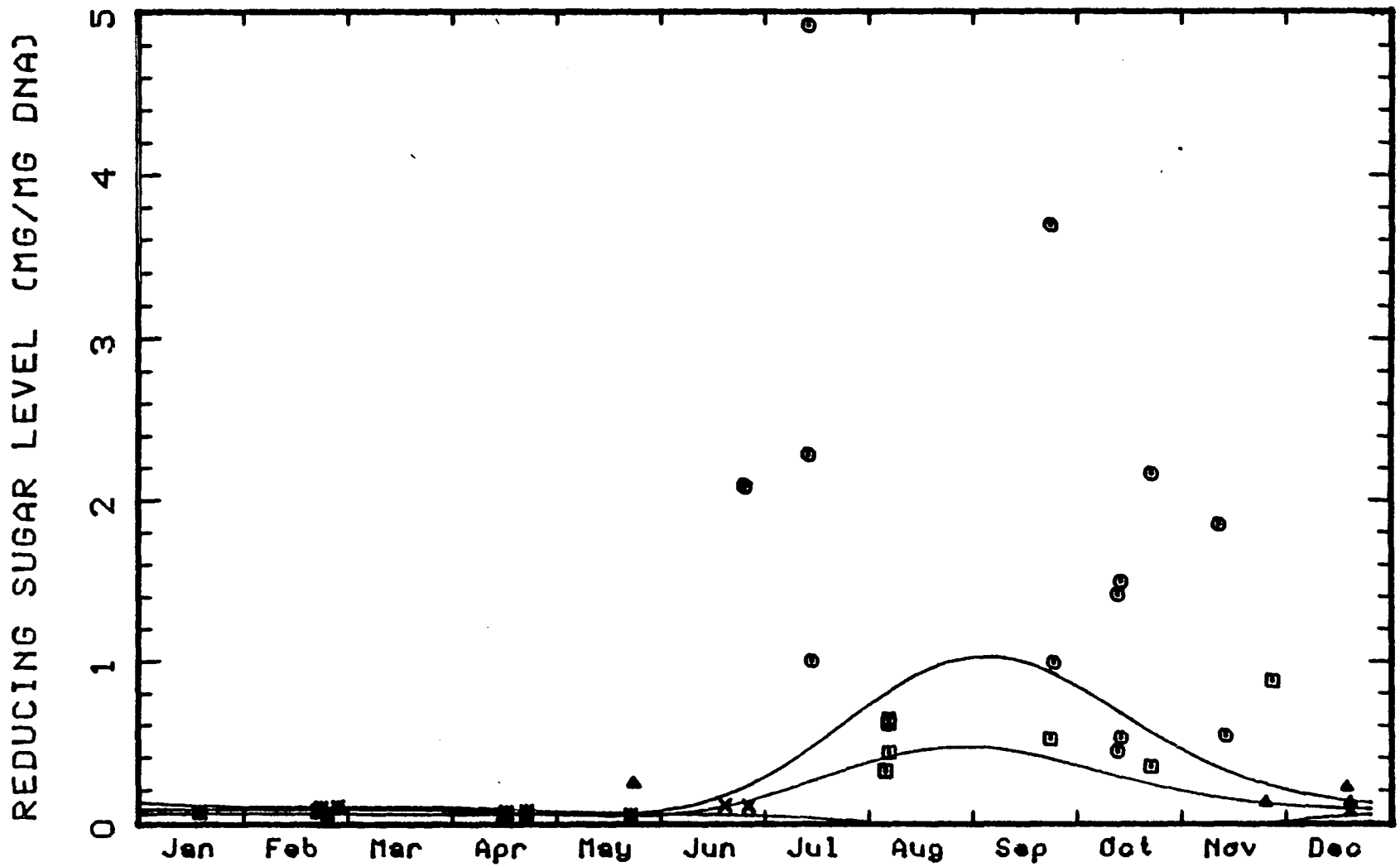

Figure 19. Annual changes in level of simple reducing sugars.

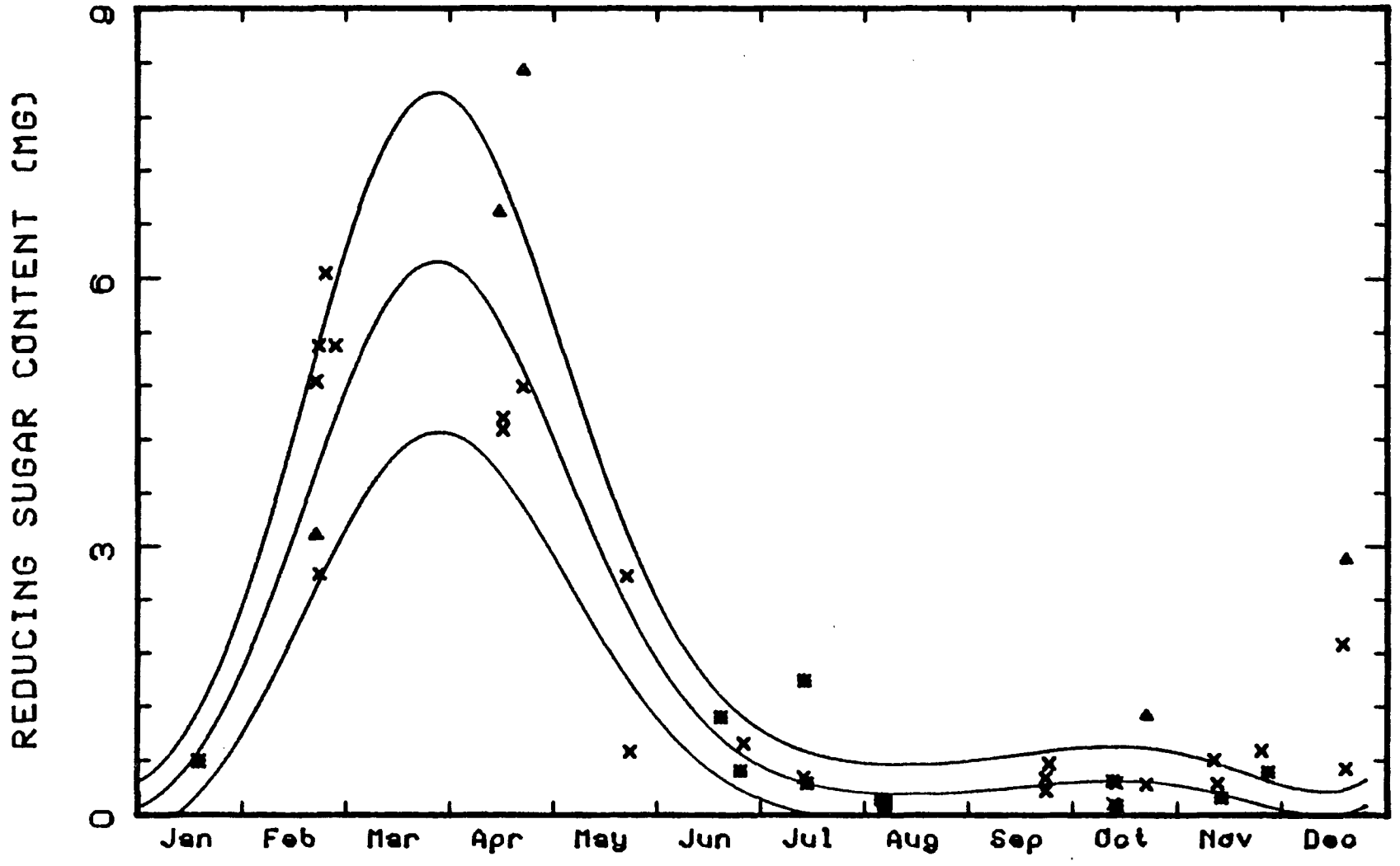

Figure 20. Annual changes in content of simple reducing sugars.

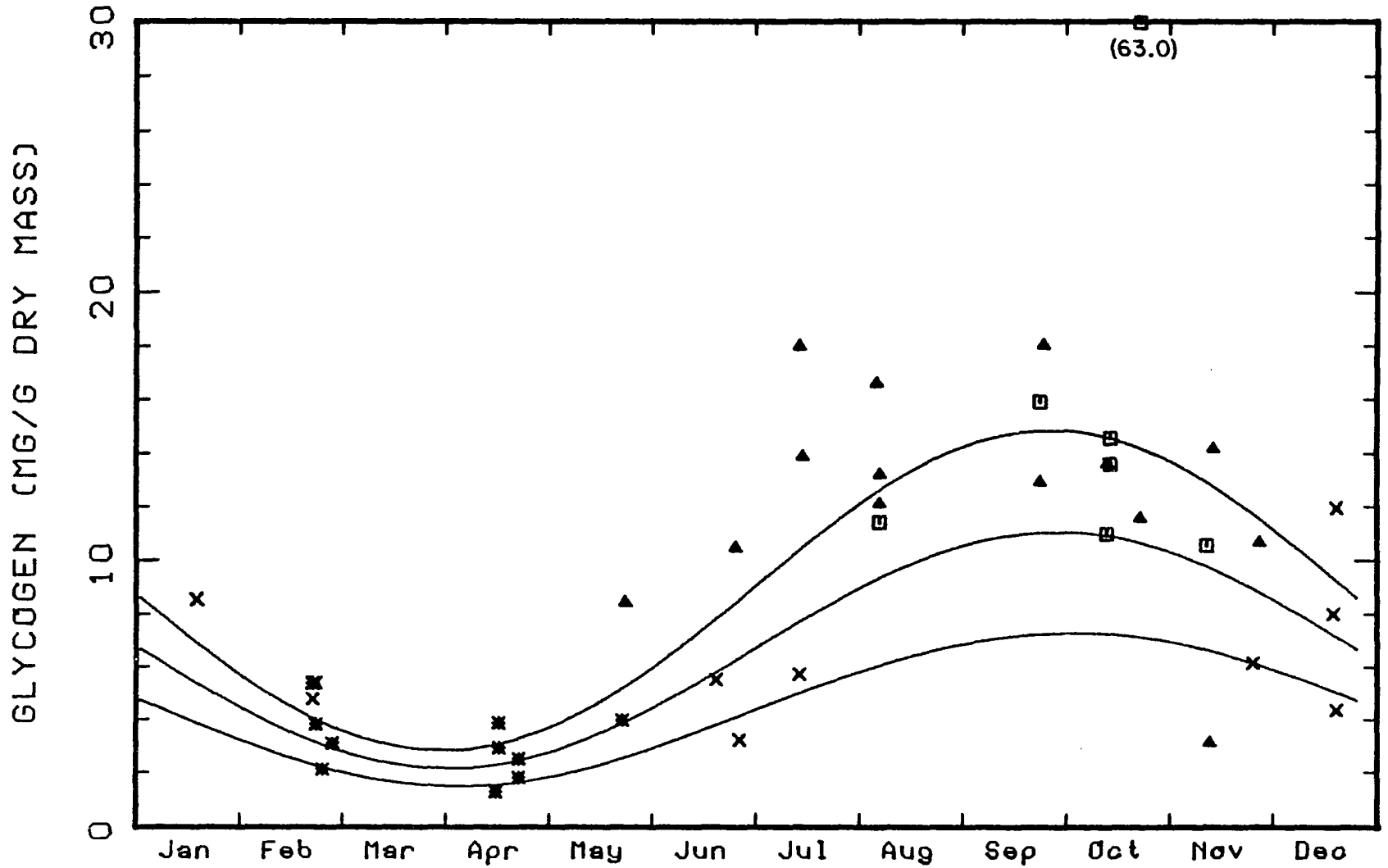

Figure 21. Annual changes in concentration of glycogen, expressed in glucose-equivalents.

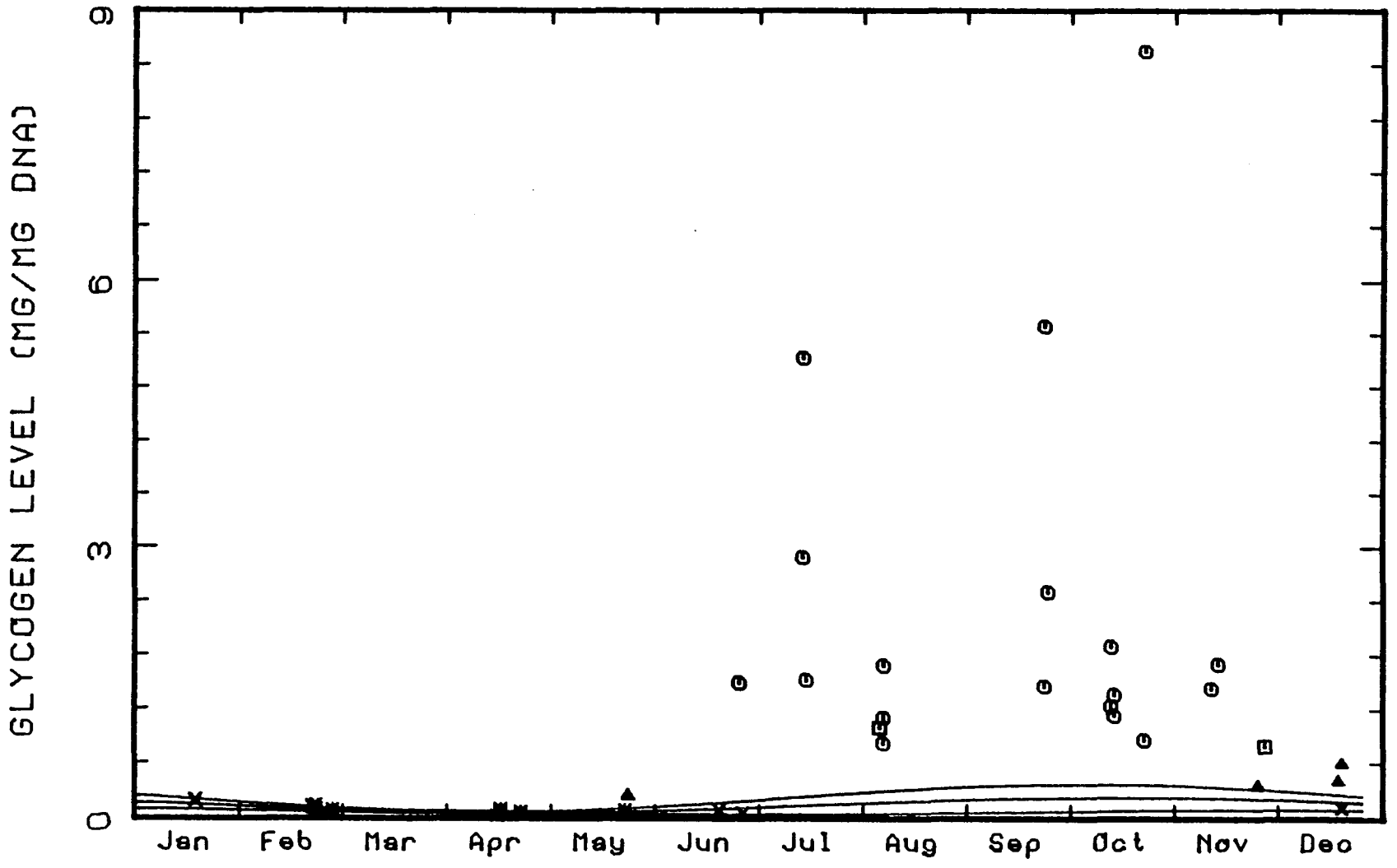

Figure 22. Annual changes in level of glycogen, in glucose-equivalents.

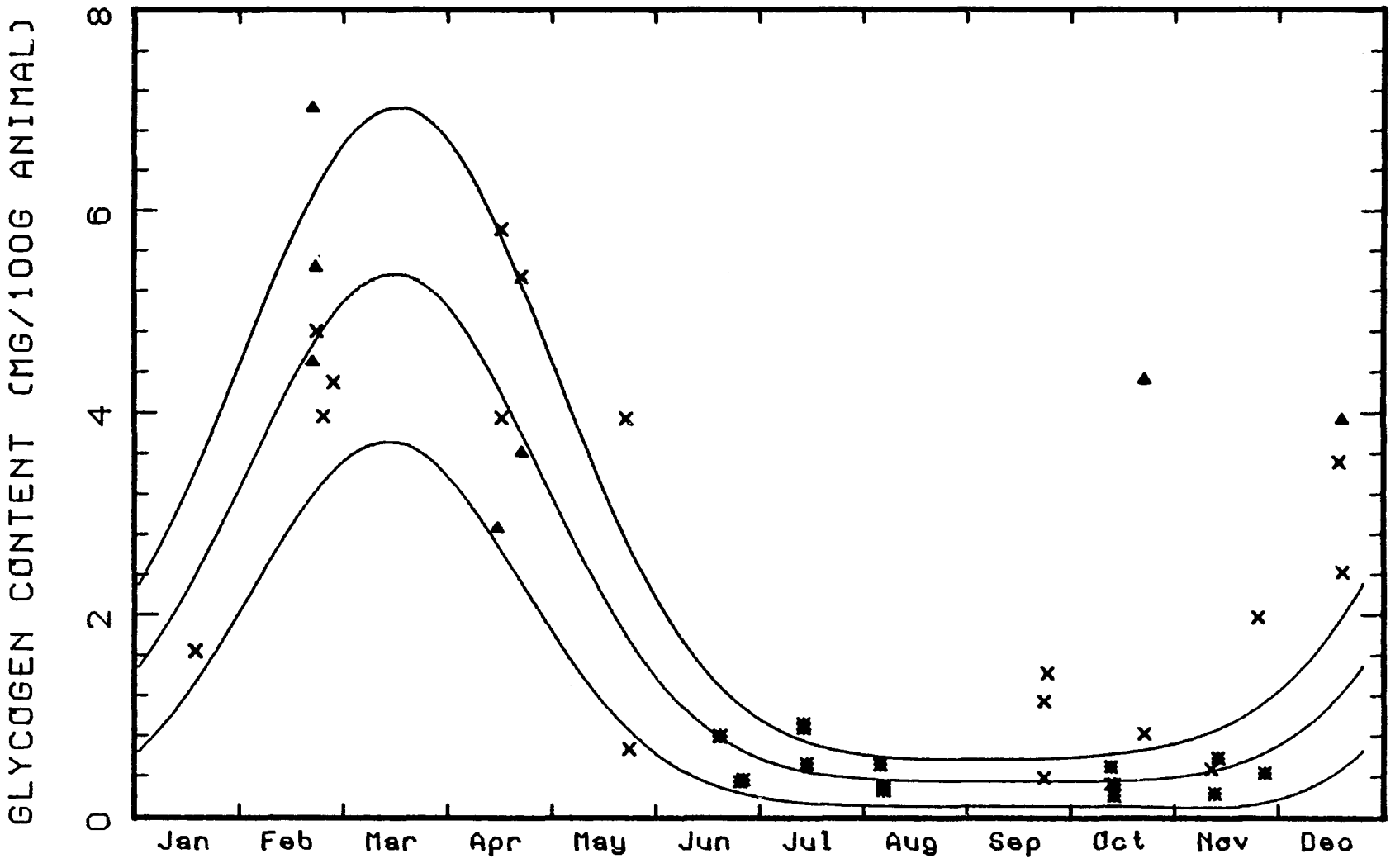

Figure 23. Annual changes in total content of glycogen in the testes, expressed as equivalent mass of glucose.

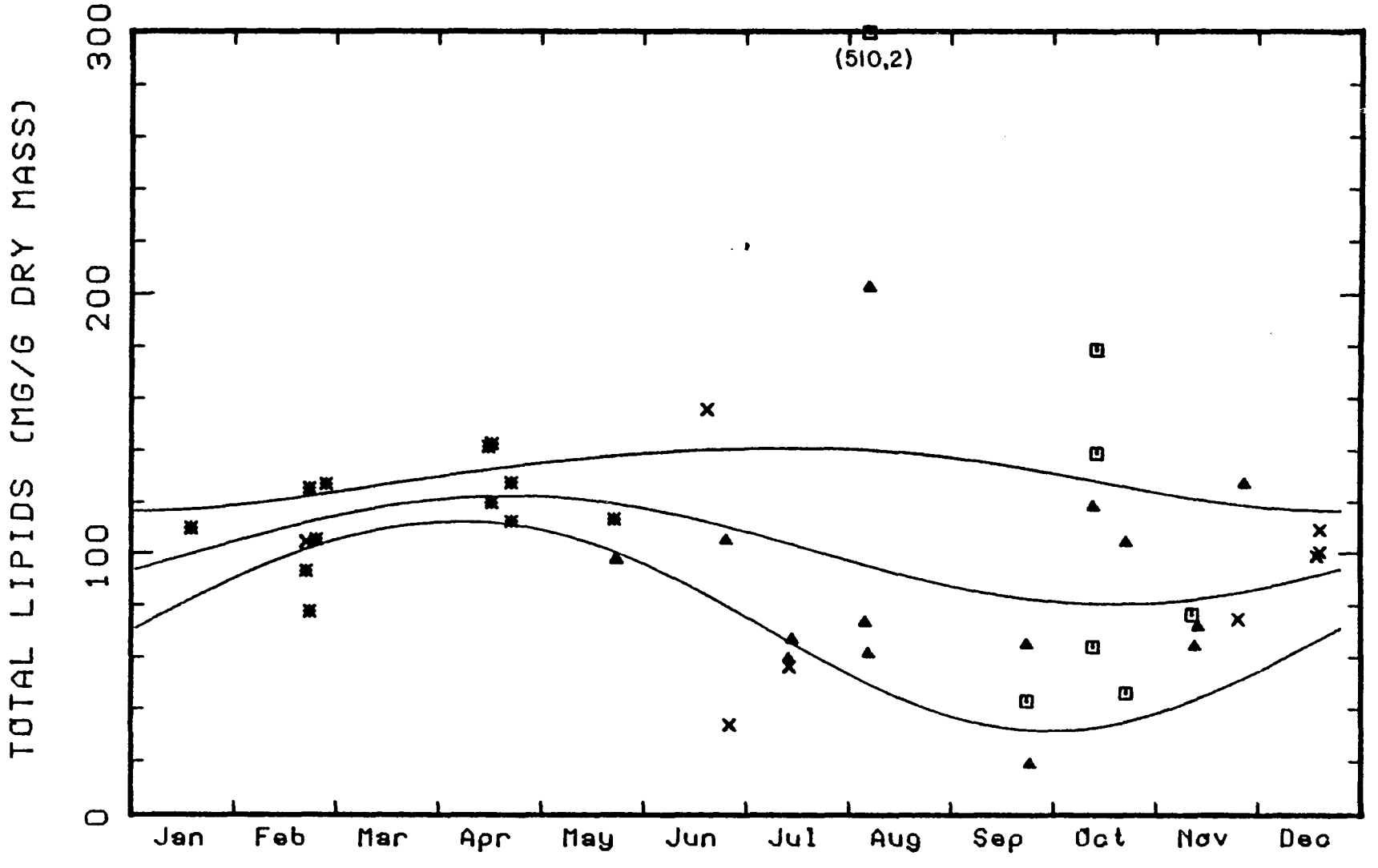

Figure 24. Annual changes in concentration of total lipids in the testes.

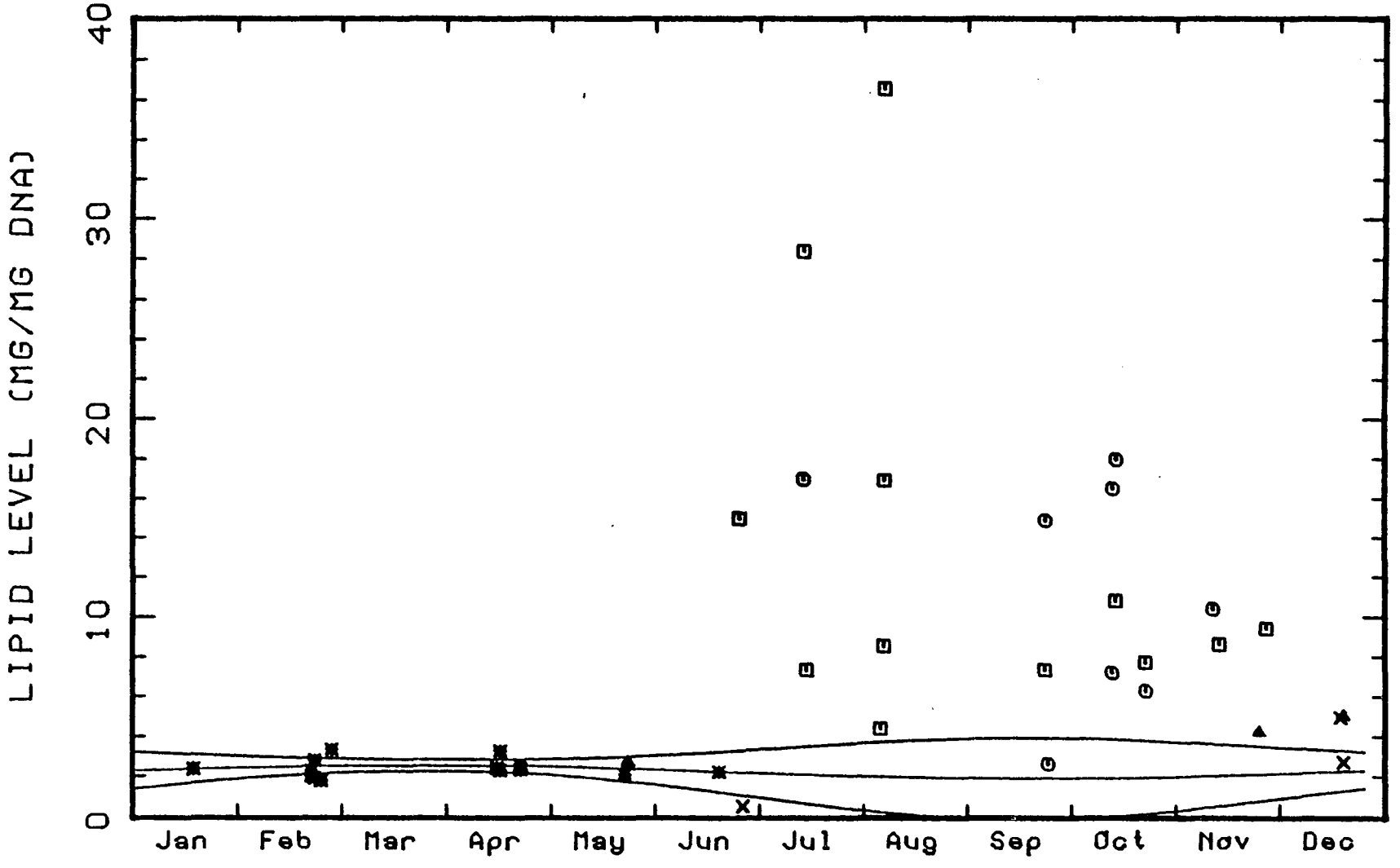

Figure 25. Annual changes in lipid level. Amplitude of the fitted curve is not significant, although it has been included in the equation graphed here.

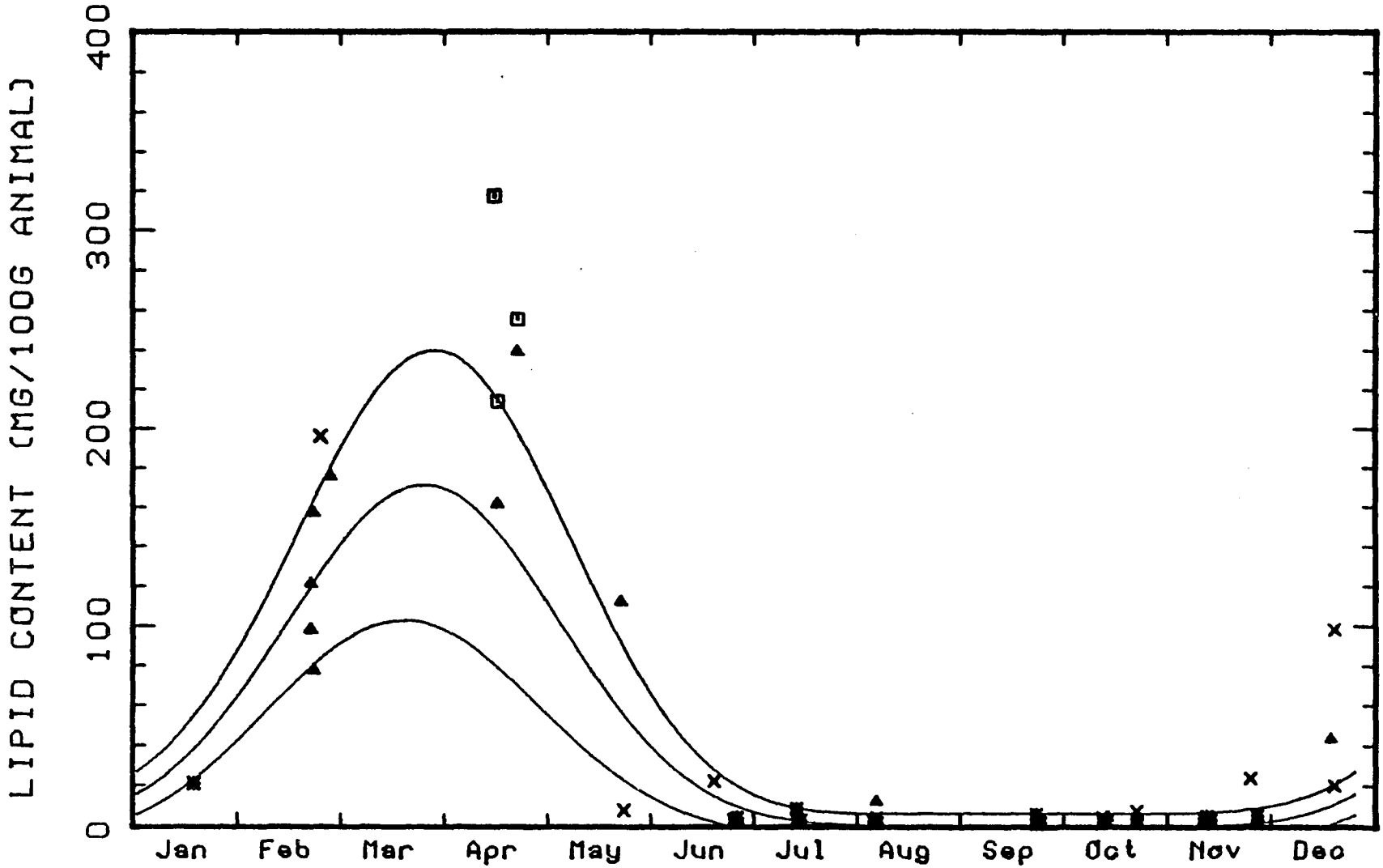

Figure 26. Annual changes in lipid content in the testes.

CHAPTER II

ACTIVITY OF ORNITHINE DECARBOXYLASE AND NUCLEIC ACID SYNTHESIS DURING GAMETOGENESIS: PRELIMINARY RESULTS

Introduction

The relationship between activity of ornithine decarboxylase, the rate-limiting enzyme of polyamine synthesis, and synthesis of nucleic acids has been well established in a variety of animal systems (e. g., Russell and Snyder 1968; Russell and Stambrook 1975; Fillingame et al. 1975; Fuller et al. 1977; Sunkara and Rao 1981). It is therefore likely that a similar relationship exists during gametogenesis in asteroids. Because nucleic acid synthesis is an essential event for both spermatogenesis and oogenesis, and because polyamine synthesis may be important in the regulation of nucleic acid synthesis (see Chapter III), an examination of temporal relationships among polyamine synthesis, nucleic acid synthesis, and related gametogenic events is important in enhancing understanding of gametogenic processes and regulation of gametogenic events. Although a relationship between ODC activity and rates of nucleic acid synthesis would be expected from research in other animal systems, it is nevertheless useful to examine these processes in some detail. Such an examination should confirm the expected correlation of polyamine synthesis with nucleic acid synthesis, as well as provide information on the variability of these measurements, thereby facilitating subsequent detailed examination of these synthetic processes during gametogenesis.

Careful examination of changes in biochemical composition of the testes during spermatogenesis indicates definite patterns of nucleic acid synthesis during spermatogenesis (see Chapter I). More direct evidence can be obtained by measuring the synthetic rates of both DNA and RNA through the incorporation of radioactively labelled thymidine and uridine. For a preliminary study, it is sufficient to examine rates of nucleic acid synthesis during several selected periods of the gametogenic cycle. Of particular interest in the study of spermatogenesis are the periods of transition from one spermatogenic phase to the next; for example, the initiation and cessation of proliferation and differentiation. Unfortunately, the small amount of testicular tissue available during the late aspermatogenic and early proliferative phases greatly complicates analysis. Therefore, for this preliminary examination, specimens were selected over the period from December to April. These specimens should include several interesting spermatogenic phases: December -- active spermatogonial proliferation; February -- period of extensive overlap of proliferation and differentiation; March -- active meiosis and differentiation; and April -- late differentiative phase. Ovaries were obtained at the same time of year, in order to compare patterns of polyamine synthesis and nucleic acid synthesis during spermatogenesis with concurrent changes during oogenesis.

