STUDIES ON DOG SEMEN
I. MORPHOLOGICAL CHARACTERISTICS
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(Received 10th May 1961)

Summary. Semen was collected by artificial vagina from three adult dogs of mixed breed twice weekly during the 1st year, once weekly during the 2nd year, and twice monthly during the 3rd year. Ejaculates were collected entire, or in separate fractions—the first or pre-sperm, the second or sperm-rich, and the third or post-sperm fractions. The average total duration of ejaculation was 11 min, the greater part of this time, 8 min, consisting of ejaculation of the third fraction. The average total ejaculate volume was 12·0 ml, the values for the first, second, and third fractions being 0·9, 2·6, and 9·2 ml, respectively. The average sperm concentration of the total ejaculate was 53 x 10⁶ cells per ml, the values for the second and third fractions being 247 x 10⁶ and 10 x 10⁶ cells per ml, respectively. In nigrosin-eosin stained preparations, the average percentage of ‘dead’ (eosin-stained) spermatozoa per ejaculate at room temperature varied from fifteen in second-fraction samples to twenty in total-ejaculate samples. The dimensions of normal unstained spermatozoa in these preparations were: total length 68 µ, head length 7 µ, head width 5 µ, midpiece length 11 µ, and tail length 50 µ. The average percentage of abnormal spermatozoa per ejaculate was fifteen. The head and tail regions of spermatozoa were affected by the highest percentage of abnormalities. Alteration in semen quality with seasonal variation or change in collection frequency was only significant (P<0·01) for the total ejaculate volume of individual dogs. Dog spermatozoa retained their motility for a greater length of time in phosphate buffered sperm suspensions than in undiluted semen, and motility was adversely affected at body temperature. The longest preservation of progressive motility at room temperature, 32 hr, was associated with the spermatozoa in a second-fraction semen sample diluted with egg-yolk citrate.

INTRODUCTION

Although the principle of the compound microscope was understood at the time that John Caius (1570) wrote his ‘De Canibus Britannicis’, it was not until more than a century later that Leeuwenhoek (1678) published the first descriptions of dog spermatozoa.

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The first successful artificial insemination with canine semen was performed in 1780 by Spallanzani (1781), who took special precautions to keep the inseminating syringe at the same temperature as the seminal fluid. His experiment was repeated by Rossi in 1782 on a bitch of the same breed. Millais renewed the attempts at artificial insemination with dog semen in 1884, and Heape (1897) recorded that in a series of experiments carried out by Millais over a period of 12 years, out of a total of nineteen artificially inseminated bitches only four failed to conceive. In the course of his own experiments, Heape observed that half the spermatozoa in a sample of dog semen sent to him by post were fully active when examined 18 hr after collection. This may be compared with Spallanzani’s observation (1803) on dog semen that ‘2 hr after the fluid came from the animal’s body, all motion was at an end’.

In 1914, Amantea (a, b) published the first description of an artificial vagina for the collection of dog semen. This was a pear-shaped, rubber receptacle into which water at 40°C could be introduced between the outer wall and an inner rubber lining. Bonadonna (1948) later evolved the more modern form of canine artificial vagina, and further modifications have been suggested by Nooder (1950) and Harrop (1954a). The use of an artificial vagina enabled Amantea to make the first observations on the fractional nature of the canine ejaculate. His observations were later supported by Antoine (1952), but not by the majority of authors, whose findings have agreed with Ivanov’s classical description of the canine ejaculate published in 1917.

Although the possibility of inducing an erection in the dog by electrical methods had been known for some time (Eckhard, 1863), the first account of a canine ejaculate obtained by electrical stimulation was supplied by Tinet in 1939. This method, however, was found to yield ejaculates of small volume contaminated with urine (Harrop, 1954a), and Christensen & Dougherty (1955) advocated the use of an anaesthetic while carrying out the procedure in the dog in order to prevent painful muscular contractions.

