COYOTE REPRODUCTION

I. THE DURATION OF THE SPERMATOGENIC CYCLE AND EPIDIDYMAL SPERM TRANSPORT

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Summary. Sexually mature, male coyotes were injected with [^3H]thymidine to estimate (1) the duration of one cycle of the seminiferous epithelium, and (2) epididymal sperm transport time.

The mean duration of spermatogenic stages 1 to 8 was estimated to be 3.4, 2.1, 0.5, 1.4, 1.8, 1.7, 1.5 and 1.3 days, respectively. The total duration of one cycle of the seminiferous epithelium was 13.6 days. Radioactive spermatozoa were observed in the caput epididymidis 40 days after [^3H]thymidine injection, but data suggested that they may arrive as early as 37.4 days. Radioactive spermatozoa first appeared in the ejaculate of one male 52 days after [^3H]thymidine injection, indicating that the epididymal sperm transport time is about 14 days.

INTRODUCTION

The coyote, *Canis latrans*, is considered to be a vertebrate pest species in many areas of the United States, and efforts to control it have been pursued for decades. The reports by Knipling (1955) and Davis (1961) on control of insects and birds through sterilization has focused attention on the possibility of using reproductive inhibitors to reduce coyote birth rates (Balser, 1964). However, the paucity of information on coyote reproduction has hampered efforts in this area. Hamlett (1938) and Gier (1957) presented information on some of the gross and microscopic changes that occur in the reproductive tract of this seasonal breeder, but there is no detailed description of gametogenesis and other physiological phenomena related to the production of offspring. The selection and evaluation of a 'birth control' chemical is difficult, if not impossible, without such information. The purpose of this study was to increase our understanding of reproduction in male coyotes; specifically, we wished to determine (1) the duration of spermatogenesis, and (2) the time required for epididymal sperm transport.

The cells of the seminiferous epithelium in mammals follow a well-defined series of cellular associations or stages collectively referred to as 'the cycle of the seminiferous epithelium' (Leblond & Clermont, 1952). The number of stages in one cycle is limited only by the number of easily definable cellular

associations. Depending on the species and the point selected for the initiation of spermatogenesis, at least four cycles of the seminiferous epithelium are required for the completion of spermatogenesis (Clermont, Leblond & Messier, 1959; Amann, 1962; Swierstra, 1968).

There are several methods available for estimating the duration of one cycle of the seminiferous epithelium, but the best involves the injection of tritiated thymidine ($[^3\text{H}]$ thymidine) into the test animals. $[^3\text{H}]$ thymidine, a precursor of DNA, is incorporated into cell nuclei during DNA synthesis before mitosis or meiosis. Testicular samples are then collected at appropriate intervals after the administration of the tracer and the development of labelled cells is followed by autoradiography (Clermont *et al.*, 1959; Swierstra & Foote, 1965; Clermont & Trott, 1969). Extension of this procedure to epididymal spermatozoa provides an estimate of the time required for epididymal sperm transport (Orgebin-Crist, 1965; Swierstra, 1968).

MATERIALS AND METHODS

Treatment of animals and experimental design

Male coyotes, caught in Colorado at 3 to 5 weeks of age and routinely raised, were divided into three groups; Group I consisted of eight adults, Group II of six juveniles, and Group III of eight adults. Except for one 4-year-old coyote, adults were about 21 months old, and juveniles about 9 months old. All males were sexually active throughout the study.

All coyotes in Groups I and II received a single intravenous injection of $[^3\text{H}]$ thymidine (Nuclear Chicago, sterile aqueous solution, sp. act. 3.0 to 5.0 Ci/mm) at the rate of 0.5 $\mu\text{Ci/g}$ body weight. Group III males were used for tissue samples to estimate the frequency of the stages of spermatogenesis and did not receive any $[^3\text{H}]$ thymidine.

Semen collection, histology, and autoradiography

Semen was collected by electroejaculation by the technique of Christensen & Dougherty (1955). The animals were anaesthetized with Surital (Parke-Davis—reference to trade names does not imply endorsement of commercial products by the Federal Government) at levels based on the manufacturer's recommendations for dogs. About 1 to 2 ml of semen was collected from each animal at each collection time.

Several semen smears were made from each ejaculate and from the caput, corpus and cauda epididymidis. Each smear was fixed immediately in absolute ethanol:acetic acid (3:1) for 5 min, then Feulgen stained, air dried and held until autoradiographs were made. All testicular and epididymal tissue was fixed in absolute ethanol:acetic acid (3:1) for 5 to 7 hr and embedded in

EXPLANATION OF PLATES 1 AND 2

Cross-section of the seminiferous tubules of sexually active coyotes. All magnifications $\times 540$. A = type A spermatogonia; B = type B spermatogonia; C = old primary spermatocytes; M = metaphase figures in maturation divisions; P = preleptotene primary spermatocytes; S = Sertoli cells; T = spermatids; Y = young primary spermatocytes; Z = spermatozoa. Figs 1–8 represent the eight stages of the spermatogenic cycle.