In this paper, ornithine decarboxylase activity throughout the spermatogenic cycle is described and the relationship to concurrent spermatogenic events discussed. In addition, ornithine decarboxylase activity and synthetic rates of RNA and DNA are determined for both

ovaries and testes during the period December to April and probable relationships of those processes to gametogenic events are suggested.

Materials and Methods

Specimens of Asterias vulgaris were collected from the Gulf of Maine by divers and maintained in the laboratory as described in Chapter I. Specimens were narcotized and dissected and their sexes determined as described in Chapter I.

Seasonal changes of ornithine decarboxylase (ODC) activity were examined using lyophilized testes from the same specimens as in Chapter I; use of these specimens facilitated comparison of resulting data to gross biochemical changes during spermatogenesis. Lyophilized testes were homogenized in ODC reaction buffer (Appendix C). Ornithine decarboxylase activity was assayed by scintillation counting of $^{14}CO_2$ released from radioactively labelled DL-[1-^{14}C]-ornithine hydrochloride (Amersham, 58 mCi/mmol) (Landy-Otsuka and Scheffler 1978; Appendix C); released $^{14}CO_2$ was trapped with 200µl of NCS (Amersham) in a suspended center well (Kontes). Protein concentration in the homogenate was determined by the method of Bradford (1976) with a bovine serum albumin standard. ODC activity was expressed as disintegrations per minute of trapped $^{14}CO_2$ per microgram of protein. Resulting data were analyzed by periodic regression and circular-linear correlation (see Chapter I and Appendices D and E).

For the examination of nucleic acid synthetic rates, fresh gonads (both testes and ovaries) were incubated for 24 hours in artificial coelomic fluid (see Chapter III) containing 1 µCi/ml of either [methyl-^{3}H]-thymidine (New England Nuclear, 6.7 Ci/mmol) or [5-^{3}H]-uridine (New England Nuclear, 26.8 Ci/mmol). After a chase period of 16 to 24

hours, gonads were placed in 10% trichloroacetic acid and stored at -16° C. Nucleic acids were separated by the method of Schmidt and Thannhauser (1945). DNA was analyzed with diphenylamine (Burton 1968) with a calf thymus DNA standard; RNA was analyzed by the modified orcinol method of Almog and Shirey (1978) with a Torula yeast RNA standard. Incorporation of radioactive label was determined by scintillation counting with a Beckman LC-7000 liquid scintillation counter. Gonads from the same animals were also homogenized in ODC reaction buffer and ODC activity was assayed as described above (and in Appendix C).

ODC activity and nucleic acid synthetic rates for both males and females in December, February, March, and April were analyzed by analysis of variance. Variance-stabilizing transformations were selected by the method of Box and Cox (1964). Logarithmic transformations were used for ODC activity and RNA synthetic rate; square root transformation was used for DNA synthetic rate. Effects attributable to month and sex were analyzed by Student-Newman-Keuls multiple range tests applied to transformed data.

Results

Periodic regression of ornithine decarboxylase activity during the spermatogenic cycle (Fig. 27) shows that ornithine decarboxylase activity is greatest during the proliferative phase and early differentiative phase. The circular-linear rank correlation coefficient (Mardia 1976) between ornithine decarboxylase activity and spermatogenic stage (determined by histological examination) of U_n = 6.12 ($n = 27$, $D_n = 0.239$) differs significantly from zero ($p < 0.05$).

The overall pattern of ODC activity relative to protein suggests an increase during the late aspermatogenic phase with high activities maintained throughout the proliferative phase. Maximum activity of ODC is attained during late February or early March; ODC activity subsequently declines to the low values seen fairly consistently in the aspermatogenic phase.

DNA synthetic rates from December to April show extreme differences attributable to both sex and month (Fig. 28; square-root transformed data in Fig. 29). Analysis of variance and multiple range testing (Table 4) indicate that the rate of DNA synthesis, expressed per unit mass of DNA present, declines greatly from December to February in males and remains relatively low through April. The data suggest that DNA synthesis again increases slightly during March, but this apparent increase is not statistically significant. The pattern observed for females during this time of year is nearly a mirror-image of that observed for males. The ovarian DNA synthetic rate is relatively low from December to March, with possibly a slight (non-significant) increase in March; DNA synthesis then increases greatly during April, to approximately the same level as that observed in testes during December.

RNA synthesis shows little difference attributable to sex (Fig. 30; logarithmically transformed data in Fig. 31). In the gonads of both males and females, RNA synthetic rate apparently decreases from high values in December to a much lower and nearly constant level from February to April; multiple range testing (Table 5) shows that decrease to be highly significant ($p<0.01$).

Specific activity of ornithine decarboxylase relative to total protein is less amenable to interpretation (Fig. 32; log-transformed data in Fig. 33). The relatively large variability and smooth transitions result in many of the data points being statistically indistinguishable by a Student-Newman-Keuls multiple range test (Table 6). Analysis of variance (Table 6), however, indicates that significant effects can be attributed to month, sex, and month-sex interaction. For all four months sampled, mean ODC activity is higher in males than in females. In addition, females show significantly greater ODC activity in March than in December or April. Although ODC activity in testes does not differ significantly among the four months sampled, the Spearman rank correlation coefficient between ODC activity and DNA synthetic rate is significantly different from zero (r_s = 0.758; $p < 0.02$), which suggests that there does exist an underlying pattern of ODC activity obscured by large variability and small sample size.

Discussion

Ornithine decarboxylase activity (Fig. 27) increases greatly during the proliferative phase of spermatogenesis, when a large proportion of testicular cells are actively dividing. This increase is entirely expected, given the requirement of polyamines for DNA synthesis (Fillingame et al. 1975; Fuller et al. 1977) and the usual pattern of changes in ODC activity during the cell cycle (Russell and Stambrook 1975; Fillingame et al. 1975; Fuller et al. 1977; Sunkara and Rao 1981). That ODC activity correlates well with spermatogenic phase is shown by the high coefficient of circular-linear rank correlation. ODC activity remains high during the early differentiative phase as

primary spermatocytes prepare for meiosis; ODC activity then declines during March so that levels of activity in April are only slightly, if at all, greater than those seen during the aspermatogenic phase. The low levels of ODC activity seen from approximately April to September are consistent with the conclusion (Chapter I) that pre-meiotic DNA synthesis is essentially complete by late March.

Because of the good correlation between ODC activity and spermatogenic stage, ODC activity would also be expected to correlate well with DNA synthetic rate. For males during the period December to April, at least, this correlation holds. Details of changes in DNA synthetic rates are difficult to determine from the data presented here; sample size and inter-sample variability obscure any real differences. Nevertheless, DNA synthetic rate clearly ($p<0.001$) declines from December to April; the decline seen from March to April is expected on the basis of prior observations (Chapter I), but is not statistically significant for this data set.

In females, ovarian DNA synthetic rate increases greatly in April. This probably corresponds to the beginning of S phase associated with oogonial proliferation (Schoenmakers *et al.* 1981a). One would expect this increase in DNA synthetic rate to be associated with increased ODC activity and RNA synthesis. The lack of any detectable increase in either ODC activity or RNA synthesis in April may be artifactual. Because RNA synthetic rate is expressed as a function of total RNA present and ODC activity is expressed relative to total protein, storage of large quantities of RNA and protein during vitellogenesis could mask any increase in activity. The data suggest an increase in ODC activity during March, although this increase is not statistically

significant. If real, this increased ODC activity may represent either polyamine synthesis in preparation for the period of DNA synthesis in April or generation of intracellular polyamine pools sufficient for early cleavage.

The results presented here are preliminary. Many apparent differences attributable to gametogenic stage or sex are statistically obscured by variability among animals. The effect of high variability among animals at a given time of year can be reduced by increasing the sample size or by using cytological covariates to more precisely specify gametogenic stage. Larger sample sizes and parallel histological or cytological observations would be essential for a subsequent study intended to confirm or elucidate relationships suggested here among ODC activity, nucleic acid synthesis, and gametogenic events. In addition, it would be extremely useful for such a study to express all data relative to DNA content rather than, or in addition to, protein or RNA content. Expressing data relative to DNA allows the data to be more readily interpreted in terms of cellular events. This is especially important for data derived from ovaries, in which the amounts of RNA and protein per cell increase greatly during vitellogenesis.

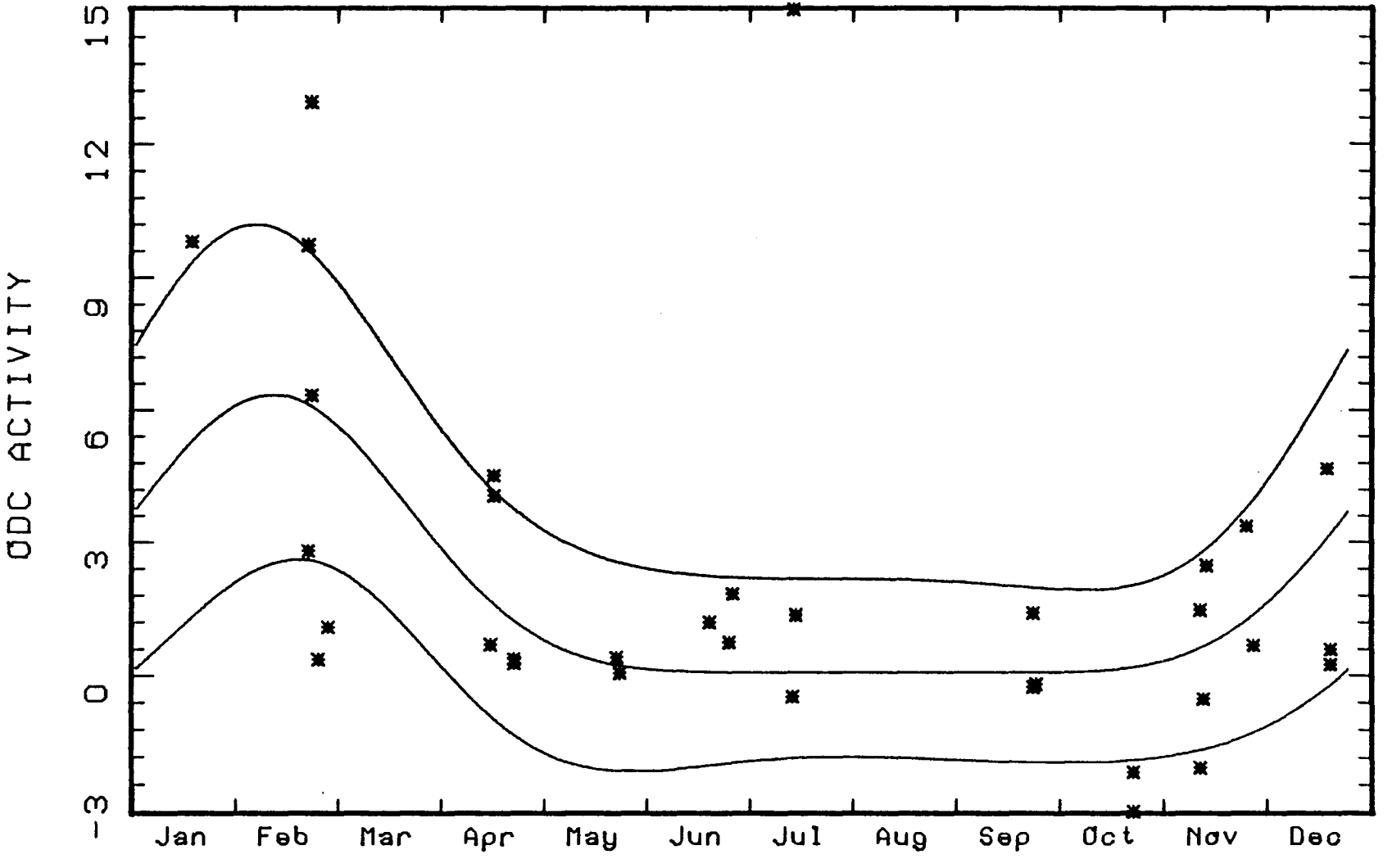

Figure 27. Ornithine decarboxylase activity during the annual spermatogenic cycle. ODC activity is expressed as cpm/μg protein. Fitted periodic regression with 95% confidence band.

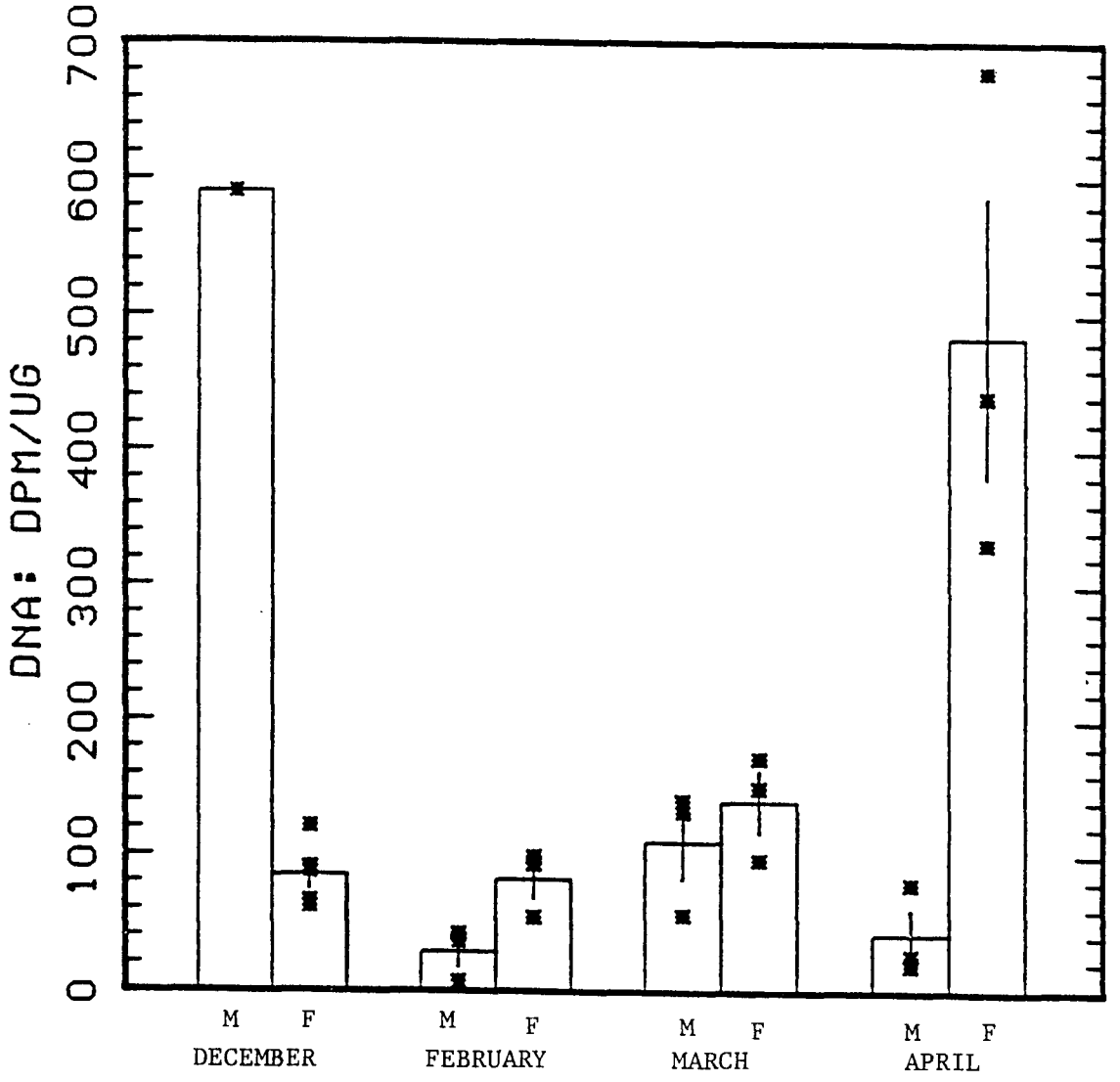

Figure 28. DNA synthetic rates from December to April. Untransformed data; bars show means, error bars represent standard errors of group means.

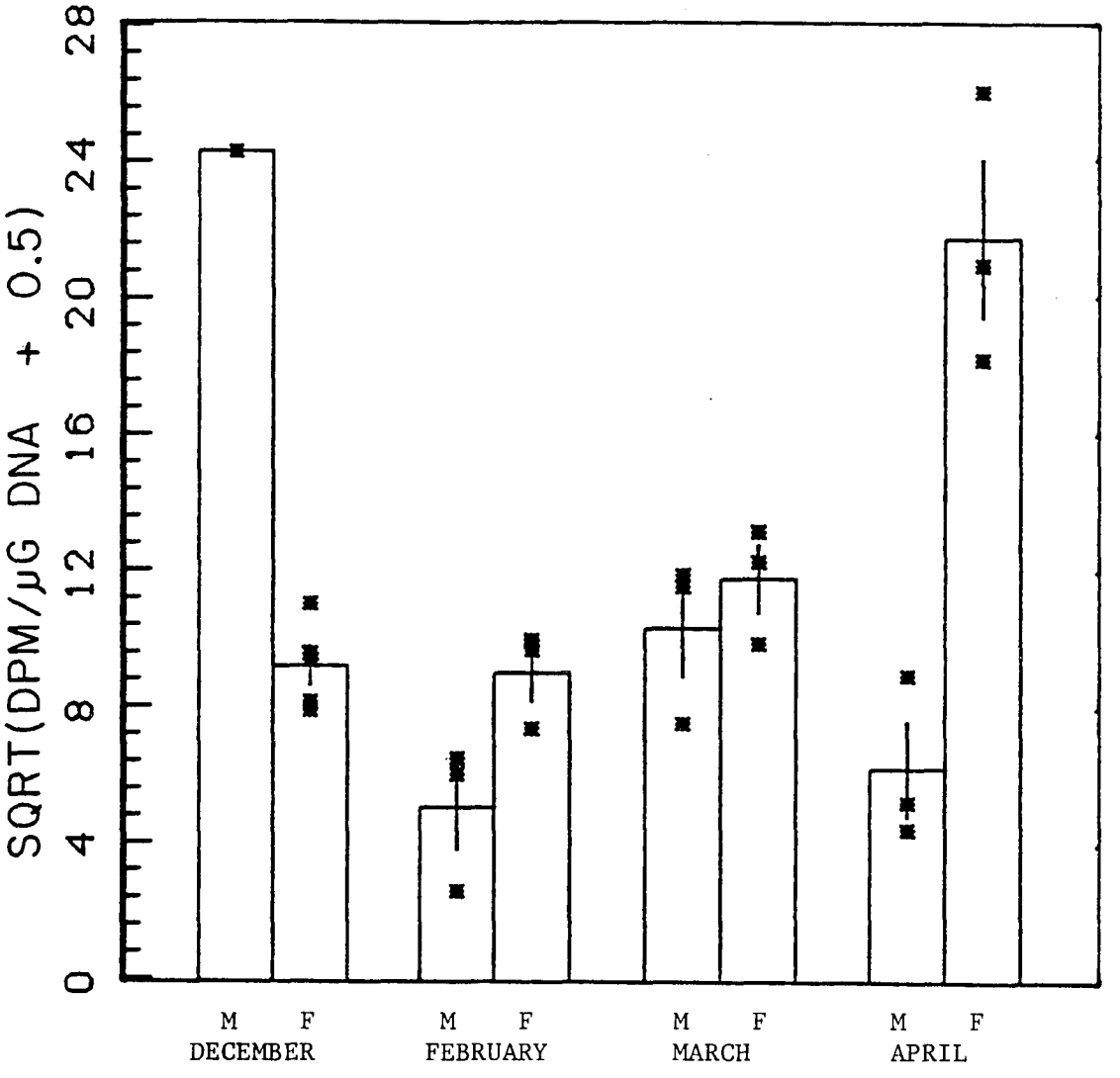

Figure 29. Square-root transformed DNA synthetic rates. Bars show group means; error bars represent standard errors of group means.

Table 4. DNA synthetic rates from December to April. Analysis of variance and Student-Newman-Keuls multiple range test applied to square-root transformed data.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F-VALUE	TAIL PROBABILITY	
MONTH	250.6030	3	83.5343	17.61	<0.0001	***
SEX	10.8338	1	10.8338	2.28	0.1491	N.S.
INTERACTION	515.1491	3	171.7164	36.21	<0.0001	***
ERROR	80.6249	17	4.7426			

MULTIPLE RANGE TEST

GROUP MEANS (ranked high to low)

	MONTH	SEX	GROUP MEAN	GROUP SIZE (n)
1.	December	Male	24.310	1
2.	April	Female	21.763	3
3.	March	Female	11.757	3
4.	March	Male	10.301	3
5.	December	Female	9.180	5
6.	February	Female	8.988	3
7.	April	Male	6.171	3
8.	February	Male	5.018	3

COMPARE	q	TAIL PROBABILITY		COMPARE	q	TAIL PROB.	
1 vs. 8	10.85	< 0.001	***	3 vs. 8	5.36	< 0.025	*
vs. 7	10.20	< 0.001	***	vs. 7	4.44	< 0.05	*
vs. 6	8.61	< 0.001	***	vs. 6	2.20	> 0.20	N.S.
vs. 5	8.96	< 0.001	***				
vs. 4	7.87	< 0.001	***	4 vs. 8	4.20	> 0.05	N.S.
vs. 3	7.06	< 0.001	***				
vs. 2	1.43	> 0.200	N.S.	5 vs. 8	3.70	> 0.05	N.S.
2 vs. 8	13.32	< 0.001	***	6 vs. 8	3.20	> 0.05	N.S.
vs. 7	12.40	< 0.001	***				
vs. 6	10.16	< 0.001	***	7 vs. 8	0.917	> 0.50	N.S.
vs. 5	11.19	< 0.001	***				
vs. 4	9.12	< 0.001	***				
vs. 3	7.96	< 0.001	***				

FEB-M	APR-M	FEB-F	DEC-F	MAR-M	MAR-F	APR-F	DEC-F

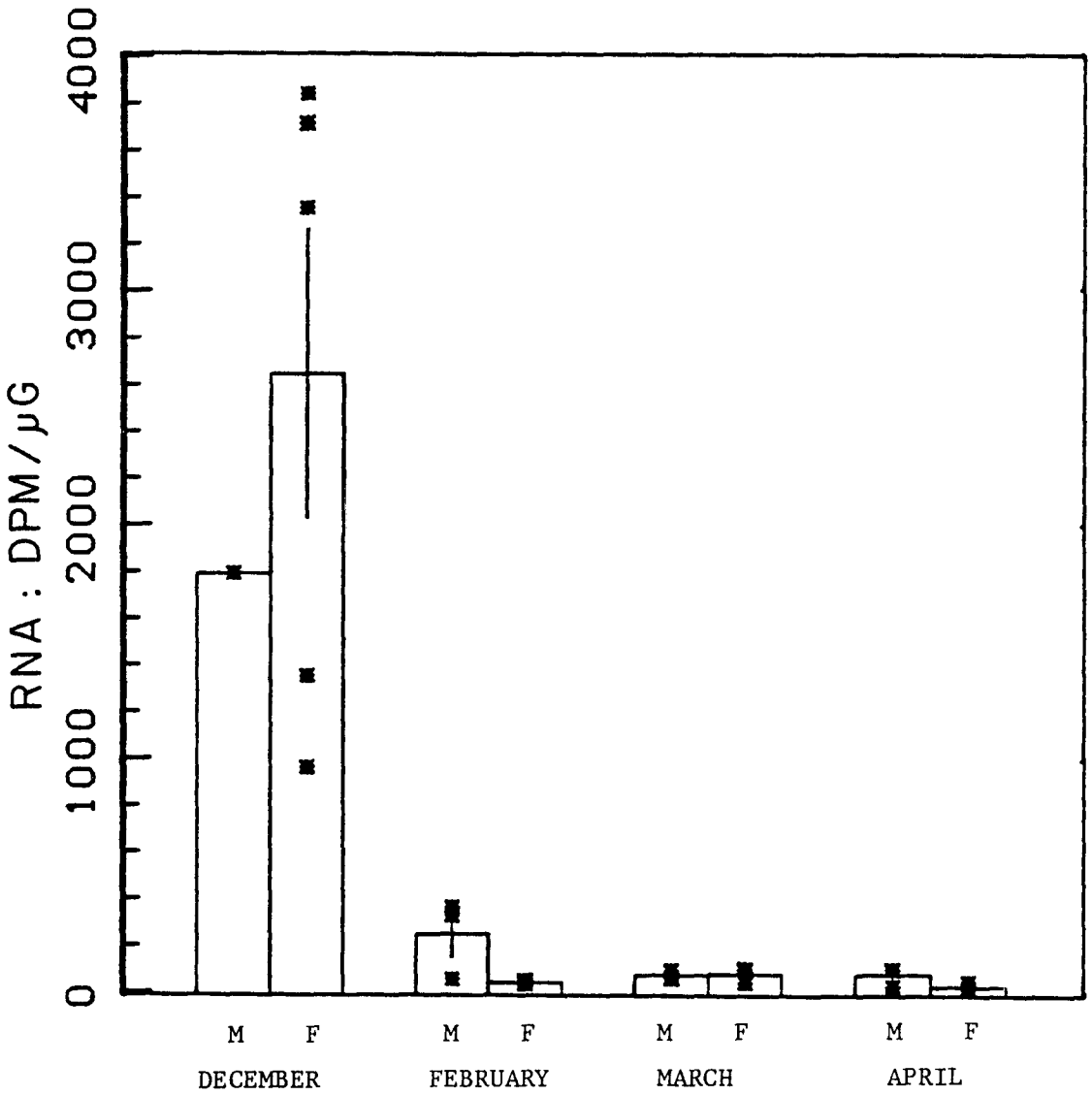

Figure 30. RNA synthetic rates. Untransformed rates of RNA synthesis expressed as dpm of ^{3}H-thymidine incorporated per µg of RNA.

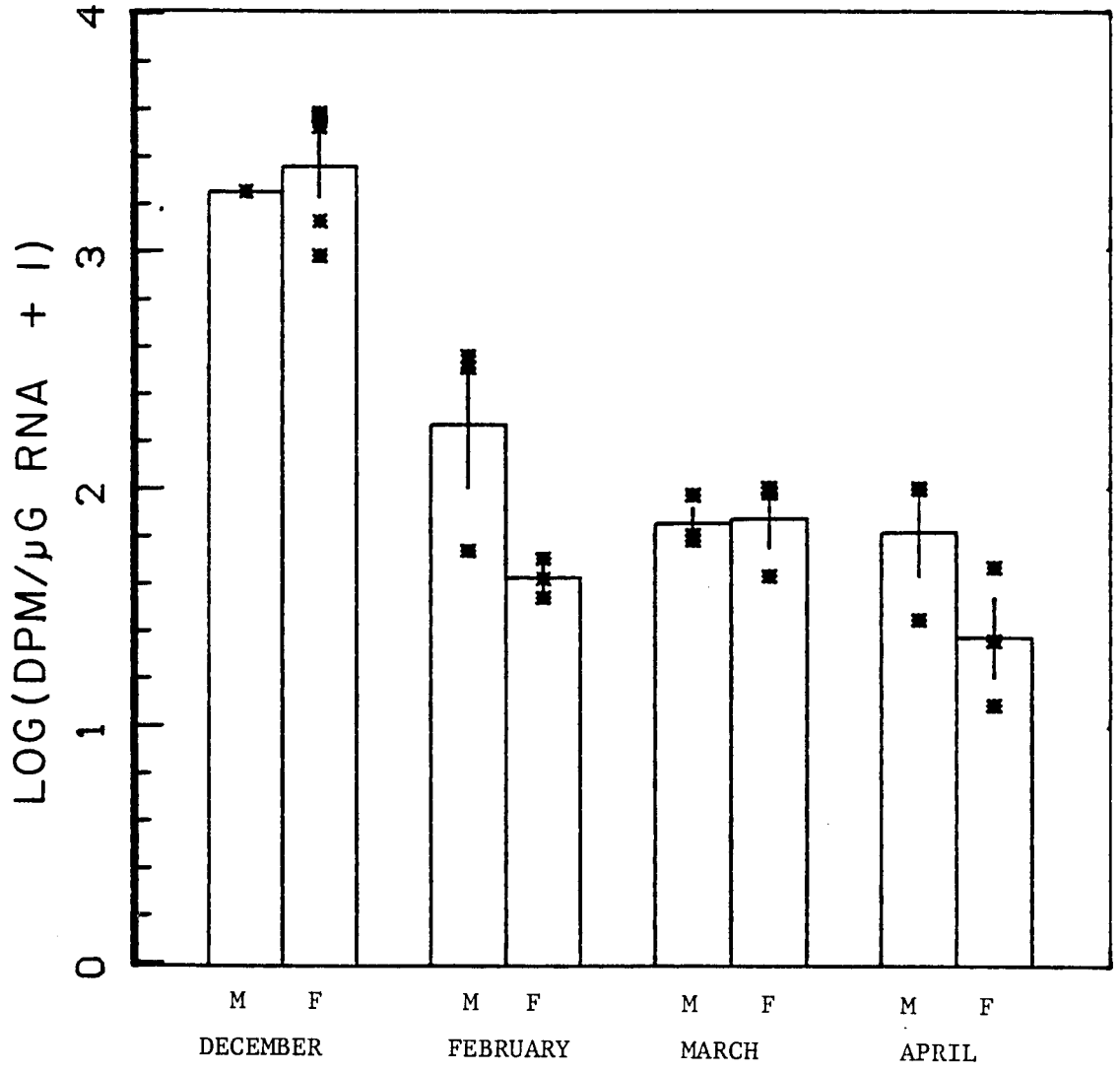

Figure 31. Log-transformed RNA synthetic rates. Bars show group means; error bars represent standard errors.