The observations of earlier workers, most of whom were Russian, on the appearance, consistency, volume, sperm concentration, motility, and percentages of dead and abnormal spermatozoa in canine ejaculates have been reviewed by Anderson (1945). More recent work has been carried out by Alvarez (1947), Hancock & Rowlands (1949), Asher & Kaemmerer (1950), Nooder (1950), Harrop (1955, 1960), Doepfmer & Freihoff (1956), Doepfmer (1956-57), Nales (1957), Garcia (1957), Letard, Szumowski & Théret (1957), Boucher, Foote & Kirk (1958), and Wales & White (1958). For the better preservation of the spermatozoa, buffered egg-yolk diluents were adapted for use with dog semen by Laurans, Malterre & Wilczynska (1947-48), Coulomb (1952), Brochart & Coulomb (1952), Nales (1957), and Bendorf & Chung (1958). The effectiveness of milk diluents was demonstrated by Harrop (1954b, 1956 a, b, c) and Davoine (1958).

The present morphological study was carried out in order to provide a basis for an investigation of the biochemical characteristics of dog semen, and as a further confirmation of the findings described in the literature.
MATERIALS AND METHODS

Three, adult, mixed-breed dogs (Dogs I, II and III), whose respective weights were 15·9, 13·2 and 14·5 kg, were used as experimental animals. The dogs were kept in unheated kennels with runs attached. The average daily food intake consisted of 660 g cooked meat mixed with ‘Winalot’ biscuit (Spiller’s product). Access to water was unrestricted.

Following a variable training period, semen was collected twice weekly during the 1st year, once weekly during the 2nd year, and twice monthly during the 3rd year of the study. Collections were carried out by means of an artificial vagina (Harrop, 1954a), the design of which permitted the transmission of pulsations to the penis via a cylindrical, inflatable, latex bladder situated within the water-space of the vagina. No preliminary lubrication of the detachable latex liner of the artificial vagina was made before semen collections.

Erections were obtained by manual stimulation in the region of the bulb of the penis. The artificial vagina was introduced and further stimulation induced thrusting movements of the penis and ejaculation. On termination of these movements, the artificial vagina was carried back between the dog’s hind legs to simulate the position assumed at natural mating. This position was maintained until the end of ejaculation. A ‘teaser’ bitch was not used.

In order to obtain the separate fractions of the ejaculate, the collecting tubes (10-ml centrifuge tubes) were changed at times coinciding with the end of the emission of one fraction and the beginning of the emission of the next. Ejaculate volumes were recorded directly from the graduated collecting tubes. The duration of ejaculation was recorded by stopwatch.

Sperm concentrations were determined in a Fuchs-Rosenthal haemocytometer after suitable dilution of the semen. The unfractioned ejaculate was diluted 1 in 50 to 1 in 100, the second fraction 1 in 100 to 1 in 300, and the third fraction 1 in 10 to 1 in 50 with 0·2 % formol-saline solution, made up by adding 0·2 ml of a saturated solution of formaldehyde (40 %) to 99·8 ml of a 9 % solution of sodium chloride. The final sperm concentration was estimated from counts made from one or more samples of diluted semen, and the average concentration calculated from the number of sperm cells counted in at least sixty-four small squares. The number of spermatozoa counted in sixteen small squares of a Fuchs-Rosenthal haemocytometer gave an estimation of the number of spermatozoa in ¼ cm² of diluted semen.

The percentage of ‘live’ (unstained) and ‘dead’ (eosin-stained) spermatozoa were calculated from semen smears stained with a solution of nigroin-eosin in either distilled water or 3 % sodium citrate dihydrate solution (Campbell, Dott & Glover, 1956). The pH of the stain mixture was checked by means of a Pye Universal pH meter, and adjusted where necessary to between 6·8 and 7·4 by the addition of requisite amounts of 3 % solutions of sodium citrate or sodium bicarbonate. The normal stain-to-semen proportions were five to one, but were varied according to the sperm concentration. The majority of stained preparations were made 30 min after collection of the semen, and the stain was allowed to act on the drop of semen for 5 min before a smear was prepared. Two stained preparations were made from each semen sample, and 400
spermatozoa were counted in each preparation. The average percentage live or dead count was calculated from a total of 800 spermatozoa.