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paraffin wax. The tissues were then cut at 6 μm , mounted on acid-cleaned, glass slides and stained with Feulgen's reagent.

Tissues and smears were coated with Kodak NTB-3 Nuclear Tract Emulsion with a stainless steel roller, air dried, put in light-excluding plastic boxes that contained CaSO_4 and stored at 5°C. Smears were exposed for 12 weeks, and tissues for 3 weeks. After exposure, the slides were developed and processed by the technique of Bélanger (1961).

Cycle of the seminiferous epithelium

The method used to determine the duration of spermatogenesis required an estimate of (1) the duration of one cycle of the seminiferous epithelium and (2) the relative frequency of each of the stages comprising one cycle of the seminiferous epithelium. In this study, the cycle of the seminiferous epithelium was divided into eight stages based on criteria similar to those used by other workers to describe the typical cellular associations observed in other species (Roosen-Runge & Giesel, 1950; Swierstra & Foote, 1965; Swierstra, 1968). A detailed description of each stage is provided in Results and illustrated in Plates 1 and 2, Figs 1 to 8.

To determine the relative frequency of each of the eight stages, tissue samples were collected from four different locations in one testis from each of the eight Group III coyotes. Biopsy samples were obtained at intervals intermediate to those discussed below and shown in Table 2, but were excluded from the study because they were small and did not contain sufficient numbers of each spermatogenic stage to be considered representative. Fifty tubular cross-sections were randomly selected in each location and classified according to stage, providing a total of 1600 observations. The frequency of occurrence of each of the eight stages, when expressed as a percentage of the total classified, indicates the relative duration of each stage (Clermont & Trott, 1969).

Estimates of the duration of one cycle of the seminiferous epithelium were based on how far labelled germinal cell nuclei progressed between the time [^3H]thymidine was injected and the tissue was collected, and was similar to the method used by other workers (Clermont & Harvey, 1965; Swierstra, 1968).

RESULTS

The criteria for classifying the eight spermatogenic stages were:

Stage 1. From the absence of spermatozoa around the lumen to the start of spermatid elongation. The spermatid nuclei are spherical and an acrosome, covering about two-fifths of the nuclear perimeter, is distinctly visible (Pl. 1, Fig. 1).

Stage 2. From the start to the finish of spermatid elongation. The elongating spermatids group together and appear to attach to the Sertoli cells (Pl. 1, Fig. 2).

Stage 3. From the end of spermatid elongation to the start of the first maturation division. Young primary spermatocytes have begun to 'move' away from the basement membrane although many still remain in that location throughout this stage (Pl. 1, Fig. 3).

Stage 4. From the start to the end of the maturation divisions. The primary

spermatocytes have begun to divide and meiotic metaphase figures as well as secondary spermatocytes and newly formed spermatids are prevalent throughout this stage (Pl. 1, Fig. 4).

Stage 5. From the end of the maturation divisions to the start of acrosome formation. The latter first appears as a small, spherical, vacuole-like protuberance tangential to the surface of the spermatid nuclei. The chromatin in the spermatid nuclei is unevenly distributed (Pl. 2, Fig. 5).

Stage 6. From the time the acrosome 'vacuole' first appears until it covers about one fifth of the perimeter of the spermatid nuclei. Type A spermatogonia are adjacent to the basement membrane and divide to form Type B spermatogonia during this stage. The chromatin material in the spermatid nuclei becomes more diffuse and appears granular toward the end of this stage (Pl. 2, Fig. 6).

TABLE 1
RELATIVE FREQUENCY AND ESTIMATED
AVERAGE DURATION OF THE STAGES OF
THE CYCLE OF THE SEMINIFEROUS
EPITHELIUM IN COYOTES

Stage	Relative frequency (%) ($\pm$ S.E.)	Duration (days)
1	25.2 $\pm$ 0.7	3.4
2	15.2 $\pm$ 0.8	2.1
3	3.5 $\pm$ 0.3	0.5
4	10.0 $\pm$ 0.8	1.4
5	13.1 $\pm$ 0.7	1.8
6	12.3 $\pm$ 0.7	1.7
7	11.0 $\pm$ 0.6	1.5
8	9.6 $\pm$ 0.8	1.3

Stage 7. From the time the acrosome 'vacuole' covers about one fifth of the perimeter of the spermatid nuclei until it covers about two fifths. The spermatozoa begin to move towards the lumen of the tubule (Pl. 2, Fig. 7).