Table 5. RNA synthetic rates from December to April. Analysis of variance and Student-Newman-Keuls test applied to logarithmically transformed data.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F-VALUE	TAIL PROBABILITY	
MONTH	6.7702	3	2.2567	30.92	<0.0001	***
SEX	0.2925	1	0.2925	4.01	0.0615	N.S.
INTERACTION	0.5017	3	0.1672	2.29	0.1148	N.S.
ERROR	1.2406	17	0.0730			

MULTIPLE RANGE TEST

GROUP MEANS (ranked high to low)

	MONTH	SEX	GROUP MEAN	GROUP SIZE (n)
1.	December	Female	3.359	5
2.	December	Male	3.253	1
3.	February	Male	2.272	3
4.	March	Female	1.873	3
5.	March	Male	1.854	3
6.	April	Male	1.817	3
7.	February	Female	1.624	3
8.	April	Female	1.372	3

COMPARE	q	TAIL PROBABILITY		COMPARE	q	TAIL PROB.	
1 vs. 8	14.24	< 0.001	***	3 vs. 8	5.77	< 0.01	**
vs. 7	12.44	< 0.001	***	vs. 7	4.16	> 0.05	N.S.
vs. 6	11.05	< 0.001	***				
vs. 5	10.79	< 0.001	***	4 vs. 8	3.21	> 0.10	N.S.
vs. 4	10.65	< 0.001	***				
vs. 3	7.79	< 0.001	***	5 vs. 8	3.09	> 0.10	N.S.
vs. 2	0.51	> 0.50	N.S.				
				6 vs. 8	2.85	> 0.10	N.S.
2 vs. 8	8.53	< 0.001	***				
vs. 7	7.38	< 0.001	***	7 vs. 8	1.62	> 0.20	N.S.
vs. 6	6.51	< 0.005	**				
vs. 5	6.34	< 0.005	**				
vs. 4	6.26	< 0.005	**				
vs. 3	4.45	< 0.01	**				

APR-F FEB-F APR-M MAR-M MAR-F FEB-M DEC-M DEC-F

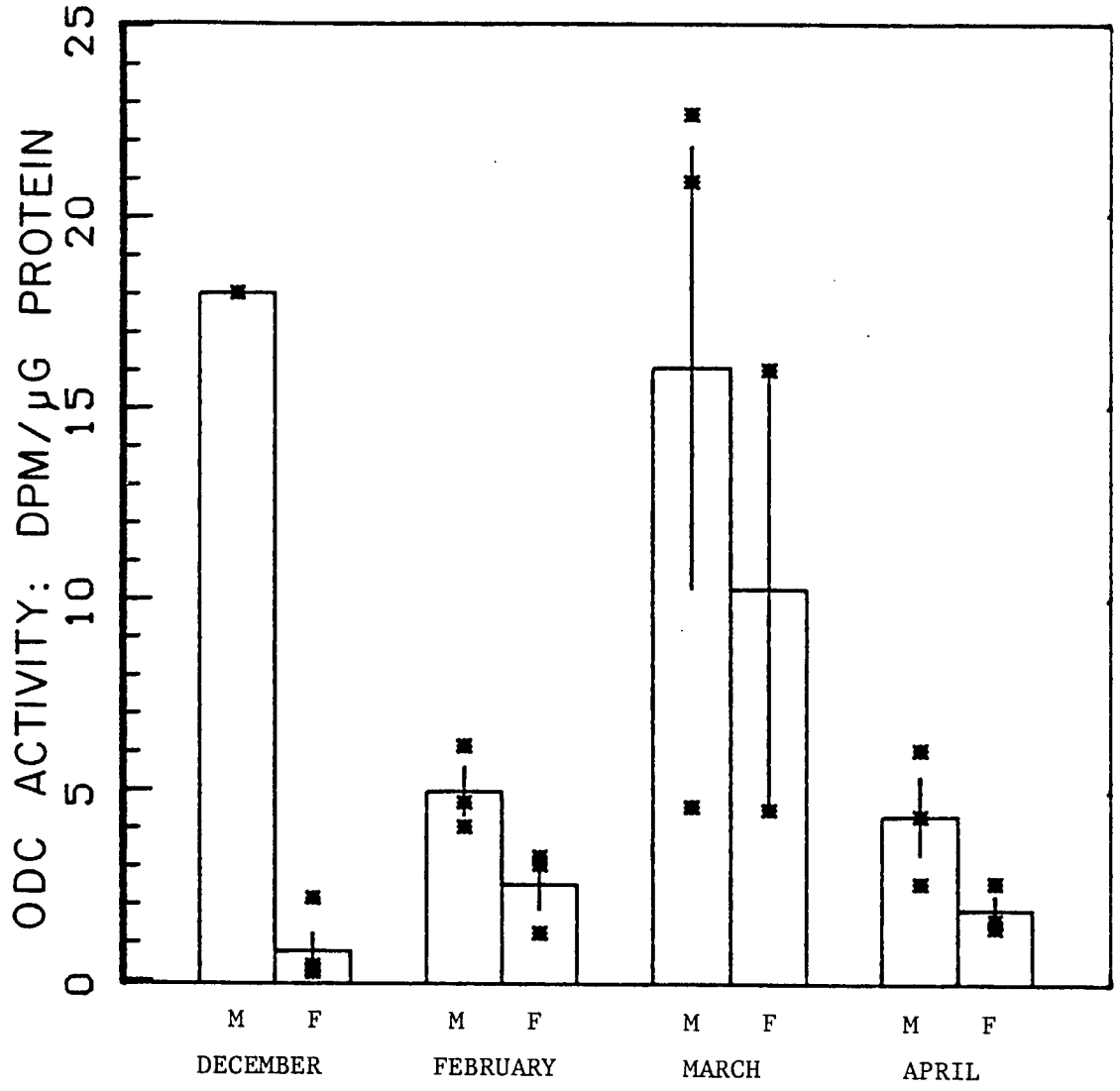

Figure 32. Activity of ornithine decarboxylase. Bars show group means; error bars represent standard errors.

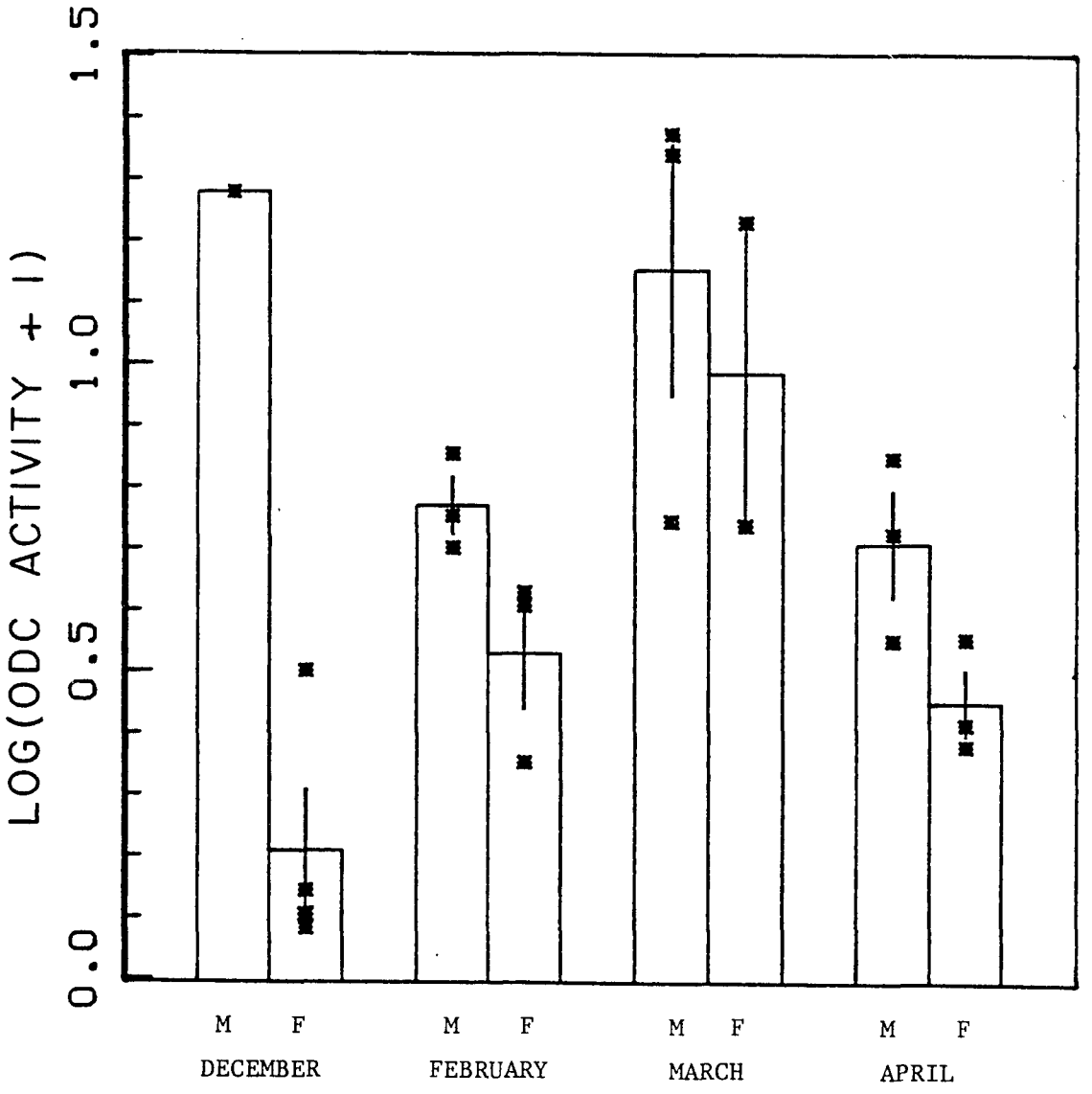

Figure 33. Log-transformed activity of ornithine decarboxylase. Bars show group means; error bars represent standard errors.

Table 6. Activity of ornithine decarboxylase from December to April. Analysis of variance and Student-Newman-Keuls multiple range test applied to logarithmic transformation of activities expressed as dpm of evolved $^{14}CO_2$ per µg protein.

ANALYSIS OF VARIANCE

SOURCE	SUM OF SQUARES	DF	MEAN SQUARE	F-VALUE	TAIL PROBABILITY	
MONTH	0.7236	3	0.2412	5.95	0.0070	**
SEX	0.8838	1	0.8838	21.79	0.0003	***
INTERACTION	0.4845	3	0.1615	3.98	0.0285	*
ERROR	0.6084	15	0.0406			

MULTIPLE RANGE TEST

GROUP MEANS (ranked from high to low)

	MONTH	SEX	GROUP MEAN	GROUP SIZE (n)
1.	December	Male	1.279	1
2.	March	Male	1.154	3
3.	March	Female	0.986	2
4.	February	Male	0.771	3
5.	April	Male	0.709	3
6.	February	Female	0.530	3
7.	April	Female	0.451	3
8.	December	Female	0.209	4

COMPARE	q	TAIL PROBABILITY		COMPARE	q	TAIL PROB.	
1 vs. 8	6.72	< 0.005	**	4 vs. 8	5.17	< 0.025	*
vs. 7	5.04	< 0.05	*	vs. 7	2.75	> 0.20	N.S.
vs. 6	4.56	> 0.05	N.S.				
				5 vs. 8	4.597	< 0.025	*
2 vs. 8	8.688	< 0.001	***	vs. 7	2.219	> 0.20	N.S.
vs. 7	6.046	< 0.01	**				
vs. 6	5.367	< 0.025	*	6 vs. 8	2.951	> 0.10	N.S.
vs. 5	3.827	> 0.05	N.S.				
				7 vs. 8	2.225	> 0.10	N.S.
3 vs. 8	6.317	< 0.005	**				
vs. 7	4.115	> 0.05	N.S.				

DEC-F APR-F FEB-F APR-M FEB-M MAR-F MAR-M DEC-M

CHAPTER III

CONTROL OF SPERMATOGONIAL PROLIFERATION BY POLYAMINES

Introduction

The identification and investigation of mechanisms underlying the regulation of cellular proliferation are fundamental to understanding the cell cycle of cells undergoing preparation for mitosis. Specifically, mechanisms regulating the commitment of a cell to undergo DNA synthesis and subsequent mitosis are incompletely known. Pardee (1974) proposed that proliferation in normal (i. e., non-transformed) cells is generally regulated by a restriction point in G_1, which limits growth in sub-optimal conditions. There is evidence that activity of ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine biosynthesis, is induced in G_1, near Pardee's restriction point, by a wide variety of mitogenic stimuli (for review see Magun and Gerner 1981). ODC activity shows a transient maximum in late G_1 and a second peak in G_2 (Fuller et al. 1977). The polyamines, spermidine and spermine, and their precursor, putrescine, have frequently been implicated as serving a regulatory role in proliferation in a wide variety of cell systems (e. g., Dion and Cohen 1972; Fillingame et al. 1975; Fuller et al. 1977; Kusunoki and Yasumasu 1978; Haddox and Russell 1981; McCann et al. 1981; Sunkara and Rao 1981). Polyamine synthesis, which can be fairly reliably predicted from ODC activity, therefore constitutes an early and consistent marker for the commitment of a cell to undergo mitosis (Haddox and Russell 1981). Because polyamine synthesis is necessary for DNA synthesis, and hence for

mitosis, to occur and because it forms an early marker for commitment to mitosis, the polyamines are likely candidates for a regulatory role. In this paper, I investigate the possibility that polyamines serve a regulatory function during spermatogenesis in the seastar Asterias vulgaris. Preliminary experiments (see Chapter IV) indicated that ornithine decarboxylase activity and hence polyamine synthesis are closely correlated with the proliferative phase of spermatogenesis. This relationship suggests that in asteroids, as in many other animals, polyamines are related to cell division.

In the testes of Asterias vulgaris, spermatogonia form a population of essentially amitotic cells that contemporaneously enter the proliferative phase of spermatogenesis, producing several trillion primary spermatocytes in a relatively short time. Because initiation of proliferation is initially separated temporally from meiosis and differentiation, the asteroid testis forms a useful model for examining controls over spermatogenic proliferation and also the initiation of mitosis in a mitotically quiescent system (Walker 1980). Although environmental (Pearse and Eernisse 1982; Pearse and Walker, in press), hormonal (Voogt et al. 1984), and cytological (Walker 1980) conditions have been investigated, proximate biochemical events influencing the initiation of spermatogonial mitosis in these animals are unknown.

Because polyamines are both necessary for cell division and are also an early marker for commitment to mitosis, they become likely candidates for a mitotic control point (see Magun and Gerner 1981; Sunkara and Rao 1981). Presumably, as has been shown in other animals, polyamines are essential for nucleic acid synthesis in asteroids; in other words, they must be present for DNA synthesis, and hence mitosis,

to occur. Furthermore, polyamines may be not merely necessary but also sufficient for the initiation of DNA synthesis in mitotically quiescent cells. If polyamine synthesis serves a regulatory role, then the presence of polyamines should satisfy a regulatory constraint and promote an increased spermatogonial mitotic rate, regardless of whether the polyamines are formed intrinsically by the normal enzymatic pathway or are supplied from an extrinsic source. Implicit in this argument is the assumption that exogenous and endogenous polyamines similarly influence the cell cycle of target cells. Goyns and Hopkins (1982) have suggested that the action of exogenous putrescine may be distinct from that of endogenous putrescine; most other researchers, however, have concluded that actions of exogenous and endogenous putrescine are sufficiently similar for this assumption to be considered reasonable.

Subject to the assumption mentioned above, the role of polyamines in regulating initiation of proliferation can be tested by supplying extrinsic polyamines to testes near the beginning of the proliferative phase, when spermatogonia are mitotically quiescent but competent, and then examining the rate of DNA synthesis measured by incorporation of ^{3}H-thymidine. Testes in the late aspermatogenic stage, at or near the beginning of the proliferative phase of spermatogenesis, provide the most desirable cells to be used in an investigation into events regulating the initiation of spermatogonial mitosis. In a testis from this time of year (approximately the month of October in the Gulf of Maine), the population of spermatogonia is presumably mitotically competent and is ready for commitment to proliferation, except for one or more regulatory requirements which keep the cells in early G1 (Walker and Larochelle 1984). Testes were incubated for at least 24

hours in the presence of mixed polyamines and putrescine and DNA synthesis measured by incorporation of tritiated thymidine.

Materials and Methods

Specimens of Asterias vulgaris collected from the Gulf of Maine by divers during September 1983 and October 1984 were maintained in the laboratory at ambient ocean temperature and were allowed to feed ad libitum on mussels. All injections and incubations were carried out in artificial coelomic fluid (ACF) based on MBL artificial sea water (Cavanaugh 1956) with salt concentrations modified to correspond with reported values from A. vulgaris coelomic fluid (Boolootian 1961) and with the addition of amino acids and glucose in concentrations similar to those indicated by Ferguson (1964, 1975a) as characteristic of seastar coelomic fluid. Final concentrations used appear in Table 7. The pH of this incubation medium was adjusted to approximately 7.2 with hydrochloric acid, penicillin and streptomycin (DIFCO Laboratories) were added to final concentrations of 100 units or µg per ml, and the solution was sterilized by filtration through a 0.25 µm Millipore filter.

In the first experiment, a total of 23 specimens were divided randomly into two groups. Animals were injected every other day for two weeks (total of 7 injections). Control specimens received sham injections of 16 µl artificial coelomic fluid per gram wet mass; animals in the other group received a similar volume of artificial coelomic fluid containing 50 nmol putrescine, 10 nmol spermidine, and 10 nmol spermine per gram wet mass. These polyamine concentrations were selected in an attempt to simulate concentrations subsequently

used in organ culture media. After two weeks of injections, animals were sacrificed and sexed. Isolated testes from each male specimen were incubated for 24 hours at 11° C in both artificial coelomic fluid and artificial coelomic fluid containing 50 μM putrscine, 10 μM spermidine, and 10 μM spermine. After an initial 24-hour incubation, 1 μCi/ml tritiated thymidine ([methyl-^{3}H]-thymidine, 6.7 Ci/mmol, New England Nuclear) was added and the testes were incubated an additional 24 hours.

For the second experiment, isolated testes from 15 male seastars were divided into four groups in a randomized block design (fifteen blocks crossed by one within-block factor with four levels). Each group, containing two or three testes from a single animal, was incubated for two days according to one of the following protocols: ACF both days; ACF with mixed polyamines (concentration as in first experiment) both days; ACF with polyamines on one of the two days and ACF without polyamines on the other. The third day of incubation used the same type of medium as the second day, with the addition of 1 μCi/ml tritiated thymidine.

After incubation in both experiments, testes were transferred to 0.5N perchloric acid and homogenized. The resulting suspension was centrifuged in an IEC Centra-7R refrigerated centrifuge (1650 g), and the pellet washed with 5% perchloric acid followed by absolute ethanol. The precipitate was then resuspended in 0.5 N KOH and heated at 37° C for one hour to hydrolyze RNA. DNA was again precipitated with perchloric acid and hydrolyzed from the resulting pellet by heating 15 minutes at 70° C in 0.5 N perchloric acid. Concentration of DNA was estimated by the diphenylamine reaction (Burton 1968) with calf thymus

DNA as a standard; tritiated thymidine incorporation was determined by scintillation counting in a Beckman LS-7000 liquid scintillation counter.

One testis from each animal was prepared for histological examination. Testes from the first experiment were fixed in Bouin's fluid, embedded in paraffin, and sectioned at 7 μm on a rotary microtome. Testes from the second experiment were fixed according to Walker (1979) with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer containing a balanced salt solution, post-fixed in 1% osmium tetroxide, embedded in Epon-Araldite, and sectioned at 0.5 to 1 μm using glass knives on a Reichert OMU3 ultramictrotome.

Specific activities of ^{3}H-thymidine labelled DNA were expressed as disintegrations per minute per microgram of DNA. Results from the first experiment were analyzed by analysis of variance and a paired t-test; results from the second experiment were subjected to two-way analysis of variance in accordance with the randomized block design used in that experiment.

Examination of residuals from analysis of variance for the second experiment suggested that a logarithmic transformation would help attain equal variances over the range of predicted thymidine incorporation rates. Findings of significance were not affected by this transformation, although precise tail probabilities changed slightly. For consistency, data from the first experiment were also subjected to logarithmic transformation. Tail probabilities for both untransformed and log-transformed data are given below and in Tables 8 and 10.

Results

First Experiment

One of the 23 animals autotomized two rays during the injection period and was discarded. Among the remainder, eleven were males; four of these had received sham injections, the other seven had received polyamine injections. Specific activities of thymidine-labelled DNA are shown in Table 8. A one-tailed paired t-test comparing incubation in the presence of extrinsic polyamines to incubation in polyamine-free medium shows a significant increase in the rate of thymidine incorporation into DNA when polyamines are present ($p=0.0132$, for log-transformed data $p=0.0308$). Prior injection of polyamines shows no significant effect ($p=0.4393$, for log-transformed data $p=0.2628$). DNA synthetic rate shows an average increase of approximately 50% in the presence of polyamines.

Second Experiment

Specific activities and transformed values are shown in Table 9; results of analysis of variance and Student-Newman-Keuls multiple range test are shown in Table 10. Analysis of factor effects by the Student-Newman-Keuls multiple range test indicates that none of the incubations with polyamines can be statistically distinguished from another, and incubation with polyamines on both days does not differ significantly from the control group incubated in the absence of polyamines; however, both incubations with polyamines on only one of the first two days show significantly greater incorporation of thymidine than the control group.

Discussion

Although an *in vitro* experimental protocol necessarily introduces artificiality to the chemical microenvironment of the testes, there are several overriding advantages. First, an *in vitro* protocol allows use of defined media. Exogenous signals can therefore be controlled precisely or eliminated, thereby helping to minimize variations among specimens as well as enabling definition of those exogenous factors that influence the germinal epithelium. In addition, rapid and precise changes in the contents of defined culture media allows selective alteration of the chemical microenvironment of cultured tissues; this selective alteration facilitates investigation of mechanisms and dynamics of regulatory phenomena. Second, an *in vitro* protocol allows the use of multiple testes (ten in *Asterias vulgaris*) in a randomized block design in which different testes from the same animal receive different experimental treatments. The randomized block design is extremely helpful for detecting differences attributable to organ culture medium. There is often considerable variability among animals, so that comparisons of different animals is difficult, at best. By using some gonads from each specimen in both experimental and control treatments, each specimen is treated as a separate block, thus accounting for some of the variability among the specimens. The remaining variability may be attributable in part to differences in the precise spermatogenic stage of an animal, or even to minor differences between testes of a single animal. To some extent, variability attributable to differences among or within animals may by reduced by placing specimens into blocks prior to experimentation on the basis of cytological observations; these observations can be made on fresh,

living tissue with interference optics. Whether these observations could be sufficiently precise and informative to significantly improve efficiency of blocking compared to treating each animal as a separate block is not known at this time.

The first experiment combined *in vivo* and *in vitro* protocols. The original intent of this experiment was to examine the effect on DNA synthesis resulting from exposure to extrinsic polyamines over both a long period (two weeks) and a short period (two to three days). Because of the difficulty involved in maintaining asteroid testes *in vitro* in a defined medium for several weeks (Walker, in press), prolonged exposure to extrinsic polyamines was accomplished by intracoelomic injection. It has been shown that material injected intracoelomically is rapidly distributed throughout the perivisceral coelom and is therefore available to all internal organs (Ferguson 1964, 1970). For short term exposure to extrinsic polyamines it was possible to use an *in vitro* protocol with its associated advantages.

Results from the *in vitro*, randomized block portion of the first experiment strongly support the conclusion that supplying extrinsic polyamines results in an increased rate of DNA synthesis. As mentioned, prior intracoelomic injection of polyamines showed no sustained effect on thymidine incorporation. After 24 hours in polyamine-free medium, thymidine incorporation was indistinguishable statistically from that of control-injected animals. This lack of a sustained effect from polyamine injection has at least two possible causes. First, polyamine concentration might not have been maintained above threshold levels. Polyamines could have been lost by leakage through the body wall after injection, by rapid diffussion through the

body wall, or by preferential uptake by non-testicular tissue. Second, any effect of polyamines might be transient and readily reversible, so that over the period of a two to three week experiment only the presence or absence of polyamines on the final day significantly affects the DNA synthetic rate. If the effect of extrinsic polyamines on DNA synthesis is in fact transient and reversible, then the presence of polyamines has not induced a fundamental change in the genetic "program" of spermatogonia: the spermatogonia are still mitotically quiescent rather than actively proliferating. Instead, reversibility would suggest that the polyamines have simply satisfied a regulatory requirement in the existing program.

However, the original experimental design was not designed to test for reversibility of the polyamine effect; the second experiment was conducted with the specific intent of testing for a reversible effect of extrinsic polyamines on DNA synthesis. In order to ensure that a known concentration of polyamines was supplied to the testes and to take full advantage of randomized block design, the second experiment used _in vitro_ protocols entirely. Analysis of variance and the Student-Neuman-Keuls multiple range test confirmed that rate of DNA synthesis increases in the presence of extrinsic polyamines. In addition, this increase is retained after 24 hours incubation in the absence of polyamines, strong evidence against the hypothesis of rapid reversibility. Hence, the first explanation for lack of effect from injection is more likely; the injection procedure used may not have delivered polyamines in adequate concentration to the testes.

The point estimate of mean thymidine incorporation is highest for testes which were incubated in the presence of polyamines on the first

day only. Although the statistical evidence is inconclusive, point estimates of mean thymidine incorporation decline as the total number of days exposure to extrinsic polyamines increases from one to three. Although this trend may simply result from sampling variability, if it is real, it may indicate a slight long-term cytotoxic effect of polyamines at the concentrations used. It would be useful, therefore, to examine possible cytotoxic effects of polyamines on echinoderm cells; a seastar oocyte bioassay (Shirai 1974) would be an appropriate method. In addition, examination of normal physiological levels of polyamines in proliferating asteroid spermatogonia would allow selection of possibly more meaningful concentrations of exogenous polyamines. Cytochemical localization of polyamines, both endogenous and exogenous, would help to further clarify the role of polyamine synthesis in the physiology of asteroid spermatogonia.

In summary, supplying extrinsic polyamines results in an increased rate of DNA synthesis. Similar enhancements of proliferative rate have been obtained by the addition of putrescine alone to cultures of Chinese hamster kidney cells (Ham 1964) and human fibroblasts (Pohjanpelto and Raina 1972). The effect observed here does not appear to be readily or rapidly reversible after incubation with extrinsic polyamines for 24 hours. The observed increase in thymidine incorporation may be interpreted as an increased rate of spermatogonial commitment to proceeding through the mitotic cell cycle. My findings thus support the hypothesis that polyamine synthesis serves a regulatory function: supplying the products of the synthesis pathway apparently satisfies a constraint upon otherwise mitotically competent cells, enhancing progress into the S phase of the cell cycle.

Table 7. Artificial coelomic fluid. Major components are added in solid form; trace components are first dissolved to make stock solutions. Stock solutions are 0.1 M, except for tryptophan (10 mM). Indicated amounts of major components and trace component stock solutions are combined and dissolved in distilled water to make a final volume of one liter.

A. Major Components

	Concentration	
	mmol/l	g/l
NaCl	425.0	24.8323
KCl	8.8	0.6535
$CaCl_2 \cdot 2H_2O$	8.6	1.2630
$MgCl_2 \cdot 6H_2O$	18.5	3.7606
$MgSO_4 \cdot 7H_2O$	25.5	6.2850
$NaHCO_3$	2.13	0.1789
Glycine	1.45	0.1088

B. Trace Components

	Stock g/100ml	Final Conc. µmol/l	ml Stock to make 1 l of ACF
Taurine	1.251	323.5	3.24
Serine	1.051	100.0	1.00
Phenylalanine	1.652	100.0	1.00
Arginine	2.107	40.0	0.40
Glucose	1.802	40.0	0.40
Tryptophan	0.204	1.0	0.10

Table 8. Thymidine incorporation after exposure to extrinsic polyamines, *in vivo* and *in vitro*. Specific activity of thymidine incorporated into DNA is given as dpm/μg DNA; also shown are data after logarithmic transformation ($x' = \ln(x)$). A one-tailed paired t-test is shown for both sets of data, testing for greater incorporation of thymidine in the presence of polyamines in incubation media. A two-sample t-test comparing specimens injected with polyamines to those injected without polyamine-free medium is also given for both untransformed and logarithmically transformed data.

	UNTRANSFORMED		LOGARITHMIC TRANSFORMATION	
INJECTION	INCUBATION		INCUBATION	
POLYAMINES	POLYAMINES	CONTROL	POLYAMINES	CONTROL
-	8.85	25.10	2.181	3.223
-	52.85	34.50	3.968	3.541
-	63.33	14.08	4.148	2.645
-	4.15	4.44	1.424	1.491
+	58.84	33.66	4.075	3.516
+	6.40	4.12	1.857	1.416
+	33.99	14.31	3.526	2.661
+	34.00	26.86	3.526	3.291
+	42.76	20.43	3.756	3.017
+	41.20	30.28	3.718	3.410
+	21.27	14.31	3.057	2.661

PAIRED T-TEST		
Mean Difference (d):	13.23	0.397
Standard Deviation (s_d):	5.09	0.189
T-Score (t):	2.602	2.104
Tail Probability (p):	0.0132 *	0.0308 *

TWO-SAMPLE T-TEST	Mean	S.D.	Mean	S.D.
Injected with polyamines:	27.32	15.1	3.106	0.744
Control injection:	25.91	22.6	2.828	1.06
T-Score (t):	0.157		0.655	
Tail Probability (p):	0.439 (N.S.)		0.263 (N.S.)	

Table 9. Thymidine incorporation (dpm/μg DNA) after exposure to extrinsic polyamines *in vitro* for one or two days. Treatments are: (1) Control, no polyamines; (2) Polyamines on day one only; (3) Polyamines on day two only; (4) Polyamines on both days one and two. Tritiated thymidine added on third day, with polyamines present or absent according to medium used on second day.

A. SPECIFIC ACTIVITY (dpm/ g DNA)

ANIMAL	TREATMENT				ROW
	1	2	3	4	MEANS
1	2048.	1273.	2235.	2655.	2053.
2	421.	817.	1331.	144.	678.
3	312.	906.	364.	573.	539.
4	741.	813.	563.	859.	744.
5	445.	1634.	1961.	934.	1244.
6	288.	956.	667.	610.	630.
7	214.	901.	501.	798.	604.
8	578.	586.	571.	186.	480.
9	327.	1000.	340.	599.	567.
10	240.	367.	701.	174.	370.
11	71.	361.	113.	402.	237.
12	62.	103.	171.	33.	92.
13	231.	437.	296.	301.	316.
14	104.	193.	182.	245.	181.
15	583.	421.	337.	473.	454.
COL. MEANS	444.	718.	689.	599.	613.

POOLED ST. DEV. = 310.

B. LN(SPECIFIC ACTIVITY)

ANIMAL	TREATMENT				ROW
	1	2	3	4	MEANS
1	7.624	7.149	7.712	7.884	7.592
2	6.042	6.706	7.194	4.970	6.228
3	5.743	6.809	5.897	6.350	6.200
4	6.607	6.701	6.333	6.756	6.599
5	6.099	7.399	7.581	6.840	6.980
6	5.662	6.863	6.502	6.414	6.360
7	5.366	6.803	6.218	6.682	6.267
8	6.360	6.373	6.348	5.227	6.077
9	5.789	6.907	5.830	6.396	6.231
10	5.479	5.905	6.552	5.158	5.773
11	4.256	5.888	4.731	5.997	5.218
12	4.121	4.633	5.144	3.506	4.351
13	5.442	6.080	5.691	5.707	5.730
14	4.648	5.264	5.206	5.500	5.154
15	6.368	6.043	5.821	6.160	6.098
COL. MEANS	5.707	6.368	6.184	5.970	6.057

POOLED ST. DEV. = 0.510

Table 10. Analysis of variance and Student-Newman-Keuls multiple range test of data in Table 9. Multiple range test performed only on treatment levels of log-transformed data.

ANALYSIS OF VARIANCE: UNTRANSFORMED DATA

DUE TO	DF	SS	MS=SS/DF	F-VALUE	P	
ANIMAL	14	13154834.	939631.	9.77	<<0.0005	***
TREATMENT	3	681722.	227241.	2.36	0.05<p<0.10	N.S.
ERROR	42	4039928.	96189.			
TOTAL	59	17876485.				

ANALYSIS OF VARIANCE: LOG-TRANSFORMED DATA

DUE TO	DF	SS	MS=SS/DF	F-VALUE	P	
ANIMAL	14	33.353	2.382	9.13	<<0.0005	***
TREATMENT	3	3.645	1.215	4.66	0.005<p<0.01	**
ERROR	42	10.943	0.261			
TOTAL	59	47.941				

MULTIPLE RANGE TEST: ANALYSIS OF TREATMENT EFFECTS (LN(SPEC. ACT.))

COMPARE	DIFFERENCE	S.E.	q	r	Tail Prob. (p)	
2 vs. 1	0.661	0.132	5.015	4	0.001<p<0.005	**
2 vs. 4	0.398	0.132	3.020	3	0.05 <p<0.01	N.S.
2 vs. 3	0.184				DO NOT TEST	
3 vs. 1	0.477	0.132	3.619	3	0.05 <p<0.025	*
3 vs. 4	0.214	0.132	1.624	2	0.5 <p<0.2	N.S.
4 vs. 1	0.263	0.132	1.996	2	0.2 <p<0.1	N.S.

(where q is the test statistic, r is the number of means in range being compared)

Treatments ranked low to high: 1 4 3 2

Means not significantly different are indicated by lines beneath.

CHAPTER IV

CONCLUSIONS

The patterns described in Chapter I for principal biochemical components of the testes show clear correlations to structural changes in the germinal epithelium during spermatogenesis. A number of conclusions about changes in biochemical microenvironment of germinal cells can be drawn from examination of those patterns (see Chapter I). Of particular applicability to the regulation of proliferation are the patterns observed for protein and nucleic acids. Total DNA content suggests that DNA synthesis begins at or near the beginning of the proliferative phase in October and is essentially complete by the end of March. However, RNA and protein both begin to increase slightly earlier, representing prior preparation for mitosis. Changes in activity of ornithine decarboxylase (Chapter II) parallel the presumed pattern of DNA synthesis. ODC activity increases in October and remains high until late March, when it declines to the lower values maintained throughout the aspermatogenic phase. Thus, polyamine synthesis correlates well with DNA synthesis, as expected from the well-documented dependence of DNA synthesis on the presence of polyamines (Haddox and Russell 1981; Sunkara and Rao 1981). Furthermore, experiments presented in Chapter III indicate that polyamines are sufficient stimulus to enhance commitment to proliferation in testes at or near the beginning of the proliferative phase of spermatogenesis. Therefore, it is likely that polyamines are involved in regulating spermatogonial mitoses.

The evidence that polyamines serve a role in regulating the initiation of mitotic proliferation in the asteroid testis can be combined with several other lines of evidence for other regulatory events to formulate an overall model. The model proposed here for regulation of spermatogonial proliferation is largely speculative at this time; in particular, there is currently no evidence showing causal linkage between these events. Nevertheless, this hypothesized mechanism provides useful guidance for subsequent research into the regulation of spermatogenesis.