Sperm dimensions were determined from normal live spermatozoa in nigrosin-eosin preparations with the aid of a micrometer eyepiece, which had been previously calibrated for the appropriate objective.

The percentage of abnormal spermatozoa per ejaculate was also estimated from nigrosin-eosin preparations (Swanson & Bearden, 1951). The smears were examined at a magnification of \( \times 1000 \) under an oil-immersion objective. A hundred or more spermatozoa were counted in each preparation (Salisbury & Mercier, 1945). The classification into primary and secondary abnormalities was based on that of Blom (1950).

‘Exhaustion tests’ were carried out with Dogs I and II, each dog being subjected to the test on one occasion only. The procedure involved the collection of ejaculates at 15-min intervals over a period of 1 to 1\( \frac{1}{2} \) hr, and the ejaculate volume, sperm concentration, and motility rating were recorded where possible for each ejaculation.

Sperm motility was assessed on a rating scale of 0 (complete immotility) to 4 (full activity) based on the visual criteria described by Emmens (1947). For purposes of comparison, the average time taken by 80 to 90\% of the spermatozoa to reach 0 motility was chosen as the criterion of sperm vitality. The normal observation period was 8 to 9 hr, but certain samples of good motility were re-examined later.

All semen samples, with or without added diluent, were set up at the beginning of each experiment in clean glass test-tubes \( (75 \times 10 \, \text{mm}) \), and aliquots were removed at intervals varying from 5 min to 1 hr for examination under a coverslip at magnifications of \( \times 100 \), and \( \times 400 \).

In experiments carried out to determine the effect of oxygen on sperm motility, second-fraction semen samples were supplied with oxygen through a narrow gauge (1-mm diameter) polythene catheter at a rate of flow of sixty bubbles of oxygen per minute.

In experiments to determine the effect of ‘temperature shock’ on sperm motility, the collecting tube containing the second fraction of the ejaculate was placed in a boiling water bath and the temperature of the semen was raised rapidly to 34° C. The tube was then transferred to an ice water bath, and the semen was rapidly cooled to 20° C, after which the tube was removed and allowed to stand at room temperature. The final rate of cooling was between 14° and 20° C in 5 min.

The calcium-free Ringer-phosphate solution that formed the basic diluent for experiments on the motility of dog spermatozoa in this study was the same as that described by Mann (1946). The diluents containing sugar in the form of glucose, fructose, or mannose were composed of 0·92 g NaCl, 0·42 g KCl, 0·03 g NaHCO\(_3\) and 1·0 g sugar in 100 ml ion-free water. The egg-yolk phosphate diluent (pH 6·75) was based on that described by Lardy & Phillips (1939) and Phillips & Lardy (1940), and the egg-yolk citrate diluent (pH 7·0) on that described by Salisbury, Knodt & Bratton (1948). The sterile milk diluent was prepared by the method described by Harrop (1954b).
RESULTS

The appearance of the unfractionated ejaculate varied according to the sperm content. It was usually greyish in colour, semi-opaque, and watery in consistency. When the ejaculate was fractionated, the first fraction was transparent and colourless. It often contained a few immotile spermatozoa and a variable amount of cellular debris. The second fraction, which contained the majority of the spermatozoa, varied in colour from cream to grey, and in consistency from thick to watery, according to the sperm density. The third fraction presented a greyish-yellow appearance and was watery in consistency. It contained a variable number of spermatozoa, probably owing to the absence of any definite intermission period between the ejaculation of the second and third fractions comparable to that which occurred between the ejaculation of the first and second fractions.

