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SPERMATOGENESIS IN THE AXOLOTL, *AMBYSTOMA MEXICANUM*

BY

Mary Jane Miltner

A thesis submitted to the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree
of Master of Science in Biology

Ottawa, Canada
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Denn was faengt man am Juengsten Tag, wenn die menschlichen Werke gewogen werden, mit drei Abhandlungen ueber die Ameisensaeure an, und wenn es ihrer dreissig waeren?! Andererseits, was weiss man vom Juengsten Tag, wenn man nicht einmal weiss, was alles bis dahin aus der Ameisensaeure werden kann.

Therefore what should be done on Judgement Day, when the works of Man are in the balance, with three theses on formic acid, or for that matter thirty?! On the other hand, what do we know of Judgment Day if, by that time, we don't even know everything that can be done with formic acid.

--Robert Musil

Acknowledgements

I would like to thank my advisor, Dr. John B. Armstrong, and the members of my committee, Dr. David Brown and Dr. James Fryer, for their help and encouragement leading to the successful completion of this project. Further, I would like to thank Sylvia Gottlob and Gillian Cooper for their valuable help in carrying out portions of the work described herein. To Mr. G. Ben-Tchavtchavadze goes my thanks for his kind help in photographic matters, and to Mr. J. Hélie for drafting some of the figures. Last, but not least, I would like to thank my friends, both in the laboratory and out, for their constructive criticisms and support, and most of all, I thank my husband for his patience and unfailing support of my undertaking.

Abstract

The duration of spermatogenesis in the axolotl has been established; it takes 60-80 days for mature sperm to be formed in the testes. The average duration of each stage is as follows; leptotene 5 days, zygotene 5 days, pachytene 22 days, diplotene 1 day, metaphase 7 days, secondary spermatocyte 10 days, spermatid 12.5 days, and sperm from 62.5 days onward. Further, testes labelled with tritiated ethyl methane sulfonate (EMS) show label confined to the late spermatid stage, and when animals are spawned at regular intervals after injection with tritiated thymidine, labelled sperm appears in spermatophores laid after 121 days.

The axolotl does not appear to follow the normal reproductive pattern found in amphibians in the wild. It seems to have become free-running. The spermatogenic cycle is, therefore, fairly constant for any given animal in the colony and varies from 60-80 days. Each animal seems to be independent of its neighbor, and the natural synchrony found in wild amphibian species is lost. This is reflected in data obtained from cyst counts done on each testis from five ramdonly chosen longitudinal sections, and in the absence of zonation of the testis, a characteristic of amphibians in their natural habitat.

Resumé

La durée de la spermatogénèse dans l'axolotl a été établi. Les résultats ont montré que la spermatogénèse dure 60-80 jours dans l'axolotl. La durée de chaque stade est la suivante; leptotène 5 jours, zygotène 5 jours, pachytène 22 jours, diplotène 1 jour, métaphase 7 jours, spermatocyte de second ordre 10 jours, spermatide 12.5 jours, et devient spermatozoide à partir de 62.5 jours.

Il semble que l'axolotl ne suit pas le cycle de reproduction normal qui est caractéristique de cette espèce à l'état sauvage. L'axolotl a établi dans le laboratoire, semble-t-il, un rythme qui est indépendent de la saison; un rythme soit-disant "free-running". Dans le laboratoire le cycle est constant d'un axolotl à l'autre. Chaque animal est indépendent de son voisin, et la synchronie naturelle des membres de cette espèce à l'état sauvage est perdue. Ceci est démontré par deux résultats; 1) par l'absence d'un testicule zonal, et 2) par la présence d'une asynchronie dans le développement des cellules spermatogénique dans un axolotl individu. De plus, d'autres expériences avec EMS trtié ont montré que la radiocactivité devient localisée seulements dans les spermatides avancés. En outre on trouve que les spermatozoides prodiuts 121 jours après une injection de thymidine tritié sont radioactifs.

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List of Abbreviations

D	diplotene
DL	dominant lethal
EMS	ethyl methanesulfonate
ESP	early spermatid
FSH	follicle stimulating hormone
F-W	fall-winter
L	leptotene
LH	leutenizing hormone
LSP	late spermatid
M	metaphase
P	pachytene
SPM	sperm
S-S	spring-summer
Z	zygotene
1° SC	primary spermatocyte
2° SC	secondary spermatocyte

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Introduction

The axolotl, or water slave as it was called by the Aztecs in Mexico, has been a biological research animal for well over a century. It was presumably imported to the Spanish court from its native Lake Xochimilco by the conquistadors in the late 16th century. The term water slave is particularly appropriate for the axolotl, as it is a neotenous salamander which never loses its feathery external gills. Studies on the axolotl have covered a wide range of topics from limb regeneration to plasma isozymes and, over the years, the axolotl has proven to be a valuable and highly useful laboratory animal. Unlike many species taken into captivity, the axolotl does not lose its ability to reproduce; indeed it can be spawned at frequent intervals throughout the year. It is an easy animal to raise in the laboratory, requiring little special care and capable of withstanding great hardship and privation (Brunst 1955).

In our laboratory, one of the main areas of research has centered around the attempt to understand how development occurs by trying to induce new developmental mutations into wild-type axolotls. One of several ways to approach this problem is treatment of an animal with chemical mutagens. Ethyl methanesulfonate (EMS) is a potent chemical that has already been proven capable of inducing mutations in the mouse and thus was chosen as the agent for mutagenesis

experiments in our laboratory.

When EMS is used to treat male mice, there is a dominant lethal effect that shows up when the males are mated after treatment. A dominant lethal (DL) mutation has been defined by Bateman (1977) as a mutation in the gamete (in this case, mouse sperm) which will be lethal to the zygote, but which does not incapacitate the gamete itself.

Ehling, Cumming and Malling (1964) found that the peak in DL induction in the mouse occurred seven to nine days after exposure to the mutagen. At this time, none of the F1 progeny survived. From previous studies on the time course of spermatogenesis in the mouse (Oakberg 1956, Monesi 1962) the authors were able to correlate this peak with a specific stage of spermatogenesis, namely the late spermatid-early spermatozoa stage, when DNA in the sperm head is being even further condensed as histones are replaced by protamines on the chromosomes. The time-dependent events following EMS treatment have since been confirmed by others (Cattanach, Pollard and Isaacson 1968, Generoso and Russell 1974), who again found that the peak incidence of DL's was between seven and nine days post treatment.

As with any chemical interacting in a whole organism, there is a very complex relationship between it and the host; EMS and the mouse are certainly no exception. The exact mode of action of EMS has long been debated. The mutagen acts as an alkylating agent and as such will react

with many chemicals in the cell. At the present time, it is thought that EMS causes a DL effect by either 1) alkylating DNA and causing chromosome translocation or breakage, or 2) alkylating the proteins (both histones and protamines) that are an integral part of chromatin. The consequences are again chromosome breakage.

When EMS reacts with DNA, three major alkylation products are formed; N-7, N-3 and O-6 ethylguanine. The N-7 produdt is most frequently formed and leads to depurination at the site of interaction because of instability of the glycosidic bond; such apurinic sites are usually recognized by enzymatic excision-repair systems in the cell nucleus and would therefore not lead to a DL effect. O-6 ethylguanine is a more stable alkylation product and leads to a GC-AT transition in the affected double helix. Considering these facts and the fact that the germ cells are only sensitive to EMS at a very specific time during spermatogenesis, an accessibility-repair model may be used to explain the DL effect.

In this model, the DNA of all germ cell stages would theoretically be vulnerable to attack by the mutagen. The early stages, however, would be repaired by existing DNA repair systems in the cell, as they continue to differentiate, whereas mature spermatozoa would not be affected because of the tight packaging of the DNA within the sperm head. The late spermatid early spermatozoa stage,

therefore, must represent that point during spermatogenesis where the damage done by EMS can no longer be repaired and it is this damage which leads to the DL effect (reviewed by Armstrong and Ortiz 1978).

Should, however, the major proteins of chromatin be the primary target of EMS, their interaction can also be understood within the framework of the accessibility-repair model outlined above. Sega and Owens (1978) feel that EMS does in fact react with certain protamine amino acid residues. Their model for EMS-chromatin interaction would follow these steps:

1) Ethylation of sulfhydryl groups found in mouse protamines- perhaps the cysteine residues;

2) Inability of normal disulfide bonds to form between protamines;

3) Improper chromosome condensation in the sperm head;

4) Stress events in the chromatin leading to breaks in the chromosome and subsequent DL effects in F1 progeny.

Thus the only stage that would be sensitive to the mutagen is the late spermatid early spermatozoa stage during which newly synthesized protamines cannot form normal disulfide bonds because of EMS alkylation. The histones present during the early stages of spermatogenesis would be damaged by EMS but would be replaced by protamines as histones are removed during spermiogenesis. Mature sperm would not be affected by EMS because disulfide bonds have

already formed between protamines.

With this background information, EMS mutagenesis was attempted in the axolotl. In experimenting with both males and females, however, Armstrong and Ortiz (1978) found that treatment of females led to suppression of ovulation and that treatment of mature sperm had no effect; fertilized eggs developed normally. Thus the only alternative left was to treat the whole male axolotl. Injection of the axolotl with EMS proved also to be ineffective because the mutagen was almost immediately excreted by the animal. Therefore, animals were placed in a bowl with a known concentration of EMS and left unchanged and unfed for three days. This method proved feasible and a DL effect was induced. As in the mouse, Armstrong and Ortiz found a maximum effect a certain number of days after treatment, followed by a recovery phase.

This initial work was confirmed by Armstrong and Gillespie (1980). At 100-130 days after exposure to 100 mg/l EMS no F1 progeny survived to hatching. However, when this time is compared to mouse EMS data, it is immediately apparent that the time scale is greatly extended. If one assumes the same mode of action of EMS in the axolotl as in the mouse, then surely this time must also represent the late spermatid early spermatozoa stage in the axolotl. But is this the case? At that time, no experiments on the time course of spermatogenesis in the axolotl had been done and

no exact correlation could be made.

It is against this background that the rationale for an experiment on spermatogenesis in the axolotl was conceived; for although amphibian spermatogenesis has been the topic of frequent articles for many years, there are but two articles which deal with spermatogenesis in laboratory bred species (Carrick 1934, Kalt 1976). The former was written to establish the chromosome number of the axolotl and the latter deals with Xenopus laevis.

The main questions to which this thesis addresses itself are thus:

1) What is the time course of spermatogenesis in the axolotl?

2) How does the axolotl fit into what is known of typical urodele spermatogenesis?

3) How best can data from ongoing mutagenesis experiments be explained as results from axolotl spermatogenesis experiments become known?

From previously published articles on amphibian spermatogenesis there are three major points that serve to describe the reproductive cycle of these animals in the wild. These are:

1) The cystic structure of the amphibian testis;

2) The concept of seasonality of the reproductive cycle which becomes apparent if one observes the changes in weight and composition of the gonad over the course of a

year;

3) The control of the spermatogenic cycle which is dependent on both hormonal and environmental cues.

The organization of the urodele testis is unlike both the mammalian and anuran. In the former, seminiferous tubules containing the germ cells are interspersed with interstitial tissue made up of connective tissue, capillaries and steroid producing Leydig cells. The Sertoli cells are contained in the seminiferous tubules and, within each Sertoli cell, the germ cells are arranged in chronological fashion. The least mature spermatogonial cells are at the bottom, contiguous with the basal lamina, and sperm cells are at the apex of the Sertoli cell. In the anuran, the seminiferous tubules are lined with a seasonally varying germinal epithelium. The tubules themselves are not arranged in an orderly fashion, but rather form a convoluted mass.

The urodele testis has as its basic unit of structure the cyst. Lofts (1974) defines a germinal cyst (nest or follicle) in the following way: "The proliferation of germ cells occurs in coordinated clusters, each cluster being enclosed within a well defined membranous capsule for much of its development." (p 116) Many cysts together form one lobule and in some urodele species, lobules can form distinct and separate lobes. There are, strictly speaking, no seminiferous tubules in urodeles, as the lobules do not have a permanent germinal epithelium. Rather, the lobules

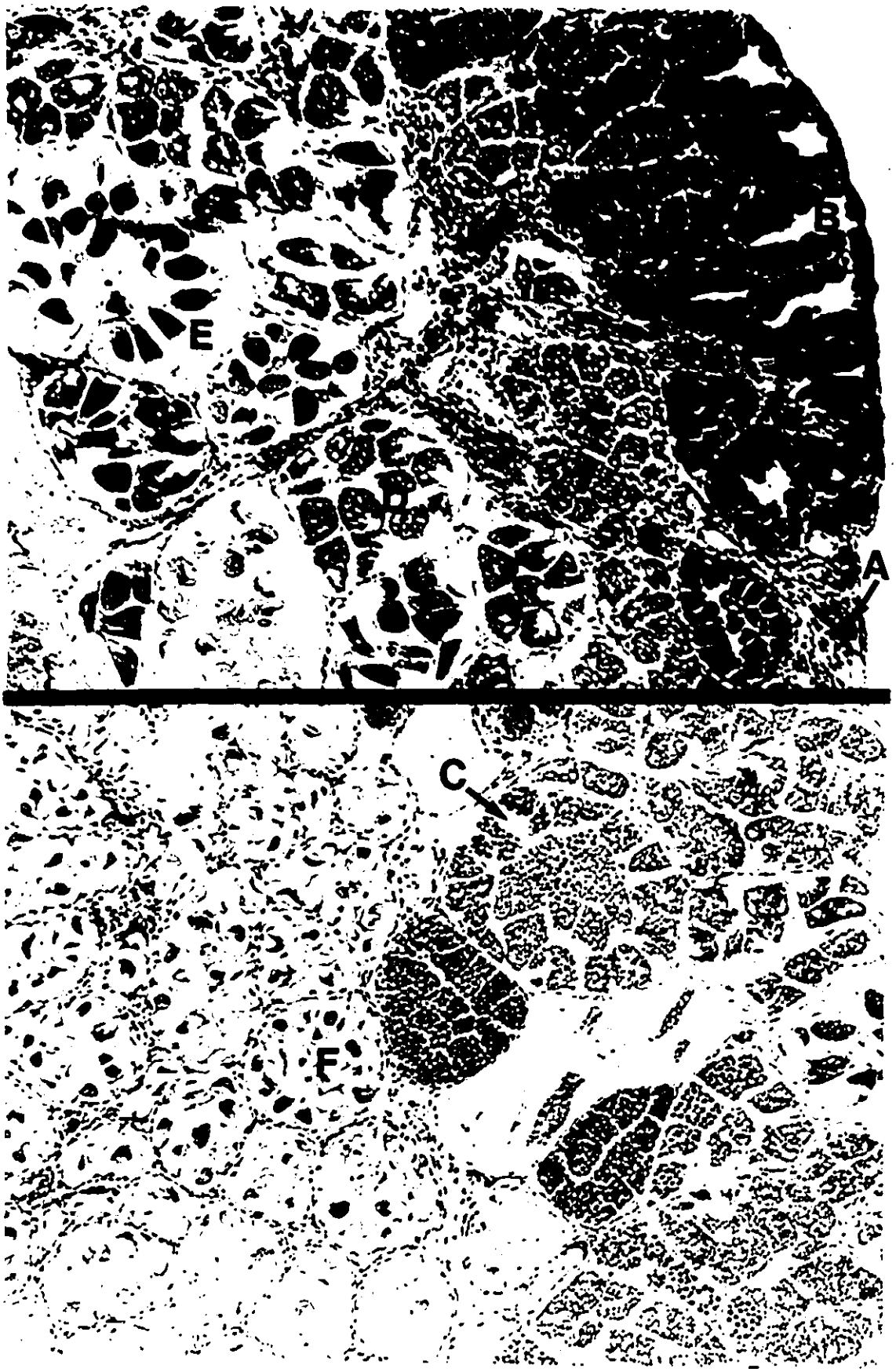
are transient structures which are renewed with each new reproductive cycle. A cyst starts out as a single stem cell or primary spermatogonium. When stained with a basic dye such as toluidine blue, these cells are characterized by large, lightly staining, irregularly shaped nuclei and are surrounded by many smaller darkly staining follicle or Sertoli cells (Figure 1A). Should incomplete cytokinesis occur after routine mitosis of a primary spermatognium, the two daughter cells will remain attached via a cytoplasmic bridge and a secondary spermatogonium is formed. All cells within a cyst will divide synchronously as they continue to differentiate and Browder (1980) speculates that the interconnection between the cells in a cyst is responsible for this phenomenon which he terms a syncytial clone.