Entrainment of gametogenic cycle is presumably accomplished by some environmental cue such as light cycle, temperature, or salinity. In Pisaster ochraceus (Pearse and Eernisse 1982) and Asterias vulgaris (Pearse and Walker in press), day length has been shown to be at least one of the functional environmental cues responsible for the entrainment of gametogenesis throughout the population. Such an environmental cue, perhaps modulated by others (such as temperature) may then be transduced into a metabolically interpretable signal by way of an unidentified neurohormone which acts upon somatic cells of the gonad (Voogt, personal communication). Alternatively, sufficient light may pass through the body wall to directly influence steroid synthesis.

There are distinct changes in progesterone and estrogen levels in the testis associated with the beginning of the proliferative period (Voogt and Dieleman 1984). Newly synthesized steroids, or the change in relative amounts of progesterone and estrone, may act upon spermatogonia in the late aspermatogenic phase, activating some metabolic pathway. It is known that later in the proliferative phase, estrogen induces or accelerates further mitotic divisions (Takahashi

1982); it may act similarly at the beginning of the proliferative phase. Steroids may induce polyamine synthesis, either directly or indirectly, as has been shown to occur in other systems (Cohen et al. 1970; Kaye et al. 1971). Synthesis of polyamines thereby satisfies an existing regulatory constraint upon the spermatogonia, and they proceed through S phase and, ultimately, mitosis.

The phenotypes expressed by somatic or germinal cells in the germinal epithelium may be influenced by the relative numbers and associations among those cells (Walker 1980; Walker and Larochelle 1984). The induction of spermatogonial mitoses results in an increased number of germinal cells; consequently, somatic accessory cells may change from the phenotype in which they surround spermatogonia, forming "follicles", to the form in which they act as the axial cell of a spermatogenic column, and they then begin to organize the primary spermatocytes into columns. Release from containment within follicles may induce or allow spermatogonia to undergo subsequent mitoses, which they continue to do until nutrient stores in the genital haemal sinus are exhausted. The organization of primary spermatocytes into columns may be sufficient stimulus to initiate pre-meiotic interphase, leaving the primary spermatocytes suspended in prophase of the first meiotic division until some signal allows them to proceed.

This model thus requires only a triggering event to initiate proliferation and is subsequently self-maintaining. This is compatible with the observation (Voogt and Dieleman 1984) that the steroid signal which may be the intra-gonadal trigger is transient, with no evidence of prolonged high steroid levels needed to maintain gametogenesis.

APPENDIX A

THE THEORETICAL BASIS OF AND STATISTICAL ALTERNATIVES TO ORGAN INDICES

The organ index, defined as 100 times the ratio of organ mass to whole animal mass (using either wet or dry masses) (Giese 1966), has been frequently, perhaps even universally, used in studies of biochemical composition of invertebrate body components for at least the last twenty years (e. g., Farmanfarmaian et al. 1958; Giese et al. 1959; Boolootian 1966; Lawrence 1973; Jangoux and Vloebergh 1973; Jangoux and van Impe 1977; Oudejans and van der Sluis 1979; van der Plas and Voogt 1982). It is a computationally straight-forward and intuitively satisfying means of correcting for the effect of animal size on organ size; it thereby purportedly allows direct comparison among animals of different sizes. Given the variability of animal sizes in a sample of conspecifics taken from the field, correction for difference in size is ultimately essential. Furthermore, organ indices seem to have worked quite well. For example, in several studies, predictions about reproductive patterns have been made on the basis of patterns observed in gonad indices, and these predictions have been supported by observations using other methods (e. g., the observation by Schoenmakers and Goedhart (1979) that ovary index is a good predictor of reproductive state).

Use of organ indices is based upon several assumptions which, although reasonable, are not certain a priori. Hence, the necessary assumptions should be tested before organ indices are used as a measure

of organ size in an attempt to correct for differences in animal size. So far as I am able to determine, every author (at least in the echinoderm literature) with the exception of Gonor (1972) either tacitly accepts those assumptions or does not explicitly test them -- it is often very difficult to tell which. In addition, many authors have then proceeded to calculate and graph 95% confidence intervals around the mean organ index. Although, again, it is very difficult to determine precisely how those confidence intervals were calculated, it is possible (and seems extremely likely) that they were calculated using the well-known equation: mean $\pm$ t*standard error. However, this equation is not valid for percentages. Percentages must first be transformed by the variance-stabilizing function $\arcsin(\sqrt{X})$ before any parametric statistics are calculated; this transformation is especially important for percentages near 0% or 100%. Thus it may be the case, but is clearly not certain, that organ indices have at least occasionally been used when they are not justified, or have been treated by invalid statistical methods. It should be noted, nevertheless, and in defense of those authors who have done so, that even if organ indices are not justified or are analyzed incorrectly, the results obtained may be approximately correct if certain other requirements are met.

My purpose in this appendix is to carefully and critically examine the theoretical background of the organ index and the principal assumptions underlying its use. I will also propose specific techniques which may be used in place of organ indices if the latter are found to be unjustified and provide, as an example, an analysis of gonad dry mass using data derived from animals used in Chapter I.

The fundamental rationale for using an organ index is that organ size is a strictly increasing monotonic function of animal size; that is, the bigger the animal, the bigger its organs, other things being equal. Because any sample of animals will include a range of animal sizes (with variability depending on the species and method of collection), comparison of organs within a sample or among several samples is much easier if the data are normalized relative to animal size. In the literature, this consideration often leads to discussion of a "hypothetical" animal of a given size (see, for example, Oudejans and van der Sluis 1979; van der Plas and Voogt 1982). (These hypothetical animals are really just organ indices in disguise, so all comments concerning organ indices apply equally to "hypothetical animals".) However, the use of an organ index further requires that the index be constant over the entire range of animal sizes investigated, other things being equal. In other words, organ size must not merely increase with animal size, but must be strictly proportional. If organ size is not proportional to body size, then the organ indices of animals of different sizes will not be equal. As the relationship between organ and animal sizes departs further from strict proportionality, an organ index becomes a progressively poorer approximation of "reality". Use of an organ index when it is not justified could increase the perceived variability of the data, thereby hiding an existing effect, or produce a spurious effect which should be, but probably would not be, ascribed solely to differences in animal size (see Gonor 1972).

The simplest variation from strict proportionality arises if organ size is an affine function of body size: that is, the graph of organ

size against body size is a straight line but does not pass through the origin. In this case, an organ index is not justified, and the amount of error introduced depends upon the relative magnitude of the Y-intercept. Consider the following hypothetical case: organ mass is an affine function of body mass and can be accurately described by the equation

$$\text{organ} = 1 + 0.1 * \text{body} \qquad \text{(A.1)}$$

Two animals are examined: one with a mass of 50, the other with a mass of 100; both animals precisely fit Equation A.1 (no sampling variability). Then the animals will have organ masses of 6 and 11, respectively, giving organ indices of 12% and 11% -- a sizable difference, especially considering that both animals can be precisely described by the same equation. Thus, use of an organ index in this case introduces variability where none actually exists.

The actual relationship between organ and body size cannot be predicted <u>a priori</u>: it may or may not be a strict proportionality. That relationship probably is a smooth function, both continuous and continuously differentiable, although it is certainly conceivable that the relationship might not be continuously differentiable. For example, an animal's gonads might be extremely small in immature specimens, and large in mature specimens; if sexual maturity is attained by all animals in the population at a distinct threshold body size, one might expect a sudden change in the relationship between gonad and body size at that threshold, with a discontinuous derivative at that point. However, if the organ-body relationship is continuously differentiable over the range of body sizes considered, and if that range is sufficiently narrow, the actual relationship will not differ

significantly (in a statistical sense) from the affine function tangent to the true relationship at the mean body size. Hence, provided that the range of body sizes is sufficiently small, one can approximate the organ-body relationship with an affine function. Nevertheless, if the Y-intercept of that affine function differs significantly from zero, use of a simple organ index would be an approximation of an approximation, at best, and could introduce unacceptable error.

The question then arises: how can one determine whether or not the data being examined allow use of an organ index, and if they do not, what alternative approach will allow valid analysis of the data. First, consider the simple case in which variation in gonad size can legitimately be ascribed entirely to variation in animal size plus sampling variability; that is, all specimens are collected from the same population, and if the organ in question is subject to seasonal variation, all specimens are collected at the same time or the same seasonal state. Animals from multiple populations or sampling dates can be separated into size classes and comparisons of organ size made within size classes to determine whether or not sampling location or time significantly affect organ size. Then, the relationship between organ size and body size can be examined through regression techniques. Initially, the data can be fitted to a simple linear (affine) function, i.e., organ = a + b * body. There are four possible outcomes for this regression:

	POSSIBLE OUTCOME			
	I	I	III	IV
Significant	a	b	a,b	-
Not Significant	b	a	-	a,b

In addition, if the data set allows (i.e., if there are multiple samples for at least some body sizes), one can test for linearity of

the relationship. If one is permitted to assume linearity, then each combination of significant regression coefficients has a slightly different interpretation:

I. Y-intercept significant, slope not significant. Organ size is essentially constant regardless of body size, and may be used directly.

II. Slope significant, Y-intercept not significant. Organ size is directly proportional to body size. An organ index is usable and is equal to the value determined for slope. If an organ index is used for statistical manipulations, values must first be transformed by an arcsin transformation. However, there are specific statistical tests which can be applied to slopes of regression lines; e. g., one can readily compare slopes of two or more regression lines (see Zar 1974, Chapter 17).

III. Both slope and y-intercept significant. Relationship is affine, and organ indices are inappropriate. Comparisons among samples are possible by analysis of covariance.

IV. Neither slope nor y-intercept are significant. Organ size, then is not significantly different from zero. Either something very strange is happening, or there is a very large sampling variability. In either case, there may not be much that can be done other than increase sample size in the hope of decreasing the effect of variability.

If the organ-body relationship differs significantly from linearity, two approaches are possible. First, as noted above, a sufficiently restricted range of body sizes will result in the observed relationship not differing statistically from a straight line.

Therefore, the range of observed body sizes could be restricted by elimination of extreme values until deviations from linearity become non-significant. In effect, one throws out data so that a straight line becomes "close enough". The disadvantages should be readily apparent, but even with those disadvantages, it may be the technique of choice. Alternatively, one can attempt to approximate the curvilinear relationship by using some other function. Choice of function, unless guided by some a priori theoretical consideration, is largely a matter of aesthetics. Depending upon the observed form of the data, one might choose to try polynomials, logarithmic, exponential, power, or any other suitable function. Analysis based on theoretical descriptions of allometric growth (Perkkiö and Keskinen 1985) could be particularly effective. Another potentially useful approach would be to fit a variety of functions and select the one which provides the best fit without introducing numerous predictor variables. If one is attempting to compare two or more samples, one could find a class of function which adequately describes each sample and then proceed by analysis of covariance. Alternatively, one could use analysis of variance of the data split into size classes to test for differences among samples without specifying the form of the organ-body relationship.

A frequent use of organ indices has been the description of seasonal changes in organ size. In this instance, where the investigator is attempting to explain variations in organ size by some factor in addition to animal size, analysis is complicated by the requirement to simultaneously account for variations resulting from both time and animal size. If the investigator is interested only in detecting differences among time periods and/or size classes, analysis

of variance or covariance is adequate. However, detailed quantitative description requires multivariate regression techniques, with organ size described as a function of both time and body size.

A potentially valuable method of describing cyclic changes in organ size over time without recourse to organ indices entails an adaptation of periodic regression as used extensively in Chapter II. For a given body size, cyclic changes in organ size can often be described very precisely by periodic regression; at a given time in the cycle, organ size can often be adequately described by a relatively simple function, such as a polynomial of low degree. These two relationships can be easily combined by considering each parameter of one relationship to be a function of the other variable. This approach makes use of the fact that non-linear relationships can often be approximated quite well by polynomials of relatively low degree; analysis is facilitated by the simplicity of the functions. For example, if organ size can be described by the simple periodic function

$$O = M + A \cdot \cos(\omega t - \phi), \qquad (A.2)$$

the parameters M, A, and ϕ (possibly even ω) can all be considered functions of body size. A compound regression function could then be determined by forward selection of predictors, using polynomial regressions for parameters of the periodic regression. For example, one might find that organ size is well described by an equation of the form

$$O = M_0 + M_1 B + M_2 B^2 + (A_0 + A_1 B)\cos(\omega t - \phi) \qquad (A.3)$$

Although not difficult, proper application of this technique could be extremely tedious and time-consuming, especially if the full periodic function (with parameters of skewness and peakedness) is used. In

addition, the number of predictors could rapidly increase; consequently, a large data set may be essential. However, the results could be very interesting, and the parameters biologically meaningful. For example, in an analysis of gonad sizes one could find that acrophase varies with body size, possibly suggesting that certain size classes breed earlier than others.

As a relatively simple example of this procedure, testicular dry mass for animals from Chapter I (see data in Appendix F) was analyzed by regression on both date and animal mass. The simple sinusoidal function of Equation A.2 was algebraically transformed into a form suitable for linear regression (Equation D.29). The three parameters, M, X, and Y, were expressed as quadratic functions of animal mass, yielding the regression function:

$$y = b_0+b_1M+b_2M^2 + (b_3+b_4M+b_5M^2)\cos(\omega t) + (b_6+b_7M+b_8M^2)\sin(\omega t), \qquad (A.4)$$

where M = animal mass. This function was fitted by stepwise regression (F to enter and F to remove both set equal to 4.0). Five parameters differed significantly from zero: b_0, b_4, b_5, b_6, and b_8. The final model has an R^2 of 97.14%, an extremely good fit. (For comparison, simple periodic regression of testicular dry mass index has an R^2 of only 72.2%.) Consequently, gonad mass is not strictly proportional to animal mass, at least at some times, and a gonad index may not be acceptably accurate. Gonad mass can be much better described as a function of both animal mass and date:

$$y = 0.339 + (0.00564\cdot M-4.363E-5\cdot M^2)\cos(\omega t) + (0.233+7.43E-5\cdot M^2)\sin(\omega t).$$

A more detailed analysis could, possibly, determine a functional dependence of testicular mass on animal mass and date even more precise

than this relatively simple one. However, the improvement gained by this simple modification of the analysis is striking.

Because forward selection of regression parameters can be readily automated in a computerized regression program (although the BMDP program library does not include automatic selection of parameters for non-linear regressions), the lengthy process of analyzing data by the technique suggested here is not a major disadvantage. A suitable program could be allowed to run unattended (overnight, perhaps?) and the final regression function produced for later examination.

Organ indices have also often been used to relate changes in total content of a component among animals in a range of sizes. This use is often seen as the representation of amount of component in the organ of a "hypothetical animal" of specified size. Provided organ indices are appropriate for the organ in question, this technique can be valuable. However, if organ indices are not appropriate, any interpretation of results becomes problematical. An alternative approach would be to use the regression analysis described above. For a given animal, the discrepancy between observed organ size and predicted organ size would be noted and used to adjust the predicted organ size of a "hypothetical animal". Thus, animals of different sizes can be compared by correcting organ size in accordance with the relationship between organ and body size that was empirically determined. For example, suppose a 50 g animal collected in January has gonads with a total mass of 12 g. Regression analysis of the population from which that animal was taken indicates that a typical 50 g animal has a total gonad mass of 10 g in January, whereas a typical 100 g animal has a total gonad mass of 25 g at the same time of year. Note that in this case, the gonad index

approach is clearly inappropriate; gonad mass is not proportional to animal mass. However, the animal in question has a gonad mass 1.2 times that of a typical animal of its size; a reasonable assumption might then be that <u>if</u> the animal had had a body mass of 100 g, its gonads would have weighed 1.2 times as much as those of a typical 100 g animal, or 30 g. Subsequent calculations could be based on this estimate of gonad size in a comparable "hypothetical" 100 g animal. The multiplicative relationship used in this example seems most likely to me; however, the relationship between observed and expected organ size could just as easily be additive, in which case the "hypothetical 100 g animal" corresponding to the observed 50 g animal would have a gonad mass of 27 g (expected gonad mass of 100 g animal + difference between observed and expected 50 g animal). Additive and multiplicative effects of body size can be readily distinguished by examination of residuals from the regression analysis. An additive effect would show constant variance of organ size over the range of body sizes, whereas a multiplicative effect would show variance of organ size proportional to body size. In the latter case, logarithmic transformation of organ sizes prior to regression would be appropriate. In general, residuals should be carefully examined to ensure homoscedascticity and an appropriate variance-stabilizing transformation applied if necessary. Effects on organ size attributable to body size will generally be additive after transformation produces variances constant over the range of body sizes.

Despite their extensive use in comparison of organs for animals over a range of body sizes, organ indices must be used with caution.

Their applicability is dependent upon several restrictive assumptions which should be examined for the species or population in question. If those assumptions are violated, organ indices are not justified mathematically. Inappropriate use of organ indices may suggest differences where none actually occur or obscure differences which are present. Regression analysis as described in this appendix serves both to verify the assumptions underlying the use of organ indices and provides a reasonable and statistically valid alternative if those assumptions do not hold. The principal disadvantage of the technique described here is the need for a fairly extensive data set relating organ size to body size and any other predictors desired.

APPENDIX B

BIOCHEMICAL SEPARATIONS

Estimation of biochemical components often requires separation of those components so that other materials do not interfere with the assay of a particular biochemical class. I used two independent separation procedures. The first, based on Schmidt and Thannhauser (1945), isolates protein, DNA, and RNA. The other separation procedure is based on Bligh and Dyer (1959) and Van Handel (1965) and produces three fractions containing (1) total lipids, (2) glycogen, and (3) simple reducing sugars and free amino acids. The use of both separation procedures on two aliquots of tissue provide six fractions with good separation of the seven major biochemical classes I examined. The two components which appear in the same fraction, simple reducing sugars and free amino acids, do not significantly interfere with one another by the assay methods used.

In the procedural descriptions which follow, suggested volumes for reagents are given. These volumes are suitable for sample aliquots up to approximately 0.1 g dry mass. Significantly larger sample aliquots may require that some or all reagent volumes be increased by the same factor. In addition, both separation procedures require three sets of test tubes calibrated to known volumes before use; volumes of 2, 3, 5, 10, and 15 ml are especially useful.

Schmidt-Thannhauser Separation

The separation procedure proposed by Schmidt and Thannhauser

(1945) makes use of differential susceptibility to hydrolysis of protein, DNA, and RNA. In their polymerized form, all three components are insoluble in cold, dilute trichloroacetic acid (TCA) or perchloric acid (PCA). RNA undergoes hydrolysis with mild heating in potassium hydroxide, conditions under which DNA and protein are stable. Both nucleic acids will hydrolyze in hot TCA or PCA. The procedure thus entails separating proteins and nucleic acids from smaller, more soluble molecules by precipitation in TCA or PCA, hydrolysis of RNA in KOH with subsequent precipitation of DNA and protein by the addition of TCA or PCA, and finally hydrolysis of DNA in PCA. Perchloric acid is chosen for the final hydrolysis of DNA because the presence of perchloric acid enhances the sensitivity of the diphenylamine assay for DNA (Burton 1968).

Reference: Schmidt and Thannhauser (1945).

Reagents:

I. 100% Trichloroacetic acid
Stock solution of TCA containing 100 g trichloroacetic acid in 100 ml aqueous solution may be stored at room temperature. Dilute solutions should be made up shortly before use.

II. 10% Trichloroacetic acid

10.0	ml	100% Trichloroacetic acid stock
100	ml	Total volume with distilled water

III. 5% Trichloroacetic acid

5.0	ml	100% Trichloroacetic acid stock
100	ml	Total volume with distilled water

IV. 0.5 N Perchloric acid

4.3	ml	Perchloric acid, conc.
100	ml	Total volume with distilled water

V. 1 M Potassium hydroxide

6.6	g	Potassium hydroxide pellets (c. 85% KOH)
100	ml	Total volume with distilled water

VI. 0.3 N Potassium hydroxide

2.0	g	Potassium hydroxide pellets (c. 85% KOH)
100	ml	Total volume with distilled water

VII. Phosphate buffer, pH 7.2, 0.005 M

VIII. Absolute ethanol

IX. 95% ethanol

X. Hydrochloric acid, concentrated

Procedure:

(1) Homogenize aliquot of lyophilized or fresh tissue (up to approximately 100 mg dry mass) in 1 ml phosphate buffer at 0°C. Transfer homogenate into test tube A; rinse homogenizer three times with 1 ml phosphate buffer. Pool rinses with homogenate.

(2) Add 100% TCA to final concentration of 10%. If homogenate volume after (1) is approximately 4 ml, add 0.444 ml 100% TCA. Chill at 0°C for at least one hour.

(3) Centrifuge, discard supernatant. (I generally used an IEC Centra-7R refrigerated bench top centrifuge at 1650G.)

(4) Wash precipitate pellet by suspending pellet, centrifuging, and discarding supernatant. Wash with:

2 ml 10% TCA, twice.

2 ml absolute ethanol (cold), twice. This step removes both remaining TCA and excess lipid.

Caution: TCA pellets tend to be extremely fragile. It may be necessary to remove the supernatant with a pasteur pipet rather than by pouring.

(5) Suspend precipitate in 1 ml 0.3 N KOH. Adequate suspension of the precipitate may require sonication or mechanical disruption. Heat at 37°C for 60 minutes.

(6) Neutralize with concentrated HCl: 40 µl concentrated HCl for 1 ml 0.3 N KOH. Add 100% TCA to a final concentration of 5%: c. 53 µl

100% TCA. Chill at 0°C for at least one hour.

(7) Centrifuge. Transfer supernatant to tube B.

(8) Wash precipitate in tube A with 1 ml cold 5% TCA. Pool supernatant from this step with that from (7).

(9) Suspend precipitate in tube A in 1 ml 0.5 N $HClO_4$. Sonication is usually sufficient for small samples; larger samples may require mechanical disruption of the pellet.

(10) Heat at 70°C for 15 min. Cool to 0°C or lower for at least one hour.

(11) Centrifuge; transfer supernatant to tube C. Wash precipitate with 1 ml 0.5 N $HClO_4$; pool supernatants in tube C.

(12) Rinse pellet in tube A with 1 ml 95% ethanol; discard supernatant.

(13) Suspend pellet in tube A in 1 M KOH.

(14) Bring all test tubes to known volume.

Result:

Tube A contains protein, tube B contains hydrolyzed RNA, and tube C contains hydrolyzed DNA.

Bligh and Dyer/Van Handel Separation

This procedure is based on a combination of the monophasic chloroform-methanol method of Bligh and Dyer (1959) and the glycogen isolation procedure proposed by Van Handel (1965). Bligh and Dyer's technique produces large yields of lipid by first suspending the sample in a monophasic mixture of chloroform, methanol, and water, and then separating the chloroform phase from the methanol-water phase by the addition of chloroform and water. The initial monophasic solution results in very efficient extraction of lipids. The Van Handel

procedure separates glycogen from simple sugars and free amino acids by the adsorption of glycogen onto sodium sulfate in an alcoholic suspension; both glycogen and sodium sulfate are insoluble in alcohol, whereas simple sugars and free amino acids are soluble.

References: Bligh and Dyer (1959); Van Handel (1965)

Reagents:

I. Sodium sulfate
Saturated aqueous solution.

II. 30% Potassium hydroxide
30 g KOH pellets
100 ml Total volume with distilled water

III. Absolute methanol

IV. Chloroform

V. 95% ethanol

Procedure:

(1) Homogenize sample with dry mass up to approximately 100 mg in 375 µl distilled water + 50 µl saturated sodium sulfate. Transfer homogenate to tube A. Rinse homogenizer twice with 175 µl water each time; pool rinses with homogenate in tube A.

(2) Rinse homogenizer three times with a total of 2.0 ml absolute methanol (1.0 ml, 0.5 ml, 0.5 ml); pool into tube A.

(3) Add 1.0 ml chloroform to tube A. Vortex. Mixture should be monophasic with Na_2SO_4 precipitate; if the mixture is biphasic, add enough methanol to make it monophasic. Let the mixture stand for at least one hour to ensure precipitation of glycogen and extraction of lipid.

(4) Centrifuge. Transfer supernatant to tube B.

(5) Rinse pellet twice with 1 ml chloroform; pool rinses in tube B. Note: the pellet will probably float on the chloroform layer, so

the rinse must be drawn off with a pasteur pipet.

(6) Suspend pellet in tube A in 0.3 ml water. Add 0.7 ml 95% ethanol (to obtain 1 ml of 66% ethanol). Let stand in freezer overnight to precipitate glycogen, then centrifuge. Transfer supernatant to tube C.

(7) Gently warm pellet in tube A to remove traces of ethanol. Add 1.0 ml 30% KOH; heat at 100°C for at least ten minutes. Add 2.0 ml ethanol and let stand in freezer overnight.

(8) Centrifuge tube A. Discard supernatant. If the supernatant is particularly dark, rinse the pellet again with 66% ethanol. (This step removes protein and nucleic acids.)

(9) Add 1 - 2 ml water to tube B; vortex. Note: strict adherence to the Bligh and Dyer procedure requires the addition of 1 ml chloroform total in (5) and 1 ml water here. Because I used a total of 2 ml chloroform in the rinses in (5), I also used 2 ml water in this step; the final outcome should be approximately the same.

(10) Centrifuge tube B to break the emulsion. Transfer methanol layer to tube C.

(11) If the methanol layer cannot be quantitatively (or nearly quantitatively) transferred, re-extract the chloroform layer in tube B with 1 ml water; pool in tube C.

(12) Bring all tubes to known volume.

Result:

Tube A contains glycogen adsorbed on sodium sulfate, tube B contains lipid dissolved in chloroform, and tube C contains both simple reducing sugars and free amino acids.

APPENDIX C

BIOCHEMICAL ASSAYS

The following biochemical assays may be readily modified by increasing or decreasing reagents and samples by the same factor; very good reasons for altering the amount of reagent used include: limited amount of sample available, minimum final volume readable in the spectrophotometer, and a need or desire to conserve reagents. In some cases, it may be possible and desirable to alter relative quantities of sample and reagent, usually increasing the amount of sample relative to reagent in order to increase assay sensitivity. Any such changes must be investigated on a case-by-case basis; where I have information on the feasibility of procedural changes, either as a result of my own efforts or from published sources, I have included that information in the detailed procedures below.

Each time an assay is performed, one should include a sequence of standards covering either the range of concentrations in the samples or, if the range of sample concentrations is unknown, the effective range of the assay. Minor and inevitable variations in technique or reagents can introduce significant variations in the standard curves that result; hence, it is important that a standard curve be generated for each performance of an assay at the time the assay is performed. Subsequently, standard curves which do not differ significantly may be pooled.

Lowry Assay for Protein

The use of the Folin-Ciocalteau phenol reagent for the spectrophotometric determination of protein was first proposed by Wu (1922), but is commonly attributed to Lowry *et al.* (1951), who described its advantages and limitations in detail. The procedure described here uses the reagent modifications suggested by Oyama and Eagle (1956).

The Lowry assay is very sensitive (to 1 µg protein or less), is relatively simple to perform, and is not subject to extreme interference by most substances encountered in biological work (Lowry *et al.* 1951). Major disadvantages to the Lowry assay include: (1) amount of color produced by a given mass of protein varies with different proteins; (2) optical density is not strictly proportional to protein concentration, requiring use of non-linear standard curves; (3) assay sensitivity can be affected by purely mechanical details of procedure, such as time from addition of phenol reagent to mixing (Oyama and Eagle 1956).

References: Lowry *et al.* (1951); Oyama and Eagle (1956)

Effective Range: c. 10 - 250 µg; sensitivity can be greatly increased by procedural modifications (see Lowry *et al.* 1951).

Reagents:

I. Protein standard.
Aqueous solutions of approximately 250 µg/ml are useful, but higher concentrations (up to about 1 mg/ml) can be used. For assay of total protein, either bovine serum albumin or bovine gamma globulin are frequently used standards; I used bovine serum albumin consistently. Store frozen.

II. Alkaline tartrate
20.0 g Na_2CO_3
4.0 g NaOH

0.2 g Sodium potassium tartrate
1.0 l Final volume in distilled water

III. 0.5% Cupric sulfate
5.0 g $CuSO_4 \cdot 5H_2O$
1.0 l Final volume in distilled water

IV. Alkaline copper tartrate
50.0 ml Alkaline tartrate solution (Reagent II)
1.0 ml 0.5% Cupric sulfate (Reagent III)
Make on day of use.

V. Folin-Ciocalteau reagent
Dilute commercial Folin-Ciocalteau phenol reagent (2N) with an equal volume of distilled water.

Procedure:

(1) Samples and standards diluted if necessary to 1 ml total volume.

(2) Add 5 ml of alkaline copper tartrate (Reagent IV) and mix well.

(3) Add 0.5 ml of Folin-Ciocalteau reagent (Reagent V) and immediately mix well. A convenient way to do this consistently is to add the phenol reagent while the sample is being vortexed. Inconsistency in this step can lead to variable (and unpredictable) assay results.

(4) Let stand at room temperature for 30 minutes. Color is stable for approximately two hours.

(5) Read absorbance in spectrophotometer at either 550 nm or 750 nm. (Sensitivity is slightly greater at the latter wavelength, which is the absorbance maximum.)

Bradford Assay for Protein

The protein assay described by Bradford (1976) is based on the color change of Coomassie Brilliant Blue G-250 upon binding of the dye to protein. The sensitivity of this assay is comparable to that of the Lowry assay. In addition, the Bradford assay has several distinct advantages. (1) It is both faster and easier to perform than the Lowry

assay. (2) It is less subject to interference from non-protein substances than the Lowry method. (3) A commercial reagent is available (Bio-Rad Laboratories), increasing consistency among assays. Its principal disadvantage is variability in response to different proteins. Like the Lowry assay, the optical density produced by a given concentration of protein varies considerably among proteins. In particular, the color development is considerably greater with bovine serum albumin than with most other proteins (Bio-Rad 1981), so that either a correction factor must be included or an alternative standard must be used for determination of absolute protein concentrations. (Any purified protein is useable if only relative values of protein concentration are required.)

Reference: Bradford (1976)

Effective Range: c. 20 - 140 µg by standard procedure; c. 1 - 20 µg by microassay procedure.

Reagents:

I. Protein standard
Aqueous solution of purified protein at a concentration of approximately 1.4 mg/ml. Store frozen.

II. Bradford dye reagent concentrate
Available commercially from Bio-Rad Laboratories. The commercial dye concentrate uses methanol rather than ethanol.
100 mg Coomassie Brilliant Blue G-250
50 ml 95% ethanol
Dissolve dye in alcohol, then add:
100 ml 85% (w/v) phosphoric acid
Dilute with distilled water to a final volume of 200 ml.

III. Diluted Bradford dye reagent
1 part Bradford dye reagent concentrate (Reagent II)
4 parts distilled water
Filter (Whatman No. 1 or equivalent) before use. May be stored at room temperature for up to two weeks.

Procedure: Standard assay (c. 20 - 140 μg protein)

(1) Samples and standards diluted with distilled water or sample buffer to 0.1 ml total volume.

(2) Add 5.0 ml diluted dye reagent (Reagent III).

(3) Vortex, avoiding excessive foaming, or mix several times by inversion.

(4) Let stand at room temperature for at least five minutes. Color is stable for approximately one hour.

(5) Read absorbance in spectrophotometer at 595 nm.

Procedure: Microassay (c. 1 - 20 μg protein, ≤ 25 μg/ml)

(1) Samples and standards diluted with distilled water or sample buffer to 0.8 ml total volume.

(2) Add 0.2 ml dye reagent concentrate (Reagent II).

(3) Vortex gently or mix by inversion.

(4) Let stand at room temperature for at least five minutes. Color is stable for approximately one hour.

(5) Read absorbance in spectrophotometer at 595 nm.

Diphenylamine Assay for DNA

First described by Dische (1930), the reaction between diphenylamine and deoxyribose is commonly used for spectrophotometric determination of DNA; the analysis is relatively simple to perform and specific for deoxyribose. The original assay procedure was modified (Burton 1956) to obtain greater sensitivity by adding perchloric acid and acetaldehyde and developing the color for 17 hours at 30°C. The procedure described here (Tillinghast, personal communication) differs from Burton's (1956) modification only in the concentration of

acetaldehyde used, which is four times that in Burton's diphenylamine reagent.

Apparently, relatively few substances seriously interfere with color development or produce spurious color (Burton 1956). Protein may significantly inhibit development of color, especially if the protein has been treated with alkali (Burton 1968). This may be the cause of one of the more frustrating features of the diphenylamine assay: at low concentrations of DNA, it is not unusual to obtain optical densities less than that of the blank, even if protein precipitate has been completely removed.