TABLE 1

AVERAGE DURATION OF EJACULATION, EJACULATE VOLUME, SPERM CONCENTRATION, TOTAL SPERM CONTENT AND PERCENTAGE OF ‘DEAD’ SPERMATOZOAS/EJACULATE ± SE FOR UNFRACTIONATED AND FRACTIONATED CANINE EJACULATES

<table>
<thead>
<tr>
<th></th>
<th>Unfractionated ejaculate</th>
<th>1st fraction</th>
<th>2nd fraction</th>
<th>3rd fraction</th>
<th>No. ejaculates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of ejaculation</td>
<td>11 ± 0·2 min **</td>
<td>27 ± 0·3 sec **</td>
<td>52 ± 0·4 sec *</td>
<td>8 ± 0·1 min **</td>
<td>332, 50, 50, 50</td>
</tr>
<tr>
<td>Ejaculate vol. ml/100 kg live weight</td>
<td>12·0 ± 0·2 **</td>
<td>0·9 ± 0·0 **</td>
<td>2·6 ± 0·1 **</td>
<td>9·2 ± 0·3 **</td>
<td>319, 131, 131, 131</td>
</tr>
<tr>
<td>Sperm conc. cells X 10⁶/ml</td>
<td>53 ± 2·1 **</td>
<td>247 ± 9·9 **</td>
<td>10 ± 0·8 **</td>
<td>139, 0, 157, 157</td>
<td></td>
</tr>
<tr>
<td>Sperm content cells X 10⁶</td>
<td>528 ± 25·8 **</td>
<td>637 ± 31·7 **</td>
<td>76 ± 6·9 **</td>
<td>139, 0, 157, 157</td>
<td></td>
</tr>
<tr>
<td>% Dead sperm at 18 to 23° C.</td>
<td>20 ± 1·0 **</td>
<td>15 ± 0·8 **</td>
<td>104, 0, 91, 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Significant difference between dogs, P<0·01
* Significant difference between dogs, P<0·05

DURATION OF EJACULATION, EJACULATE VOLUME, SPERM CONCENTRATION, TOTAL SPERM CONTENT AND DIFFERENTIAL ‘LIVE-DEAD’ STAINING

The duration of ejaculation, ejaculate volume, sperm concentration, total sperm content and percentage of ‘dead’ spermatozoa in unfractionated canine ejaculates, and in the separate fractions of the ejaculate, are shown in Table 1. The final column of this Table, and of subsequent Tables, records the numbers of unfractionated ejaculates or ejaculate fractions on which the values in the preceding columns are respectively based. Unfractionated ejaculate volumes
have been expressed both in millilitres and in ml/100 kg live weight. No values have been recorded for the sperm concentration or total sperm content of the first fraction of the ejaculate since this fraction, with the exceptions noted in the Discussion, was normally water-clear and almost devoid of spermatozoa.

Experiments were carried out in order to assess the effect of body temperature on sperm viability in eight unfractionated ejaculates and the second fractions of thirty-five fractionated ejaculates. After collection, the semen sample was divided into two portions, one of which was held at room temperature (18 to 23° C), while the other was incubated at 37° C for 2 hr. An aliquot was removed from each of these portions at varying times between 1/2 and 2 hr after collection, and the percentages of 'dead' spermatozoa were compared in nigrosin-eosin preparations made from these samples. The following results, expressed as the average percentage of 'dead' spermatozoa per ejaculate ± SE, were obtained: at room temperature—unfractionated ejaculate 18 ± 4·5, second fraction 18 ± 1·3; at 37° C—unfractioned ejaculate 31 ± 7·1, second fraction 27 ± 2·2.