The secondary spermatogonium is committed to meiosis and must eventually become a sperm cyst. Secondary spermatogonia undergo a multiplication phase and can divide mitotically up to eight times, all the while remaining enclosed by a membranous cyst. A cyst of secondary spermatogonial cells will stain more darkly than primary spermatogonial cells; their nuclei are smaller and have a more uniform appearance. An "older" secondary spermatogonial cyst will often have a clear area at its center such that the cyst has a doughnut like appearance (Figure 1B).

Figure 1: A longitudinal section through the testis of the axolotl. The section is embedded in glycol methacrylate, sectioned at 3 μ, and stained with 0.1% Toluidine blue, pH 4.4.

Legend: A=primary spermatogonia
B=secondary spermatogonia
C=primary spermatocyte
D=secondary spermatocyte
E=spermatid
F=sperm

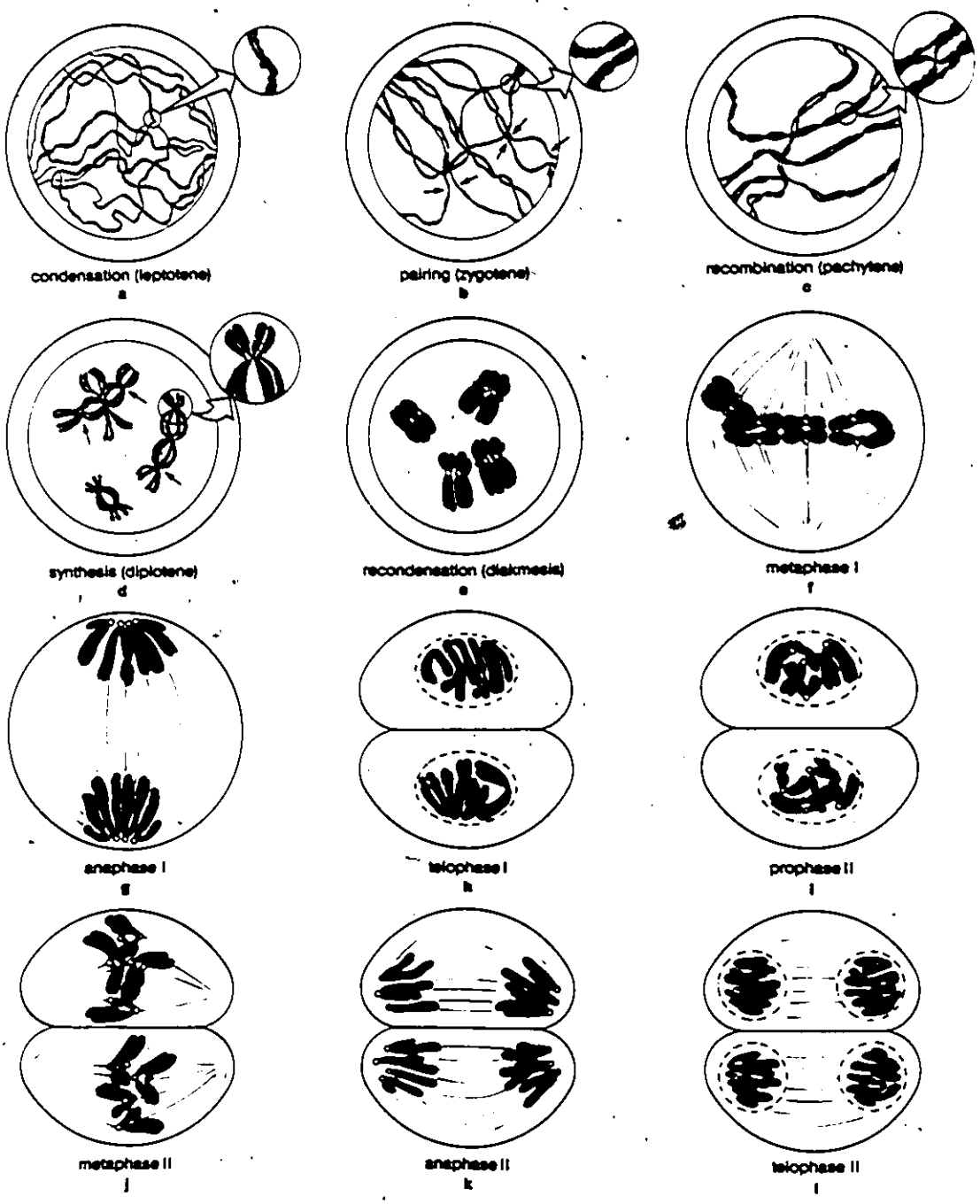
In the lower photograph, C refers to a primary spermatocyte cyst in meiosis; the cyst is partially filled with secondary spermatocytes. Magnification is 96X.



The term maturation period is used to characterize the transition from secondary spermatogonium to primary spermatocyte. All nuclei within a primary spermatocyte cyst look the same. The cells fill the cyst and the large clear area characteristic of a secondary spermatogonial cyst has disappeared (Figure 1C). The individual stages of first meiotic prophase can be visualized in good sections of primary spermatocyte cysts and with the completion of this first meiotic or reduction division, the cysts become secondary spermatocytes (Figure 1D).

The first and most important step in meiosis is the duplication of DNA which takes place during S phase of the cell cycle. It is only during leptotene of first meiotic prophase that the chromosomes become visible and although they appear unduplicated when viewed under the microscope, they are in fact already doubled (Figure 2A). While still in S phase, the chromosomes begin to form lateral elements which are an integral part of the synaptonemal complex. As leptotene succeeds premeiotic S phase, the lateral elements elongate until they run the whole length of the chromosome. The formation of these lateral elements is an essential prerequisite to the pairing and subsequent synapsis of homologous chromosomes which begins at zygotene (Figure 2B).

Figure 2: The stages of meiosis, reprinted from Wolfe (1980).


condensation (leptotene)
a
pairing (zygotene)
b
recombination (pachytene)
c
synthesis (diplotene)
d
recondensation (diakmesis)
e
metaphase I
f
anaphase I
g
telophase I
h
prophase II
i
metaphase II
j
anaphase II
k
telophase II
l

During zygotene, homologous chromosomes form bivalents; the four chromatids are brought into close association by the synaptonemal complex which now extends from telomere to telomere. Almost all sexually reproducing organisms have a synaptonemal complex and it seems to be a sine qua non for recombination. The synaptonemal complex is a proteinaceous structure composed of an axial core, a central element and two lateral elements. It is interesting to note that female Drosophila have a synaptonemal complex and can undergo recombination whereas male Drosophila spermatocytes have no synaptonemal complex and consequently no genetic recombination. The first step in the formation of the synaptonemal complex takes place at the telomeric ends of the chromosome as the chromatin rotates to expose the lateral elements formed on the sister chromatids. The central and transverse elements are then able to form and hold the two strands together.

Rasmussen and Holm (1980), in their paper on the mechanics of meiosis, give evidence in support of a unit, not unlike a ribosome, that is capable of mediating the whole process of recombination by attaching itself to the synaptonemal complex. This unit is called a recombination nodule and is postulated to function in two ways:

1) By coordinating the enzymatic requirements of recombination events and;

2) By regulating the number and distribution of cross-over events in the nucleus.

Crossing-over and recombination between four chromatids are events that take place during pachytene, the third step of first meiotic prophase (Figure 2C). The chromosomes have shortened; consequently, they look thicker under the light microscope and the homologues appear as one.

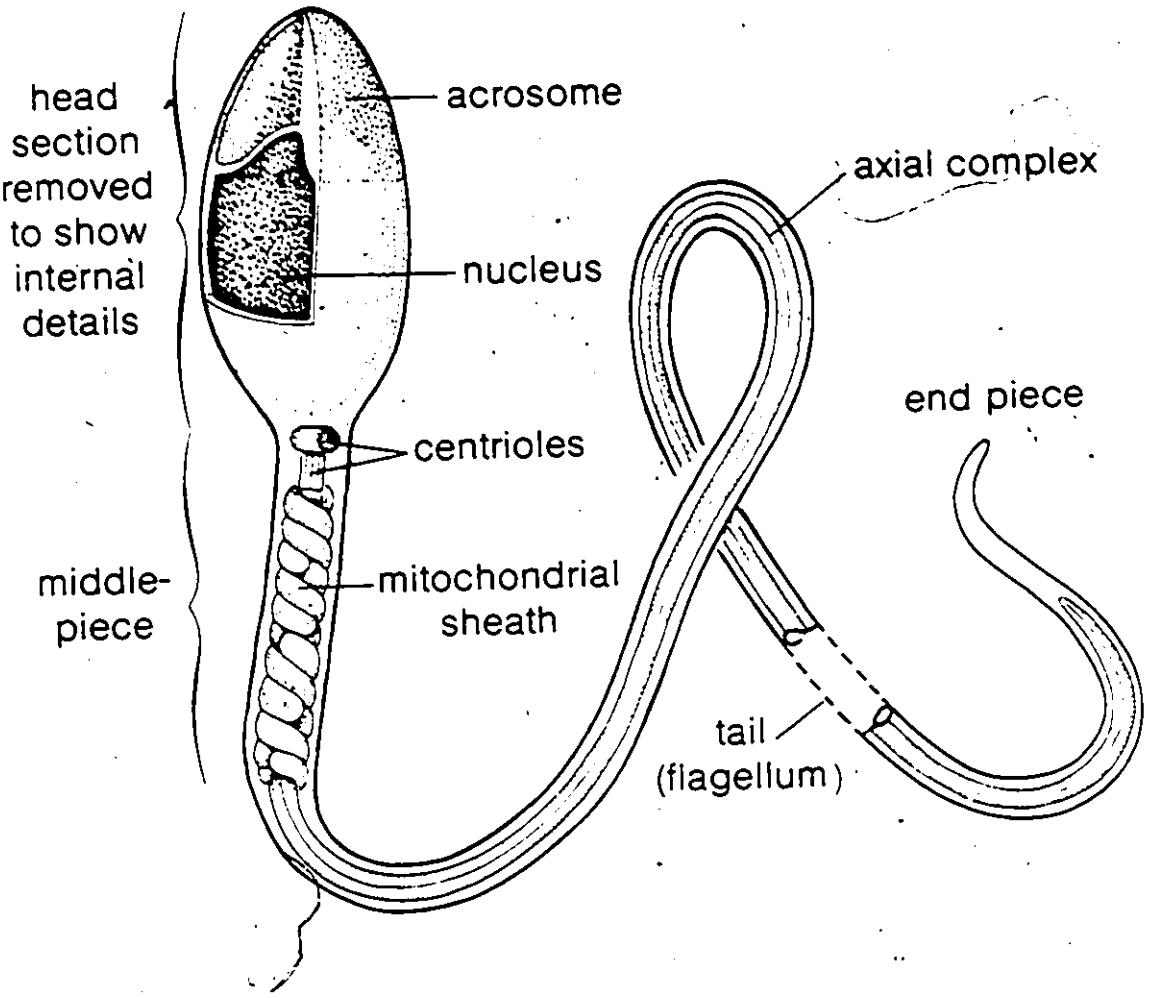
During diplotene the four chromatids of the bivalent become distinct because they are starting to separate. As the chromosomes separate along their entire length, the synaptonemal complex is no longer functional. Chiasmata hold the chromosomes together at points where crossing-over has occurred (Figure 2D). In many species chromosome decondensation will take place during diplotene and the chromosomes may look almost as though they are in leptotene. This is called the diffuse stage in male spermatocytes and the lampbrush stage in female oocytes. This period represents a developmental hold during meiosis and in some species it can last a few days or weeks; in man, it can last up to 40 years (Bennett 1977). The diffuse or lampbrush stage represents a time of transcription; in females numerous mRNAs are being stored to aid the egg during its development immediately after fertilization. In males it seems most likely that mRNAs are being formed for subsequent synthesis of protamines (Dixon and Iatrou 1978).

At the end of diplotene, the lateral loops characteristic of the lampbrush chromosome regress. Diakinesis follows as the chromosomes continue to contract and the chiasmata move

to the ends of the bivalents. This is known as terminalization and signals the last stage of first meiotic prophase before dissolution of the nuclear membrane and movement of the chromosomes onto the spindle fibers at first metaphase (Figure 2E and F). Separation of homologous pairs and the formation of dyads are characteristic of metaphase 1. The dyads orient themselves on the spindle such that each chromosome migrates to the opposite pole of the cell. Independent assortment of non-homologous pairs occurs here; a form of interchromosomal recombination. Intrachromosomal recombination took place during crossing-over at pachytene.

A second meiotic division, usually much quicker than the first one, converts secondary spermatocytes into spermatids. Again reduction in the size of the nucleus of the spermatid cell is the most obvious histological feature of the spermatid cyst just after it has been formed. This is the critical stage when histones are removed from the chromatin, the nucleosome structure is disrupted and protamines repackage the DNA into a very condensed, transcriptionally inactive form within the sperm head. As spermatids become sperm they elongate and late spermatid cysts contain many differently shaped cells (Figure 1E). The process whereby mature sperm are formed from spermatids is called spermiogenesis and can be divided into three stages which involve:

Figure 3: Generalized sperm structure, reprinted from Wolfe (1980).


head section removed to show internal details
acrosome
nucleus
axial complex
end piece
centrioles
middle-piece
mitochondrial sheath
tail (flagellum)

1) Formation of the acrosome at the very head of the sperm;

2) Formation of the sperm midpiece;

3) Differentiation of the sperm tail.

Generalized sperm structure is illustrated in Figure 3.

The acrosome contains hydrolytic enzymes which will be used to dissolve the membranes surrounding the egg so that sperm can penetrate. It appears that the acrosome is derived from the Golgi complex of the cell by a coalescense of proacrosomal granules on the nuclear envelope. It takes its final shape as the chromatin within the nucleus is condensed.