Reference: Burton (1956, 1968)

Effective Range: c. 10 - 200 µg/ml

Reagents:

I. DNA Standard. Approximately 200 µg/ml.
Dissolve DNA at a precisely known concentration of approximately 400 µg/ml in 0.005 N NaOH. Sonication is useful for rapid solution of DNA. Add an equal volume of 1 N $HClO_4$ and heat at 70°C for 15 min. Store frozen.

II. 0.005 N Sodium hydroxide
20 mg Sodium hydroxide
100 ml Total volume with distilled water

III. 1 N Perchloric acid
8.6 ml Perchloric acid ($HClO_4$), concentrated (c. 70%)
100 ml Total volume with distilled water

IV. 0.5 N Perchloric acid
4.3 ml Perchloric acid, concentrated
100 ml Total volume with distilled water

V. Acetaldehyde, 32 mg/ml
2.0 ml Acetaldehyde (cold: 0 - 4°C)
50.0 ml Distilled water
Add acetaldehyde to water with a cooled pipet. Solution is stable for several months at 4°C in a well-stoppered bottle.

VI. Diphenylamine reagent. Adjust quantities to provide adequate amount for assays.
Mix in order:
100 ml Glacial acetic acid

1.5 g Diphenylamine
1.5 ml Sulfuric acid, concentrated
1.0 ml Acetaldehyde, 32 mg/ml aqueous solution
Make on day on use, add acetaldehyde just before use.

Procedure:

(1) Samples and standards up to 1 ml volume, diluted with 0.5 N perchloric acid to 1 ml.

(2) Add 2 ml diphenylamine reagent.

(3) Seal with stopper or parafilm.

(4) Incubate at 30°C for approximately 18 hours (although acid-treated DNA achieves maximum color in about 7 hours).

(5) Read in spectrophotometer at 600 nm.

Orcinol Assay for RNA

Dische and Schwarz (1937) first applied the orcinol reaction for determination of RNA. The standard procedure for the assay, as described by Munro and Fleck (1966), is subject to interference from simple sugars and DNA; therefore, these substances must first be removed from the sample (e. g., by the Schmidt-Thannhauser method described in Appendix B). Almog and Shirey (1978) described a modification of the standard assay which is much less sensitive to interference by DNA. The modified orcinol procedure relies upon difference in reaction products when DNA and RNA are incubated in acid. Under acidic conditions, ribose sugar in RNA is converted to furfural, which reacts with orcinol; under similar conditions, deoxyribose sugar in DNA is converted mainly to levulinic acid, which does not subsequently react with orcinol.

Both the standard orcinol assay and the modified procedure of Almog and Shirey (1978) are described here. The standard orcinol assay

was used for analysis of RNA in Chapter II; the modified procedure was used for RNA analysis in Chapter IV. The principal advantage of the modified procedure is that RNA and DNA need not be separated for analysis. Thus, a simpler separation procedure can be used. However, the modified procedure requires an additional 24-hour incubation.

Reference: Munro and Fleck (1966); Almog and Shirey (1978)

Effective Range: Standard procedure: c. 5 - 100 µg/ml

Modified procedure: c. 10 - 300 µg/ml

Reagents:

I. 0.5 N Potassium hydroxide
- 3.3 g KOH pellets, c. 85% KOH
- 100 ml Total volume with distilled water

II. 85% Sulfuric acid
- 15.0 ml Distilled water
- 85.0 ml Sulfuric acid, concentrated

III. Ferric chloride stock
- 10 g $FeCl_3 \cdot 6H_2O$
- 100 ml Total volume with distilled water

IV. Acid ferric chloride
- 0.5 ml Ferric chloride stock
- 100 ml Hydrochloric acid, conc.

V. Alcoholic orcinol
- 0.6 g Orcinol
- 10 ml 95% ethanol

VI. 6% orcinol, aqueous solution
- 0.6 g Orcinol
- 10.0 ml Distilled water

VII. Standard orcinol reagent

Optional: can either combine reagents first, or add to sample separately. (I usually did the latter.) 60 ml

Acid ferric chloride
- 4 ml Alcoholic orcinol

VIII. Modified orcinol reagent
- 0.35 ml 6% orcinol, aqueous solution
- 5.00 ml Hydrochloric acid, concentrated

IX. RNA standard

Dissolve known quantity of RNA in 0.5 N KOH at a concentration of approximately 100 µg/ml (for modified orcinol procedure, a higher concentration is desireable, up to c. 500 µg/ml). Allow hydrolysis to proceed at room temperature for 24 - 48 hours.

Standard Procedure:

(1) Samples and standards up to 1 ml, diluted to 1 ml total volume with distilled water.

(2) Add 2 ml acid ferric chloride and 0.133 ml alcoholic orcinol (or 2.133 ml orcinol reagent). Note: standard procedure as described by Almog and Shirey (1978) uses 6% aqueous solution of orcinol rather than alcoholic orcinol.

(3) Heat at 100°C for 20 min. Cool.

(4) Read absorbance in spectrophotometer at 660 nm.

Modified Procedure:

(1) Samples and standards up to 1.0 ml, diluted to 1.0 ml with distilled water.

(2) Add 4.0 ml 85% sulfuric acid. Incubate at 40°C for 24 h.

(3) Add 0.1 ml of modified orcinol reagent. Mix thoroughly.

(4) Incubate at 100°C for 30 min.

(5) Read absorbance in spectrophotometer at 500 nm.

Anthrone Assay for Sugars (Glycogen and Simple Sugars)

Conditions and procedures necessary for the simple and reproducible determination of carbohydrates with anthrone were described by Seifter et al. (1950). The most important requirement is that all assay tubes be heated uniformly. This condition can be conveniently met by adding reagent to sample and standard solutions while the assay tubes are in an ice-water bath, and mixing reagent and

test solutions completely while keeping the tubes cold. The second major condition described by Seifter et al. (1950) is that incubation in boiling water proceed for only 9-10 min., with the tubes promptly returned to ice-water at the end of incubation.

If glucose is used as a standard, assayed carbohydrates will be given as "glucose-equivalents." If, for example, absolute amounts of glycogen are desired, it is necessary to convert this "glucose-equivalent" mass to glycogen mass. The appropriate conversion factor (Morris 1948) is 1.11; i. e., glycogen mass equals estimated glucose equivalent divided by 1.11.

In some analyses for simple sugars, I noticed that the normal bright green produced by reaction of carbohydrate with anthrone was replaced with dull olive or occasionally orange-red. Both of these unusual colors can be attributed to the presence of tryptophan in the sample (Seifter et al. 1950); olive coloration probably results from combination of orange-red color attributed to tryptophan with the usual green carbohydrate product. The orange-red color produced by reaction of tryptophan with anthrone has maximum absorbance at 515 nm and negligible absorbance at 620 nm (Seifter et al. 1950). Hence, presence of tryptophan probably does not seriously interfere with determination of carbohydrates.

Reference: Seifter et al. (1950)

Effective Range: c. 5 - 300 µg/ml

Reagents:

I. 95% Sulfuric acid.
5 ml Distilled water
95 ml Sulfuric acid, conc.
Add acid to water slowly, in an ice bath.

II. Anthrone reagent

0.2	g	Anthrone
100	ml	95% sulfuric acid

III. Glucose standard

Prepare glucose standard in water at a concentration of approximately 0.3 mg/ml.

Procedure:

(1) Samples and standards up to 1 ml, diluted with water to a total volume of 1 ml.

(2) Cool all assay tubes and anthrone reagent to 0°C in an ice bath. Leave assay tubes in an ice bath while reagent is added.

(3) Add 2 ml anthrone reagent.

(4) Swirl assay tubes in an ice bath to mix. Complete mixing by vortexing.

(5) Heat at 100°C for 10 minutes. Cool in an ice bath to below room temperature.

(6) Read in spectrophotometer at 620 nm.

Note: Precision of the assay depends upon consistent heating among all assay tubes. Because of the high heat of solution for the 95% sulfuric acid reagent, the tubes should be left in an ice bath while the anthrone reagent is added and the contents are mixed.

Ninhydrin Assay for Amino Acids

Ninhydrin is commonly used as a reagent for the spectrophotometric determination of amino acids; the assay is extremely sensitive. Colored reaction products are formed not only from amino acids, but from any substances containing free amino groups. Thus, assay of free amino acids requires the removal of proteins from the sample.

A potentially troublesome disadvantage for quantitative work is that the color yield varies for different amino acids. Fortunately,

the color yields for most amino acids falls into a fairly narrow range (Moore and Stein 1949). Consequently, unless the amino acid composition is subject to considerable variation, differences in color yield will not greatly affect relative concentrations. Leucine is often used as the standard; I used glycine because it is the major amino acid present in asteroid tissues (Ferguson 1975a).

Reference: Moore and Stein (1949); Moore (1968)

Effective Range: c. 1 - 20 µg

Reagents:

I. Ninhydrin reagent
Available commercially (Sigma Chemical Company); the commercial reagent uses dimethyl sulfoxide as an organic solvent (Moore 1968). Store under nitrogen at 0 - 4 °C.

II. Diluent
Mix equal volumes of n-propanol and distilled water.

III. Amino acid standard
Aqueous solution of desired amino acid at a concentration of approximately 200 µg/ml.

Procedure:

(1) Samples and standards diluted as necessary to a final volume of 0.1 ml.

(2) Add 0.5 ml ninhydrin reagent.

(3) Place in water bath at 100°C for 20 min.

(4) Cool assay tubes in room temperature water.

(5) Add 2.5 ml diluent.

(6) Let stand at room temperature for 15 min.

(7) Read optical density at 600 nm in spectrophotometer. Color is stable for approximately one hour.

Note: samples with higher concentrations can be easily handled by increasing all volumes, thereby allowing greater dilution without loss

of precision. Samples with lower concentrations could probably be assayed by increasing the relative amount of sample to ninhydrin reagent (e.g., using 0.2 ml sample with 0.5 ml ninhydrin) provided standards are treated similarly. Assay tubes with optical densities greater than about 1.0 may be diluted with additional diluent; again, standards should be treated similarly to provide an appropriate standard curve. Although I have not tried it, I suspect that optical densities could be read against a diluent blank. All assays, including assay blanks, would then have higher optical densities. However, addition of diluent to samples with high optical densities should reduce absorbance in precise proportion to the additional dilution; thus, the same standard curve could be used for both diluted and undiluted samples without introducing significant error.

Ornithine Decarboxylase Assay

As the rate-limiting enzyme in polyamine synthesis, ornithine decarboxylase (ODC) is commonly assayed as a means of estimating relative rates of polyamine synthesis. Ornithine labeled with ^{14}C at the carboxyl group is the substrate; carbon dioxide freed by the enzyme is trapped by a strong base and the amount of labeled CO_2 is determined by scintillation counting. Variations of the procedure differ mainly in the precise formulation of reaction buffer and the base used for trapping CO_2. The reaction buffer described below is based on Landy-Otsuka and Scheffler (1978). Activity of ornithine decarboxylase is dependent on the presence of pyridoxal phosphate as a cofactor.

Preliminary experiments (with a very small sample size) using lyophilized testes from the proliferative phase, with 0.2 M NaOH for

CO_2-trapping, indicated that:

(1) Enzyme reaction, as measured by evolved CO_2, is approximately linear for at least 90 minutes.

(2) ODC is not sensitive to temperature, at least in the range 10 - 37°C. CO_2 evolution increases exponentially with temperature, as would be predicted by simple reaction kinetics. Therefore, in order to optimize CO_2 evolution, and hence sensitivity of the assay, reactions were allowed to proceed for one hour at 37°C.

(3) Three CO_2-trapping agents were tried: 0.2 M NaOH, hyamine hydroxide (New England Nuclear), and NCS (Amersham). Highest efficiency was obtained with NCS, so it was used in subsequent assays. Other CO_2-trapping agents, such as phenethylamine, could also be used and may be more efficient.

A major problem which can arise in this assay is chemiluminescence resulting from interaction between the base used for CO_2-trapping and emulsifiers in the liquid scintillation cocktail. This effect can be minimized by using non-aqueous scintillation cocktails (without emulsifiers) and leaving the scintillation vials in the dark overnight before counting; subsequent exposure to light should be avoided as much as possible. I used Beckman Ready-Solv NA scintillation cocktail, but any emulsifier-free cocktail should serve adequately.

<u>Reference:</u> Landy-Otsuka and Scheffler (1978)

<u>Reagents:</u>

I. Reaction Buffer

0.7888 g	Tris-HCl	(50mM)
0.0771 g	Dithiothreitol	(5mM)
5.844 mg	EDTA (as free acid; adjust if salts are used)	(0.2mM)
1.326 mg	pyridoxal 5-phosphate monohydrate	(50µM)

Dissolve in slightly less than 100 ml distilled water. Adjust pH to 7.1 with hydrochloric acid. Bring volume up to

100 ml. Divide into portions (approximately 10-15 ml each) and store frozen.

II. 10% Trichloroacetic Acid

III. DL-[1-^{14}C] Ornithine hydrochloride
I used labelled ornithine from Amersham Internation, Ltd., with a specific activity of 58 mCi/mmol.

Procedure:

(1) Homogenize tissue in reaction buffer (at least 5:1 (v/w) buffer:tissue). Most authors (including Landy-Otsuka and Scheffler 1978) centrifuge homogenates at 10,000 to 50,000 G and use supernatant only; I generally just filtered the homogenate through Nitex cloth to remove large pieces of unhomogenized tissue.

(2) Place in conical-bottomed test-tube:

150 µl homogenate

5 µl ^{14}-C ornithine (= 5 µCi)

Include several process blanks to determine background and counting efficiency.

(3) Place 200 µl NCS or other CO_2-absorber into center well suspended from double-seal rubber stopper (both from Kontes). Seal all reaction tubes with center well - stopper combination.

(4) Incubate at 37° C for 60 minutes.

(5) Stop reaction by injecting 0.5 ml 10% TCA through the stopper. (Note: avoid contaminating the center well with acid).

(6) Continue incubation at 37° C overnight, to ensure absorption of all CO_2.

(7) Cut center well directly into mini-scintillation vials; add 4 ml non-aqueous counting fluid.

(8) Leave overnight in dark; count in scintillation counter as

appropriate for ^{14}-C samples (*e. g.*, on Beckman LC-7000, I used program 4 with 20 minute counting time).

APPENDIX D

DATA REDUCTION AND STATISTICAL ANALYSIS

After tissue samples have been separated into component biochemical classes (see Appendix B) and those biochemical classes have been quantitatively analyzed (see Appendix C), the resulting raw data must be reduced to a manageable and interpretable form and subjected to appropriate statistical analysis. This appendix describes the methods of data reduction and statistical analysis used for interpretation of data on biochemical composition of the testes (Chapter I), together with some of the underlying assumptions and reasons for particular choices of techniques. Specific procedural details and programming code used for data reduction and statistical analysis are given in Appendix E.

The analysis discussed here occurs in several discrete steps, each of which will be described in detail. First, standard curves for spectrophotometric assays must be derived by regression analysis. These standard curves are then used for quantitative estimation of biochemical components from observed optical densities. From these estimates, it is possible to calculate component concentration (in μg/mg dry mass), generating an estimate of error (or uncertainty) at the same time. Estimates of component concentration in terms of dry mass can be combined to provide estimates of component level (mg/mg DNA) and total content. The overall pattern of changes in a particular component can then be described by regression analysis; results from regression can then be used to calculate confidence bands around the

fitted regression function. The function, confidence bands, and data are then plotted in order to allow easy interpretation of the resulting regression function.

Standard Curves

Standard curves (or calibration curves) for spectrophotometric data can be conveniently determined by regression analysis using any computer-based statistical package. Simple linear regression is sufficient in many cases, as predicted by Beer's law. However, the range of standard concentrations may be so great that Beer's law no longer precisely holds. The most convenient way to test linearity of the standard curve, and to produce a better fitting standard curve when Beer's law is violated, is through polynomial regression. Generally, a quadratic equation will adequately fit the empirical standard curve and has the advantage of being easily invertible (unlike cubics, for example). Therefore, both linear and quadratic equations should be fitted to standard curve data and the quadratic form used if it provides significantly better fit.

Concentration of the standard is the independent variable (here designated X); absorbance or optical density is the dependent variable (Y). Thus, the desired regression functions are:

$$\hat{Y} = b_0 + b_1 X \qquad (D.1)$$

and

$$\hat{Y} = b_0 + b_1 X + b_2 X^2. \qquad (D.2)$$

Significance of parameters (b_1) can be determined either by a t-test (Minitab, for example, provides t-values for each parameter) or by an F-test comparing regression with the parameter included to regression with the parameter excluded. For example, significance of b_2 in

Equation D.2 can be tested by calculating

$$F = (SSE_1 - SSE_q) / MSE_q \qquad (D.3)$$

where SSE_1 = residual sum of squares for linear regression

SSE_q = residual sum of squares for quadratic regression

MSE_q = residual mean square for quadratic regression

with degrees of freedom for the numerator equal to 1 and for the denominator equal to residual degrees of freedom from the quadratic regression. Alternatively, b_2 from Equation D.2 can be tested for significance by use of the t-value provided by the regression program, with degrees of freedom equal to residual DF.

If more than one set of standards have been used in determinations of a particular component, combination of resulting standard curves may be desireable. Provided that several sets of standard can be considered statistically indistinguishable, accuracy of the standard curves may be improved by combining the sets and fitting a single common standard curve (either linear or quadratic, as appropriate). To test the hypothesis that k sets of standards can be described by a single standard curve, perform regressions on each set and on all of the sets taken together. All regression functions must be identical; thus, if the "total" regression or any of the k separate regressions are quadratic, all must be quadratic regardless of significance of the b_2s. Residual sums of squares and residual degrees of freedom are determined for all k+1 regressions; sums of squares and degrees of freedom for the k separate regressions are summed. An F statistic with $(m+1)(k-1)$ and DFE_p degrees of freedom is then calculated:

$$F = \frac{\frac{(SSE_t - SSE_p)}{(m+1)(k-1)}}{\frac{SSE_p}{DFE_p}} \qquad (D.4)$$

where SSE_t = residual sum of squares for all data together

SSE_p = "pooled" sum of squares of separate regressions, i. e.,

$= \sum_i SSE_i$

$DFE_p = \sum_i DFE_i$

m = number of predictors (= 1 for linear, 2 for quadratic)

k = number of regressions being tested for coincidence.

This F test only determines whether the k separate regressions can be considered statistically coincident. Unfortunately, I know of no multiple comparison test for coincident regressions; therefore, if the k separate regression differ significantly, one must examine all possible subsets in order to determine which groups of regressions are coincident. Preliminary plots of the sets of standards can help to determine which regressions lie close together, so that not all possible subsets need be examined. An additional complication may arise if several regressions are near the statistical limit for coincidence. Because a finding of non-significance (i. e., coincident regressions in this case) is a non-transitive relationship, one regression may be coincident with each of two other regressions which are not themselves coincident. In other words, regression A may be coincident with regression B, and regressions B and C may be coincident, but A and C or all three taken together may differ significantly. Generally, this rather confusing situation arises only if the "coincident" regressions barely miss being significantly different. Thus, a reasonable approach to resolving the dilemma is to

consider all of the regressions involved to be non-coincident (A, B, and C are thus treated as separate regressions despite coincidence of two pairs out of the three curves).

When the sets of standards have been separated into groups of coincident regressions, regression coefficients and mean square errors for both linear and quadratic curves (if the latter is significant) must be noted. Also needed are the $(\mathbf{X}'\mathbf{X})^{-1}$ matrices for each group of coincident standard curves (the program REGVA.BAS in Appendix E calculates $(\mathbf{X}'\mathbf{X})^{-1}$ matrices from X-vectors of standard concentrations).

Inverse Prediction

Determination of component concentration from spectrophotometric data requires that the independent variable, concentration, be predicted from an observed value of the dependent variable, optical density. Because finding the regression of an independent variable as a function of a dependent variable is generally inappropriate, inverse prediction is used instead. The regression function of Y on X is determined in the usual way, and the resulting equation is solved for X. In addition, it may be useful to obtain a preliminary estimate of error associated with predicted concentration. Standard error of predicted X based on uncertainty of the regression function can be calculated directly for a linear regression function, although numerical methods seem to be simpler for quadratic regression functions.

Linear calibration curve

If the regression function of Y on X is a straight line, Y would be predicted from X by the equation

$$\hat{Y} = b_0 + b_1 X \qquad (D.5)$$

or equivalently $$\hat{Y} = \bar{Y} + b_1(X - \bar{X}) \quad (D.6)$$

where $\bar{X}$ and $\bar{Y}$ are determined from the calibration standards. X would then be predicted from an observed Y by

$$\hat{X} = (Y - b_0)/b_1. \quad (D.7)$$

or $$\hat{x} = y/b_1 \quad (D.8)$$

where $x=(X-\bar{X})$ and $y=(Y-\bar{Y})$. Although no exact expression for the standard error of predicted $\hat{X}$ is known, confidence limits can be derived from the confidence limits of $\hat{Y}$ given X (Snedecor and Cochran 1980):

$$Y = \bar{Y} + b_1 x \pm t\sqrt{MSE(1 + 1/n + x^2/\Sigma x^2)} \quad (D.9)$$

where $\bar{X}$, $\bar{Y}$, Σx^2, MSE, and n are based on the set of calibration standards. Equation D.9 is solved as a quadratic equation in x for given Y, with the substitutions $\hat{x}=(Y-\bar{Y})/b_1$ (Eqn. D.8) and $c=ts_b/b_1=(t/b_1)\sqrt{MSE/\Sigma x^2}$:

$$x = \frac{\hat{x} \pm (t/b_1)\sqrt{MSE\{(1+1/n)(1-c^2)+x^2/\Sigma x^2\}}}{1 - c^2} \quad (D.10)$$

The calculated confidence limits are asymmetrical around $\hat{X}$. However, if b_1 is highly significant (which will usually be the case for standard curves), s_b will be small relative to b_1. Therefore, c^2 will be negligible and the confidence limits simplify to

$$x = \hat{x} \pm (t/b_1)\sqrt{MSE(1 + 1/n + \hat{x}^2/\Sigma x^2)} \quad (D.11)$$

which are symmetric around $\hat{x}$. Thus,

$$s_{\hat{x}} \simeq (1/b_1)\sqrt{MSE(1 + 1/n + \hat{x}^2/\Sigma x^2)}. \quad (D.12)$$

Quadratic calibration curve

Even if the calibration curve differs significantly from a straight line, it can often be adequately describe by a quadratic

function. Higher order polynomials or non-polynomial functions may occasionally provide better descriptions, but subsequent analysis is considerably more complex; I did not find it necessary to use calibration curves more complex than quadratic functions for any of the spectrophotometric assays performed. Should more complex functions be required, analysis would proceed analogously to that described here.

For a quadratic regression function, the relationship between Y and X,

$$\hat{Y} = b_0 + b_1X + b_2X^2, \tag{D.13}$$

can be solved as a quadratic equation in X for a given observed Y; thus,

$$\hat{X} = \{-b_1 \pm \sqrt{b_1^2 - 4b_2(b_0 - Y)}\} \; / \; (2b_2). \tag{D.14}$$

(In every case I analyzed, the appropriate root was obtained by addition at '±' in Equation D.14.)

Matrix notation is often used to simplify and generalize multiple regression models, including the quadratic regression function considered here. Matrix notation is described in detail in Neter and Wasserman (1974). Several definitions are important for the present discussion, however. **X** is a $n \times p$ matrix (n = number of points used in fitting the regression function; *i. e.*, the number of standards assayed, including the blank. p = total number of parameters in the regression function; 3 for a quadratic function) in which each of the n rows contain a set of values for the independent variables corresponding to one data point. The first column of **X** is filled with ones, representing the "dummy" variable associated with b_0, the Y-intercept. $\mathbf{X}'$ is the usual notation for the transpose of a matrix. Thus, for a quadratic function:

$$\mathbf{X} = \begin{bmatrix} 1 & X_1 & X_1^2 \\ 1 & X_2 & X_2^2 \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ 1 & X_n & X_n^2 \end{bmatrix} \qquad \mathbf{X}' = \begin{bmatrix} 1 & 1 & \cdots & 1 \\ X_1 & X_2 & \cdots & X_n \\ X_1^2 & X_2^2 & \cdots & X_n^2 \end{bmatrix}$$

In addition, **b** is the column vector of regression coefficients, and Y_h is an observed value of the dependent variable (*e. g.*, optical density of a sample assay) with corresponding values for the independent variables contained in the column vector $\mathbf{X_h}$. Thus, the quadratic regression function (Eqn. D.13) could be restated in matrix terms as

$$\hat{Y}_h = \mathbf{X_h'}\,\mathbf{b} \tag{D.15}$$

The analogue in matrix notation to Equation D.9 for confidence limits around a newly observed Y is

$$Y_h = \hat{Y}_h \pm t\sqrt{MSE\{1 + \mathbf{X_h'}(\mathbf{X'X})^{-1}\mathbf{X_h}\}} \tag{D.16}$$

Unlike the algebraic form in Equation D.9, Equation D.16 is, at best, difficult to solve for $\mathbf{X_h}$. However, it can be readily solved by numerical methods. If t is set equal to one, confidence limits are one standard error around the mean. Given reasonably good initial estimates for the standard error of X resulting from inverse prediction, numerical solution of Equation D.16 will provide precise estimates. Because quadratic standard curves are generally quite close to the linear standard curves for the same set of standards, Equation D.11 can be used to provide initial estimates of confidence limits around an $\hat{x}$ calculated by Equation D.14. The final confidence limit calculated by numerical solution of Equation D.16 will not generally be symmetric around $\hat{X}$. However, the extremely good fit of most standard curves results in little asymmetry, and a good estimate of $s_{\hat{X}}$ can be obtained by simply averaging the deviations above and below $\hat{X}$.

Data reduction: Calculation of concentrations

Inverse prediction, as described in the preceeding section, provides estimates of the amount of a component of interest based on spectrophotometric assay for that component. In addition, an estimate of error attributable to uncertainty of the standard curve is provided for each replicate sample. Because a specimen will generally have several replicate assays for a single component, results from the replicates must be combined to find an average estimate. In addition, spectrophotometric assays will provide only amount of component present in a replicate. For the data to be interpretable, estimates of amount of component present in the replicates must be converted to amount present in the entire sample and ultimately to concentration (per μg dry mass, per μg DNA, or any other measure of concentration).

For the following discussion, component estimates derived from inverse predction (as above) and associated quantities for replicate j of specimen i will be symbolized as:

X_{ij} = amount of component (μg) determined by spectrophotometry and inverse prediction

$s_{(r)ij}$ = standard error of X_{ij}, based on uncertainty of standard curve

V_{ij} = volume of replicate analyzed (ml)

Then the concentration of component in its fraction (μg/ml) is estimated by each replicate as

$$C_{ij} = X_{ij}/V_{ij} \qquad (D.17)$$

with a standard error estimated by usual formulae for propagation of error (Beers 1957) as

$$s_{(c)ij} = s_{(r)ij}/V_{ij}. \qquad (D.18)$$

The "best" estimate of concentration is an average of all replicates. However, standard errors may in general be different for each replicate, primarily because of differences in replicate volume, V_{ij}. Under these circumstances, the "best" estimate is obtained by a weighted average in which each replicate is weighted by the inverse of its variance. Thus,

$$\bar{C}_i = \sum_j (C_{ij}/s^2_{(c)ij}) / \sum_j (1/s^2_{(c)ij}) \qquad (D.19)$$

The appropriate method to estimate standard error of $\bar{C}_i$ is less clear. There are several possibilities. If uncertainty in standard curves adequately accounts for variability among replicates, so that all replicates for a single specimen are reasonably close in terms of $s_{(c)}$, then the formula from Beers (1957) is probably appropriate:

$$s^2_{\bar{c}} = 1/\sum_j (1/s^2_{(c)ij}). \qquad (D.20)$$

However, this equation does not consider actual variation of replicates from their mean and thus appears to underestimate the standard error of $\bar{C}_i$ when replicate variability exceeds standard curve variability (as appears to have been the case in at least some specimens or some components). On the other hand, calculating standard error for each specimen individually by the usual (root-mean-square) formula produces an extremely large range of standard errors, probably as a result of sampling variability combined with inefficiency of standard error estimation for small samples. A large range of standard errors is undesireable for weighted regression, because the relatively few data points with low standard errors (and therefore high weights) largely determine the shape of the fitted regression. If one assumes that variability of X_{ij} around $\bar{X}_i$ is nearly constant for all specimens, one

can estimate standard error of $\bar{X}_i$ from the pooled deviations of all replicates from their means. This assumption is reasonable, because most of the final relative variability of component measures is attributable to subsequent calculations (correction for dilution, etc.). Given a weighted mean concentration from Equation D.19, one can calculate mean values for replicates by inverting Equation D.17; thus

$$\bar{X}_{ij} = \bar{C}_i \cdot V_{ij} \cdot \tag{D.21}$$

Standard error of X_i can then be estimated as the root-mean-square of deviations of X_{ij} from X_{ij} over all replicates of all specimens:

$$s_X = \sqrt{\sum_{ij} [(X_{ij}-\bar{X}_{ij})^2 / s^2_{(c)ij}] / [\sum_{ij} (1/s^2_{(c)ij})]}. \tag{D.22}$$

To convert s_X into a standard error of mean concentration, one can multiply by the average dilution factor for a specimen. Average dilution factor is calculated as a weighted mean of $1/V_{ij}$ using the same weights as in Equation D.19. So average dilution factor is

$$\bar{d}_i = \sum_j (1/(V_{ij} \cdot s^2_{(c)ij})) / \sum_j (1/s^2_{(c)ij}) \tag{D.23}$$

and estimated standard error of concentration (Eqn. D.19) is

$$s_{(c)i} = \bar{d}_i \cdot s_X \cdot \tag{D.24}$$

Although I used Equation D.24 to estimate standard errors for Chapter I, it is nevertheless true that most of the range in standard errors is attributable to range of dilutions and specimen dry masses. Because weighted regression only requires relative weights, it probably makes little difference in the final form of the fitted curves whether one uses Equation D.24 or Equation D.20.

Once concentration of the component of interest (in μg/ml) has been determined by Equation D.19 and standard error of that concentration estimated by either Equation D.20 or D.24, it is possible

to convert the data to concentration per unit dry mass and, then, to compare concentrations of different components (*e. g.*, calculation of level as ratio of one component to DNA). A measurement of μg/ml is converted to μg/mg dry mass by multiplying C_i by total volume of that component's fraction (from separation procedure of Appendix B) and dividing by dry mass of sample analyzed. If measurements of total volume and sample dry mass can be considered exact, standard error of the resulting concentration is estimated simply by multiplying $s_{(c)i}$ by the same factors as C_i. However, if it is desireable to include uncertainty of total volume or dry mass in final estimates of standard error, the following equation from Beers (1957) is applicable:

If $V = xy$ or $V = x/y$, then

$$s_V/V = \sqrt{(s_x/x)^2+(s_y/y)^2}. \qquad (D.25)$$

This equation (D.25) is also applicable when concentration of one component is divided by concentration of another component, as in calculation of level.

Periodic Regression

Selection of Regression Function

Quantitative description of changes in a measurement over a range of values for one or more independent variables can be accomplished most effectively by regression. Analysis of variance followed by multiple comparison tests can provide similar information provided that values for the independent variables fall naturally into several groups and the precise shape of the relationship between the dependent variable and the independent variables is not of interest. One of the major initial problems in regression analysis is selection of a regression function. In some cases, selection of regression function

can be based on a priori considerations. (E. g., a first or second degree polynomial is generally appropriate for standard curves. Beer's law predicts a first degree polynomial if concentration range is sufficiently narrow; a second degree polynomial is convenient and effective if Beer's law is not strictly followed, although one must beware of extrapolation.) In the absence of theoretical predictions of regression function, it may be necessary to try several different functions and select a function or family of functions which adequately describes the data. Selection of regression function under these circumstances must be guided by two conflicting desiderata (Neter and Wasserman 1974): (1) the fitted regression function should adequately describe the data, and (2) the number of parameters should be kept as small as possible. In addition, parameters used in a descriptive (rather than purely predictive) model should ideally have some biologically meaningful interpretation.