**SIZE OF SPERMATOZOA AND SPERM ABNORMALITIES**

The average dimensions, ±SE, of twenty 'live' spermatozoa with no detectable abnormalities, were: total length 68 μ ± 0·3, head length 7 μ ± 0·0, head width 5 μ ± 0·1, midpiece length 11 μ ± 0·2, tail length 50 μ ± 0·3.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Total spermatozoa</th>
<th>'Live' spermatozoa</th>
<th>'Dead' spermatozoa</th>
<th>No. ejaculates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal spermatozoa</td>
<td>15 ± 1·6 n.s.</td>
<td>5 ± 0·8 **</td>
<td>10 ± 1·2 n.s.</td>
<td>30</td>
</tr>
<tr>
<td>(6 ± 0·8 **</td>
<td>(60 ± 4·6 **)</td>
<td></td>
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<td></td>
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<tr>
<td>Head abnormalities</td>
<td>11 ± 1·4 n.s.</td>
<td>3 ± 0·6 **</td>
<td>8 ± 1·2 n.s.</td>
<td>30</td>
</tr>
<tr>
<td>(4 ± 0·7 **</td>
<td>(48 ± 5·0 n.s.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neck abnormalities</td>
<td>5 ± 1·5 **</td>
<td>1 ± 0·4 n.s.</td>
<td>4 ± 1·2 **</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(1 ± 0·7 n.s.)</td>
<td></td>
<td>(21 ± 4·5 **)</td>
<td></td>
</tr>
<tr>
<td>Midpiece abnormalities</td>
<td>21 ± 5·0 **</td>
<td>20 ± 4·8 **</td>
<td>2 ± 0·3 n.s.</td>
<td>30</td>
</tr>
<tr>
<td>+ c.d.</td>
<td>(24 ± 5·9 **)</td>
<td>(1 ± 0·4 n.s.</td>
<td>(15 ± 2·7 n.s.)</td>
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<tr>
<td></td>
<td>(2 ± 0·5 *</td>
<td></td>
<td>(1 ± 0·3 n.s.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8 ± 2·3 n.s.)</td>
<td></td>
</tr>
<tr>
<td>Midpiece abnormalities</td>
<td>2 ± 0·5 n.s.</td>
<td>1 ± 0·4 n.s.</td>
<td>1 ± 0·3 n.s.</td>
<td>30</td>
</tr>
<tr>
<td>- c.d.</td>
<td>(2±0·5 *)</td>
<td></td>
<td>(8±2·3 n.s.)</td>
<td></td>
</tr>
<tr>
<td>Tail abnormalities</td>
<td>7 ± 0·8 n.s.</td>
<td>4 ± 0·6 **</td>
<td>3 ± 0·4 n.s.</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(5 ± 0·8 *)</td>
<td></td>
<td>(17 ± 2·6 n.s.)</td>
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</tbody>
</table>

** Significant difference between dogs, P<0·01
* Significant difference between dogs, P<0·05
n.s. No significant difference between dogs, P>0·05

'Live' and 'dead' sperm abnormalities are expressed as a percentage of the total sperm population, and also, in parentheses, as a percentage of the 'live' or 'dead' sperm in the total population. c.d. indicates the inclusion (+) or the exclusion (−) of cytoplasmic droplets as abnormalities.

Sperm abnormalities were analysed on the basis of (a) the average total percentage of abnormalities per ejaculate, (b) the average percentage of
abnormal ‘live’ spermatozoa per ejaculate, and (c) the average percentage of abnormal ‘dead’ spermatozoa per ejaculate. The results of these analyses based on thirty ejaculates, ten from each of Dogs I, II and III, are shown in Table 2. With the exception of abnormalities affecting the neck and midpiece regions of spermatozoa, there were no significant differences in the percentage of sperm abnormalities affecting the total sperm population of ejaculates from different dogs. The high incidence of neck and midpiece abnormalities was largely influenced by one of the three dogs (Dog II), and the number of midpiece abnormalities, in turn, was largely influenced by the presence of numerous cytoplasmic droplets. When these droplets were not included as a form of sperm abnormality, the difference between the percentages of midpiece abnormalities occurring in ejaculates from different dogs was no longer significant.

Although the number of ‘live’ and ‘dead’ sperm abnormalities expressed as a percentage of the total sperm population suggests that a slightly higher percentage of most abnormalities, except those affecting the sperm tail, are found in association with ‘dead’ than with ‘live’ spermatozoa, the further analysis of the same data in Table 2, expressing the number of abnormalities affecting ‘live’ or ‘dead’ spermatozoa as a percentage of the number of ‘live’ or ‘dead’ spermatozoa counted in the total population, shows that a much higher percentage of abnormalities is associated with ‘dead’ than with ‘live’ spermatozoa. An exception to this was the incidence of cytoplasmic droplets, which were commonly found in association with ‘live’ rather than ‘dead’ spermatozoa.