Stage 8. From the time the spermatozoa line the lumen until their release. The acrosome 'vacuole' disappears during this stage and instead, a distinct acrosomal line appears on the perimeter of each spermatid. Type B spermatogonia divide during this stage and form young preleptotene primary spermatocytes (Pl. 2, Fig. 8).

The relative frequency and duration of the eight stages of spermatogenesis are shown in Table 1. Stage 1 was the most frequent (25.2%) and Stage 3 the most infrequent (3.5%). Analysis of variance (with arcsin-transformed data) indicated that the frequency of the stages differed significantly among coyotes ($P < 0.05$).

One hour after [^3H]thymidine injection, leptotene primary spermatocytes in Stage 1, Cycle 2, were the most mature germ cells labelled. Six hours after [^3H]thymidine, the tubules containing the most mature labelled germ cells had advanced to Stage 2 (Table 2). As the time after [^3H]thymidine injection

increased, the most mature labelled germ cells advanced until at 34 days, the spermatids in Stage 6, Cycle 4 (Table 3) were labelled. The duration of one cycle of the seminiferous epithelium, based on the progress of the most mature

TABLE 2
PERCENTAGE OF STAGES 1 AND 2 WITH LABELLED
LEPTOTENE PRIMARY SPERMATOCYTES IN COYOTES

Hours after [³ H]thymidine injection	No. of tubules observed		Percentage of tubules labelled*	
	Stage 1	Stage 2	Stage 1	Stage 2
1	119	52	30.2	0
6	85	43	23.5	9.3

* Labelled = at least one nucleus with at least four grains.

TABLE 3
ESTIMATES OF THE DURATION OF ONE CYCLE OF THE SEMINIFEROUS EPITHELIUM
IN COYOTES

Group	Animal no.	Testis removal (days after [³ H]thymidine injection)	Cycle and stage of most advanced cell type labelled			No. of cycles covered	Estimated duration of one cycle (days)
			Cell type	Stage*	Cycle		
I	1	6	Prim. sp-cyte	5	2	0.43	14.0
	2	18	Prim. sp-cyte	4	3	1.31	13.7
	3	30	Spermatid	2	4	2.15	14.0
II	4	3	Prim. sp-cyte	3	2	0.25	12.0
	5	6	Prim. sp-cyte	5	2	0.43	14.0
	5	8	Prim. sp-cyte	6 L	2	0.59	13.6
	6	13	Prim. sp-cyte	1	3	0.95	13.7
	6	15	Prim. sp-cyte	2 E	3	1.11	13.5
	7	21	Spermatid	6	3	1.56	13.5
	7	23	Spermatid	7 L	3	1.70	13.5
	8	32	Spermatid	4	4	2.31	13.8
	8	34	Spermatid	6 E	4	2.52	13.5
Mean $\pm$ S.E.							13.6 $\pm$ 0.15

* Mid-stage unless identified with an L (late stage) or E (early stage).

labelled cells observed at various intervals between 3 and 34 days, was 13.6 days. Labelled spermatozoa were first observed in the caput, corpus and cauda epididymidis at 40, 43 and 50 days, respectively, and in the ejaculate of one male at 52 days, following the injection of [³H]thymidine.

DISCUSSION

The relative frequency of each of the eight stages of spermatogenesis and the duration of each based on a 13.6-day cycle is presented in Table 1. Analysis of variance indicated that the frequency of Stages 5 to 8 differed significantly among coyotes ($P < 0.05$). It seems unlikely that the duration of each stage is

peculiar to each animal while the cycle duration is similar among animals. The relative frequency of the stages in the seminiferous epithelium differs among species but is generally constant among animals within a species (Courrot, Hochereau-de Reviers & Ortavant, 1970). Hochereau (1963) did find some variation in stage frequency among bulls and among samples both within and between testes from the same bull, but the differences were not significant. It is unknown why the differences observed in this study were significant. It may be due to a sampling bias but, in view of the large areas sampled in each testis, it is doubtful if this was the cause. It should be noted that coyotes differ from other species studied so far in being seasonal breeders and it is possible this factor could be responsible for the differences observed.