Polynomial regression provides an obvious family of regression functions for description of a complex pattern, and I performed some preliminary analysis (not presented here) of biochemical components by polynomial regression of component on date. There are several major advantages to polynomial regression. First, it is computationally simple. Any good statistical package for a computer can perform polynomial regression; in fact, the calculations are simple enough that they can be feasibly performed with nothing more than a simple calculator. Second, selection of terms for inclusion in the final model follows a well-defined statistical procedure, generally forward selection (Zar 1974). Third, the fitted curve can be made to fit observed data as precisely as desired simply by increasing the power of

the polynomial, although in practice a forward selection procedure is used to determine degree of the fitted polynomial. However, several disadvantages to polynomial regression may override its advantages. Polynomial regression does not allow effective extrapolation, particularly if the underlying relationship is not really a polynomial. In addition, behavior of a fitted function within the range of the independent variable may depart excessively from the actual relationship, either because the fitted curve is too smooth or because it includes extraneous local extrema (i. e., interpolation must also be performed cautiously). In the case of biological components analyzed for this dissertation, parameters are often not readily interpretable biologically. Although the coefficient of the first power of time, the slope of the fitted line, can be viewed as the rate of change of a component with time, the meaning of coefficient of higher powers is less clear. (Coefficient of date^2 presumably indicates concavity of the fitted curve, i. e., rate of change of slope, but higher powers serve mainly to provide a "good" fit without contributing to interpretation.) In addition, polynomial regression explicitly ignores the inherently cyclical nature of a reproductive cycle and thus seems to me to be counter-intuitive.

The asteroid spermatogenic cycle is intrinsically cyclical, with the testes annually passing through the same sequence of states at approximately the same times of year (subject to sampling variability and, possibly, differences attributable to environmental conditions). Of course, certain biochemical measurements, notably estimates of total testicular content of a component, undergo cataclysmic changes at spawning which may result in an extremely steep or possibly

discontinuous relationship. For these components, it may be appropriate to consider the cycle to have a distinct beginning and end. In other cases, however, there may be no evidence for discontinuity, in which case a non-periodic regression function is inappropriate. For any cyclical phenomenon, time is properly considered to be a circular variable (Batschelet 1981), and periodic regression is an appropriate statistical tool.

A simple sine curve is the fundamental model for periodic (i. e., circular-linear) regression, serving an approximately analogous role to that of a straight line in linear regression. The basic equation assumes that the period (T) of the independent variable (usually time) is known a priori.

$$y = M + A \cdot \cos(\omega(t-t_0)) \qquad (D.26)$$

In addition to the specified angular frequency, $\omega = 2\pi/T$, this model has three parameters determined by regression: M = "mean level" or "mesor", the average value attained by the dependent variable; A = "amplitude", assumed to be greater than zero; t_0 = "peak phase" or "acrophase", a circular variable (modulo T) which specifies the time at which the fitted dependent variable attains its maximum value. Equation D.26 is occasionally written

$$y = M + A \cdot \cos(\omega t-\phi) \qquad (D.27)$$

where the acrophase angle, $\phi = \omega t_0$, is substituted for acrophase; this substitution makes certain subsequent computations clearer.

Relationships between two linear variables that are more complex than a simple straight line can often be adequately described by a polynomial. In the same sense, Equation D.26 (or D.27) can be generalized for periodic regression to a trigonometric polynomial:

$$y = M + A_1\cos(\omega t-\phi_1) + A_2\cos(2\omega t-\phi_2) + \ldots + A_k\cos(k\omega t-\phi_k) \quad (D.28)$$

This trigonometric polynomial corresponds to a partial Fourier series in which shorter periods which fit exactly into the principal period (T/2, T/3, etc.) are added to the fundamental sine curve. These "higher harmonics" are analogous to higher power terms in normal polynomial regression.

For computation, Equation D.26 is usually transformed algebraically into

$$y = M + X\cdot\cos(\omega t) + Y\cdot\sin(\omega t) \quad (D.29)$$

where $X = A\cdot\cos(\phi)$ and $Y = A\cdot\sin(\phi)$. The advantage of Equation D.29 is that it is linear in all three parameters and both predictors. Thus, straightforward techniques of multiple linear regression can be applied, with parameters M, X, and Y determined from regression on the predictors $\cos(\omega t)$ and $\sin(\omega t)$. After Equation D.29 is fitted to data, Equation D.27 can be produced by solving the definitions of X and Y for A and ϕ:

$$A = \sqrt{(X^2 + Y^2)} \quad (D.30)$$

$$\phi = \begin{cases} \arctan(Y/X) & \text{if } X>0 \\ \pi + \arctan(Y/X) & \text{if } X<0 \end{cases} \quad (D.31)$$

Equation D.28 can be treated similarly and terms added by forward selection as in polynomial regression.

Preliminary trials of trigonometric polynomial regression as a means of describing patterns of biochemical changes during spermatogenesis revealed the advantages and disadvantages of this approach. Trigonometric polynomials (Eqn. D.28) are not much more complex computationally than simple linear polynomials and generally have only a few additional parameters. Furthermore, they explicitly

recognize the periodic nature of the relationships described. In addition, trigonometric polynomials allow for the occurrence of more than one peak or trough during the fundamental period. Their principal disadvantage is that the parameters are often not biologically meaningful; it is not clear that a three-month period, for example, has any inherent meaning in the annual spermatogenic cycle. If one is willing to forgo biologically meaningful parameters, trigonometric polynomials can provide good descriptions of cyclic phenomena.

Provided that there is only one peak and one trough in the fundamental cycle, a highly versatile regression function can be derived (Batschelet 1981) which adds at most two additional parameters:

$$y = M + A\cdot\cos\{\omega(t-t_0) + \nu_s\cdot\cos(\omega(t-t_0)) + \nu_p\cdot\sin(\omega(t-t_0))\}. \qquad (D.32)$$

Equation D.32 is derived from the sine curve of Equation D.26 by the addition of two internal terms, $\nu_s\cdot\cos(\omega(t-t_0))$ and $\nu_p\cdot\sin(\omega(t-t_0))$. The two added parameters, ν_s and ν_p, are called the parameters of skewness and peakedness, respectively. The parameter of skewness determines whether the function's peak is closer to the preceeding ($\nu_s>0$) or following ($\nu_s<0$) trough. Batschelet (1981) recommends that ν_s be restricted to the range $[-30^o,30^o]$ in order to ensure a sufficiently smooth function, but I did not find this restriction necessary. The parameter of peakedness determines whether the fitted curve is relatively sharply peaked ($\nu_p>0$) or flat-topped ($\nu_p<0$) compared to a simple sine curve. Batschelet (1981) recommends that ν_p be restricted to the range $[-60^o,60^o]$ in order to avoid secondary peaks and troughs. The regression function given in Equation D.32 is extremely versatile and able to adequately describe a wide variety of

cyclical patterns. All of the parameters are readily interpretable in terms of the fitted pattern, thereby facilitating biological interpretation. Because of these advantages, Equation D.32 was extensively used for regression analysis of biochemical data in Chapter I; addition of ν_s and ν_p to the model was accomplished through forward selection. A minor disadvantage, provided computer assistance is available, is that Equation D.32 is not linear in the parameters and cannot be algebraically transformed to a linear form; consequently, non-linear regression is required. Another disadvantage of Equation D.32 is its apparent inability to model extremely rapid or discontinuous changes. Consequently, it may be less appropriate for cyclical patterns demonstrably involving such rapid changes, although careful examination of residuals can largely correct this limitation. It may be possible to add a term, or modify an existing term, in order to allow for extremely rapid changes in dependent variable over a short period of time.

There are a number of alternative regression functions and other methods of analyzing data such as that presented in Chapter I. I have not examined any of these alternatives in detail, but for completeness I will briefly describe several possibilities for future investigation. As mentioned above, there may be an additional term, or modifications of existing terms, that can be added to Equation D.32 to allow for more rapid changes in dependent variable. If it considered appropriate to consider time as a linear (rather than circular) variable for measurements which show a distinct discontinuity at some point in the cycle, other classes of regression function can be investigated in place of polynomials; some such functions may have more easily

interpreted parameters (see, for example, Perkkiö and Keskinen 1985). In addition, either the independent or dependent variable (or both) can be logarithmically transformed, and polynomial regression performed with the transformed data.

Another set of procedures worthy of consideration do not involve regression are thus are not amenable to statistical quantification but can provide good visual impressions of temporal patterns. Provided the data points are equally spaced, or nearly so, data smoothing (see Velleman and Hoaglin 1981 for review) is extremely effective at removing experimental noise and revealing underlying patterns. Because data smoothing is less effective at endpoints, data at each "end" of the cycle can be appended to the other end in order to ensure that an entire cycle is properly smoothed. For irregularly spaced data, I think a method analogous to data smoothing by hanning (Velleman and Hoaglin 1981) in which regularly spaced, partially smoothed data are generated by a running mean of raw data is intuitively appealing and could be very useful. For a specified time, data occurring near that time (within a previously specified range of times) would be weighted by some function that varies inversely with temporal distance. Alternatively, regularized data could be generated by a running median of all data within a previously specified temporal range.

Correction for Heteroscedasticity

One of the assumptions underlying regression analysis is that error variance is constant for all observations (homoscedasticity). When that assumption is violated, a condition called "heteroscedasticity", proper application of regression analysis requires some form of correction for non-constant variances (Neter and

Wasserman 1974). In many cases, variance-stabilizing transformations can be selected by examination of the relationship between residuals and either dependent or independent variables (see virtually any good introductory statistics text for details); such transformations often also help correct for violations of other assumptions of regression analysis (*e. g.*, normality of errors and linearity of the fitted function). An equivalent procedure that does not affect assumptions of normality of errors or shape of the regression function is weighted regression (Neter and Wasserman 1974; Dixon *et al.* 1983). In weighted regression, each data point is assigned a weight proportional to the inverse of its residual variance.

In the case of biochemical measurements analyzed in Chapter I, error variances can be assigned to data points on the basis of propagation of error, as previously described. These error variances, which represent relative uncertainty of the data points, generally have no intrinsic functional relationship to either the dependent or independent variables. Consequently, there is no transformation effective in satisfying a requirement for homoscedasticity. Weighted regression, however, satisfies that requirement. Therefore, regression analysis in Chapter I involved weighting each data point by the inverse of its estimated error (from Equation D.24ff.) for regression with the function in Equation D.32.

Confidence Bands

Confidence bands are extremely useful in providing simple visual interpretation of relative uncertainties in fitted regression functions and indicating an overall region in which the regression function lies. For linear regression (including multiple linear regression and

linearizable functions such as Equation D.27, which can be expressed in the equivalent linear form of Equation D.29), 1-α confidence limits can be calculated by substituting Working and Hotelling's W for t in Equation D.16 (Neter and Wasserman 1974):

$$W^2 = pF_{(1-\alpha;p,n-p)} \qquad (D.33)$$

where p is the total number of parameters in the regression function and n is the number of data points. For non-linear regression functions, an analogous form of Equation D.16 can be used:

$$Y_h = \hat{Y}_h \pm W \cdot s(\hat{Y}_h), \qquad (D.34)$$

where W is calculated by Equation D.33 and $s(\hat{Y}_h)$ is the standard error of a predicted Y, often provided by the regression program for each data point (Dixon et al. 1983).

For data in Chapter I which were fitted to the non-linear periodic regression function of Equation D.32, upper and lower confidence limits were calculated by applying Equation D.34 for each data point. Upper and lower limits for confidence bands were produced by fitting the same regression function (Eqn. D.32) to the two sets of points thus produced.

Correction of Mesor and Acrophase

The addition of parameters of skewness and peakedness (ν_s and ν_p) to periodic regression (Eqn. D.32) results in the parameters M and t_0 no longer representing the true mean level and acrophase. Batschelet (1981) describes calculations necessary to calculate true values of mean level and acrophase when either ν_s or ν_p (but not both) is added to the model. These methods can be easily extended to include cases in which both ν_s and ν_p are included in the regression function. Essentially, the true mesor of a specified function is calculated by

integrating the function over one period, and the true acrophase calculated by differentiating the function to find an absolute maximum. Thus, the true mean level or mesor is

$$M = \frac{1}{365} \int_{0}^{365} [\hat{Y}_t]dt \qquad (D.35)$$

where $\hat{Y}_t$ is calculated by Equation D.32, or equivalently, by defining $\psi = \omega t - \phi$,

$$M = M + (A/2\pi) \cdot \int_{1}^{2\pi} \cos(\psi + \nu_s \cos\psi + \nu_p \sin\psi) d\psi \; . \qquad (D.36)$$

For the regression function of Equation D.32, or its equivalent form

$$y = M + A \cdot \cos[\psi + \nu_s \cos\psi + \nu_p \sin\psi], \qquad (D.37)$$

local maxima can be determined by differentiation. However, because acrophase is the function's absolute maximum, a conceptually simpler approach is possible. For ease of notation, define

$$\xi = \psi + \nu_s \cos\psi + \nu_p \sin\psi. \qquad (D.38)$$

Then Equation D.37 can be re-expressed as

$$y = M + A \cdot \cos(\xi) \qquad (D.39)$$

which attains an absolute maximum of M+A if and only if $\cos(\xi)=1$. But $\cos(\xi)=1$ if and only if $\xi=0$; therefore the true acrophase of Equation D.39 (and hence of Equations D.37 and D.32) can be found by solving

$$\psi + \nu_s \cos\psi + \nu_p \sin\psi = 0 \qquad (D.40)$$

for ψ. The acrophase is given as a Julian date by applying the definition of ψ, i. e.

$$t_a = t_0 + \psi/\omega. \qquad (D.41)$$

Acrophase date (t_a) can also be found directly by substituting the definition $\psi=\omega(t_a-t_0)$ into Equation D.40 and solving for t_a.

Circular Correlation

The intent of correlation analysis is to examine interdependence of two or more variates. If any of the variates involved belong to a circular distribution, normal methods of linear correlation are not generally applicable; instead, circular correlation analysis must be applied (for review see Batschelet 1981). Unfortunately, multivariate correlation analysis has not yet been developed (Batschelet 1981), so only bivariate methods are now available. Several distinct tests for correlation can be applied, depending upon whether one or both variates are circular and whether certain assumptions about the distributions of the variates hold. The methods of circular correlation used in this dissertation include: rank correlation of two circular variates (Mardia 1975); parametric correlation of two circular variates, without an assumption that both variates are uniformly distributed (Jupp and Mardia 1980); parametric circular-linear correlation, with the assumption that the underlying relationship is approximately sinusoidal (Mardia 1976); circular-linear correlation by ranks (Mardia 1976). All of these methods, and several others, are described in detail in Batschelet (1981).

When methods using ranks were applied to data from Chapter I, ties (especially in spermatogenic stage) were resolved by assigning ranks equal to the mean of the ranks that would have been assigned to those ranks had they not been tied. For example, if the third and fourth highest values are identical, they are assigned ranks of $(3+4)/2 = 3.5$. For circular variates, an arbitrary origin is chosen for assignment of ranks.

APPENDIX E

COMPUTER APPLICATIONS FOR STATISTICAL ANALYSIS

This appendix contains several independent programs and sequences of instructions for commercial programs which I found especially useful in applying the statistical procedures described in Appendix D. The information contained here may allow similar analyses to be pursued without the attendent (and often extensive) labor required to produce necessary computer programs. The specific programming included here is not necessarily the most efficient possible; in particular, it might be useful to combine all of the program units into a single larger program, thereby eliminating many of the intervening steps and minimizing input-output requirements. In addition, the programs given in this appendix often do not check input data for errors or unreasonable values. My goal here is not to present the best possible set of programs, but simply to present a set that works for the analyses described.

Analysis of data as outlined in Appendix D was performed with routines in five "languages". Most of the analyses were performed on the University of New Hampshire's DECsystem-1090 using (1) Minitab (Ryan et al. 1976), (2) BMDP (Dixon et al. 1983), and (3) BASIC (for several specific tasks not easily accomplished with the standard statistical packages). In addition, some analyses were performed with a NorthStar Advantage microcomputer programmed with FORTRAN-80 (released by Microsoft). Plots were prepared on a CalComp plotter with the aid of a plotting program (UPLOT, by Alan L. Baker of UNH).

Fundamental familiarity with both statistical packages and the two programming languages is assumed here. Because plotting commands are likely to be installation-specific, details of the plotting routines are not included in this appendix. Also, details of many of the necessary intervening steps have not been included; given minimal familiarity with the commercial packages and their procedures, the intervening steps should be reasonably clear.

Programming described here follows the sequence in Appendix D, which was also approximately the sequence of analysis. Within program units, double semi-colons, ";;", are used to mark explanatory comments; these comments are only included for clarification in this appendix and would not be included in actual computer code. (The statistical packages in particular are not well adapted to internal documentation.)

Standard Curves

Given amount of standard and associated optical density for several replicates covering the range of a spectrophotometric test, a standard curve (either Equation D.1 or D.2 as required) is easily calculated and its significance determined with Minitab or any other statistical package. Testing equivalence of several standard curves by Equation D.4 is also straightforward, although possibly tedious. Once coincident standard curves are combined, methods of inverse prediction are used to determine amount of component and associated standard error for sample replicates.

Inverse Prediction

With the help of Minitab and a text editor, a data file was constructed for each group of coincident standards. Each data file

contained: power of the fitted standard curve (p = 1 or 2), number of data points used to construct the standard curve (n), a list of the n standards used to construct the standard curve (X-vector), p+1 regression coefficients, mean square error of standard curve regression, and a record for each replicate associated with that group of standards. Each replicate was represented by three numbers: (1) observed optical density of that replicate, (2) $\hat{X} - s_{\hat{X}}$, and (3) $\hat{X} + s_{\hat{X}}$, where $\hat{X}$ was determined by either Equation D.7 or D.14 and $s_{\hat{X}}$ by Equation D.12. This data file was used as input to REGVA.BAS, which applies numerical methods to solve Equation D.16 for confidence limits one standard error above and below X_h for Y_h from each replicate.

The BASIC program REGVA.BAS follows:

```
00010   DIM X(60,3),X1(3,60),X2(3,3),X4(3),X5(1,3)
00015   DIM X6(1,3),X7(2,2),X8(2,2),B(3)
    ;;   Note: if n > 60, dimensions of the arrays X and X1 must be
    ;;   adjusted accordingly.
00020   '
00030   '    OPEN DATA FILE FOR SEQUENTIAL INPUT AND OUTPUT
00040   '
00050   PRINT "FILENAME FOR INPUT:";
00060   INPUT F$
00070   FILE #1,F$
00072   PRINT "FILENAME FOR OUTPUT:";
00074   INPUT F$
00076   FILE #2,F$
00078   SCRATCH #2
00080   '
00090   '    SET POWER OF REGRESSION LINE (LINEAR OR QUADRATIC)
00100   INPUT #1, P
00110   '
00120   '    READ IN X COORDINATES OF REGRESSION DATA; SET UP MATRICES
00130   INPUT #1, N
00140   MAT X = ZER(N,P+1)
00150   MAT B = ZER(P+1)
00160   MAT X4 = CON(P+1)
00170   FOR I = 1 TO N
00180        X(I,1) = 1
00190        INPUT #1, X(I,2)
00200        IF P=1 THEN 220
00210             X(I,3) = X(I,2)**2
00220        NEXT I
```

```
00230   MAT X1 = TRN(X)                    '   X1=X'
00240   MAT X2 = X1*X                      '   X2=X'X
00250   MAT X2 = INV(X2)                   '   X2=X'X-INVERSE
00260   PRINT "X'X-INVERSE"
00270   PRINT
00280   MAT PRINT X2
00290   PRINT
00300   '
00310   '    READ IN REGRESSION COEFFICIENTS AND MEAN SQUARE ERROR
00320   FOR I = 1 TO P+1
00330        INPUT #1, B(I)
00340        NEXT I
00350   INPUT #1, S2
00360   '
00370   '    INPUT CASE FOR ANALYSIS
00380   INPUT #1, Y,E1,E2
00390   '
00400   '    CALCULATE PREDICTED X          ;; X3 = predicted X
00410   IF P=2 THEN 440
00415   '         LINEAR
00420        X3=(Y-B(1))/B(2)
00430        GOTO 450
00435   '         QUADRATIC
00440        X3=(-B(2)+SQR(B(2)**2-4*B(3)*(B(1)-Y)))/(2*B(3))
00450   '
00460   'SOLVE FOR LIMITS BY SECANT METHOD (HORNBECK 1975)
00470   D = E1 - X3
00480   F2 = FNF(X3)
00490   F1 = FNF(E1)
00495   PRINT F1,F2,D
00500   D = -F1*D/(F1-F2)
00510   E1 = E1 + D
00520   IF ABS(D)<ABS(X3)*1E-6 THEN 550
00530   F2 = F1
00540   GOTO 490
00550   D=E2-X3
00560   F2=FNF(X3)
00570   F1=FNF(E2)
00580   D=-F1*D/(F1-F2)
00590   E2=E2+D
00600   IF ABS(D)<ABS(X3)*1E-6 THEN 630
00610   F2=F1
00620   GOTO 570
00630   PRINT #2,Y;X3;E1;E2,((E2-E1)/2)**2
00640   GOTO 380                           '    NEXT CASE
00650   STOP
00660   DEF FNF(X)
     ;;   The function FNF(X) is an algebraic re-arrangement of
     ;;   Equation D.16, placing all terms on the same side of the
     ;;   "equals" sign and eliminating square root (as well as
     ;;   computational ambiguity of Equation D.16).  Solution by
     ;;   secant method requires that the equation be expressed in the
     ;;   form f(x)=0.  FNF provides values of f(x) at intermediate
     ;;   estimates of x.
```

```
00670       X4(1)=1                    ;; Array X4 = Xh
00680       X4(2)=X
00690       IF P=1 THEN 710
00700           X4(3)=X**2
00710       MAT X5 = TRN(X4)
00720       MAT X6 = X5*X2
00730       MAT X7 = X6*X4
00740       MAT X8 = X5*B
00750       FNF = (Y - X8(0,0))**2 - S2*(1 + X7(0,0))
                                   ;; X7 = Xh'(X'X)^-1 Xh
                                   ;; X8 = Xh' b = Yh
                                   ;; FNF = (Yh - Ŷh)2 - MSE(1+Xh'(X'X)^-1 Xh)
00760       FNEND
00770   END
```

This program, run interactively, requests names for input and output files and prints out the $(\mathbf{X}'\mathbf{X})^{-1}$ matrix. The output file (generated in line 630) contains a row for each replicate, with five columns: (1) optical density, Y_h; (2) amount of component by inverse prediction, $\hat{X}_h$; (3) final numerical estimate of $\hat{X}_h - s_{\hat{X}}$; (4) final numerical estimate of $\hat{X}_h + s_{\hat{X}}$; (5) variance of $\hat{X}_h$ estimated as the square of deviation of (3) and (4) from (2). Although only the second and fifth columns are needed for subsequent calculations, the other columns can sometimes be useful in debugging.

Data Reduction

Data reduction as described in Appendix D can be performed relatively easily with the aid of Minitab or an equivalent statistical package. Use of Minitab's "STORE" and "EXECUTE" commands is especially useful for accomplishing repetitive tasks such as calculating weighted averages for each specimen (Equation D.19) and subsequent application of Equations D.21 to D.24. For these calculations, all replicates for a given specimen must be isolated (*e. g.,* by means of Minitab's "PICK" or "CHOOSE" commands) to allow summations for that specimen. Final data reduction to concentration is also straightforward.

Periodic Regression

Periodic regression using the regression function given as Equation D.32 requires a statistical package capable of non-linear regression, such as BMDP. Most non-linear regression routines require initial estimates of regression coefficients; for the data analyzed here, initial estimates can be obtained by linear regression with the function D.29. Regression coefficients for Equation D.29 can then be converted to initial estimates of M, A, and t_0 by means of Equations D.30 and D.31. Because Minitab is able to perform weighted linear regression, it can be used very effectively to provide initial estimates for non-linear regression by BMDP; ν_s and ν_p have initial estimates of zero.

For each biochemical measurement, the data reduction procedure produced a data file containing a row for each specimen and three columns. The first column contained date (as number of days from beginning of calendar year), the second column contained the biochemical measurement (concentration, level, or content), and the third column contained an estimate of the standard error of measurements in the second column. These files were used as input to the program BMDPAR (Dixon et al. 1983). Significance of ν_s and ν_p were tested against models in which those parameters were not included with an F test:

$$F = \frac{\dfrac{\text{SSE(reduced model)} - \text{SSE(full model)}}{\text{DFE(reduced)} - \text{DFE(full)}}}{\text{MSE(full model)}}$$

with (DFE(r)-DFE(f)) and DFE(f) degrees of freedom. Based on tests of significance with this F statistic, ν_s and ν_p were added by forward selection; consequently BMDPAR was executed up to four times for each

biochemical measurement. The following sequence of instructions for BMDPAR were contained in a separate control language file, modified as necessary for specific analyses:

```
/PROBLEM
        TITLE IS 'NONLINEAR PERIODIC REGRESSION'.
/COMMENT
        'SUBSTITUTE CORRECT FILE NAME INTO  INPUT.
        SUBSTITUTE REASONABLE INITIAL VALUES INTO PARAMETER.'.
/INPUT
        VARIABLES ARE 3.
        FORMAT IS FREE.
        FILE IS '??????.DAT'.
/VARIABLE
        NAMES ARE ID, CONCEN, SE, DATE, CASEWT.
          ;;   Some data sets had first column as an identification
          ;;   number, formed with date as integral portion with a
          ;;   sequence number for that date appended after the decimal
          ;;   point.
        ADD = 2.
        MISSING = 3*0.
/TRANSFORMATION
        DATE = INT(ID).
        CASEWT = 1/SE**2.
/SAVE
        FILE = '??????.SAV'.
        NEW.
        CODE = ??????.
/REGRESS
        DEPENDENT = 2.
        PARAMETER = 5.
        WEIGHT = CASEWT.
/PARAMETER
        FIXED ARE SK,PK.            ;;   This line modified depending on
                                    ;;   which of νs and νp, if any, are
                                    ;;   to be included in the
                                    ;;   regression model.
        NAMES ARE M, AMP, ACR, SK, PK.
        INITIAL = ?, ?, ?, 0, 0.
        MINIMUM = (5)-1.0471976.
        MAXIMUM = (5)1.0471976.
/FUN
        OMEGA = 2*3.1415926536/365.
        TMP1  = OMEGA*(DATE-ACR).
        TMP2  = SK*COS(TMP1).
        TMP3  = PK*SIN(TMP1).
        F     = M + AMP*COS(TMP1+TMP2+TMP3).
/END
```

In the control language shown here, entries appropriate for the specific data set analyzed must be inserted wherever question-marks appear. The "SAVE" paragraph is only required for the final form, in order to save estimates of predicted Y and standard errors of predicted Y. The data files produced by the SAVE paragraph provide input to a similar set of control language instructions; upper and lower limits for the confidence bands are estimated by fitting the same regression function to data calculated from predicted Y and standard error of predicted Y by Equation D.34.

True values of mean level and acrophase are calculated by numerical solution of Equations D.36 and D.40, as in the following FORTRAN program.

```
C       THIS PROGRAM WORKS INTERACTIVELY TO ESTIMATE TRUE VALUES FOR
C       MEAN LEVEL AND TIME OF MAXIMUM VALUE (ACROPHASE) OF A PERIODIC
C       REGRESSION.  USER SUPPLIES REGRESSION PARAMETERS.  PEAK IS
C       FOUND BY SECANT METHOD, FINDING ROOT OF XI (= PSI+SK*COS(PSI)
C       +PK*SIN(PSI), WHICH IS POINT AT WHICH FITTED FUNCTION ATTAINS
C       VALUE OF MESOR+AMP.  PROGRAM TABULATES XI OVER THE RANGE
C       (-PI, PI], USER THEN SUPPLIES INITIAL ESTIMATES FOR LIKELY
C       ROOTS.  MEAN LEVEL IS FOUND BY ROMBERG INTEGRATION OF
C       REGRESSION FUNCTION.
C
C       FRANK F. SMITH          26 FEBRUARY 1985
C
        INTEGER  I, J, K, L, N
        REAL TOL, PI, OMEGA, MESOR, AMP, ACR, SK, PK, PSI, XI, T1,
     1          ROOT(10), DELTA, FOLD, FNEW, A, B, T(15,15), FUN, MEAN
        FUN(A) = MESOR + AMP*COS(A+SK*COS(A)+PK*SIN(A))
C
C       GLOSSARY
C
C       TOL     TOLERANCE FOR CONVERGENCE
C       PI      3.1415926
C       OMEGA   ANGULAR FREQUENCY: 2*PI/365 FOR ANNUAL CYCLE
C       MESOR   REGRESSION PARAMETER
C       AMP     "
C       ACR     "
C       SK      "
C       PK      "
C       PSI     INTERMEDIATE ANGLE, = OMEGA*(T-ACR)
C       XI      ANOTHER INTERMEDIATE, = PSI+SK*COS(PSI)+PK*SIN(PSI)
C       T1      TIME, IN DAYS (JULIAN)
```

```
C       ROOT    ARRAY TO HOLD ROOTS OF EQUATION XI=0 (10 SPACES SHOULD
C               BE MORE THAN ENOUGH.
C       DELTA   X-INTERVAL
C       FOLD    USED IN SECANT ESTIMATION OF ROOTS
C       FNEW    "
C       A,B     INTEGRATION RANGE, [0, 2*PI]
C       T       ARRAY HOLDS INTEGRAL ESTIMATES FOR ROMBERG INTEGRATION
C       MEAN    ESTIMATED TRUE MEAN LEVEL
C       FUN     STATEMENT FUNCTION TO CALCULATE REGRESSION FUNCTION AT
C               A SPECIFIED VALUE OF PSI
C
        TOL = 1E-4
        PI = 3.1415926
        OMEGA = 2*PI/365.0
5       READ(1,10) MESOR, AMP, ACR, SK, PK
10          FORMAT(5F10.6)
        WRITE(1,20)
20          FORMAT('     T      PSI      XI')
        DO 40 I=1,20
            PSI = PI*(I/10.0 - 1)
            XI = PSI + SK*COS(PSI) + PK*SIN(PSI)
            T1 = PSI/OMEGA + ACR
            WRITE(1,30) T1, PSI, XI
30              FORMAT(F7.1,F7.3,F8.4)
40          CONTINUE
        WRITE(1,45)
45          FORMAT(' NUMBER OF ROOTS:')
        READ(1,50) N
50          FORMAT(I3)
        WRITE(1,60)
60          FORMAT(' INPUT LOWER ESTIMATE OF T FOR EACH ROOT')
        READ(1,70) (ROOT(I),I=1,N)
70          FORMAT(10F3.1)
        DO 90 I=1,N
            DELTA = PI/10.0
            PSI = OMEGA*(ROOT(I)+DELTA-ACR)
            FNEW = PSI + SK*COS(PSI) + PK*SIN(PSI)
75          FOLD = FNEW
            PSI = OMEGA*(ROOT(I)-ACR)
            FNEW = PSI + SK*COS(PSI) + PK*SIN(PSI)
            DELTA = -FNEW*DELTA/(FNEW-FOLD)
            ROOT(I) = ROOT(I) + DELTA
            IF (ABS(DELTA).GE.TOL) GOTO 75
            WRITE(1,80) ROOT(I)
80              FORMAT(F10.4)
90          CONTINUE
C
C       NUMERICAL INTEGRATION
C
        A = 0
        B = 2*PI
        T(1,1) = (FUN(A)+FUN(B))*(B-A)/2
        T(1,2) = T(1,1)/2 + FUN((A+B)/2) * (B-A)/2
        T(2,1) = (4*T(1,2) - T(1,1))/3
```

```
      J=3
100   DELTA = (B-A)/2**(J-1)
      X = A - DELTA
      N = 2**(J-2)
      SUM = 0
      DO 105 I=1,N
          X = X + 2*DELTA
          SUM = SUM + FUN(X)
105       CONTINUE
      T(1,J) = T(1,J-1)/2 + DELTA*SUM
      L = 2
110   K = J + 1 - L
      T(L,K) = (4**(L-1) * T(L-1,K+1) - T(L-1,K))/(4**(L-1)-1)
      IF (L.EQ.J) GOTO 120
          L = L + 1
          GOTO 110
120   IF (ABS((T(J,1)-T(J-1,1))/T(J,1)).LT.TOL) GOTO 150
          J = J + 1
          IF (J.LE.15) GOTO 100
              WRITE(1,130)
130               FORMAT('0CONVERGENCE NOT REACHED IN 15 ITERATIONS')
              J = 15
150   MEAN = T(J,1)/(2*PI)
      WRITE(1,160)
160       FORMAT('0ROMBERG INTEGRATION TABLE')
      DO 168 K=2,J
          I = J-K+1
          DO 167 L=1,I
              N=I-L+1
              T(K,N+1)=T(K,N)
167           CONTINUE
168       CONTINUE
      DO 180 K=1,J
          WRITE(1,170) (T(I,K), I=1,K)
170           FORMAT(15F10.4)
180       CONTINUE
      WRITE(1,190) MEAN
190       FORMAT('0MEAN LEVEL IS ',F10.4)
      WRITE(1,200)
200       FORMAT('0ANOTHER EQUATION (Y/N)')
      READ(1,210) I
210       FORMAT(A1)
      IF (I.EQ.'Y') GOTO 5
      END
```

APPENDIX F

BIOCHEMICAL DATA

This appendix contains original and processed data for the biochemical components discussed in Chapter I. All information necessary for analyzing the data has been included, so that other methods of analysis can be applied to the same data for comparison of different analytical approaches. In standard curves presented here, amounts of standards and associated optical densities are given for each set of standards; the sets of standards are followed by regression functions for coincident groups of standards. In these regression functions, amount of component (usually in μg) is indicated by X. Regression coefficients for X^2 are often given in scientific notation, with an 'E' followed by a power of ten. Quadratic terms are included only if they significantly improve fit compared to a simple linear regression. Raw data for each component is given as a sequence of optical densities. Each specimen is identified by an identification number formed from both date (days from beginning of calendar year) and a sequence number for that date. Thus, the third animal processed on 2 February would receive the identification number '33.3'. Sample volumes (V_{ij} of Equation D.17) are given in parentheses following the associated optical densities; if several consecutive O.D.s from the same specimen have identical sample volumes, that volume is only given once, following all O.D.s with that volume (or occasionally at the top of a column). Also shown are total volume for the fraction containing that component and dry mass of sample analyzed. Fully processed data

(concentration, level, and content), along with other necessary measurements (such as gonad indices), appears as a single table following the entire set of raw data.