Although the different regions of dog spermatozoa may present a considerable variety of abnormalities, the principal abnormalities affecting sperm heads were as follows: (1) Primary abnormalities: macrocephaly, microcephaly, double heads, pointed heads, indented heads, and finally opaque heads, which were usually deformed, and in which no detailed structure was visible (Pl. 1, Figs. 1 to 6). (2) Secondary abnormalities: free heads, bent or detaching heads, swollen or detaching acrosomes (Pl. 1, Figs. 7, 8 and 9). Three stages involving the detachment of the acrosome were discernible; a primary stage of early swelling of the acrosome, a secondary stage of marked swelling and early detachment, and a final stage at which the acrosome was no longer visible. Since, however, only the second of these stages was clearly defined, the first and third were discarded as criteria of abnormality.

The principal abnormalities affecting sperm necks were as follows: (1) Primary abnormalities: thickened neck, eccentric insertion (Pl. 1, Figs. 10 and 11). (2) Secondary abnormalities: disintegration of the neck region, cytoplasmic droplets (Pl. 1, Figs. 12 and 13). Neck cytoplasmic droplets were found in only four of the thirty ejaculates examined, and in association with only 2% of the spermatozoa in these ejaculates.

The principal abnormalities affecting sperm midpieces were as follows: (1) Primary abnormalities: irregularly thickened or thinned midpiece, coiled midpiece, kinked midpiece, double midpiece (Pl. 1, Figs. 14 to 18). (2) Secondary abnormalities: bent midpiece, extraneous material surrounding midpiece, proximal, mid, or distal cytoplasmic droplets (Pl. 1, Figs. 19 to 23). The mid-
pieces of 'dead' spermatozoa frequently presented the appearance of central grooving, or separation into two distinct filaments (Pl. 1, Fig. 24). This appearance was not observed in 'live' spermatozoa.

The principal abnormalities affecting sperm tails were as follows: (1) Primary abnormalities: thinned tail, double or treble tail (Pl. 1, Figs. 25, 26 and 27). (2) Secondary abnormalities: coiled tail, looped tail, kinked tail, folded tail, detached tail, cytoplasmic droplets (Pl. 1, Figs. 28 to 33). Tail cytoplasmic droplets were found in only five of the thirty ejaculates examined, and in association with only 1% of the spermatozoa in these ejaculates.

**Table 3**

AVERAGE VALUES, ± SE, FOR THE VOLUME EXPRESSED IN MILLILITRES, AND THE SPERM CONCENTRATION EXPRESSED AS NUMBER OF CELLS × 10⁶/ML, OF UNFRACTIONATED EJACULATES AND SECOND FRACTIONS OF EJACULATES COLLECTED FROM DOGS I AND II DURING QUARTERLY PERIODS OVER 3 YEARS