The sperm midpiece is formed from mitochondria in the cell which elongate and wrap themselves around the axoneme in a helical fashion. The axoneme is the source of propulsion of the mature sperm, providing it with mobility. Two microtubules are found at the center of the axoneme, and nine doublets surround them. The actual generation of sperm flagellar motion is by the axoneme found in the sperm tail. The distal centriole of the sperm nucleus is responsible for the elongation of the axoneme; it orients itself parallel to the long axis of the cell and microtubule subunits are added to the distal end of the axoneme. The differentiation of the sperm cell is now complete. The distinctive shape which the sperm nucleus finally assumes is species specific and it is thought that either the microtubules surrounding the nucleus or the particular pattern of chromatin condensation may be

responsible (Browder 1980).

The cyst which surrounded the early spermatid cells ruptures as the spermatid cells begin to elongate. The developing sperm heads become embedded in the cytoplasm of the follicle cells which originally surrounded the primary spermatogonium. The follicle cell at this time is analagous to the mammalian Sertoli cell; its cytoplasm becomes enriched with mitochondria and endoplasmic reticulum and it begins to secrete androgens. After the cyst has ruptured the follicle cell with its swirl of maturing sperm attached will migrate to the lobule wall. The close association between follicle cell and sperm continues until the mature gamete is released from the testis and stored in the vas deferens until it is needed. The ruptured cysts begin to degenerate and interstitial cells become prominent during this phase of spermatogenesis.

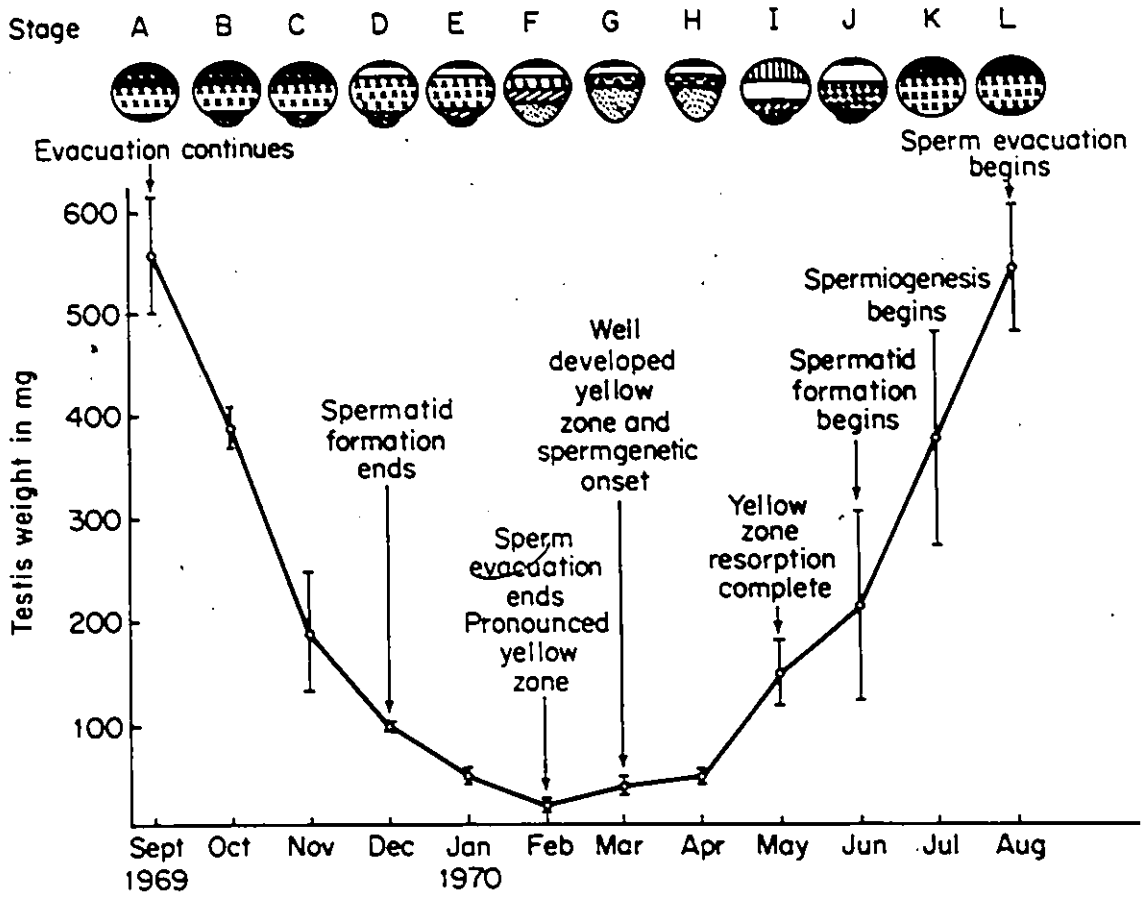
Humphrey (1921) studied the relationship between interstitial cells and spermatogenesis in urodeles more closely. Interstitial cells are usually found between cysts as these rupture and sperm leaves the testis. They are thought to function as phagocytic cells, removing the detritus from the testis and providing space for the proliferation of a new germ cell generation. As the cyst begins to grow and more secondary spermatogonia are formed, interstitial cells which had surrounded the primary spermatogonium become pushed to the periphery of the cyst.

They become less and less prominent as spermatogenesis proceeds. Interstitial cells are renewed annually in wild urodeles as degenerative changes in the testis occur. Humphrey feels that interstitial cells are temporary modifications of stromal cells, appearing only when needed during the spermatogenic cycle.

As mentioned above, the events of spermatogenesis cannot be studied without reference to the environment. The biological clock in the amphibian poikilotherm is very strong and its reproductive cycle can be closely correlated with the seasons of the year. Lofts (1974) followed the time course of spermatogenesis in Triturus hongkongensis and his data can be taken as representative of the urodele in the wild (Figure 4).

Starting the year in the spring, one finds that this is the time of renewal of germinal tissue in the male gonad. A new generation of secondary spermatogonia arise from primary spermatogonia which have proliferated in regions where old cysts ruptured and degenerated. Meiotic figures predominate in March through to May; the testis is full of secondary spermatogonia and primary spermatocytes. This is usually the active breeding season for most temperate urodeles, and sperm stored in the vas deferens from the previous winter are laid in spermatophores.

Figure 4: Seasonal changes in testicular weight and zonation in Triturus hongkongensis, reprinted from Lofts (1974).



Seasonal changes in testicular weight and zonation
(only one testicular lobe is shown)

- Yellow zone
- Evacuated lobule
- Spermatozoa
- Spermatids
- 2^{y} spermatocytes
- 1^{y} spermatocytes
- 1^{y} and 2^{y} spermatogonia

In the summer months, the later stages of spermatogenesis predominate and secondary spermatocytes and spermatids fill the testis. By August, mature sperm have been formed throughout and very few remaining spermatogonia and spermatocytes can be found. The testis is swollen and has nearly doubled in weight. Sperm are released into the vas deferens in the autumn and early winter months, ruptured cysts degenerate and interstitial cells begin to form once more.

By February, almost all mature sperm will have left the testis and it will be full of interstitial cells. The weight of the gonad declines as most of the degenerating tissue is resorbed by the salamander in preparation for a new reproductive cycle.

In urodeles, spermatogenesis proceeds through the testis in a wave-like motion; this phenomenon has been termed zonation. Basically it means that an animal taken from the wild at any particular time of year will show a progression of spermatogenic stages through the testis in a cephalo-caudal direction. The most mature stages will be posterior to the less mature ones; thus sperm will be found at the bottom and spermatogonial cells at the top. A longitudinal section would show this progression with cysts in a particular zone all being at the same developmental stage.

In most urodele species, this wave will progress from top to bottom in a year's time. However, there are some species

where spermatogenesis proceeds more slowly and does not involve the whole gonad during a yearly cycle. Humphrey (1922) showed that this effect is paramount in the development of a multi-lobed testis in Desmognathus fusca. The slow movement of the spermatogenic wave coupled with slow regeneration of emptied lobules leads to a constricted region between cysts containing only quiescent spermatogonial cells. Thus a number of cysts become isolated from each other and a multi-lobed testis is formed.

In addition to the above evidence showing the correlation between seasons and spermatogenic cycle, there is also evidence for internal control via pituitary hormones. Regulation of spermatogenesis in mammals is the result of interplay between two distinct hormones: FSH (follicle stimulating hormone) which regulates spermatogenesis itself; and LH (leutenizing hormone) which regulates the interstitial cell cycle. This same pattern of regulation has been found in several amphibian species.

Van Oordt (1960) cites an LH-like hormone as being responsible for spermatogenesis. He feels that seasonality is the result of changes in sensitivity of the germinal epithelium to the hormone and to ambient temperature. During the cooler months of the year, LH predominates, more interstitial cells are present in the testis and, in corollary, during the warmer months FSH predominates, spermatogenesis proceeds and interstitial cells atrophy.

Specker and Moore (1980) have carried these results even further and have demonstrated that in the rough-skinned newt, plasma androgens vary annually and can be correlated with testicular composition. Spermatogenesis occurs at high FSH and low LH, T and DHT concentrations (T=testosterone, DHT=dihydrotestosterone). On the other hand, spermiogenesis or spermiation occurs at low FSH and high LH, T and DHT concentrations. The authors propose that androgen synthesis is common to interstitial cells either in conjunction with or independent of pituitary secretions. The interdependence of season, hormonal concentration and testicular composition can best be visualized in Table 1.

To summarize what has been said so far:

1) Amphibian spermatogenesis is a cyclic event, each cycle being completed in one year;

2) Spermatogenesis is regulated both by external and internal factors. With respect to the former, temperature seems to be the most important variable and with respect to the latter, two pituitary hormones seem to exert control over testicular events.

Recently, however, spermatogenesis has been looked at from an entirely different viewpoint. Several researchers have been interested in the timing of events during meiosis and it is these studies that are the most relavent to establishing the time course of axolotl spermatogenesis. The technology that enables such studies to be done revolves

around the labelling of DNA with tritium in a nucleotide precursor molecule. The most frequently used nucleotide for DNA studies is tritiated thymidine.

When radioactive thymidine is administered to living tissue, either in vivo or in vitro it will be incorporated into the DNA of replicating cells. If cells in the testis are labelled, there will be several different classes of cells that become radioactive. The first of these is the spermatogonial cell in premitotic S phase. Similarly, spermatogonial cells entering a much slower premeiotic S phase will also be labelled. It is this class of cells at premeiotic S that will subsequently enter meiosis and in which label will be "seen" to progress from one stage of spermatogenesis to the next. A third class of cells which may be labelled are those cells in which late DNA replication is taking place. In the lily, Stern and Hotta (1977) found that there is delayed DNA replication during leptotene of first meiotic prophase. Thus when a pulse of radioactive thymidine is given, these cells may also be labelled.

There is some controversy about other cellular compounds becoming labelled when tritiated thymidine is administered. Baserga (1969) states that thymidine will be incorporated only into DNA and this within 30-40 minutes of exposure to the label. After this time any unused nucleotide in the intact animal will be rapidly catabolized. In addition, the

usual processes of fixation remove most soluble cellular components such as proteins which may have retained a tritium moiety, whereas labelled DNA remains insoluble and so is retained in the fixed cell. Certainly in my experience with tritiated thymidine, label has been confined to the DNA.

Labelled DNA can be seen by autoradiography; under the light microscope "hot" cells will have black dots over their nuclei. Applying this technique specifically to the study of spermatogenesis, a number of animals can be injected at the same time with a specific dose of tritiated thymidine. The gonads are then removed at specific time intervals thereafter, fixed, sectioned, and processed by autoradiography. As label proceeds through the different stages of spermatogenesis, it becomes possible to establish a time course by noting the first appearance of labelled cells at each of these stages.

Table 2 summarizes the results of three such experiments. Two of these papers report the timing of meiosis in wild urodeles: Triturus vulgaris (Callan 1968), and Plethodon cinereus (Morgan 1979), whereas the third (Kalt 1976) has been done on a laboratory bred anuran, Xenopus laevis. This last study is of particular interest to this thesis as it is the only one tracing meiosis in a captive amphibian.

Table I

INTERDEPENDENCE OF SEASON, HORMONE, AND CELL TYPE IN AMPHIBIAN SPERMATOGENESIS

	Spring	Summer	Fall	Winter
Predominant Cell Type	2° Spermatogonia 1° Spermatocyte	2° Spermatocyte Spermatid	Spermatid Sperm	Sperm Int. Cell
Major Pituitary Hormone	FSH	FSH	LH	LH
Plasma Androgen Concentration (T and DHT)	Low	Low	High	High

Note: This table is based on the work done by Van Oordt (1969) and Specker and Moore (1980).

It is interesting to note that even though different species of amphibians are represented here, the timing of meiosis in these animals is fairly comparable. Bennett (1977), in his excellent article on the timing and duration of meiosis, feels that the C-value of a particular species is a determining factor in the length of meiosis for that species and that is also a reflection on its life style. Morgan (1979), however, argues that Bennett's correlation between haploid nuclear DNA content and the length of meiosis is not linear. Bennett had found that just such a linear relationship existed for diploid angiosperms and perhaps also for animals in the same Class. Yet the C-value for Xenopus laevis is 3.2 pg DNA compared with 20 and 24 pg DNA for P. cinereus and T. vulgaris respectively. Xenopus has the longest meiosis of these three animals where, according to Bennett's theory, it should have the shortest. There are, however, other factors which can affect the duration of meiosis including genotype and ambient temperature (Bennett 1977). In addition, the length of the diffuse stage between pachytene and diplotene, which is a developmental 'hold' during meiosis, can greatly alter the duration of meiosis in individual species.

Table II

COMPARISON OF THE TIME COURSE OF MITOSIS

IN THE SPERMATOCYTES OF THREE AMPHIBIAN SPECIES

	L	Z	P	D	M	2° SC	SP?	SP1
P. cinereus (20° C)	1	8	13	24	26	27	-	-
T. vulgaris (16° C)	1	6	14	19	20	23	-	-
X. laevis (18° C)	4	5	10	22	23	23	26	27

Note: The duration of spermatogenesis is in days.
The work on P. cinereus was done by Morgan (1979), the work on T. vulgaris by Callan and Taylor (1968) and the work on X. laevis by Kalt (1976).

With this background information and additional data from EMS mutagenesis experiments, three consequtive experiments to establish the time course of spermatogenesis in the axolotl were started. In the fall and winter of 1980-1981 seven adult axolotls averaging 4.5 years of age were injected with a dose of tritiated thymidine. This first set of experiments showed that first metaphase is labelled after 40 days and sperm after 80 days. A second set of experiments was carried out in the summer of 1981 with younger animals to see if either season and or age had any influence on the duration of spermatogenesis even though the axolotls in the laboratory are kept under constant conditions of light and temperature. The results from this set of experiments showed first metaphase labelled at 30 days and sperm at 60 days. A third set of experiments was run during the fall and winter of 1981-1982 again with young animals to establish more precisely the timing of first meiotic prophase. The results from this set of experiments indicate that first metaphase is reached at 36 days and sperm is labelled at 80 days.

Materials and Methods

Animals

The animals used in the following experiments were raised in the laboratory of Dr. J.B. Armstrong. They were kept under constant environmental conditions of 12 h light and 12 h dark at 18° C. Each animal was housed in his own bowl in a 50 % modified Holtfreter's saline and was changed and fed every second day with raw beef heart. Modified Holtfreter's saline (100 %) contains, per liter dechlorinated water 3.45 gm NaCl, 200 mg $MgSO_4$, 200 mg $NaHCO_3$, 100 mg $CaCl_2$, and 50 mg KCl.