The method used in this study for estimating the duration of one cycle of the seminiferous epithelium with [^3H]thymidine is similar to the one described by Swierstra & Foote (1965) to estimate cycle duration in the rabbit. In this method, it is necessary to determine the most mature labelled germ cell at any two sampling intervals after the administration of the tracer and to classify the respective tubules according to the stage of the cycle (1 to 8) and the generation or cycle in which it is located (1 to 4). This will indicate the number of cycles of the seminiferous epithelium that have elapsed between the two sampling periods and since the time elapsed is known, the duration of one cycle can be readily calculated. In this study, the most mature labelled germ cell was located in tissue collected shortly after the administration of [^3H]thymidine and this served as a basis for comparing all subsequent samples. In tissue collected 1 hr after injection, 30% of the Stage-1 tubules showed labelled leptotene primary spermatocytes; neither cells in Cycle 2, nor the comparable cells in Stage 2 were labelled (Table 2). Five hours later (6 hr after injection), 9% of the Stage-2 tubules showed labelled primary spermatocytes. This suggests that spermatocyte DNA synthesis occurs during the last 30% of Stage 1, Cycle 2, and terminates sometime within 6 hr of the end of Stage 1. Therefore, a point which was 70% into Stage 1 was arbitrarily selected as the time of [^3H]thymidine incorporation, although the selection of any point during the last 30% of Stage 1 could be equally correct. A total of twelve samples was collected at intervals ranging from 3 to 34 days after injection and in each, the most mature labelled germ cell was located and the respective tubules classified as mentioned above. For calculation purposes, all tubules classified in this latter group were considered to be at the mid-point of the stage unless it could be determined that it was late or early in the stage. Twelve independent estimates of cycle duration were calculated and are presented in Table 3.

To illustrate how each estimate was derived, consider animal No. 6 in Table 3. It was established that 1 hr after [^3H]thymidine injection the most mature labelled germ cells were leptotene primary spermatocytes in Stage 1, Cycle 2 and it is assumed that the same obtains for other coyotes similarly treated. The left testis of animal No. 6 was sampled 13 days after [^3H]thymidine injection and the most mature labelled germ cells observed were primary spermatocytes in Stage 1, Cycle 3. Thus, in 13 days the labelled cells advanced through the remainder of Cycle 2 and as far as the mid-point of Stage 1 in the next cycle or Cycle 3. This represents 0.95 cycles and was calculated as follows:

$$\begin{array}{rcc} \text{A} & \text{B} & \text{C} \\ [(1.00) - (0.70 \times 0.25)] & + & [0.50 \times 0.25] = \text{number of cycles} \\ [0.825] & + & [0.125] = 0.95 \text{ cycles} \end{array}$$

where A represents one cycle, Cycle 2 in this case, B is the proportion of Stage 1 in Cycle 2 that occurred before the spermatocytes were labelled, and C the proportion of Stage 1, Cycle 3, that occurred before the sample was obtained. The estimate for the duration of one cycle is the time elapsed divided by the number of cycles (or 13 days $\div$ 0.95 cycles), providing an estimate of 13.7 days/cycle. The mean of twelve estimates calculated in this way was 13.6 days.

Assuming four cycles of the seminiferous epithelium are required for stem spermatogonia to develop into spermatozoa, the duration of spermatogenesis is 4×13.6 , or 54.4 days. However, labelled spermatozoa can be expected to appear in the caput epididymidis much sooner, at 37.4 days. This is due to the fact the tracer can be incorporated in developing germ cells as late as Stage 1, Cycle 2, a point 1.3 cycles into spermatogenesis. Thus, only 2.7 cycles remain before these labelled cells develop into spermatozoa released for passage to the epididymides and 2.7×13.6 days is 37.4 days. Labelled spermatozoa were first observed in the caput epididymidis 40 days after injection, the earliest interval after 37.4 days that epididymal spermatozoa were collected.

The limited number of animals available for this study made it difficult to estimate precisely the time required for epididymal sperm transport. Labelled spermatozoa first appeared in the ejaculate of one male 52 days after injection. The two other males at this phase of the study did not have any labelled spermatozoa in the ejaculate 51 days after injection but many labelled spermatozoa were found in the cauda epididymidis of these males at this time. Assuming labelled spermatozoa reach the caput epididymidis by about 38 days after injection, it suggests that the epididymal sperm transport time is 52 – 38, or 14 days.

These estimates of the duration of spermatogenic events in the male coyote—the spermatogenic stages, the cycle of the seminiferous epithelium, the spermatogenic cycle, and the epididymal sperm transport time—should permit one to evaluate the effects of chemicals that interfere with male reproduction. Such chemicals could affect the cellular associations of one or more of the eight stages of spermatogenesis and influence the duration of one or more of the above events. The results also provide a basis for estimating how long treated animals would remain fertile before the treatment took effect, and the dosing interval necessary to maintain the desired degree of reduced fertility in a target population.

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