DNA

Standard Curves

Set A		Set B		Set C		Set D	
µg DNA	O.D.	µg DNA	O.D.	µg DNA	O.D.	µg DNA	O.D.
0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000
10.0	0.075	10.0	0.082	4.76	0.028	2.38	0.013
20.0	0.120	20.0	0.125	11.91	0.082	4.76	0.031
40.0	0.230	30.0	0.210	16.67	0.106	7.15	0.051
60.0	0.370	40.0	0.240	23.82	0.158	11.91	0.072
80.0	0.480	50.0	0.260	35.73	0.227	16.67	0.111
100.0	0.620	50.0	0.300	47.64	0.358	23.82	0.148
150.0	0.870	60.0	0.330	59.55	0.398	35.73	0.226
200.0	1.155	70.0	0.400	71.46	0.482	47.64	0.309
300.0	1.523	80.0	0.430	83.37	0.547	59.55	0.390
		90.0	0.500	95.28	0.636	71.46	0.467
		100.0	0.525	119.1	0.788	83.37	0.557
		120.0	0.620	142.9	0.932	95.28	0.627
		140.0	0.730	166.7	1.076	119.1	0.772
		160.0	0.824	190.6	1.194	142.9	0.921
		180.0	0.903				
		200.0	0.971				
		230.0	1.155				
		260.0	1.222				
		300.0	1.337				

Curves	Regression Function	Mean Square Error
A	$-0.00939 + 0.0067323\ X - 5.30382\ E\text{-}6\ X^2$	0.0003344
B	$0.014229 + 0.0056588\ X - 3.94613\ E\text{-}6\ X^2$	0.0003574
C+D	$-0.004648 + 0.0069647\ X - 3.25062\ E\text{-}6\ X^2$	0.0001054

Raw Data

ID#	Dry Mass (mg)	Total Volume	Replicates: Optical Density (Sample Volumes)		
Standard Set A					
360.8	105.6	5.0 ml	0.510 (0.1 ml)	0.959 (0.2 ml)	1.40 (0.3 ml)
54.2	106.7	5.0 ml	0.590 (0.1 ml)	1.050 (0.2 ml)	1.53 (0.3 ml)

59.1	127.2 mg	5.0 ml	0.585 (0.1 ml)	1.10 (0.2 ml)	1.495(0.3 ml)
108.1	113.7	5.0	0.760 (0.1)	1.44 (0.2)	1.82 (0.3)
109.1	102.6	5.0	0.550 (0.1)	1.046 (0.2)	1.40 (0.3)
109.2	101.7	5.0	0.630 (0.1)	1.22 (0.2)	1.54 (0.3)
115.1	115.1	5.0	0.810 (0.1)	1.40 (0.2)	1.77 (0.3)
115.1	109.5	5.0	0.690 (0.1)	1.25 (0.2)	1.68 (0.3)

Standard Set B

335.1	60.9 mg	5.0 ml	0.140	0.128	0.135	0.135	(0.1 ml)
359.2	15.6	5.0	0.185	0.185	0.185	0.192	(0.5)
359.3	34.3	5.0	0.260	0.253	0.261		(0.2)
360.4	12.7	5.0	0.144	0.160	0.155	0.162	(0.5)
53.2	82.5	5.0	0.415	0.425	0.425	0.412	(0.1)
53.3	108.7	5.0	0.550	0.540	0.525	0.540	(0.1)
54.1	51.8	5.0	0.455	0.460	0.455	0.468	(0.2)
115.2	176.0	5.0	0.425	0.430	0.430	0.410	(0.05)
146.3	76.1	5.0	0.452	0.450	0.450	0.450	(0.1)
147.2	9.5	5.0	0.205	0.206	0.210	0.199	(0.5)
271.2	5.2	5.0	0.032	0.032	0.031	0.030	(1.0)
271.3	8.1	5.0	0.092	0.095	0.098	0.093	(1.0)
272.2	5.7	5.0	0.060	0.062	0.060	0.060	(1.0)
301.1	4.8	5.0	0.090	0.087	0.085	0.085	(1.0)
301.2	4.1	5.0	0.050	0.048	0.046	0.049	(1.0)
321.2	4.5	5.0	0.050	0.047	0.049	0.060	(1.0)
321.3	1.6	5.0	0.020	0.018	0.021	0.031	(1.0)
323.3	2.4	5.0	0.040	0.032	0.035	0.040	(1.0)
337.2	7.2	5.0	0.120	0.120	0.120	0.130	(1.0)
18.1	52.6	5.0	0.520	0.526	0.526	0.525	(0.2)

			Standard Set C	Set D	
174.2	11.75	3.0	0.174	0.192	(0.1 ml)
180.3	5.513	3.0	0.100	0.069	(1.0)
181.2	14.78	3.0	0.218	0.220	(0.1)
199.2	14.38	3.0	0.065	0.058	(1.0)
199.3	4.948	3.0	0.030	0.041	(1.0)
200.2	5.080	5.0	0.054	0.065	(1.0)
222.2	7.497	3.0	0.268	0.292	(1.0)
223.1	4.014	3.0	0.092	0.120	(1.0)
223.2	5.074	3.0	0.067	0.092	(1.0)
223.3	3.036	3.0	0.079	0.107	(1.0)
291.1	1.638	3.0	0.020	0.038	(1.0)
291.2	2.818	3.0	0.028	0.056	(1.0)
292.1	10.58	3.0	0.462	0.496	(1.0)
292.2	1.594	3.0	0.027	0.037	(1.0)
292.3	2.263	3.0	0.049	0.076	(1.0)
56.1	421.4	10.0	0.139	0.196	(0.01)

RNA

Standard Curves

Curve A		Curve B				Curve C		Curve D	
µg RNA	O.D.	µg RNA	O.D.	µg RNA	O.D.	µg RNA	O.D.	µg RNA	O.D.
0	0.00	0	0.00	80	0.56	0	0.00	0	0.00
10	0.07	10	0.067	90	0.61	11.57	0.116	11.57	0.127
30	0.218	20	0.159	100	0.66	23.14	0.232	23.14	0.252
50	0.347	25	0.186	110	0.72	34.71	0.360	34.71	0.380
70	0.487	30	0.225	120	0.77	46.28	0.475	46.28	0.508
100	0.66	40	0.29	125	0.796	57.85	0.602	57.85	0.631
120	0.75	50	0.37	130	0.824	69.42	0.724	69.42	0.740
150	0.88	60	0.44	140	0.866	80.99	0.833	80.99	0.845
		70	0.505	150	0.917	92.56	0.939	92.56	0.950
		75	0.54			104.1	1.046	104.1	1.051
						115.7	1.149	115.7	1.137

Curve	Regression Function	Mean Square Error
A	$-0.005259 + 0.0078927\ X - 1.3184E\text{-}5\ X^2$	4.262 E-5
B	$-0.003897 + 0.0080848\ X - 1.3312E\text{-}5\ X^2$	4.845 E-5
C	$-0.008538 + 0.0109823\ X - 0.8123E\text{-}5\ X^2$	4.545 E-5
D	$-0.006958 + 0.0119148\ X - 1.7161E\text{-}5\ X^2$	2.795 E-5

Raw Data

ID#	Dry Mass (mg)	Total Volume	Replicates: Optical Density (Sample Volumes)				
Set A							
360.8	105.6 mg	15.0 ml	0.45	0.44 (0.3 ml)		0.695	0.698 (0.5 ml)
54.2	106.7	15.0	0.427	0.39 (0.3 ml)		0.725	0.64 (0.5 ml)
59.1	127.2	15.0	0.41	0.39 (0.3 ml)		0.62	0.60 (0.5 ml)
108.1	113.7	15.0	0.32	0.322 (0.3 ml)		0.521	0.505 (0.5 ml)
109.1	102.6	15.0	0.43	0.44 (0.3 ml)		0.685	0.676 (0.5 ml)
109.2	101.7	15.0	0.392	0.434 (0.3 ml)		0.622	0.68 (0.5 ml)
115.1	115.1	15.0	0.32	0.297 (0.3 ml)		0.57	0.566 (0.5 ml)
115.1	109.5	15.0	0.35	0.33 (0.3 ml)		0.508	0.500 (0.5 ml)
Set B							
335.1	60.9 mg	15.0 ml	0.350	0.372	0.375	0.380	(0.3 ml)
359.2	15.6	10.0	0.170	0.175	0.175	0.174	(0.3 ml)
359.3	34.3	15.0	0.255	0.265	0.250	0.250	(0.3 ml)
360.4	12.7	10.0	0.138	0.148	0.145	0.164	(0.3 ml)
53.2	82.5	15.0	0.440	0.437	0.440	0.440	(0.3 ml)
53.3	108.7	15.0	0.560	0.545	0.543	0.562	(0.3 ml)

54.1	51.8	15.0	0.340	0.323	0.338	0.332	(0.3 ml)
115.2	176.0	15.0	0.701	0.710	0.688	0.699	(0.3 ml)
146.3	76.1	15.0	0.260	0.257	0.250	0.250	(0.3 ml)
147.2	9.5	10.0	0.317	0.307	0.310	0.312	(0.5 ml)
271.2	5.2	10.0	0.055	0.054	0.052	0.065	(0.5 ml)
271.3	8.1	10.0	0.120	0.130	0.120	0.119	(0.5 ml)
272.2	5.7	10.0	0.075	0.075	0.075	0.078	(0.5 ml)
301.1	4.8	10.0	0.095	0.085	0.082	0.080	(0.5 ml)
301.2	4.1	10.0	0.048	0.051	0.047	0.051	(0.5 ml)
321.2	4.5	10.0	0.040	0.040	0.038		(0.5 ml)
321.3	1.6	10.0	0.040	0.048	0.046	0.043	(1.0 ml)
323.3	2.4	10.0	0.070	0.066	0.065	0.068	(1.0 ml)
337.2	7.2	10.0	0.098	0.100	0.100	0.096	(0.5 ml)
18.1	52.6	15.0	0.062	0.060	0.061	0.062	(0.3 ml)

			Set C	Set D	
174.2	11.75 mg	3.0 ml	0.420	0.538	(1.0 ml)
180.3	5.513	3.0	0.279	0.356	(1.0 ml)
181.2	14.78	3.0	0.490	0.599	(1.0 ml)
199.2	14.38	3.0	0.655	0.764	(1.0 ml)
199.3	4.948	3.0	0.262	0.310	(1.0 ml)
200.2	5.080	3.0	0.265	0.307	(1.0 ml)
222.2	7.497	3.0	0.807	0.886	(1.0 ml)
223.1	4.014	3.0	0.279	0.360	(1.0 ml)
223.2	5.074	3.0	0.391	0.472	(1.0 ml)
223.3	3.036	3.0	0.306	0.371	(1.0 ml)
291.1	1.638	3.0	0.151	0.188	(1.0 ml)
291.2	2.818	3.0	0.188	0.231	(1.0 ml)
292.1	10.58	3.0	0.991	1.131	(1.0 ml)
292.2	1.594	3.0	0.152	0.172	(1.0 ml)
292.3	2.263	3.0	0.207	0.223	(1.0 ml)
56.1	421.4	15.0	0.300	0.726	(0.1 ml)

Protein

Standard Curve

Set A		Set B		Set C		Set D	
µg	O.D.	µg	O.D.	µg	O.D.	µg	O.D.
0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000
25.0	0.125	25.0	0.135	5.0	0.028	0.0	0.016
50.0	0.235	50.0	0.268	10.0	0.050	12.3	0.017
75.0	0.34	75.0	0.370	15.0	0.081	24.6	0.045
100.0	0.47	100.0	0.460	20.0	0.110	36.9	0.071
125.0	0.51	125.0	0.555	25.0	0.134	49.2	0.128
150.0	0.595	150.0	0.65	30.0	0.160	61.5	0.152
175.0	0.69	175.0	0.75	35.0	0.180	73.8	0.167
200.0	0.77	200.0	0.84	40.0	0.205	86.1	0.201
225.0	0.84	225.0	0.92	45.0	0.230	98.4	0.215
250.0	0.93			50.0	0.258	110.7	0.226
				55.0	0.275	123.0	0.250

Set E			
µg	O.D.	µg	O.D.
0.0	0.000	159.9	0.318
12.3	0.016	172.2	0.335
24.6	0.049	184.5	0.357
36.9	0.076	196.8	0.390
49.2	0.103	209.1	0.413
61.5	0.132	221.4	0.442
73.8	0.154	233.7	0.465
86.1	0.174	246.0	0.470
98.4	0.200	258.3	0.502
110.7	0.231	270.6	0.519
123.0	0.243	282.9	0.527
135.3	0.256	295.2	0.576
147.6	0.293	307.5	0.613

Set F	
µg	O.D.
0.0	0.000
12.3	0.035
24.6	0.067
36.9	0.087
49.2	0.124
61.5	0.152
73.8	0.183
98.4	0.238
123.0	0.290
147.6	0.349
172.2	0.399
196.8	0.450
221.4	0.501
246.0	0.547
270.6	0.593
295.2	0.632
70.0	0.335
75.0	0.360
147.6	0.300
184.5	0.334
215.3	0.411
246.0	0.450
307.5	0.543
369.0	0.633
430.5	0.706
492.0	0.793
553.5	0.866
615.0	0.939

Curves	Regression Function	Mean Square Error
A	$0.0114 + 0.0046076\ X - 3.97203\ E\text{-}6\ X^2$	0.0003088
BC	$0.007748 + 0.00505838\ X - 4.59692\ E\text{-}6\ X^2$	0.00006157
D	$0.013855 + 0.00199167\ X - 8.19399\ E\text{-}7\ X^2$	0.0001848
E	$0.005641 + 0.00192804\ X$	0.000076371
F	$0.00115 + 0.00254497\ X - 1.34559\ E\text{-}6\ X^2$	0.000008046

Raw Data

ID#	Dry Mass (mg)	Total Volume	Replicates: Optical Density (Sample Volumes)						
Set A									
360.8	105.6 mg	5.0 ml	0.625	0.66	0.59	0.61	0.60	0.58	(0.02)
54.2	106.7	5.0	0.63	0.61	0.65	0.64	0.63	0.61	(0.02)
59.1	127.2	5.0	0.75	0.76	0.78	0.75	0.70	0.71	(0.02)
108.1	113.7	5.0	0.595	0.65	0.64	0.69	0.64	0.65	(0.02)
109.1	102.6	5.0	0.615	0.545	0.55	0.60	0.62	0.56	(0.02)
109.2	101.7	5.0	0.445	0.60	0.43	0.45	0.38	0.57	(0.02)
115.1	115.1	5.0	0.665	0.66	0.71	0.70	0.70		(0.02)
115.1	109.5	5.0	0.67	0.65	0.68	0.67	0.65	0.64	(0.02)

Set B

109.2	101.7 mg	5.0 ml	0.625	0.62	0.63	0.65	0.64	0.61	(0.02)
115.1	115.1	5.0	0.75	0.71	0.72	0.69	0.68	0.74	(0.02)
115.1	109.5	5.0	0.67	0.68	0.69	0.71	0.71	0.68	(0.02)

Set C

335.1	60.9 mg	5.0	0.180	0.177	0.161	0.175	(0.02)
359.2	15.6	5.0	0.154	0.144	0.140	0.140	(0.05)
359.3	34.3	5.0	0.303	0.290	0.292	0.290	(0.05)
360.4	12.7	5.0	0.116	0.103	0.110	0.102	(0.05)
53.2	82.5	5.0	0.315	0.304	0.304	0.315	(0.02)
53.3	108.7	5.0	0.375	0.382	0.372	0.370	(0.02)
54.1	51.8	5.0	0.193	0.202	0.198	0.207	(0.02)
115.2	176.0	10.0	0.352	0.339	0.330	0.320	(0.02)
146.3	76.1	5.0	0.309	0.327	0.330	0.330	(0.02)
147.2	9.5	5.0	0.080	0.085	0.083	0.084	(0.05)
271.2	5.2	2.0	0.068	0.068	0.069	0.065	(0.05)
271.3	8.1	2.0	0.122	0.122	0.129	0.122	(0.05)
272.2	5.7	2.0	0.078	0.081	0.080	0.080	(0.05)
301.1	4.8	2.0	0.090	0.100	0.092	0.098	(0.05)
301.2	4.1	2.0	0.068	0.066	0.070	0.068	(0.05)
321.2	4.5	2.0	0.060	0.060	0.054	0.058	(0.05)
321.3	1.6	2.0	0.032	0.030	0.028	0.030	(0.05)
323.3	2.4	2.0	0.032	0.035	0.034	0.036	(0.05)
337.2	7.2	2.0	0.150	0.150	0.150	0.160	(0.05)
18.1	52.6	5.0	0.252	0.251	0.249	0.255	(0.02)

174.2	11.75 mg	2.0ml	0.410	0.411	0.424	0.416	(0.1)
180.3	5.513	2.0	0.173	0.173	0.180	0.184	(0.1)
181.2	14.78	2.0	0.492	0.517	0.535	0.511	(0.1)
199.2	14.38	2.0	0.358	0.383	0.382	0.380	(0.1)
199.3	4.948	2.0	0.159	0.162	0.179	0.170	(0.1)
200.2	5.080	2.0	0.170	0.167	0.184	0.182	(0.1)
222.2	7.497	2.0	0.281	0.290	0.282	0.287	(0.1)
223.1	4.014	2.0	0.136	0.153	0.154	0.156	(0.1)
223.2	5.074	2.0	0.178	0.188	0.194	0.192	(0.1)
223.3	3.036	2.0	0.094	0.117	0.107	0.115	(0.1)
291.1	1.638	2.0	0.096	0.119	0.116	0.117	(0.1)
291.2	2.818	2.0	0.041	0.064	0.052	0.052	(0.1)
292.1	10.58	2.0	0.365	0.440	0.410	0.412	(0.1)
292.2	1.594	2.0	0.042	0.073	0.061	0.063	(0.1)
292.3	2.263	2.0	0.067	0.091	0.079	0.081	(0.1)
56.1	421.4	15.0	0.228	(0.01) 0.465	0.474	0.486	(0.02)

Free Amino Acids

Standard Curves

Set A		Set B		Set C		Set D	
μg	O.D.	μg	O.D.	μg	O.D.	μg	O.D.
0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000
4.55	0.117	4.55	0.117	2.275	0.113	2.275	0.123
9.10	0.252	9.10	0.234	4.55	0.241	4.55	0.238
13.65	0.382	13.65	0.360	6.825	0.344	6.825	0.362
18.2	0.490	18.2	0.479	9.10	0.435	9.10	0.480
22.75	0.619	22.75	0.590	11.38	0.539	11.38	0.585
27.30	0.724	27.30	0.706	13.65	0.642	13.65	0.688
31.85	0.883	31.85	0.818	15.93	0.777	15.93	0.801
36.40	0.951	36.40	0.921	18.20	0.963	18.20	0.914
40.95	1.051	40.95	1.018	20.48	1.027	20.48	1.009
45.5	1.137	45.5	1.097			22.75	1.114

Set E		Set F		Set G	
μg	O.D.	μg	O.D.	μg	O.D.
0.0	0.000	0.0	0.000	0.0	0.000
2.275	0.112	2.275	0.118	4.55	0.129
4.55	0.233	4.55	0.215	9.10	0.234
6.825	0.343	6.825	0.329	13.65	0.378
9.10	0.436	9.10	0.437	18.2	0.462
11.38	0.551	11.38	0.557	22.75	0.595
13.65	0.656	13.65	0.668	27.30	0.682
15.93	0.764	15.93	0.775	31.85	0.810
18.20	0.873	18.20	0.873	36.40	0.879
20.48	0.963	20.48	1.004	40.95	1.046
22.75	1.056	22.75	1.108	45.5	1.114

Curves	Regression Function	Mean Square Error
A	$-0.01145 + 0.030211\ X - 0.00010393\ X^2$	0.0002589
E	$0.000084 + 0.0506954\ X - 0.00017925\ X^2$	0.00003918
BG	$0.009627 + 0.0245724\ X$	0.00037623
CDF	$0.009627 + 0.0491447\ X$	0.00037623

Note: Sets B, C, D, F, and G are all coincident after C, D, and F are transformed by a factor of two. Regression was performed on all five sets together, with C, D, and F subsequently back-transformed.

Raw Data

ID#	Dry Mass (mg)	Total Volume	Replicates: Optical Density (Sample Volumes)								
			Set A				Set B				
53.2	28.7 mg	10.0 ml	1.032	(0.1)	0.208		0.229		(0.02)		
53.3	79.1	10.0	0.610				0.580	0.602	(0.02)		
54.2	85.8	10.0	0.658				0.662	0.676	(0.02)		
56.1	196.7	15.0	0.839				0.857	0.845	(0.02)		
59.1	117.1	10.0	0.851				0.824	0.833	(0.02)		
108.1	98.1	10.0	0.618				0.652	0.629	(0.02)		
109.1	113.9	10.0	0.793				0.796	0.775	(0.02)		
109.2	93.9	10.0	0.658				0.680	0.652	(0.02)		
115.1	67.6	10.0	0.419				0.481	0.432	(0.02)		
115.2	167.7	10.0	0.924				1.000	0.947	(0.02)		
174.2	39.3	10.0	0.799	(0.1)	0.162		0.160		(0.02)		
Set C											
			(0.02)		(0.05)	(0.10)					
180.3	11.9	10.0	0.097		0.195	0.394					
181.2	18.4	10.0	0.066		0.129	0.247					
223.1	7.4	10.0	0.112		0.240	0.482					
223.2	12.2	10.0	0.067		0.152	0.307					
223.3	4.9	10.0	0.131		0.316	0.583					
291.1	3.9	10.0	0.042		0.088	0.178					
291.2	7.4	10.0	0.068		0.153	0.276					
292.2	4.2	10.0	0.044		0.087	0.149					
292.3	4.5	10.0	0.057		0.138	0.235					
335.1	23.4	10.0	0.246		0.662	1.149					
			Set D				Set E				
			(0.02)		(0.05)	(0.10)	(0.02)		(0.05)	(0.10)	
147.2	6.4	10.0	0.118		0.173	0.299	0.053		0.124	0.249	
199.2	37.6	10.0	0.229		0.588	1.036	0.193		0.510	1.009	
199.3	8.4	10.0	0.075		0.335	0.332	0.040		0.150	0.284	
200.2	11.2	10.0	0.091		0.218	0.410	0.052		0.171	0.349	
271.2	2.9	10.0	0.038		0.077	0.127	0.011		0.043	0.090	
272.2	6.6	10.0	0.086		0.200	0.372	0.050		0.162	0.333	
301.1	7.2	10.0	0.082		0.162	0.331	0.042		0.153	0.299	
321.2	4.9	15.0	0.053		0.124	0.188	0.012		0.077	0.142	
323.3	8.7	10.0	0.113		0.252	0.470	0.066		0.209	0.423	
337.2	11.8	15.0	0.100		0.229	0.421	0.063		0.194	0.363	
			Set F				Set G				
			(0.02)		(0.05)	(0.10)					
18.1	116.2	10.0	1.155				0.620	0.604	(0.02)		
54.1	67.6	10.0	0.967				0.525	0.502	(0.02)		
146.3	62.9	10.0	0.652				0.350	0.325	(0.02)		
222.2	6.8	10.0	0.0706		0.182	0.348					
271.3	9.6	10.0	0.102		0.260	0.495					
301.2	2.7	10.0	0.0123		0.0414	0.102					
322.3	13.6	10.0	0.128		0.339	0.644					

			(0.02)	(0.05)	(0.02)	(0.05)
359.2	31.6	10.0	0.370	0.886	0.207	0.352
360.4	27.4	10.0	0.309	0.783	0.176	0.430
360.8	34.4	10.0	0.459	1.013	0.225	0.537

Simple Reducing Sugars

Standard Curves

Set A		Set B		Set C		Set D	
μg	O.D.	μg	O.D.	μg	O.D.	μg	O.D.
0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000
2.95	0.046	3.02	0.051	3.02	0.022	3.02	0.052
5.90	0.084	6.04	0.091	6.04	0.065	6.04	0.094
8.85	0.122	9.06	0.133	9.06	0.112	9.06	0.148
11.8	0.164	12.1	0.200	12.1	0.192	12.1	0.198
23.6	0.322	18.1	0.287	18.1	0.269	18.1	0.285
29.5	0.423	24.2	0.382	24.2	0.368	24.2	0.397
35.4	0.490	30.2	0.449	30.2	0.470	30.2	0.493
41.3	0.573	36.2	0.545	36.2	0.568	36.2	0.597
47.2	0.640	42.3	0.633	42.3	0.654	42.3	0.754
53.1	0.733	45.3	0.690	48.3	0.738	48.3	0.914
59.0	0.810	48.3	0.719	54.4	0.807	54.4	0.860
		54.4	0.839	60.4	0.900	60.4	0.951
		60.4	0.955				

Set E		Set F		Set G		Set H	
μg	O.D.	μg	O.D.	μg	O.D.	μg	O.D.
0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000
3.02	0.054	3.02	0.050	3.02	0.046	3.02	0.028
6.04	0.106	6.04	0.108	6.04	0.101	6.04	0.079
9.06	0.153	9.06	0.150	9.06	0.152	9.06	0.117
12.1	0.205	12.1	0.197	12.1	0.183	12.1	0.169
18.1	0.306	18.1	0.281	18.1	0.285	18.1	0.252
24.2	0.408	24.2	0.387	24.2	0.392	24.2	0.346
30.2	0.517	30.2	0.497	30.2	0.497	30.2	0.452
36.2	0.583	36.2	0.604	36.2	0.593	36.2	0.539
42.3	0.690	42.3	0.695	42.3	0.697	42.3	0.613
48.3	0.796	48.3	0.788	48.3	0.783	48.3	0.714
54.4	0.896	54.4	0.845	54.4	0.876	54.4	0.793
60.4	0.971	60.4	0.991	60.4	0.951	60.4	0.879

Set I		Set J		Set K		Set L		Set M	
μg	O.D.	μg	O.D.	μg	O.D.	μg	O.D.	μg	O.D.
0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.000
6.55	0.098	6.55	0.0862	6.55	0.0726	6.55	0.0937	6.55	0.083
13.10	0.189	13.10	0.192	13.10	0.155	13.10	0.180	13.10	0.169
19.65	0.291	19.65	0.276	19.65	0.242	19.65	0.273	19.65	0.271
26.20	0.377	26.20	0.378	26.20	0.350	26.20	0.362	26.20	0.352
32.75	0.478	32.75	0.472	32.75	0.455	32.75	0.458	32.75	0.465

39.30	0.554	39.30	0.565	39.30	0.544	39.30	0.547	39.30	0.564
45.85	0.648	45.85	0.658	45.85	0.614	45.85	0.636	45.85	0.638
52.40	0.752	52.40	0.747	52.40	0.708	52.40	0.717	52.40	0.724
58.95	0.807	58.95	0.827	58.95	0.790	58.95	0.810	58.95	0.821
65.50	0.914	65.50	0.928	65.50	0.876	65.50	0.896	65.50	0.917

Curves	Regression Functions	Mean Square Error
AL	$0.000294 + 0.0140765\ X - 6.158\ E\text{-}6\ X^2$	0.0000224
BC	$-0.004442 + 0.0153421\ X$	0.0002304
DEFG	$0.003040 + 0.0162796\ X$	0.0005466
H	$-0.009830 + 0.0148391\ X$	0.0000644
IJ	$-0.002141 + 0.0148622\ X - 1.2833\ E\text{-}5\ X^2$	0.0000625
K	$-0.010609 + 0.0136663\ X$	0.0001482
M	$-0.0065 + 0.0140874\ X$	0.0000783

Raw Data

ID#	Dry Mass (mg)	Total Volume	Replicates: Optical Density (Sample Volumes)					
			Set A		Set B			
53.2	28.7 mg	10.0 ml	0.134		0.147	0.155	(1.0 ml)	
53.3	79.1	10.0	0.410		0.412	0.447	(1.0)	
54.2	85.8	10.0	0.500		0.533	0.518	(1.0)	
56.1	196.7	15.0	0.590		0.632	0.642	(1.0)	
59.1	117.1	10.0	0.614		0.658	0.644	(1.0)	
108.1	98.1	10.0	0.409		0.481	0.434	(1.0)	
109.1	113.9	10.0	0.468		0.519	0.513	(1.0)	
109.2	93.9	10.0	0.417		0.461	0.451	(1.0)	
115.1	67.6	10.0	0.388		0.490	0.501	(1.0)	
115.2	167.7	10.0	0.523		0.579	0.580	(1.0)	
174.2	39.3	10.0	0.411		0.418	0.430	(1.0)	
			Set C		Set D			
180.3	11.9	10.0	0.262	0.260	0.297	0.296	(1.0)	
181.2	18.4	10.0	0.341	0.066	0.130	0.139	(1.0)	
223.1	7.4	10.0	0.083	0.073	0.112	0.112	(1.0)	
223.2	12.2	10.0	0.072	0.075	0.113	0.105	(1.0)	
223.3	4.9	10.0	0.033	0.038	0.074	0.072	(1.0)	
291.1	3.9	10.0	0.025	0.006	0.043	0.041	(1.0)	
291.2	7.4	10.0	0.113	0.105	0.127	0.135	(1.0)	
292.2	4.2	10.0	0.085	0.087	0.126	0.127	(1.0)	
292.3	4.5	10.0	0.033	0.034	0.085	0.091	(1.0)	
335.1	23.4	10.0	0.071	0.065	0.117	0.119	(1.0)	

			Set E		Set F		
147.2	6.4	10.0	0.088	0.093	0.097	0.098	(1.0 ml)
199.2	37.6	10.0	0.590	0.588	0.616	0.616	(1.0)
199.3	8.4	10.0	0.113	0.110	0.132	0.095	(1.0)
200.2	11.2	10.0	0.174	0.168	0.163	0.172	(1.0)
271.2	2.9	10.0	0.050	0.048	0.052	0.064	(1.0)
272.2	6.6	10.0	0.079	0.079	0.087	0.074	(1.0)
301.1	7.2	10.0	0.058	0.058	0.051	0.063	(1.0)
321.2	4.9	15.0	0.067	0.070	0.091	0.073	(1.0)
323.3	8.7	10.0	0.070	0.066	0.063	0.065	(1.0)
337.2	11.8	15.0	0.152	0.148	0.158	0.158	(1.0)
18.1	116.2	10.0	0.554	0.578	0.515	0.531	(1.0)
54.1	67.6	10.0	0.291	0.285	0.262	0.257	(1.0)
146.3	62.9	10.0	0.271	0.268	0.251	0.231	(1.0)
222.2	6.8	10.0	0.058	0.0595	0.0491	0.0381	(1.0)
271.3	9.6	10.0	0.068	0.068	0.055	0.0555	(1.0)
301.2	2.7	10.0	0.0778	0.0899	0.056	0.0516	(1.0)
322.3	13.6	10.0	0.111	0.113	0.0926	0.0716	(1.0)
359.2	31.6	10.0	0.157	0.158	0.147	0.239	(1.0)
360.4	27.4	10.0	0.118	0.111	0.0899	0.0947	(1.0)
360.8	34.4	10.0	0.184	0.184	0.152	0.151	(1.0)

Glycogen

Glycogen standard curves are shown above, under simple reducing sugars.