At the start of each experiment, the required number of animals was injected intraperitoneally with a dose of 40 µCi of ^3H-thymidine. To make up the dosage used, 0.25 ml of 20 mCi/mmol of tritiated thymidine (New England Nuclear) was diluted to 1.25 ml with sterile water. Each animal was injected with 0.2 ml of this solution.

The schedules for the three spermatogenesis experiments are outlined in Table 3. For the first set of experiments, done in the fall and winter of 1980-1981, the animals averaged 4.5 years of age and time points were at ten day intervals. Standard histological procedures with paraffin-embedding were used and, sections were cut at 10 µ.

Table III

SCHEDULE FOR SPERMATOGENESIS EXPERIMENTS

Fall-Winter 1980-1981

Days	Animal	Date	Days	Animal	Date
0	31-4A	10-22-80	80	47-1A	1-10-81
10	31-4B	11-1	90	51-2B	1-20
20	51-2A	11-11	100	31-6B	1-30
30	31-6A	11-21	110	46-1B	2-9
40	46-1A	12-1	120	44-1B	2-19
50	44-1A	12-11	130	44-5B	3-1
60	44-5A	12-21	140	47-1B	3-11
70	40-1A	12-31			

Spring-Summer 1981

5	113-3A	5-27-81	45	133-15A	7-6-81
10	133-4	6-1	50	109-4	7-11
15	113-8A	6-6	55	113-3B	7-16
20	113-9	6-11	60	112-1	7-21
25	113-10	6-16	66	113-12B	7-27
30	113-11	6-21	70	119-10	7-31
35	113-12	6-26	75	113-15B	8-5
40	113-13	7-1			

Fall-Winter 1981-1982

2	119-2A	10-15-81	30	119-5B	11-12-81
6	119-5A	10-19	33	119-6B	11-15
9	119-6A	10-22	36	124-4B	11-18
12	119-7A	10-25	39	124-5B	11-21
15	124-4A	10-28	50	s.c.1B	12-2
18	124-5A	10-31	60	s.c.2B	12-12
21	s.c.1A	11-3	70	105-11A	12-22
24	s.c.2A	11-6	80	105-11B	1-4-82
27	119-2B	11-9			

The second set of experiments was done in the spring and summer of 1981. The animals used for this series were younger and averaged two years of age. The same procedures were followed, except that time points five days apart were chosen. In addition, in an attempt to better segregate the population of labelled cells at various times during spermatogenesis, the STAPUT method (Miller and Phillips 1969) was tried. Unfortunately, with the exception of one test run, this method seemed to be unsuitable for the axolotl.

A third experimental series was started in the fall and winter of 1981-1982, using one and one-half year old animals. Instead of paraffin embedding, glycol-methacrylate was tried and sections were cut at 3 μ, allowing for the visualization of much greater cellular detail. The time points chosen for this set of experiments varied from three to ten days. Until first meiotic metaphase was reached at 40 days, three day time points were taken. After that time, up to 80 days, the interval was ten days. In addition, five animals were injected with tritiated thymidine, but were not operated upon. After 50 days, spermatophores were collected at weekly intervals from one of the five in an attempt to see what the absolute time is for labelled sperm to appear in a spermatophore. This sperm was collected, squashed on a slide and then processed by autoradiography.

The gonads were removed from axolotls that had been

anesthetized in a solution of 1 gm/l MS 222 (Sigma, St. Louis, Mo.). An incision of 1.5-2 cm in length was made to the right or the left of the midline in the lower quadrant of the abdomen. Using colibri retractors (Fine Science Tools), the intestines were carefully held aside and the testis was exposed. The entire testis was excised, surrounding fatty tissue was carefully cut away, and the testis was weighed. The incision was closed with three or four running stitches of sterile 00 silk sutures, and the recovering animal was placed in a fresh solution of 50 % modified Holtfreter's.

Paraffin Embedding

The excised testis was immediately fixed in a freshly made Clark's solution (3 parts ethanol: 1 part glacial acetic acid). The tissue was usually fixed overnight at 4° C, but it can remain in the fixative indefinitely.

Dehydration of the tissue was done in two changes of 95% ethanol for 1/2 hour each, followed by 1/2 hour in 99% ethanol. Clearing was done overnight in methyl benzoate (Baker). The clearing agent was removed just prior to infiltration with a 1/2 hour rinse in xylene, and the tissue was transferred to a small plastic beaker containing melted Paraplast Plus (Lancer, St. Louis Mo.). Paraplast Plus was usually placed in a 60°C oven the night before infiltration to ensure that it was thoroughly melted. The infiltrating medium was changed twice for a total infiltration of 1 1/2

hours.

Some molten Paraplast Plus was poured into a plastic mold (Tissue Tek, Miles Laboratories), and a block holder was fitted on top. The tissue was oriented in the mold and as soon as a thin film had formed over the block, the whole block was submerged in cold water to ensure rapid and uniform cooling. When the block had cooled and hardened, the plastic mold was simply peeled off and the block was ready for sectioning.

Sectioning

The block containing the material to be sectioned was first trimmed so that the excess paraffin was removed, but enough was left to allow for ease in ribbon formation and handling of the sectioned material. Longitudinal sections were cut at seven to ten microns on a rotary microtome (American Optical) with a steel knife. To ensure better sections, the block was cooled in an ice bath for 15-20 minutes. After a ribbon of sectioned material had been made, it was floated on distilled water and lifted onto a clean, albuminized slide. The sections were expanded on a slide warmer at 45-55° C for a short time and then left overnight at 35° C.

Glycol Methacrylate Embedded Sections

The testes were fixed in a freshly made up solution of 4% gluteraldehyde (J.B. EM Services) in phosphate buffer at pH 7.4 and fixed overnight. Dehydration, infiltration, and

embedding were done according to the method outlined in the Sorvall Instruction Manual (1980). Usually infiltration lasted one day, but larger specimens were infiltrated for up to three days at 4° C. A small vacuum jar was used to speed up polymerization, and blocks were cured for 1-3 hours at 35° C prior to sectioning at 3 μ. Sections were cut on a Sorvall JB-4 microtome, floated on distilled water, and picked up on a clean glass slide. They were dried on a slide warmer at 35-40° C for several minutes before being processed for autoradiography.

Autoradiography

The slides used for autoradiography were cleaned prior to use by soaking them for three hours at 60° C in a mild detergent solution, i.e. 4% Decon 75 (BDH Chemicals, Toronto). They were then rinsed under running hot water for one hour, air-dried, and stored in a slide box until needed.

Before the sections were coated with emulsion, the paraffin was removed from them. This was done by taking the slides to water. They were first rinsed in xylene for 20-30 minutes, followed by 15 minutes each in graded ethanol solutions, from 95% to 35%. Finally, the slides were rinsed in cold water for 15-30 minutes.

The required amount of NTB 2 liquid emulsion (Kodak) was removed from its package in complete darkness , placed in a small vial, and melted for 45 minutes in a 37° C water bath.

When it was melted it was diluted 1:1 with 2% glycerol and thoroughly mixed. The red safety light may be turned on for this procedure. Enough emulsion was poured into a small plexiglass container and the slides were dipped and coated with emulsion. They were air-dried, upright, for 20 minutes, then stored in light tight slide boxes with some $CaSO_4$. The sections were exposed for 2-3 weeks at 4° C, if they were 10 μ thick, and for 4-5 weeks, if they were 3 μ thick.

Developing

Kodak Dektol or D-19 developer was prepared according to the instructions on the package. Just prior to use it was diluted 1:1 with water. The slides were removed from their boxes in complete darkness and developed for 2-2.5 minutes. They were then dipped quickly in water and fixed in Kodak Fixer for 5-7 minutes. At this time the red safety light was turned on. After fixing, the slides were washed under running water for 20 minutes.

Staining

Paraffin sections were stained for 5-10 minutes in 0.1% Toluidine blue (Fisher Scientific, Ottawa). They were subsequently dehydrated in two changes of 95% ethanol and 99% ethanol for two minutes each. After clearing in xylene for 5 minutes, the slides were mounted in Permount (Fisher.) and viewed with the light microscope.

Plastic-embedded sections were also stained with Toluidine blue, except that the solution was made in a 0.02M sodium benzoate buffer at pH 4.4. The slides were stained for 1-5 minutes, washed in water, air-dried, and mounted in Permount.

Cell Separation at Unit Gravity

The procedure for the 1 g separation of cells with the STAPUT apparatus (Johns Scientific, Toronto) is outlined in Dixon (1972). With the exception of a few minor changes, his procedure was followed.

The testis was prepared for the gradient in the following way. After its removal and weighing, the gonad was forced through a fine wire mesh screen in a petri dish containing 5 ml of 0.5% BSA (Sigma, St.Louis Mo.) in PBS. The solution was homogenized with a Pasteur pipette and then transferred to a test tube containing 10 ml of 0.5% BSA in PBS. Five ml of fetal calf serum (Gibco) was layered underneath the cell suspension and larger debris was allowed to settle out for one minute. The supernatant was pipetted off, transferred to another test tube and fetal calf serum was again layered underneath. These steps were repeated until a homogeneous suspension, free of cellular debris, was obtained. Prior to layering this suspension onto the STAPUT, a small aliquot was removed and the cells were counted with a hemocytometer.

The running time of the STAPUT varied from 3 to 5 hours,

and 7-8 ml fractions were collected at a flow rate of 20 ml/min.

Cyst Counts

Five randomly chosen longitudinal sections were scored and the number of cysts of each different stage of spermatogenesis were counted with a stereomicroscope at 5x. Spermatogonial cysts containing more than two large nuclei were scored as secondary spermatogonia.

Sperm Squashes

An average of 5 spermatophores were collected from one of five injected males at weekly intervals, starting 50 days after labelling with tritiated thymidine. The sperm packet was removed from each spermatophore and frozen at -20°C until processed. After the sperm were thawed out, they were placed on a clean, abluminized slide. A siliconized cover slip was applied, and gentle pressure was used to squash the sperm packet; one sperm packet per slide. The slide was placed on a bed of dry ice for 5-10 minutes, and after the cover slip was gently flipped off, the slides were ready to be processed by autoradiography.

Labelling of Testes using Tritiated EMS

One animal was injected intraperitoneally with a dose of 41 μCi of tritiated EMS (New England Nuclear). A 1 ml water sample taken from the bowl was removed hourly, mixed with 10

ml Biofluor (New England Nuclear), and counted in a liquid scintillation counter (Beckman). When the excretion of ^{3}H-EMS had levelled off, five hours post injection, the animal was anesthetized and one testis was removed. The testis was fixed in 4% glutaraldehyde (J.B. EM Services) and processed with glycol methacrylate. Sections were cut at 7 µ, coated with Kodak NTB 2 liquid emulsion and stored for three weeks at 4° C.

Results

Time Course of Spermatogenesis

The duration of spermatogenesis in the axolotl was established through three separate experiments. Two of these experiments were run the fall and winter (1980, and 1981, respectively), whereas the third was run during the intervening spring and summer. At the start of each experiment, a group of axolotls was injected with a dose of 40 μCi of ^{3}H-thymidine. One testis was removed from each animal at specific intervals thereafter and the sectioned material was processed for autoradiography.

Figure 5 shows the progression of radioactivity through the different spermatogenic stages.

The first series, summarized in Table IV, showed a progression of spermatogenesis ending with labelled sperm in the testis at 80 days. The 10 day time intervals used in this experiment did not allow for an accurate estimation of the time spent in each of the stages of first meiotic prophase. However, it was possible to identify some of these stages; first metaphase was observed 40 days post labelling, pachytene 30 days, zygotene 20 days, and leptotene 3 days after injection. The time points at 20 and 90 days were lost because the animal failed to incorporate tritiated thymidine when first injected.

Figure 5: The following photographs illustrate the typical labelling pattern observed throughout the timing experiments. The photographs were taken from sections embedded in glycol methacrylate, sectioned at 3 μ, and stained with 0.1% Toluidine blue, pH 4.4. Figure 5C was taken from a section embedded in Paraplast Plus and sectioned at 10 μ. The stain was 0.1% Toluidine blue in water. Magnification is 500x, except for Figure 5C, which is 125x.

Legend: A=primary spermatogonium surrounded by follicle cells. Note that the follicle cells are much more heavily labelled than the two primary spermatogonial cells.

B=secondary spermatogonial cyst.

C=primary spermatocyte cyst. This photograph shows label confined to the primary spermatocytes in the cyst. Secondary spermatocytes in the left hand portion of the photograph are not labelled. The most heavily labelled primary spermatocytes are from spermatogonial cells that were just entering premeiotic S when the label was given.

D=primary spermatocytes, pachytene stage.

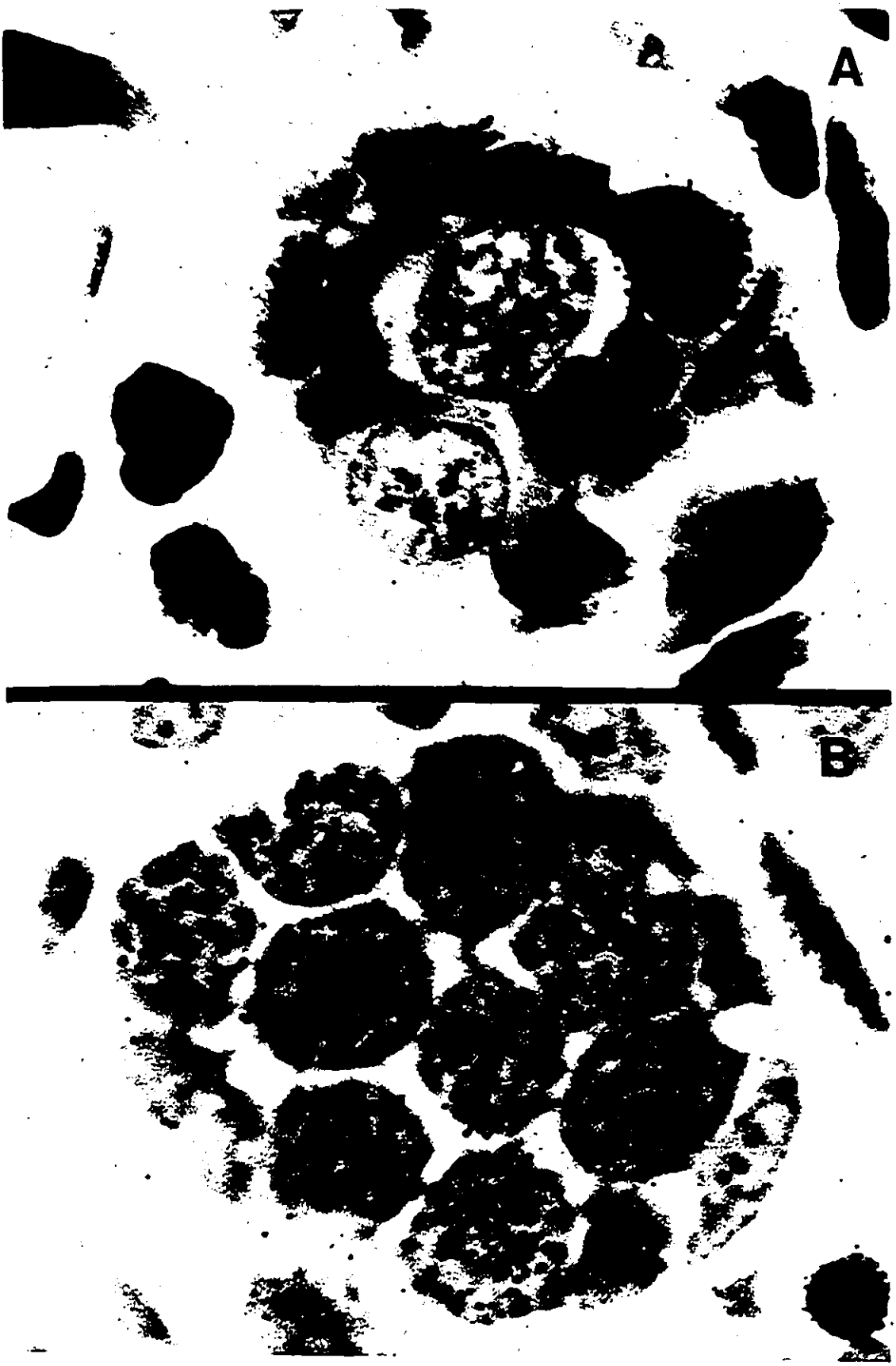
E=primary spermatocytes, diplotene and metaphase stages.

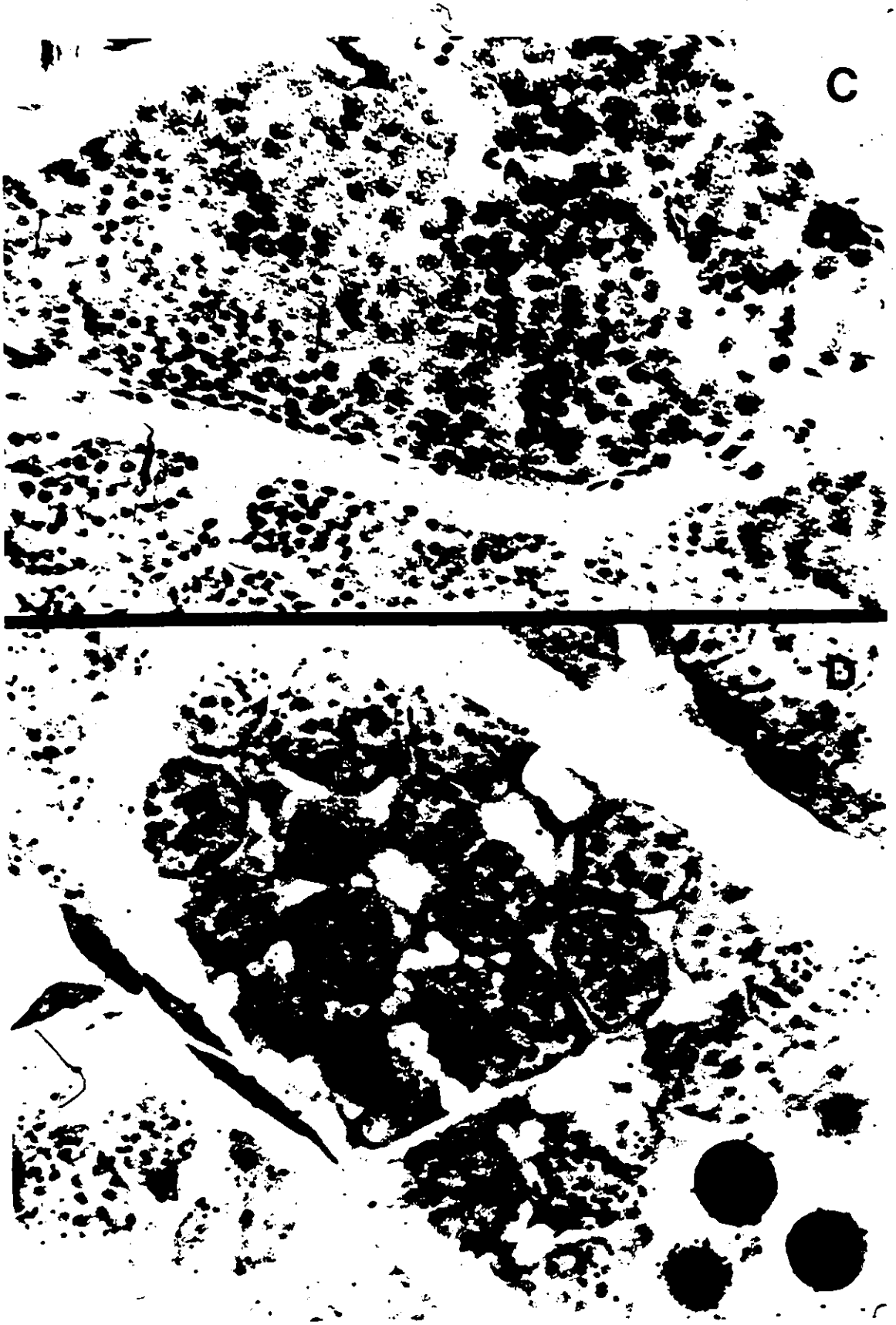
F=secondary spermatocytes

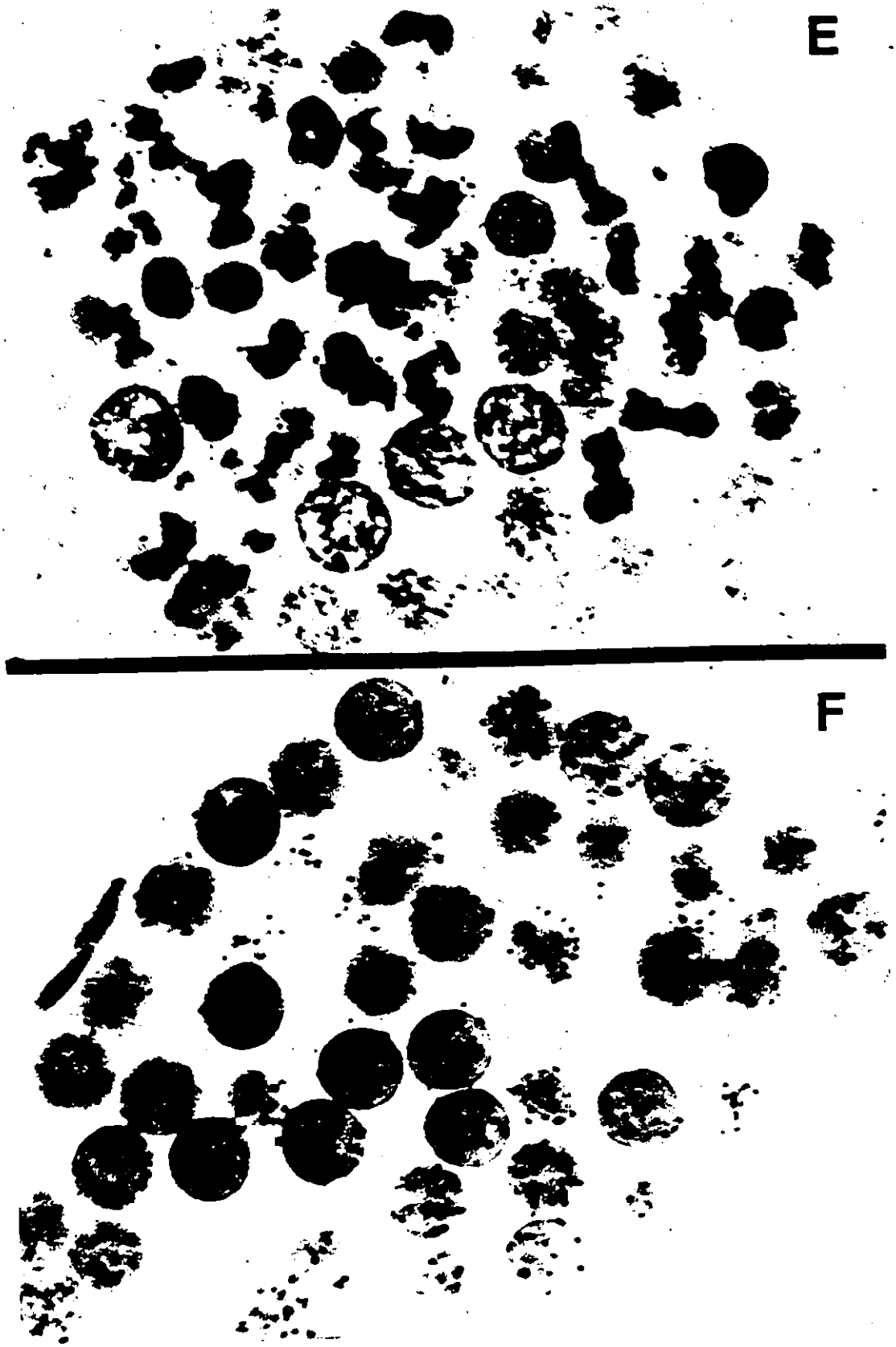
G=late spermatid cells

H=spermatozoa

I=spermatid cells, ^{3}H-EMS labelled


A
B


C
D


E
F


G
H

Table IV

PROGRESSION OF LABEL THROUGH

SPERMATOGENIC STAGES,

FALL-WINTER 1980-81

Animals	Days after injection	Most advanced stage labelled
31-4A	3 hrs.	leptotene
31-4B	10	zygotene
31-6A	30	pachytene
46-1A	40	metaphase
44-1A	50	2° spermatocyte
44-5A	60	early spermatid
40-1A	70	late spermatid
47-1A	80	sperm

Although this experiment was designed to continue for 140 days in the expectation that spermatogenesis in the axolotl might be a very lengthy process, no further progression of the label was seen after 80 days. Interstitial cells were labelled throughout the experiment and at the later dates, between 110 and 140 days, it was possible to see clusters of labelled cells in regenerating regions of the testis. It was not possible to see the start of a new germ cell generation at these later times. Primary spermatogonial cells labelled during mitosis and then entering premeiotic S phase would form the basis for a new cycle of spermatogenesis, but apart from the clusters mentioned above, I was not able to document the beginning of a new spermatogenic wave. Presumably, dilution of the label from these cells as they underwent subsequent divisions made it impossible to follow the renewed cycle.

In the second set of experiments, done in the spring and summer of 1981, two basic changes in experimental procedure were made. First, time points were taken every five days. Second, separation of cells at unit gravity, or STAPUT, (Miller and Phillips 1969) was tried in an attempt to better define the population of labelled cells at different times during spermatogenesis. The experiments were set up so that one testis was processed for paraffin sectioning every five days, and the STAPUT was used every ten days.

The closer time intervals chosen for this experiment

Table V

PROGRESSION OF LABEL THROUGH

SPERMATOGENIC STAGES,

SPRING-SUMMER 1981

Animals	Days after injection	Most advanced stage labelled
113-3A	5	leptotene
113-4	10	pachytene
113-8A	15	pachytene
113-9	20	diplotene
113-10A	25	pachytene
113-11	30	metaphase
113-13	40	2° spermatocyte
113-15A	45	2° spermatocyte
113-3B	55	early spermatid
112-1	60	sperm

permitted a clearer understanding of the time spent in first meiotic prophase. Overall the results (Table V) showed a slightly faster time for spermatogenesis than in the fall and winter of 1980-81. Sperm cysts were labelled after only 60 days, and first metaphase was reached at 30 days. The 35 and 65 day time points were lost because no label was found in sections examined, and at 50 days sections contained only sperm cysts, as yet unlabelled.

The procedure for separation of cells at unit gravity was tried every ten days. There were many procedural difficulties associated with this method and only on one occassion was it possible to obtain satisfactory results whereby the separated cells fractions could be positively correlated with radioactivity in those fractions. This run was done 20 days post-injection and showed a clear separation of spermatogenic cells into six major fractions corresponding to primary and secondary spermatogonial cells, primary and secondary spermatocytes, spermatids, and mature spermatozoa. Radioactivity was counted in primary and secondary spermatogonial cells as well as in primary spermatocytes. Results from autoradiography of paraffin-embedded sections also showed label in primary spermatocytes thus nicely complementing the results from the STAPUT.

As part of the preparation of a sample for use on the STAPUT gradient, a measurement of live spermatogenic cells was made. The diameter of primary spermatogonial cells

ranged from 6.5 to 10 μ; secondary spermatogonial cells had a diameter of 4.8 to 5.6 μ. Primary spermatocyte cells were approximately 4 μ and secondary spermatocyte cells 2.4 μ in diameter. Early spermatid cells ranged from 0.8 to 1.6 μ diameter and spermatozoa were extremely long, ranging in length from 64 to 120 μ.

The final series of experiments on the time course of spermatogenesis in the axolotl was started in the fall of 1981. For this set of experiments, three day time points were taken until 40 days, with ten day time points taken thereafter. Glycol methacrylate was used to embed the samples and sections were cut at 3 μ as compared to 7-10 μ for paraffin-embedded sections. The animals used were younger than those in the first series of experiments and yet the timing of spermatogenesis (Table VI) was found to closely parallel that found in the first experimental series. Sperm cysts were labelled at 80 days and first metaphase was reached at 36 days post injection. The 24 day time point was unusable because sections contained only unlabelled sperm cysts.

Two subsidiary experiments were done in addition to the timing experiments discussed above. The first involved the injection of one animal with tritium-labelled EMS at a dose of 100 mg/kg. The excretion of label into the water was measured at hourly intervals after injection. When the level

Table VI

PROGRESSION OF LABEL THROUGH

SPERMATOGENIC STAGES,

FALL-WINTER 1981/82

Animals	Days after injection	Most advanced stage labelled
119-2A	2	leptotene
119-5A	6	zygotene
119-6A	9	zygotene
124-4A	15	pachytene
124-5A	18	pachytene
sc 1A	21	pachytene
119-2B	27	pachytene
119-5B	30	pachytene
119-6B	33	diplotene
124-4B	36	metaphase
124-5B	39	2° spermatocyte
sc 1B	50	early spermatid
sc 2B	60	spermatid
105-11A	70	late spermatid
105-11B	80	sperm

of excretion began to slow down, after five hours, the animal was anesthesized and one testis was removed. The gonad was fixed and sectioned using glutaraldehyde and glycol methacrylate and was then processed for autoradiography. After three weeks exposure, the slides were developed, stained and examined under the light microscope. Lightly labelled late spermatid cells were observed and no other stages were labelled. The labelling pattern is seen in Figure 5I and is similiar to that seen in Figure 5G.