Raw Data

ID#	Dry Mass (mg)	Total Volume	Replicates: Optical Density (Sample Volumes)				
			Set C		Set D		
			(0.1)	(1.0)			
53.2	28.7 mg	5.0	0.038	0.389	0.478	0.486	(1.0 ml)
53.3	79.1	5.0	0.128	1.30	0.308	0.298	(0.2)
54.2	85.8	5.0	0.079	1.004	0.242	0.224	(0.2)
56.1	196.7	5.0	0.117	1.276	0.318	0.295	(0.2)
59.1	117.1	5.0	0.098	1.108	0.253	0.253	(0.2)
108.1	98.1	5.0	0.018	0.386	0.397	0.411	(1.0)
109.1	113.9	5.0	0.137	1.347	0.297	0.298	(0.2)
109.2	93.9	5.0	0.061	0.842	0.185	0.179	(0.2)
115.1	67.6	5.0	0.009	0.380	0.372	0.407	(1.0)
115.2	167.7	5.0	0.097	1.292	0.266	0.288	(0.2)
174.2	39.3	5.0	0.052	0.664	0.156	0.144	(0.2)

			Set E		Set F	
			(0.2)	(1.0)	(0.2)	(1.0)
180.3	11.9	5.0	0.098	0.409	0.123	0.399
181.2	18.4	5.0	0.051	0.211	0.047	0.180
223.1	7.4	5.0	0.085	0.318	0.064	0.321
223.2	12.2	5.0	0.115	0.488	0.107	0.476
223.3	4.9	5.0	0.043	0.200	0.039	0.170
291.1	3.9	5.0	0.040	0.149	0.040	0.132
291.2	7.4	5.0	0.086	0.330	0.066	0.328
292.2	4.2	5.0	0.078	0.190	0.041	0.180
292.3	4.5	5.0	0.060	0.227	0.065	0.199
335.1	23.4	5.0	0.104	0.475	0.105	0.469

			Set I	Set J	Set K	Set L	Set M
			(0.2)	(0.5)	(0.2)	(1.0)	(1.0)
147.2	6.4	5.0	0.0419	0.0737	0.0141	0.147	0.163
199.2	37.6	5.0	0.146	0.297	0.0975	0.599	0.602
199.3	8.4	5.0	0.0942	0.192	0.0575	0.424	0.423
200.2	11.2	5.0	0.106	0.208	0.067	0.431	0.442
271.2	2.9	5.0	0.0287	0.0506	0.0031	0.132	0.125
272.2	6.6	5.0	0.066	0.131	0.0334	0.351	0.293
301.1	7.2	5.0	0.067	0.0926	0.0315	0.239	0.222
321.2	4.9	5.0	0.0273	0.0580	0.0088	0.149	0.137
323.3	8.7	5.0	0.0555	0.138	0.0391	0.362	0.309
337.2	11.8	5.0	0.0726	0.162	0.0453	0.356	0.346

			Set I	Set J	Set K	Set L	Set M
18.1	116.2	5.0	0.541	0.548	0.532	.569	.548 (.2)
54.1	67.6	5.0	.238 (.2)	.476 (.5)	.170 (.2)	.530	.488 (.5)
			(0.2)	(0.5)	(0.2)	(1.0)	(1.0)
146.3	62.9	5.0	0.143	0.320	0.117	0.706	0.674
222.2	6.8	5.0	0.0778	0.146	0.0545	0.314	0.323
271.3	9.6	5.0	0.0737	0.207	0.0458	0.341	0.343
301.2	2.7	5.0	0.102	0.222	0.458	0.469	0.460
322.3	13.6	5.0	0.0424	0.0595	0.0097	0.111	0.141
359.2	31.6	5.0	0.160	0.297	0.148	0.701	0.728
360.4	27.4	5.0	0.182	0.417	0.161	0.903	0.939
360.8	34.4	5.0	0.0937	0.197	0.0665	0.418	0.425

Lipid

Because amount of lipid was determined gravimetrically, there are no standard curves. Each specimen has two replicates; both tare and tare+lipid mass are shown for each replicate (as a difference: mass of vial including lipid - mass of empty vial). For each specimen, sample volume was 2.0 ml and total volume of the chloroform fraction was 5.0 ml. Because standard error of all replicates was approximately

identical, unweighted averages were used to calculate lipid concentration in chloroform fractions (g/ml). Thus, differences in standard error of final concentration (g/mg dry mass) is attributable to propagation of error upon division by dry mass (Equation D.25)

Raw Data

ID#	Dry Mass	Replicates (Total mass - tare, in g)	
53.2	28.7 mg	4.7154 - 4.7143	4.7855 - 4.7842
53.3	79.1	4.7402 - 4.7373	4.7526 - 4.7496
54.2	85.8	4.7777 - 4.7738	4.7329 - 4.7282
56.1	196.7	4.8560 - 4.8478	4.7901 - 4.7817
59.1	117.1	4.7402 - 4.7345	4.7633 - 4.7571
108.1	98.1	4.7985 - 4.7930	4.7239 - 4.7183
109.1	113.9	4.8060 - 4.7995	4.7783 - 4.7718
109.2	93.9	4.7222 - 4.7177	4.7020 - 4.6975
115.1	67.6	4.7931 - 4.7898	4.7240 - 4.7204
115.2	167.7	4.8066 - 4.7991	4.7854 - 4.7778
174.2	39.3	4.7428 - 4.7403	4.7734 - 4.7710
180.3	11.9	4.7642 - 4.7637	4.7648 - 4.7643
181.2	18.4	4.7865 - 4.7861	4.7602 - 4.7601
223.1	7.4	4.7247 - 4.7241	4.7523 - 4.7517
223.2	12.2	4.7946 - 4.7942	4.7960 - 4.7958
223.3	4.9	4.7354 - 4.7345	4.7552 - 4.7541
291.1	3.9	4.7266 - 4.7264	4.7579 - 4.7579
291.2	7.4	4.7375 - 4.7371	4.7337 - 4.7334
292.2	4.2	4.7568 - 4.7566	4.6733 - 4.6729
292.3	4.5	4.7740 - 4.7735	4.7318 - 4.7318
335.1	23.4	4.7019 - 4.7011	4.7457 - 4.7451
147.2	6.4	4.7215 - 4.7210	4.7566 - 4.7566
199.2	37.6	4.7665 - 4.7655	4.7048 - 4.7041
199.3	8.4	4.7488 - 4.7486	4.8091 - 4.8089
200.2	11.2	4.7195 - 4.7189	4.7206 - 4.7206
271.2	2.9	4.7684 - 4.7683	4.7677 - 4.7677
272.2	6.6	4.7034 - 4.7033	4.7202 - 4.7202
301.1	7.2	4.7367 - 4.7362	4.6945 - 4.6944
321.2	4.9	4.7081 - 4.7079	4.7877 - 4.7876
323.3	8.7	4.7386 - 4.7385	4.8100 - 4.8096
337.2	11.8	4.7284 - 4.7275	4.8245 - 4.8242
18.1	116.2	4.7435 - 4.7387	5.4763 - 5.4709
54.1	67.6	4.7809 - 4.7787	5.2773 - 5.2753
146.3	62.9	4.7498 - 4.7475	4.7618 - 4.7584
222.2	6.8	4.6917 - 4.6917	4.7549 - 4.7545
271.3	9.6	4.7760 - 4.7758	4.8033 - 4.8030
301.2	2.7	4.7150 - 4.7151	4.7461 - 4.7460
322.3	13.6	4.7838 - 4.7836	4.7656 - 4.7651
359.2	31.6	4.6620 - 4.6609	4.7402 - 4.7388
360.4	27.4	4.8192 - 4.8184	4.7734 - 4.7720
360.8	34.4	4.7658 - 4.7644	4.7232 - 4.7216

Processed Data

Final form of analyzed data from Chapter I is shown here. Concentration, level, and content of each component is given, together with estimated standard errors for those measurements. In addition, animal mass, gonad mass (both wet and dry), dry mass index of gonad, percentage water content in gonad, relative spermatogenic stage (ordinal scale rankings of testes based on histological observations), and ornithine decarboxylase activity are shown. In this table, row number can be used to compare measurements from the same animal which are not adjacent.

General Information

ROW	YEAR	DATE	#	MASS	WET MASS	DRY MASS	GDMI	%WATER	STAGE
1	1982	18	1	190.8	2.2169	0.3669	0.192	83.4	8
2	1981	53	2	68.6	3.5937	0.6451	0.940	82.0	8
3	1981	53	3	66.1	4.9869	0.8609	1.302	82.7	7
4	1981	54	1	58.6	3.6470	0.5873	1.002	83.9	7
5	1981	54	2	90.5	6.4267	1.1330	1.252	82.4	8
6	1982	56	1	386.2	41.6511	7.1886	1.861	82.7	9
7	1981	59	1	81.7	6.4491	1.1324	1.386	82.4	9
8	1981	108	1	64.3	7.9710	1.4435	2.245	81.9	11
9	1981	109	1	60.3	4.8352	0.9046	1.500	81.3	9
10	1981	109	2	59.5	4.1634	0.8030	1.350	80.7	9
11	1981	115	1	39.3	4.1908	0.7878	2.004	81.2	10
12	1981	115	2	245.4	29.9398	5.2209	2.128	82.6	12
13	1981	146	3	52.8	3.4245	0.5233	0.991	84.7	11
14	1981	147	2	38.8	0.2917	0.0309	0.080	89.4	13
15	1983	174	2	55.7	0.5214	0.0802	0.144	84.6	2
16	1983	180	3	81.1	0.1068	0.0268	0.033	74.9	2
17	1983	181	2	73.5	0.3506	0.0832	0.113	76.3	2
18	1983	199	2	46.3	0.2515	0.0705	0.152	72.0	2
19	1983	199	3	36.3	0.0805	0.0186	0.051	76.0	1
20	1983	200	2	64.7	0.1317	0.0246	0.038	81.3	2
21	1983	222	2	50.9	0.0844	0.0159	0.031	81.2	4
22	1983	223	1	51.2	0.0726	0.0103	0.020	85.9	3
23	1983	223	2	75.9	0.1018	0.0183	0.024	82.0	4
24	1983	223	3	30.8	0.0437	0.0072	0.024	83.4	4
25	1981	271	2	52.3	0.1066	0.0128	0.024	88.0	5
26	1981	271	3	46.2	0.2880	0.0409	0.089	85.8	5
27	1981	272	2	37.0	0.1994	0.0292	0.079	85.3	5
28	1983	291	1	39.5	0.0604	0.0115	0.029	80.9	3
29	1983	291	2	40.6	0.0730	0.0148	0.036	79.7	1
30	1983	292	1	52.1	0.1573	0.0336	0.065	78.6	5

31	1983	292	2	34.1	0.0265	0.0080	0.024	69.7	*
32	1983	292	3	51.4	0.0358	0.0073	0.014	79.7	3
33	1981	301	1	48.9	0.2093	0.0348	0.071	83.4	*
34	1981	301	2	40.1	0.2002	0.0275	0.069	86.3	5
35	1981	321	2	76.2	0.1721	0.0340	0.045	80.2	5
36	1981	321	3	93.2	0.0591	0.0107	0.011	81.9	5
37	1980	322	3	63.1	0.3471	0.0467	0.074	86.5	*
38	1981	323	3	50.0	0.1125	0.0206	0.041	81.7	5
39	1980	335	1	63.3	1.3537	0.2023	0.320	85.1	*
40	1981	337	2	118.0	0.2483	0.0476	0.040	80.8	6
41	1980	359	2	40.4	1.0969	0.1777	0.440	83.8	*
42	1980	359	3	37.4	1.1393	0.1824	0.488	84.0	*
43	1980	360	4	43.3	0.5672	0.0877	0.203	84.5	*
44	1980	360	8	120.2	6.3483	1.0837	0.902	82.9	*

Concentrations of Biochemical Components

ROW	DNA	SD-DNA	RNA	SD-RNA	PROT	SD-PROT
1	45.93	0.86	7.76	2.69	480.6	24.57
2	45.79	1.02	36.92	1.72	383.6	15.83
3	45.81	0.83	36.39	1.30	359.2	12.20
4	40.32	0.84	43.47	2.74	380.5	24.75
5	44.84	0.58	28.76	0.99	361.8	12.41
6	59.31	3.59	18.20	1.01	379.6	9.14
7	37.99	0.49	21.91	0.82	371.6	10.63
8	58.56	0.73	19.76	0.91	349.6	11.68
9	43.66	0.57	30.67	1.02	342.7	12.81
10	51.76	0.64	29.20	1.03	334.7	12.90
11	54.61	0.68	20.74	0.93	359.8	11.84
12	43.43	0.96	29.39	0.81	392.3	14.52
13	53.72	1.13	22.21	1.86	437.1	17.21
14	36.36	1.67	88.21	6.04	317.5	53.52
15	69.67	4.02	11.67	0.75	313.1	11.65
16	7.01	0.83	16.33	1.56	270.4	19.58
17	66.19	3.25	10.56	0.60	319.3	10.52
18	1.99	0.32	14.32	0.64	229.7	9.09
19	3.51	0.92	16.23	1.74	287.7	21.70
20	9.11	1.49	15.76	1.69	294.5	21.26
21	16.68	0.66	33.10	1.26	321.0	15.72
22	11.96	1.14	22.60	2.15	313.9	26.43
23	7.19	0.90	24.11	1.72	312.6	21.44
24	13.95	1.51	31.45	2.84	293.8	34.17
25	2.90	1.45	29.10	10.90	183.7	39.39
26	8.84	0.94	39.57	7.01	231.4	25.83
27	7.21	1.33	35.15	9.95	202.4	36.08
28	8.88	2.77	29.37	5.20	571.6	63.48
29	7.16	1.61	20.99	3.03	145.0	36.13
30	20.37	0.55	30.04	0.95	340.6	12.82
31	9.93	2.84	28.52	5.35	306.5	64.02
32	12.85	2.01	26.36	3.78	281.6	45.32
33	13.47	1.60	46.95	11.83	292.1	43.27
34	7.36	1.85	32.42	13.82	235.0	50.10
35	7.36	1.69	23.98	12.59	178.2	45.42

36	4.44	4.72	37.60	17.84	220.8	127.50
37	*	*	*	*	*	*
38	8.32	3.16	37.22	11.89	175.5	84.85
39	17.72	1.25	41.30	2.33	277.1	20.98
40	13.47	1.07	35.95	7.88	326.7	29.65
41	19.98	1.00	48.71	6.06	355.5	32.74
42	32.40	1.15	49.44	4.13	348.6	15.21
43	19.98	1.21	51.20	7.44	317.1	40.09
44	40.36	0.54	30.65	1.00	353.1	12.50

ROW	CHO	SD-CHO	GLY	SD-GLY	AA	SD-AA	LIPID	SD-LIP
1	3.087	0.064	8.550	0.299	102.1	1.46	109.7	4.09
2	3.329	0.250	4.791	0.247	139.6	1.77	104.5	15.98
3	3.723	0.094	5.397	0.122	146.1	2.14	93.2	5.86
4	2.683	0.108	5.422	0.219	146.7	2.47	77.6	6.81
5	4.198	0.087	3.835	0.105	150.3	2.00	125.3	5.48
6	3.253	0.056	2.132	0.049	126.8	1.28	105.5	2.56
7	3.792	0.065	3.101	0.079	140.3	1.53	127.0	4.11
8	3.001	0.076	1.274	0.072	124.6	1.74	141.4	4.88
9	2.961	0.066	3.874	0.086	135.4	1.55	142.7	4.26
10	3.195	0.079	2.931	0.093	136.9	1.83	119.8	5.02
11	4.147	0.110	1.800	0.104	124.4	2.44	127.6	6.89
12	2.252	0.045	2.512	0.057	112.7	1.10	112.6	2.95
13	2.686	0.116	3.983	0.127	104.6	2.58	113.3	7.36
14	8.731	1.144	8.436	1.180	80.0	6.58	97.7	71.52
15	7.508	0.190	5.542	0.217	76.1	1.15	155.9	11.75
16	14.655	0.627	10.482	0.659	65.6	3.56	105.0	38.48
17	6.971	0.398	3.236	0.418	26.4	2.28	34.0	24.87
18	9.793	0.201	5.747	0.209	56.7	1.23	56.5	12.18
19	8.007	0.871	17.984	0.940	70.8	5.00	59.5	54.48
20	9.116	0.655	13.873	0.698	63.4	3.77	67.0	40.86
21	5.292	1.071	16.600	1.144	101.3	6.33	73.5	67.30
22	7.613	0.988	13.198	1.059	129.6	5.93	202.7	61.93
23	4.395	0.597	12.116	0.648	49.1	3.45	61.5	37.51
24	6.000	1.488	11.425	1.587	241.7	9.91	510.2	94.10
25	10.690	2.532	15.909	2.640	63.2	14.37	43.1	157.80
26	4.558	0.758	12.928	0.805	103.4	4.50	65.1	47.67
27	7.140	1.106	18.040	1.185	101.8	6.50	18.9	69.33
28	3.899	1.865	10.968	1.991	86.6	10.94	64.1	117.34
29	10.116	0.992	13.590	1.061	74.5	5.74	118.2	61.87
30	*	*	*	*	*	*	*	*
31	14.826	1.766	13.585	1.860	69.4	10.08	178.6	109.04
32	6.713	1.621	14.557	1.741	104.8	9.57	138.9	101.74
33	4.647	1.011	11.585	1.060	83.9	5.88	104.2	63.58
34	15.945	2.755	63.006	3.697	63.6	15.65	46.3	169.48
35	13.583	2.241	10.557	1.544	89.9	12.70	76.5	93.40
36	*	*	*	*	*	*	*	*
37	4.569	0.536	3.122	0.552	95.4	3.18	64.3	33.66
38	4.445	0.837	14.164	0.891	98.9	4.98	71.8	52.61
39	2.216	0.311	6.174	0.334	102.1	1.92	74.8	19.57
40	11.788	0.929	10.674	0.653	95.6	5.38	127.1	38.81
41	4.323	0.231	7.995	0.254	109.3	2.51	98.9	14.52

42	*	*	*	*	*	*	*	*
43	2.512	0.266	11.952	0.311	116.4	2.89	100.4	16.73
44	3.162	0.212	4.360	0.224	121.6	2.34	109.0	13.35

Levels of Biochemical Components

ROW	RNA/DNA	SD-R/D	PROT/DNA	SD-P/D	CHO/DNA	SD-CH/D	GLY/DNA	SD-G/D
1	0.17	0.036	10.46	0.57	0.0672	0.0019	0.1862	0.0074
2	0.81	0.034	8.38	0.39	0.0727	0.0058	0.1046	0.0059
3	0.79	0.029	7.84	0.30	0.0813	0.0025	0.1178	0.0034
4	1.08	0.047	9.44	0.64	0.0665	0.0030	0.1345	0.0061
5	0.64	0.024	8.07	0.30	0.0936	0.0023	0.0855	0.0026
6	0.31	0.025	6.40	0.42	0.0548	0.0034	0.0359	0.0023
7	0.58	0.025	9.78	0.31	0.0998	0.0021	0.0816	0.0023
8	0.34	0.017	5.97	0.21	0.0512	0.0014	0.0218	0.0013
9	0.70	0.025	7.85	0.31	0.0678	0.0017	0.0887	0.0023
10	0.56	0.021	6.47	0.26	0.0617	0.0017	0.0566	0.0019
11	0.38	0.018	6.59	0.23	0.0759	0.0022	0.0330	0.0020
12	0.69	0.026	9.03	0.39	0.0518	0.0015	0.0578	0.0018
13	0.41	0.027	8.14	0.36	0.0500	0.0024	0.0742	0.0028
14	2.43	0.130	8.73	1.53	0.2401	0.0333	0.2320	0.0341
15	0.17	0.016	4.50	0.31	0.1078	0.0068	0.0796	0.0055
16	2.33	0.328	38.57	5.35	2.0905	0.2628	1.4952	0.2002
17	0.16	0.014	4.82	0.29	0.1053	0.0079	0.0489	0.0068
18	7.20	1.212	115.43	18.90	4.9201	0.7884	2.8874	0.4708
19	4.63	1.267	82.07	22.32	2.2843	0.6466	5.1305	1.3673
20	1.73	0.317	32.34	5.77	1.0011	0.1786	1.5236	0.2603
21	1.98	0.104	19.25	1.21	0.3173	0.0655	0.9954	0.0792
22	1.89	0.218	26.24	3.34	0.6363	0.1026	1.1031	0.1378
23	3.36	0.458	43.50	6.21	0.6115	0.1129	1.6859	0.2295
24	2.25	0.272	21.06	3.34	0.4302	0.1163	0.8190	0.1441
25	0.04	5.155	63.39	34.53	3.6884	2.0430	5.4888	2.8953
26	4.47	0.562	26.16	4.04	0.5154	0.1018	1.4616	0.1802
27	4.87	0.999	28.06	7.20	0.9897	0.2384	2.5006	0.4897
28	3.31	1.062	64.39	21.30	0.4392	0.2508	1.2356	0.4455
29	2.93	0.702	20.25	6.79	1.4123	0.3463	1.8974	0.4514
30	1.47	0.062	16.72	0.77	*	*	*	*
31	2.87	0.855	30.87	10.94	1.4930	0.4630	1.3681	0.4341
32	2.05	0.355	21.92	4.92	0.5225	0.1504	1.1331	0.2232
33	3.49	0.487	21.69	4.12	0.3449	0.0855	0.8600	0.1290
34	4.40	1.216	31.92	10.51	2.1655	0.6601	8.5570	2.2068
35	3.26	0.889	24.23	8.30	1.8467	0.5214	1.4353	0.3901
36	8.46	9.043	49.71	60.12	*	*	*	*
37	*	*	*	*	*	*	*	*
38	4.47	1.750	21.10	12.98	0.5345	0.2267	1.7029	0.6561
39	2.33	0.186	15.64	1.62	0.1251	0.0196	0.3485	0.0310
40	2.67	0.298	24.25	2.93	0.8751	0.0980	0.7924	0.0795
41	2.44	0.173	17.80	1.86	0.2164	0.0158	0.4002	0.0236
42	1.53	0.083	10.76	0.60	*	*	*	*
43	2.56	0.207	15.87	2.23	0.1257	0.0153	0.5983	0.0396
44	0.76	0.027	8.75	0.33	0.0783	0.0054	0.1080	0.0057

ROW	AA/DNA	SD-AA/D	AA/WATER	SD-AA/W	LIP/DNA	SD-L/D
1	2.222	0.052	20.25	0.290	2.39	0.100
2	3.048	0.078	30.53	0.388	2.28	0.353
3	3.188	0.074	30.48	0.447	2.04	0.133
4	3.637	0.097	28.15	0.474	1.93	0.174
5	3.352	0.062	32.17	0.429	2.79	0.128
6	2.137	0.131	26.45	0.268	1.78	0.116
7	3.694	0.062	29.89	0.326	3.34	0.116
8	2.128	0.040	27.55	0.384	2.42	0.088
9	3.102	0.054	31.17	0.358	3.27	0.106
10	2.645	0.048	32.72	0.437	2.31	0.101
11	2.277	0.053	28.79	0.564	2.34	0.130
12	2.594	0.063	23.79	0.233	2.59	0.089
13	1.948	0.063	18.87	0.466	2.11	0.144
14	2.201	0.207	9.47	0.780	2.69	1.971
15	1.092	0.065	13.82	0.211	2.24	0.212
16	9.350	1.217	21.91	1.198	14.98	5.767
17	0.399	0.040	8.22	0.708	0.51	0.377
18	28.473	4.567	22.09	0.483	28.39	7.605
19	20.207	5.470	21.30	1.518	16.98	16.163
20	6.959	1.209	14.55	0.871	7.35	4.646
21	6.075	0.450	23.50	1.490	4.41	4.040
22	10.832	1.149	21.33	1.032	16.94	5.424
23	6.838	0.981	10.79	0.763	8.55	5.329
24	17.325	2.003	48.02	2.269	36.58	7.819
25	21.793	11.986	8.59	1.957	14.87	54.948
26	11.685	1.344	17.13	0.749	7.36	5.447
27	14.106	2.754	17.48	1.120	2.63	9.623
28	9.754	3.279	20.39	2.593	7.22	13.409
29	10.406	2.472	18.95	1.474	16.51	9.401
30	*	*	*	*	*	*
31	6.993	2.245	30.19	4.441	17.98	12.128
32	8.157	1.478	26.71	2.516	10.81	8.098
33	6.230	0.860	16.75	1.176	7.73	4.808
34	8.638	3.037	10.13	2.493	6.29	23.072
35	12.228	3.291	22.17	3.134	10.40	12.920
36	*	*	*	*	*	*
37	*	*	14.85	0.498	*	*
38	11.895	4.561	22.12	1.131	8.64	7.126
39	5.762	0.421	17.94	0.338	4.22	1.144
40	7.098	0.692	22.69	1.279	9.44	2.978
41	5.472	0.300	21.13	0.486	4.95	0.767
42	*	*	*	*	*	*
43	5.828	0.383	21.29	0.529	5.02	0.892
44	3.013	0.071	25.03	0.482	2.70	0.333

Total Testicular Content of Biochemical Components

ROW	DNA:TOT	SD-DNA:T	RNA:TOT	SD-RNA:T	PRT:TOT	SD-P:T
1	8.83	0.165	1.49	0.517	92.43	4.72
2	43.03	0.964	34.70	1.617	360.45	14.88
3	59.67	1.088	47.40	1.704	467.86	15.90

4	40.41	0.840	43.56	2.743	381.28	24.81
5	56.14	0.732	36.00	1.240	452.96	15.55
6	110.41	6.683	33.87	1.878	706.52	17.02
7	52.66	0.679	30.37	1.135	515.11	14.75
8	131.46	1.647	44.35	2.051	784.78	26.24
9	65.50	0.860	46.01	1.536	514.13	19.24
10	69.85	0.882	39.41	1.391	451.67	17.42
11	109.47	1.388	41.57	1.864	721.31	23.80
12	92.40	2.045	63.60	1.724	834.66	30.90
13	53.24	1.124	22.02	1.845	433.25	17.08
14	2.89	0.134	7.02	0.482	25.26	4.26
15	10.03	0.580	1.68	0.108	45.07	1.68
16	0.23	0.027	0.54	0.052	8.92	0.65
17	7.49	0.369	1.20	0.068	36.15	1.19
18	0.30	0.048	2.18	0.097	35.00	1.39
19	0.18	0.047	0.83	0.089	14.75	1.12
20	0.35	0.057	0.60	0.064	11.20	0.81
21	0.52	0.022	1.03	0.041	10.02	0.50
22	0.24	0.023	0.45	0.044	6.30	0.54
23	0.17	0.022	0.58	0.042	7.54	0.52
24	0.33	0.036	0.74	0.069	6.91	0.82
25	0.07	0.035	0.71	0.266	4.48	0.96
26	0.78	0.083	3.51	0.621	20.51	2.29
27	0.57	0.105	2.78	0.786	15.99	2.85
28	0.26	0.081	0.86	0.152	16.66	1.87
29	0.26	0.059	0.76	0.111	5.29	1.32
30	1.32	0.036	1.94	0.062	21.98	0.83
31	0.23	0.067	0.67	0.127	7.22	1.52
32	0.18	0.029	0.37	0.054	3.98	0.65
33	0.96	0.114	3.34	0.843	20.81	3.08
34	0.50	0.127	2.22	0.949	16.12	3.44
35	0.33	0.075	1.07	0.562	7.96	2.03
36	0.05	0.054	0.43	0.205	2.53	1.46
37	*	*	*	*	*	*
38	0.34	0.130	1.53	0.489	7.22	3.49
39	5.66	0.400	13.20	0.744	88.56	6.71
40	0.54	0.043	1.45	0.318	13.18	1.20
41	8.79	0.438	21.42	2.665	156.36	14.41
42	15.80	0.563	24.11	2.015	170.02	7.43
43	4.05	0.246	10.37	1.507	64.23	8.12
44	36.39	0.486	27.63	0.898	318.37	11.28

ROW	CHO:TOT	SD-CHO:T	GLY:TOT	SD-GLY:T	AA:TOT	SD-AA:T	LIP:TOT	SD-LIP:T
1	0.594	0.0124	1.644	0.058	19.6	0.281	21.10	0.0145
2	3.129	0.2386	4.502	0.233	131.1	1.679	98.23	0.1461
3	4.849	0.1224	7.029	0.159	190.2	2.807	121.44	0.1856
4	2.689	0.1087	5.434	0.219	147.0	2.488	77.83	0.1348
5	5.256	0.1095	4.802	0.132	188.1	2.518	156.86	0.1753
6	6.056	0.1051	3.968	0.091	236.0	2.390	196.36	0.0512
7	5.256	0.0902	4.298	0.109	194.5	2.136	176.07	0.2174
8	6.736	0.1700	2.860	0.162	279.7	3.927	317.52	0.4954
9	4.443	0.0986	5.812	0.129	203.2	2.356	214.03	0.3571
10	4.311	0.1069	3.955	0.126	184.8	2.489	161.68	0.2738

11	8.313	0.2207	3.608	0.209	249.3	4.924	255.75	0.6531
12	4.791	0.0953	5.345	0.122	239.7	2.350	239.45	0.0980
13	2.662	0.1154	3.948	0.126	103.7	2.568	112.28	0.2155
14	0.695	0.0911	0.671	0.094	6.4	0.525	7.77	0.0433
15	1.081	0.0274	0.798	0.031	11.0	0.168	22.44	0.0588
16	0.483	0.0209	0.346	0.022	2.2	0.118	3.47	0.0218
17	0.789	0.0451	0.366	0.047	3.0	0.258	3.84	0.0095
18	1.492	0.0310	0.876	0.032	8.6	0.189	8.61	0.0280
19	0.411	0.0448	0.922	0.049	3.6	0.259	3.05	0.0288
20	0.347	0.0250	0.527	0.027	2.4	0.144	2.55	0.0170
21	0.165	0.0335	0.518	0.036	3.2	0.201	2.30	0.0250
22	0.153	0.0199	0.264	0.022	2.6	0.126	4.06	0.0634
23	0.106	0.0144	0.292	0.016	1.2	0.084	1.48	0.0134
24	0.141	0.0351	0.269	0.038	5.7	0.268	12.00	0.2801
25	0.261	0.0619	0.388	0.065	1.5	0.351	1.05	0.0136
26	0.404	0.0672	1.146	0.072	9.2	0.401	5.77	0.0253
27	0.564	0.0875	1.425	0.094	8.0	0.516	1.50	0.0091
28	0.114	0.0544	0.320	0.058	2.5	0.321	1.87	0.0270
29	0.369	0.0364	0.495	0.039	2.7	0.211	4.31	0.0480
30	*	*	*	*	*	*	*	*
31	0.349	0.0423	0.320	0.044	1.6	0.240	4.20	0.0917
32	0.095	0.0230	0.206	0.025	1.5	0.140	1.96	0.0441
33	0.331	0.0720	0.825	0.076	6.0	0.420	7.42	0.0378
34	1.094	0.1892	4.323	0.255	4.4	1.074	3.18	0.0227
35	0.607	0.1002	0.472	0.069	4.0	0.568	3.42	0.0192
36	*	*	*	*	*	*	*	*
37	0.338	0.0397	0.231	0.041	7.1	0.237	4.77	0.0179
38	0.183	0.0344	0.582	0.037	4.1	0.208	2.95	0.0265
39	0.708	0.0993	1.973	0.107	32.6	0.618	23.90	0.0426
40	0.476	0.0376	0.431	0.026	3.9	0.217	5.13	0.0194
41	1.901	0.1018	3.516	0.112	48.1	1.112	43.49	0.1150
42	*	*	*	*	*	*	*	*
43	0.509	0.0538	2.421	0.063	23.6	0.589	20.33	0.0592
44	2.850	0.1910	3.931	0.201	109.6	2.114	98.28	0.0838

Ornithine Decarboxylase Activity (relative scale)

ROW	DATE	CPM/UGP
1	18	9.81
2	53	9.73
3	53	2.81
4	54	6.34
5	54	12.95
6	56	0.37
7	59	1.10
8	108	0.70
9	109	4.53
10	109	4.07
11	115	0.28
12	115	0.39
13	146	0.41
14	147	0.06
15	174	1.21

16	180	0.75
17	181	1.85
18	199	-0.46
19	199	20.32
20	200	1.37
25	271	1.42
26	271	-0.25
27	272	-0.19
33	301	-2.13
34	301	-3.04
35	321	-2.03
36	321	1.48
37	322	-0.51
38	323	2.48
39	335	3.36
40	337	0.68
41	359	4.68
43	360	0.25
44	360	0.60

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