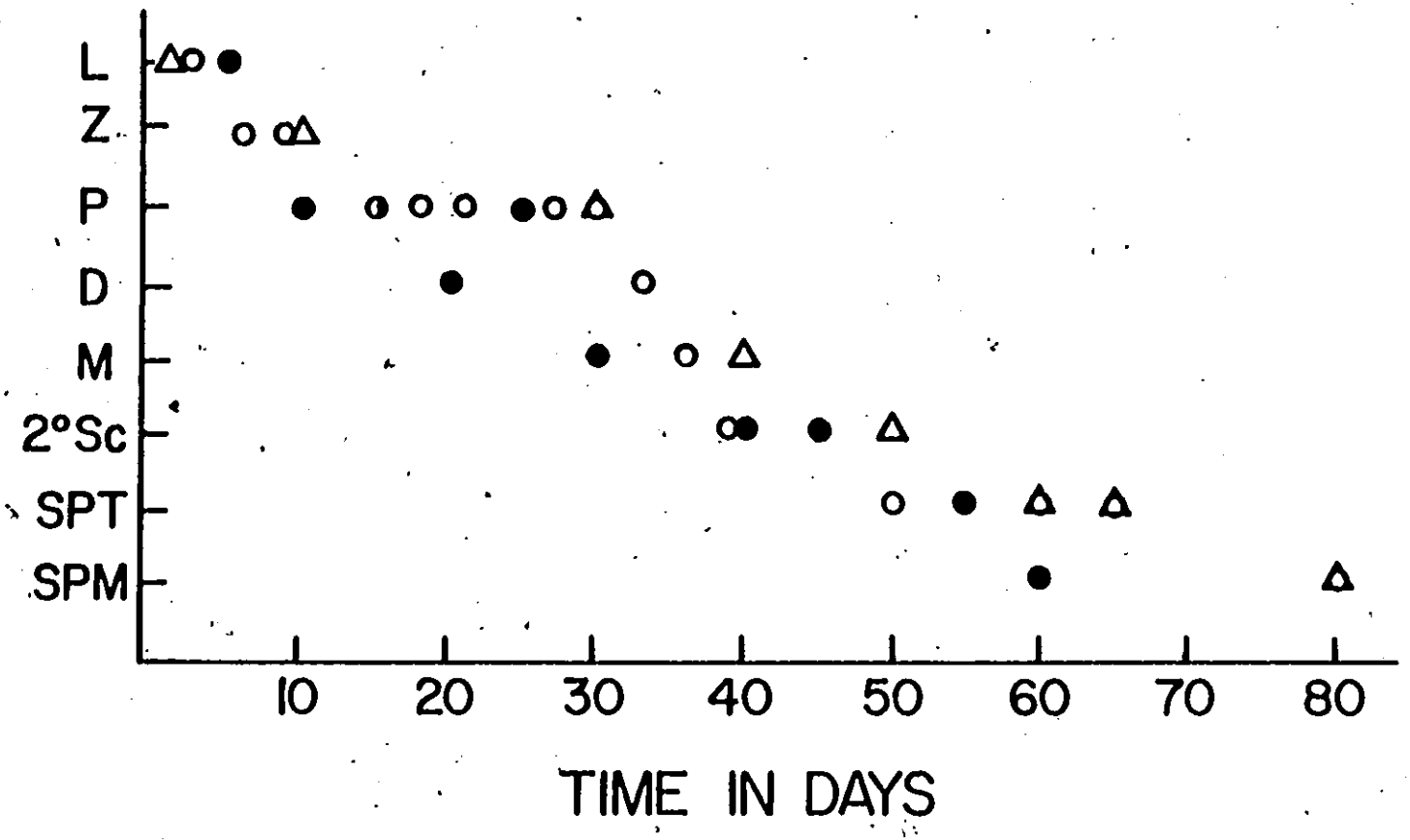
The second experiment involved the collection of spermatophores from one of five animals injected with tritiated thymidine at the start of the third experiment in the fall of 1981. At weekly intervals, starting 50 days after injection, one animal was spawned and a maximum of five spermatophores were collected and frozen. When enough samples were collected, these were processed according to the procedure outlined in the materials and methods section. Examination of the squash preparations after an exposure time of two to three weeks showed labelled sperm in the spermatophore 121 days after injection. Spermatophores collected after this time are still being exposed and have not yet been examined.

Figure 6 This figure shows the stage that was labelled at each time point in the three experimental series.

Legend: △ =Fall-Winter 1980-1981

● =Spring-Summer 1981

ȯ =Fall-Winter 1981-1982


L
Z
P
D
M
2°Sc
SPT
SPM
10
20
30
40
50
60
70
80
TIME IN DAYS

Testis Weights

Table VII gives the mean weights recorded for each experimental series, broken down according to the age of the animal, and first or second testis. There was a rather large age differential in these experiments and older animals had significantly larger gonads than the younger ones. However, there was no significant difference seen in the weights of first and second testis when animals of the same group were compared.

Cyst Counts

As mentioned in the introduction, amphibians in the wild show a seasonal progression of spermatogenic stages termed zonation. Cysts of each stage of spermatogenesis will predominate in the testis at different times of the year; for example, sperm cysts are most prevalent in the late summer and autumn, whereas 2° spermatogonial cysts and 1° spermatocytes will peak in the spring. I had noticed a change in the proportion of each cell type when examining sections taken from different animals and wanted to see if there was any trend in the variability. For this reason, five randomly chosen longitudinal sections were counted from each animal and the number of cysts at each stage of spermatogenesis was scored as a percentage of the total number of cysts counted. The results of these cyst counts are graphed on the following pages.

Table VII

MEAN AND STANDARD DEVIATION OF TESTIS WEIGHTS, IN GRAMS

Series	Age	1st	2nd
F-W 80-81	4.5	0.7735 ±0.1543	0.9938 ±0.2720
S-S 81	2	0.3914 ±0.2186	0.2820 ±0.1034
F-W 81-82	1.5	0.2912 ±0.1195	0.2269 ±0.0760

There was a large predominance of sperm cysts throughout the year, even though there were large fluctuations in the percentages found in individual animals. Sperm cysts accounted for roughly 30-100 % of the cysts counted, with the exception of two animals who had abnormally low sperm counts. The cross-hatched regions on the graph represents animals whose sections could not be counted because their sperm cysts had ruptured and mature spermatozoa were being evacuated into the vas deferens. Because the cyst boundaries had ruptured, their definition in the section was lost, and no accurate count of their number was possible. In the first series, the fall and winter of 1980-81, there were three such animals. In the second series, the spring and summer of 1981, there were seven animals. There were eight animals in the final series, the fall and winter of 1981-82. This period of evacuation of the gonad seemed to occur at two distinct times of the year, the months of mid-June to mid-August and the months of mid-October to mid-December. However, there were no animals counted in September and early October and it may be possible that this trend continues during that time period, thus forming a single space of time during which sperm are evacuated from the testes.

The predominance of sperm cysts overshadowed the number of cysts in other classes. These were found to contain a correspondingly low percentage of cysts at any particular time of the year. One exception noted was in the number of

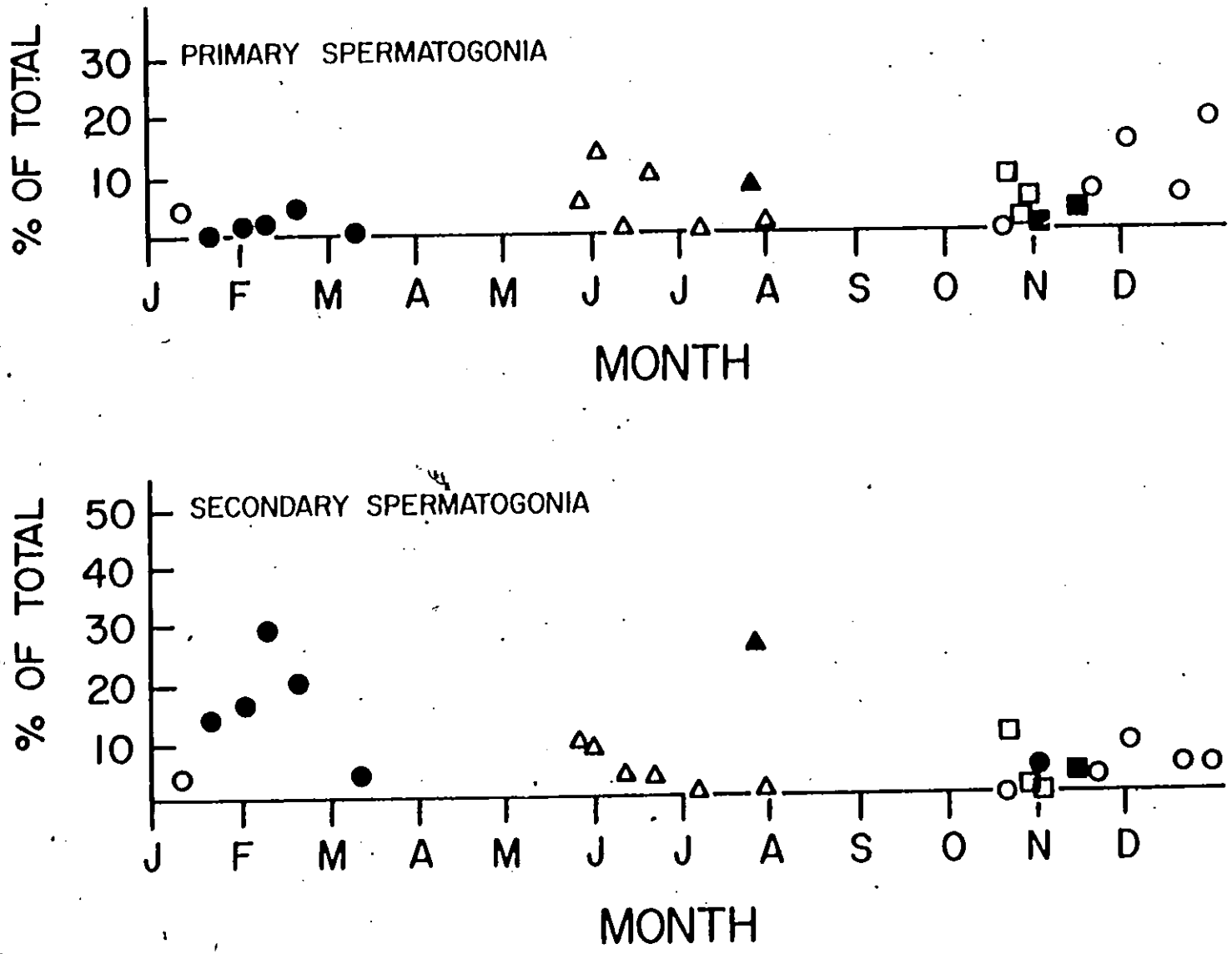
primary spermatocyte cysts scored in the fall, when the percentage rose above 50 %.

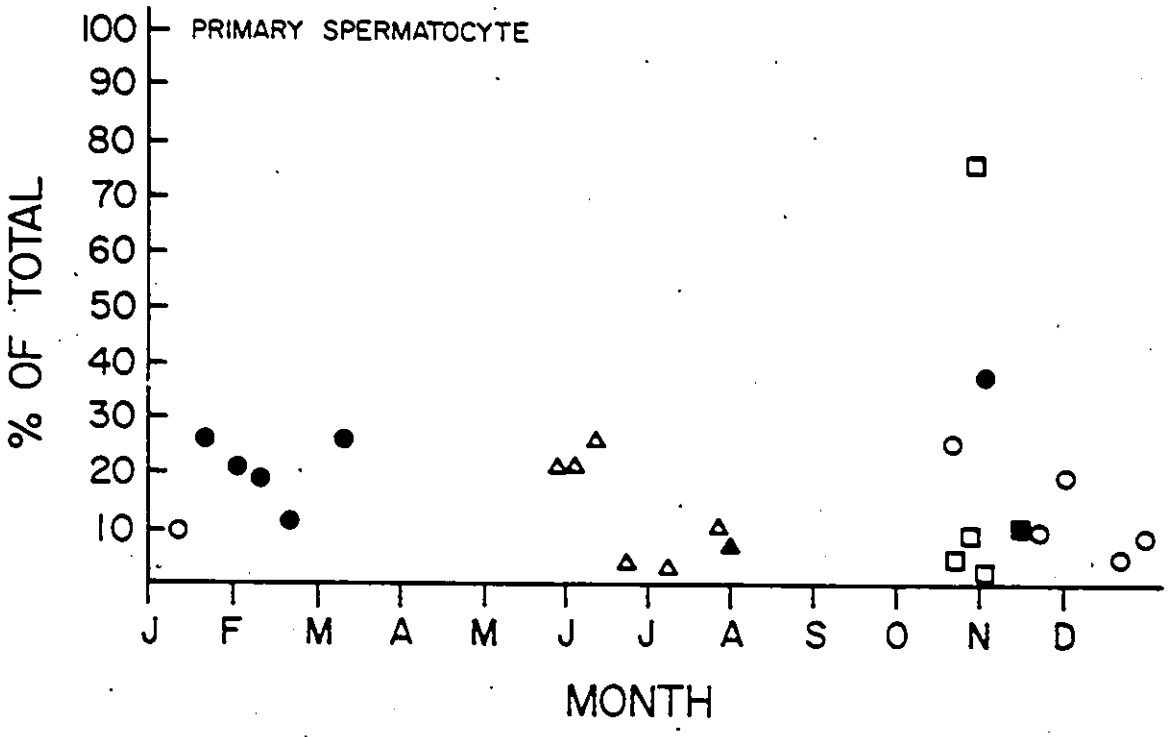
Another interesting development was observed in the percentage of secondary spermatogonial cysts. A peak was formed in the late winter months from mid-January to March. Another high count showed up in August, but this was confined to only one animal.

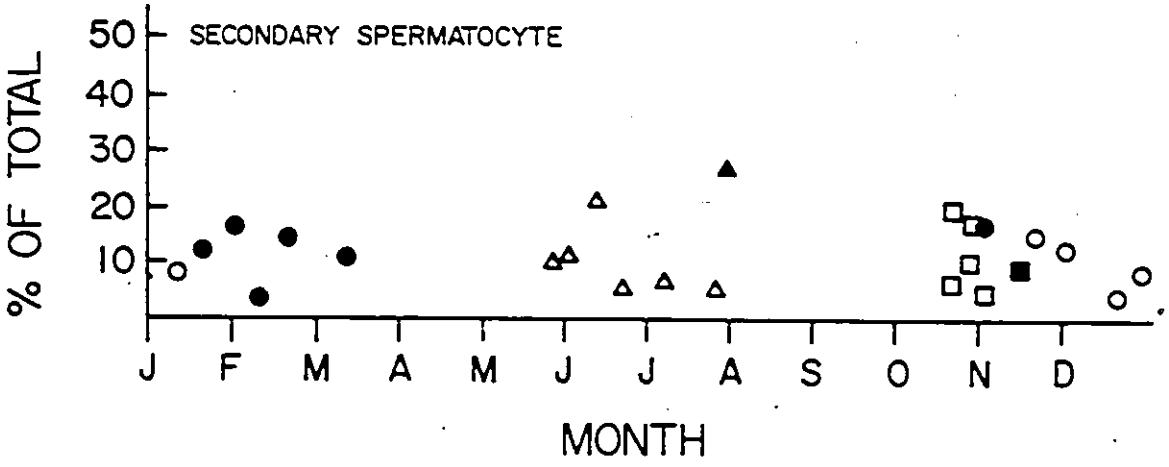
Figure 7: Cyst counts were done on five randomly chosen longitudinal sections from each animal. Six classes of spermatogenic cells were counted; primary and secondary spermatogonial cysts, primary and secondary spermatocyte cysts, spermatid and sperm cysts. The percentage in each stage present was calculated from the total number of cysts scored in the five sections.

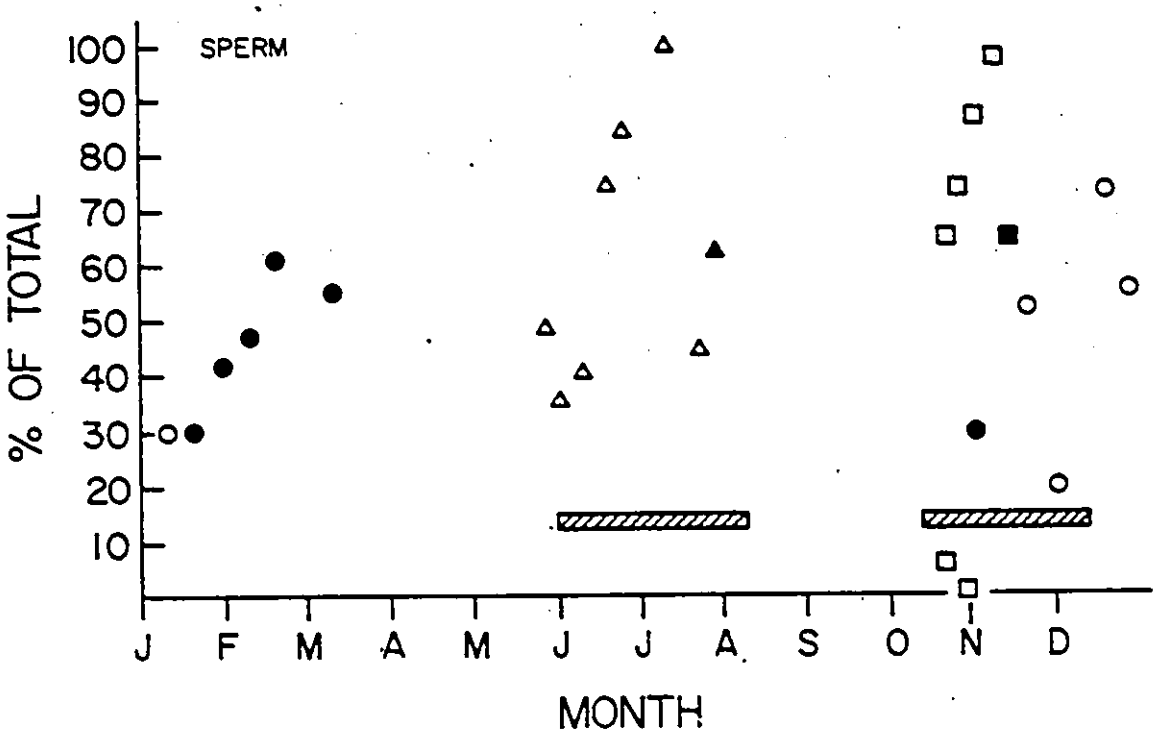
Legend: ○--F-W 80-81 △--S-S 81 □--F-W 81-82

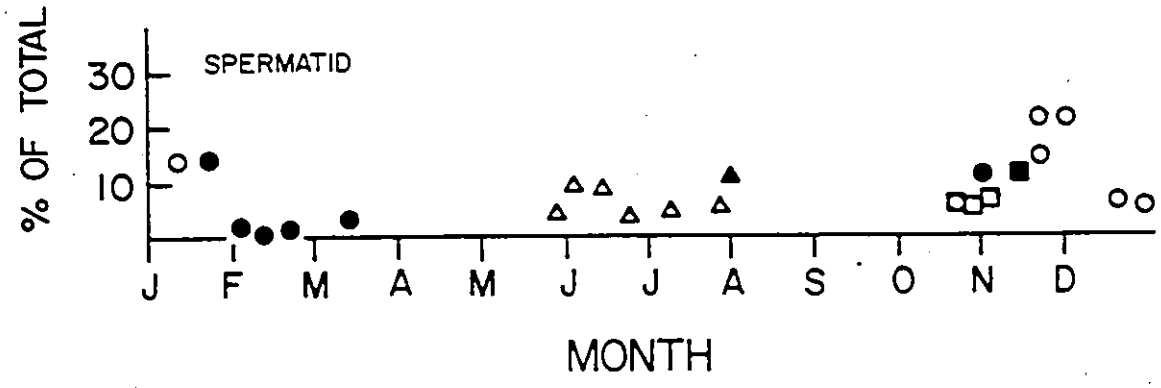
The closed circles, triangles and squares are cyst counts taken from the second testis of the axolotl, whereas the open ones are from first testes. The cross-hatched regions represent samples that could not be counted because of ruptured sperm cysts.


PRIMARY SPERMATOGONIA
% OF TOTAL
30
20
10
J F M A M J J A S O N D
MONTH
SECONDARY SPERMATOGONIA
% OF TOTAL
50
40
30
20
10
J F M A M J J A S O N D
MONTH


PRIMARY SPERMATOCYTE
% OF TOTAL
100
90
80
70
60
50
40
30
20
10
J F M A M J J A S O N D
MONTH


SECONDARY SPERMATOCYTE
% OF TOTAL
50
40
30
20
10
J F M A M J J A S O N D
MONTH


SPERM
% OF TOTAL
100
90
80
70
60
50
40
30
20
10
J F M A M J J A S O N D
MONTH


SPERMATID
% OF TOTAL
30
20
10
J F M A M J J A S O N D
MONTH

Discussion

The experiments described in this thesis were carried out with three objectives in mind:

1) To establish the time course of spermatogenesis in the axolotl;

2) To correlate this with what is known about spermatogenesis in other amphibians;

3) To see whether the answers to these questions can provide additional help in interpreting data from EMS mutagenesis experiments.

Over the course of fifteen months, three experiments on the timing of spermatogenesis in the axolotl were done using radioactive thymidine as a marker and autoradiography as the main analytical tool. The data from these experiments provides an answer to the first question: Spermatogenesis in the axolotl takes between 60-80 days. This is the absolute time needed for an immature spermatogonial, or stem cell to complete its differentiation and become a mature spermatozoon in the testis. Of these three experiments, two were run in the fall and winter and show a nearly identical course, ending with labelled sperm in the testis at eighty days. The other experiment was run in the intervening spring and summer and shows, overall, a faster course of spermatogenesis ending with labelled sperm in the testis at sixty days.

The question which immediately comes to mind when considering these three times, is whether or not they are significantly different from each other. Do they represent two discrete times for spermatogenesis in the axolotl, and can this be due to a seasonal effect despite the fact that the axolotl is an animal born and bred in captivity?

This apparent discrepancy in the data may have its roots in the very complex workings of the biological clock. Indeed, before taking a closer look at the axolotl in the laboratory, it is perhaps wise to examine the environmental conditions its ancestors might have faced in Lake Xochimilco near Mexico City.

Mexico City itself is at a relatively high altitude and the year-round temperature flucuations are not that great. The mean monthly high variation is between 19-25°C and the mean monthly low varies between 6-8°C during the year. The winter is dry, rains start in the spring and are the heaviest in the late summer months.

Amphibians in the wild have developed a yearly, or circannual rhythm as far as their breeding habits are concerned. Their breeding season is in the spring and is a reflection of what are called ultimate and proximate factors in the environment. The most common ultimate factor in breeding behavior is the availability of enough food for the offspring. Thus most animals will have their young when their natural habitat can provide the most food, ensuring

optimal survival of the new generation. Proximal factors to which animals of all types will respond, include changes in temperature, rainfall, length of daylight and tidal or lunar phases. Amphibians appear not to have a well-defined photoperiod control mechanism as a determinant of their reproductive behavior, yet they do seem to respond to changes in temperature and rainfall. In fact experiments have been done with amphibians kept in complete darkness and these have shown a) that normal spermatogenic cycles do occur, and, b) that elevations in ambient temperature will stimulate and indeed accelerate gametogenesis (Lofts 1970).

Thus it is not unreasonable to suppose that the axolotl, were it in its native habitat, would cue to an increase in rainfall in the spring, and/or an increase in water temperature when starting its spawning season in the spring.

But what happens to this natural rhythm when the axolotl is removed from its natural habitat and exposed to constant environmental conditions in the laboratory? Animals facing just this sort of displacement have been known to establish what is referred to as a free-running rhythm. Moreover, they will keep remarkably close to their natural biorhythms over a long period of time. Brady (1979) speaks of both exogenous and endogenous factors as being responsible for the free-running phenomenon, be it with respect to circadian, circa-lunar or circannual rhythms.

Exogenous control is understood to include the influence of naturally occuring phenomena that are not usually controlled in a laboratory setting. These would include variations in the magnetic field, gamma radiation and changes in barometric pressure. The establishment of a free-running rhythm would thus be a response to these subtle environmental cues. Endogenous control, on the other hand, refers to physiological changes in the displaced animal, usually of a hormonal nature, that aid it in keeping to its normal periodicity. The maintenance of the organism's normal circadian and other rhythms in the face of constant conditions, i.e. the free-running, is most often cited as the major reason for postulating endogenous control of biological functions. In addition, temporal homeostasis, or the keeping to relatively constant cycles despite flucuations in temperature is more evidence for endogenous control. Most physiological reactions have a Q 10 of 2; for every 10° C change the rate of reaction is doubled. However, for circadian, circa-lunar or circannual rhythms, the Q 10 is much closer to 1. Over a large range of physiologically acceptable temperatures, poikilotherms will try to stick to their natural cycles (Brady 1979). A third piece of evidence for endogenous control is one that is quite familiar to humans and occurs in many other species as well, and that is the problem of jet lag. When animals, human or otherwise are displaced from one time zone to another far away, they will, for a short period stick to

their "old" habits.

How then can the variations in the length of the spermatogenic cycle of the axolotl be best explained with these points in mind? The axolotl is kept under constant conditions in our laboratory; the temperature is 18° C and day and night are each 12 hours long. If the axolotl reacts to constant conditions the same way that other species seem to do, then the 60 to 80 days for spermatogenesis must represent a close approximation of the natural cycle of the animal; it has become free-running. As such, each animal is independent of the other, the synchrony which the animals would have in the wild is lost. Although the absolute time for the spermatogenic cycle should be the same in each animal, each is at a different point in the yearly reproductive cycle. Thus cyst counts from many animals over a period of time would not be expected to show any trend similar to that shown in Figure 3 in the introduction. This is, in fact, the observed result. The Figures 5-7 in the results section show a scattering of points over the course of a year, but no discernable trend.

The phenomenon of zonation which is characteristic of amphibians in the wild, would also be lost if the animal became free-running. Indeed, instead of finding an orderly progression of spermatogenic stages in the testis of the axolotl, no such pattern is observed in the longitudinal sections counted. This observation was also made by Carrick

in 1934 and Lazard in 1979, although they offer no explanation for it. The cysts in the testis of the axolotl seem to be independent of each other and adjacent cysts can be at opposite ends of the spermatogenic range (Figure 1).

The data obtained from cyst counts done on sections from the axolotl further support the argument that the animal is free-running and that it does not show an orderly progression of spermatogenic stages through the testis. In an amphibian in the wild, there is a non-random entry of spermatogonial cells into meiosis. As seen from Figure 3, the testis in the early spring contains the largest proportion of spermatogonial and primary spermatocyte cells. This is the beginning of the spermatogenic wave and is followed by a synchronized progression of all cysts through meiosis so that in the summer the testis is full of secondary spermatocytes. Spermatid formation or spermiogenesis occurs in the late summer, and the evacuation of mature sperm from the testis begins.

If the axolotl were in any way still attached to this schedule, then one would expect a similar pattern to show up in sections from animals examined over the course of the year. However, as Figure 7 clearly shows, this is not the case. Apart from a small peak of secondary spermatogonial cells in February and March (Figure 7B) there is no analogy to be made with the amphibian in the wild.

Figure 7E shows the fluctuations in sperm cysts over the course of a year. The cross-hatched regions on the figure indicate sections taken from animals over that time span that could not be scored because the sperm cysts had ruptured. This period of evacuation of mature sperm occurs between the months of August and February in amphibians in the wild and it would seem that the axolotl shows the same phenomenon at the same time. However, a much more likely explanation for this observation, in view of the fact that the axolotl is free-running, is that the sampled population is too small. If enough animals were tested, there should be some in each month of the year that show evacuation of sperm from the testis, and the cross-hatched regions in Figure 7E would eventually form a continuous bar covering all 12 months of the year.

The peak observed in the secondary spermatogonial cells may, in fact, be due to another phenomenon known to occur in amphibians in the wild. The peak itself consists almost entirely of counts taken from the second testis of the axolotl. There is a well-known effect of testicular compensation after partial castration in amphibians (van Oordt 1960), and the question arises whether or not this may be responsible for the observed rise in secondary spermatogonial cysts. Specker and Moore (1980) find that spermatogenesis is in part regulated by FSH secretions from the pituitary and that androgen secretions from interstitial cells in the gonad exert an inhibitory effect on FSH levels.

If one testis is removed, the total amount of androgens in the animal will decrease and the effect of FSH on the remaining gonad will be increased. This will result in increased spermatogonial and meiotic activity, thus accounting for the observed upward trend in Figure 7B. However, this increased activity should not have any effect on the time needed for the completion of meiosis, only on the number of cysts entering meiosis.

Compensation following partial castration also leads to hypertrophy of the remaining gonad; it will increase in weight. This effect is not found in the axolotl (Table VII); most probably the time between removal of the first and second testes was too short to allow full compensation to occur. For this reason, in the third experimental series, both first and second testes were used, whereas in the first two series, care was taken to ensure that each time point, with two exceptions, was based on results from the first testis of the animal.

The question remains whether or not it is justifiable, in light of the evidence just presented, to propose that the observed 60 and 80 day cycles are separable. Do they, in fact, represent a faster summer cycle versus a slower winter one? The answer, however, must be no; the points from all three experimental series must be taken together as

Table VIII

COMPARISON OF THE TIME COURSE OF MITOSIS IN THE SPERMATOCYTES OF THREE AMPHIBIAN SPECIES

	L	Z	P	D	M	2° SC	SPT	SPM
P. cinereus (20° C)	1	8	13	24	26	27	-	-
T. vulgaris (16° C)	1	6	14	19	20	23	-	-
X. laevis (18° C)	4	5	10	22	23	23	26	27
A. mexicanum (18° C)	1	5	10	32	33	39	50	62.5

Note: The duration of spermatogenesis is in days. The work on P. cinereus was done by Morgan (1972), the work on T. vulgaris by Callan and Taylor (1968), and the work on X. laevis by Kalt (1976).

representative of the spermatogenic cycle in the axolotl. The discrepancy in the appearance of the same labelled stage at different times is a reflection of;

1) the individual variation among the animals tested, and

2) the fact that daily time points were not taken.

Ideally, in an experiment of this kind, animals should be sacrificed at daily intervals after labelling in order to get an accurate transition from stage to stage in spermatogenesis. But due to the small size of the colony, this was not possible, and thus the duration of some of the stages shown in Figure 6 may vary by several days.

Up to this point the discussion has centered mainly on the final time point; the time at which labelled sperm are found in the testis. But this is only a small part of the experiment and, indeed, the two final experimental series were done in order to understand more about the timing of the individual stages of meiosis. Figure 6 is a composite of all three experimental series and shows the progression of label through the various meiotic stages and beyond to mature sperm. The figure is not intended to be an absolute indicator of the time spent in each stage, nonetheless, it gives a good indication of the average time spent in each stage.

In determining the duration of a stage, previous workers have taken a fairly simple approach; that is, the duration of a stage is the time from the first appearance of label in

that stage to the first appearance of label in the next stage. However, even assuming that controls, seasonal or otherwise, in the number of cysts entering spermatogenesis have no effect on the duration of spermatogenesis, one can nevertheless expect some variation in duration between animals. This will inevitably cause some overlaps in the data such as seen in Figure 6. For example, in one animal the label has progressed as far as diplotene in 20 days, yet in another animals label has progressed only as far as pachytene after 25 or even 30 days. The same situation is repeated in the transition fom spermatid to sperm; one animal shows labelled sperm at 60 days, whereas another shows labelled spermatids as late as 65 days. There seems to be no greater validity in defining the beginning of a stage by the first animal to show label in that stage than there would be in defining it by the last animal to show label in the preceeding stage. However, the median point between these two times is a useful parameter that can be used to estimate the "average" duration of a stage. Though animals from the summer group were the first to reach pachytene, diplotene, metaphase and sperm, the differences are neither large enough nor consistent enough to support a claim that spermatogenesis is faster in these animals. Neither is there any evidence that the age of the animals has any significant effect.

Taking all this into account, and combining the results from all three experimental series, the "average" duration

of the stages of spermatogenesis would be as follows; leptotene lasts from 0-5 days, zygotene from 5-10, pachytene from 10-25, diplotene from 25-31.5, metaphase from 31.5-39.5, secondary spermatocyte from 39.5-50, spermatid from 50-62.5, and sperm from 62.5 days onward. The duration of diplotene, however, may be greatly over-estimated because label in this stage alone was only observed twice; it was most often seen together with metaphase, as in Figure 5E. If the animal showing diplotene labelled at 20 days is regarded as an anomaly, the transition from pachytene to metaphase would be very quick, and the duration of diplotene would be so short to be almost negligible. This would mean that pachytene is in fact longer than indicated; lasting from 10-32 days, with diplotene lasting only for one day, from 32-33 days. If this is compared with the duration of spermatogenesis in other amphibians (Table VIII), they, too, show a very long pachytene stage and a very rapid cycling through diplotene to metaphase.

Pachytene is the longest stage for two reasons; 1) it is the time when recombination takes place between the four chromatids, and 2) the diffuse stage at the end of pachytene is considered by many to be a developmental hold during meiosis during which transcription occurs. In some papers the diffuse stage, which is analogous to the lampbrush stage in oocytes, is considered a part of pachytene (Bennett 1977, Owen 1973). In other references, however, chromosome decondensation which is characteristic of this stage is

considered part of diplotene (Browder 1980). In these experiments, I have considered the clear separation of homologous chromosomes and the formation of chiasmata to be the criteria for classifying a cell as being in diplotene. The more evenly staining cell, which looks like a cell in leptotene, was taken as a pachytene cell in the diffuse state.

Iatrou and Dixon (1978) describe the life history of a protamine mRNA molecule in the trout. They pinpoint the synthesis of these mRNAs only as taking place during the primary spermatocyte stage. Morgan (1979), in his article on spermatogenesis in P. cinereus points to the fact the ^{3}H-uridine incorporation is highest during the diffuse stage, part of first meiotic prophase. Thus it seems reasonable to assume that pachytene, ie. the diffuse stage, is the time of synthesis of protamine mRNA. Once synthesized, these mRNA's are stored in the cytoplasm of trout spermatocytes as mRNP particles. They become activated during the mid to late spermatid stage and begin the synthesis of the protamine molecules that replace the histones on the chromosomes and allow the spermatid nucleus to be condensed even further (Iatrou and Dixon 1978).

The events that take place during pachytene are thus of a dual nature, and represent a time of great metabolic activity in the spermatocyte cell. On the one hand, the recombination nodule and its enzymatic components must be

synthesized to aid in recombination. On the other hand, the transcription and storage of protamine mRNA molecules takes place. These two events do not appear to occur simultaneously, but rather follow each other and can be observed in the cell. The pachytene cell (Figure 5D) hasregions of darkly staining chromatin in it, presumably reflecting the cross-over points of the chromosomes, whereas the cell in the diffuse stage is much more evenly staining and individual homologues are not visible.

When the length of meiosis in the axolotl is compared to that of three other amphibians, both similarities and differences appear (Table VIII). The leptotene stage is in most cases labelled almost immediately and this is taken to mean that there is late DNA synthesis which takes place at this time. In the case of X. laevis, leptotene is only labelled after four days; presumably the late replicating DNA period does not occur and premeiotic S phase is completed before the cell enters first meiotic prophase. Zygotene is the time of formation of the synaptonemal complex and this process appears to take from four to eight days in the four amphibians being compared here. This stage is followed by pachytene, which, as already mentioned, is the longest of the stages of first meiotic prophase. Among the four amphibians, the axolotl has the longest pachytene period, 22 days. In X. laevis pachytene lasts for 12 days, in P. cinereus for 11 and in T. vulgaris it lasts only for 5 days. The lengthy hold at pachytene no doubt contributes to

the greater overall length of spermatogenesis in the axolotl, but the evolutionary reasons for this diversity are not clear.

One popular approach to this problem is the attempt to correlate the length of meiosis with the amount of DNA in the germ cell. Bennett (1977), in his article on the timing and duration of meiosis, proposes that there is a positive correlation between the length of meiosis and the C-value of an organism. The C-value is understood to be the haploid DNA content of a germ cell. Thus, for increasing C-values, the time needed for meiosis should also increase when organisms within the same Class are compared. Bennett found that such a relationship exists in diploid angiosperms. (In order for such comparisons to be correct, meiosis must be measured at the same temperature. The Q 10 for meiosis is 2, which means that for every 10 degree increase in temperature, the time needed for meiosis will be halved. In this way, meiotic duration measured at different temperatures can be converted to a standard temperature and thereby compared.)

Morgan (1979) in his study on spermatogenesis in P. cinereus compares the C-values of several species of amphibians and discusses possible reasons for the lack of a linear relationships. Figure 8 shows C-value versus duration of meiosis. There is no linear relationship to be found here, and meiotic duration appears to be independent

of the C-value of the organism. Morgan argues that perhaps the genes controlling meiosis are not well conserved in this particular group of animals, and that this is reflected not only in the lack of positive correlation between C-value and meiosis, but also in the differing lengths of the diffuse stage. The axolotl has the longest diffuse stage, followed by X. laevis and P. cinereus.

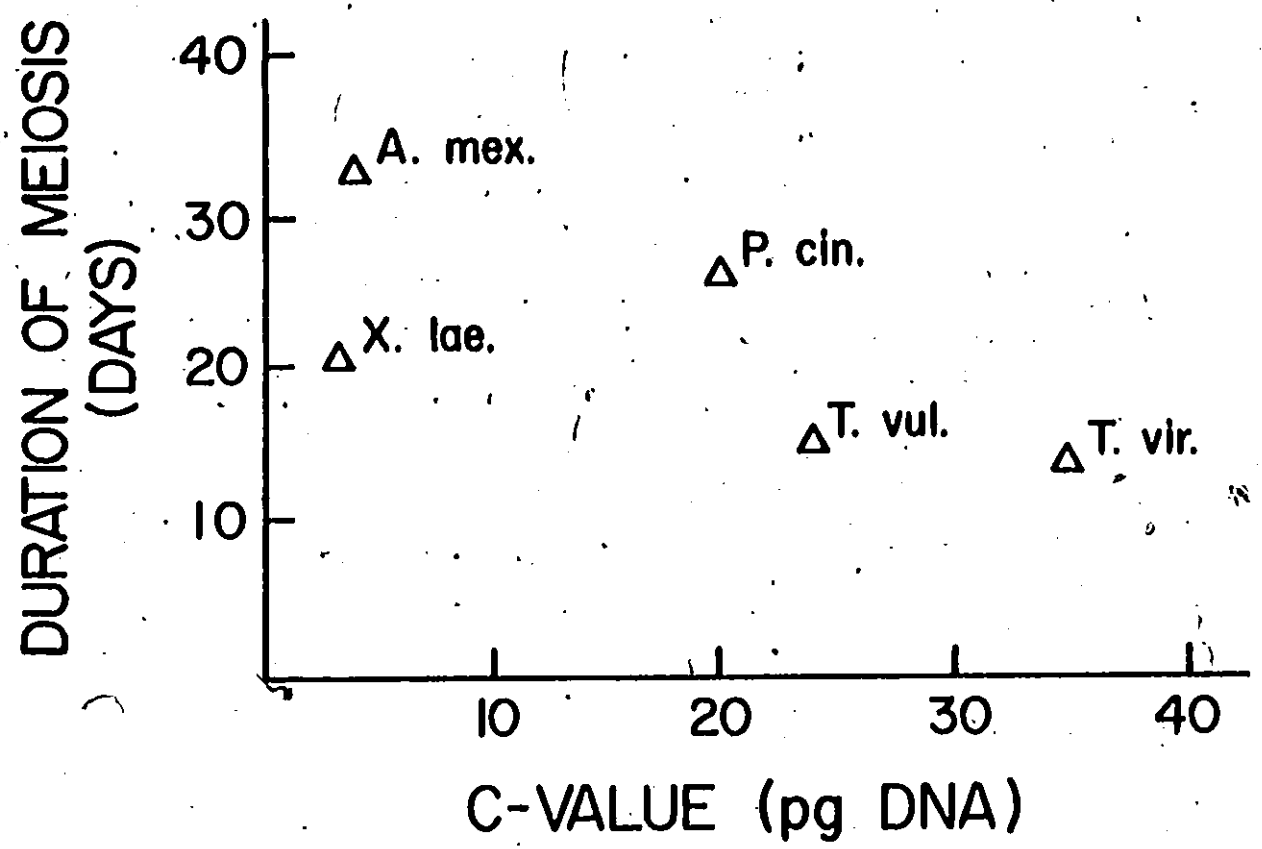
Bennett (1977) also cites environmental factors are being important determinants of meiotic duration and one might argue that the different life styles of these amphibians is the major reason for the great range in the timing of spermatogenesis found in this group. In their different habitats, each species has evolved a pattern of meiosis most compatible with its surroundings. Without meiotic data from many more amphibians, the reasons behind the observed disparity between C-values and meiotic duration, if indeed such a disparity exists when a much larger population is compared, must remain speculative.

As mentioned in the introduction, only one study has been done on spermatogenesis in a captive amphibian. This is the study by Kalt (1976) on X. laevis. How does his analysis of spermatogenesis in Xenopus compare with the results obtained from the axolotl? Both animals are found to have a continuous spermatogenic cycle and both have presumably established a free-running rhythm under constant laboratory

Figure 8 A comparison of C-Value (pg DNA) versus duration of meiosis at 20° C for five amphibians. Key: Plethodon cinereus = P. cin.

Triturus vulgaris = T. vul. Triturus viridescens = T. vir.

Ambystoma mexicanum = A. mex. Xenopus laevis = X. lae.


DURATION OF MEIOSIS (DAYS)
40
30
20
10
A. mex.
P. cin.
X. lae.
T. vul.
T. vir.
10
20
30
40
C-VALUE (pg DNA)

conditions. This is further strengthened by the fact that Kalt did not find any trend in cyst counts made on sections taken from Xenopus, and this is the same result as that found for the axolotl. In this regard, both species have lost their natural synchrony, and the zonation of the gonad, which is so characteristic of amphibians in the wild, is found in neither species. There is a continuous entry of varying numbers of spermatogonial cysts into meiosis, but without the very specific timing of this event which is found in nature. Thus both animals have retained a spermatogenic cycle whose duration is close to the one they would be expected to show in the wild.

All this information gives a good understanding of the functioning of spermatogenesis in the axolotl. To summarize, the cycle itself takes 60 to 80 days to produce mature sperm in the testes. The axolotl has lost its natural synchrony and in its place it has established a free-running rhythm with a continuous entry of varying numbers of spermatogonial cysts into meiosis. Each animal is independent of its neighbors and can be at any point in the "natural" yearly reproductive cycle at a given time.

The axolotl does not show zonation of the gonad; this is reflected in the lack of any visible trend in cyst counts made from longitudinal sections.

The important questions which remain, however, are; 1) how long does it take for sperm to leave the testis and be laid in a spermatophore (ie., how long is the storage period), 2)

can this information be used to interpret data from EMS mutagenesis experiments.

In conjunction with the main timing experiments, two other experiments were carried out. One of these involved labelling several animals with tritiated thymidine, and rather than removing the testes, spermatophores were collected at frequent intervals thereafter. The samples were processed by autoradiography and examined to see when labelled sperm showed up. The second experiment involved injecting one animal with tritiated EMS at a dose of 100 mg/kg, removing one testis, processing it the same way, doing autoradiography and looking to see the cell type or types in the testis that were labelled by the EMS.

The former experiment shows that labelled sperm is in the spermatophore 121 days after labelling. The latter experiment shows that the late spermatid cell is labelled with ^{3}H-EMS to the exclusion of all other cell types in the testis. This result complements data already obtained in the mouse. There the late spermatid-early spermatozoa stage was the most mutagen sensitive. This stage is postulated as the time of histone-protamine exchange. The histones are removed from the nucleosome, protamines are synthesized from activated message in the cytoplasm and complex with the DNA. The DNA helix is exposed and vulnerable to alkylation by EMS. As there is otherwise very little metabolic activity in the nearly mature germ cell,

the alkylation damage cannot be repaired. If the animal is mated after EMS treatment, a dominant lethal (DL) effect will show up in the F1 generation. The DL trough is, therefore, the time required for late spermatids to become mature sperm which are used in a mating.

Over a period of five years, 20 axolotls have been mutagenized with EMS doses ranging from 10 to 500 mg/kg. When all animals are compared, the results of treatment with the mutagen fall into three groups. Group one, at 10 and 25 mg/kg EMS, show no DL trough; group two, at 100 and 250 mg/kg EMS show a DL trough at 60 to 110 days; group three, at 500 mg/kg EMS, shows a lethal effect with only two of the four animals surviving the treatment. These, however, also show a DL trough; the survival of their progeny was lowest at 60 days after exposure to EMS.

In the laboratory the axolotl is presumably on a free-running cycle and each animal is essentially independent of its neighbor. Since each animal is holding to his own biological clock, he can be at any point in his reproductive cycle at month X. His neighbor may or may not be "in phase" with him. Thus an animal, if mutagenized when his sperm production is at a maximum, a time of high LH influence, would have to lay more spermatophores before the treated ones appeared; damaged sperm would be stored for a time and the trough would be expected at 110 days. On the other hand, an animal mutagenized when sperm production is at a

minimum, a time of high FSH influence, would show essentially no storage effect; sperm released from the testis would be laid almost immediately and the trough would be expected at 60 days. Given individual variation in the duration of spermatogenesis spoken of earlier, troughs between 60 and 110 days could also be expected to occur.

In the same way, the finding of radioactive sperm at 121 days after labelling with tritiated thymidine lends credence to this hypothesis. The expected time for late-spermatid cell differentiation to occur is 60 days. Thus if a late spermatid cell is damaged by the mutagen alkylating the "exposed" DNA, the earliest time that a DL trough could appear would also be about 60 days thereafter. This assumes the animal is not producing very much sperm at the time of treatment so that treated cells appear "rapidly" in the spermatophores. If, on the other hand, an animal were producing large numbers of sperm at the time of treatment, there would be a delay in the laying of damaged sperm, and the DL trough would occur at a later time. Thus the 121 days is the time needed for a cell labelled with tritiated thymidine at premeiotic S to differentiate into a mature sperm and be laid in a spermatophore. I think that it also represents the faster time for spermatogenesis, and that this time could be longer for other animals. When the remaining sperm squashes are examined under the light microscope, I would expect labelled sperm to appear after 121 days.

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