<table>
<thead>
<tr>
<th></th>
<th>Dog I</th>
<th>Dog II</th>
<th>Dogs I &amp; II</th>
<th>No. ejaculates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unfractionated ejaculate volume</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>January to March</td>
<td>12·8±0·6</td>
<td>10·5±0·3</td>
<td>11·6±0·4</td>
<td>39, 38, 77</td>
</tr>
<tr>
<td>April to June</td>
<td>13·3±1·2</td>
<td>10·6±0·2</td>
<td>11·8±0·6</td>
<td>33, 39, 72</td>
</tr>
<tr>
<td>July to September</td>
<td>15·6±0·8</td>
<td>10·1±0·3</td>
<td>13·0±0·6</td>
<td>36, 32, 68</td>
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<tr>
<td>October to December</td>
<td>16·9±0·5</td>
<td>9·1±0·4</td>
<td>13·1±0·6</td>
<td>34, 32, 66</td>
</tr>
<tr>
<td>Seasons</td>
<td>**</td>
<td>**</td>
<td>n.s.</td>
<td></td>
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<tr>
<td>Dogs</td>
<td></td>
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<tr>
<td><strong>Sperm concentration</strong></td>
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<tr>
<td>January to March</td>
<td>45·1±4·0</td>
<td>67·9±3·6</td>
<td>57·9±3·5</td>
<td>11, 14, 25</td>
</tr>
<tr>
<td>April to June</td>
<td>63·8±7·5</td>
<td>73·4±3·9</td>
<td>67·8±4·8</td>
<td>10, 7, 17</td>
</tr>
<tr>
<td>July to September</td>
<td>62·1±5·7</td>
<td>55·1±0·4</td>
<td>56·3±3·5</td>
<td>10, 12, 22</td>
</tr>
<tr>
<td>October to December</td>
<td>45·0±8·6</td>
<td>60·8±11·7</td>
<td>54·8±7·9</td>
<td>8, 13, 21</td>
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<tr>
<td>Seasons</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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<td>Dogs</td>
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<tr>
<td><strong>Second fraction volume</strong></td>
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<tr>
<td>January to March</td>
<td>2·9±0·2</td>
<td>2·5±0·2</td>
<td>2·7±0·1</td>
<td>22, 21, 43</td>
</tr>
<tr>
<td>April to June</td>
<td>2·8±0·3</td>
<td>2·0±0·1</td>
<td>2·3±0·1</td>
<td>10, 18, 28</td>
</tr>
<tr>
<td>July to September</td>
<td>3·2±0·2</td>
<td>2·5±0·2</td>
<td>2·9±0·2</td>
<td>18, 12, 30</td>
</tr>
<tr>
<td>October to December</td>
<td>2·9±0·1</td>
<td>2·0±0·1</td>
<td>2·5±0·1</td>
<td>18, 11, 29</td>
</tr>
<tr>
<td>Seasons</td>
<td>n.s.</td>
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<tr>
<td>Dogs</td>
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<tr>
<td><strong>Sperm concentration</strong></td>
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<tr>
<td>January to March</td>
<td>243·6±21·4</td>
<td>209·1±18·5</td>
<td>227·1±14·5</td>
<td>24, 22, 46</td>
</tr>
<tr>
<td>April to June</td>
<td>265·1±38·9</td>
<td>275·6±23·9</td>
<td>271·9±18·7</td>
<td>10, 18, 28</td>
</tr>
<tr>
<td>July to September</td>
<td>269·2±26·4</td>
<td>196·9±5·5</td>
<td>236·3±17·4</td>
<td>18, 15, 33</td>
</tr>
<tr>
<td>October to December</td>
<td>337·8±39·2</td>
<td>271·7±46·2</td>
<td>312·8±30·0</td>
<td>18, 11, 29</td>
</tr>
<tr>
<td>Seasons</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Significant difference between seasons or dogs, P<0·01
* Significant difference between seasons or dogs, P<0·05
n.s. No significant difference between seasons or dogs, P>0·05

EFFECT OF SEASONAL VARIATION AND FREQUENCY OF COLLECTION ON SEMEN QUALITY

The data obtained from Dogs I and II were analysed in order to determine the effects on semen quality, as assessed by ejaculate volume and sperm density,
of (a) seasonal variation over a period of 3 years, and (b) two different frequencies of collection over 2 successive yearly periods. The results are shown in Tables 3 and 4.

The quarterly variations in volume and sperm density over the 3-year period were not significant \((P > 0.05)\) for the combined unfractionated ejaculate values for both dogs, although there were significant differences \((P < 0.01)\) for the quarterly variations in the unfractionated ejaculate volume for individual dogs over the same period. The quarterly variations in volume and sperm density of the combined values for the second fractions of both dogs were significant \((P < 0.05)\) over the 3-year period, but, with the exception of the values for the second-fraction volume of Dog II, were not significant for individual dogs over the same period. Differences between dogs over the 3-year period were significant at the 1\% level for ejaculate-volume values,

### Table 4

**Average values, \(\pm SE\), for the volume expressed in millilitres, and the sperm concentration expressed as number of cells \(\times 10^8/\text{ml}\), of unfractionated ejaculates and second fractions of ejaculates collected from Dogs I and II twice weekly and once weekly over successive periods of 1 year**

<table>
<thead>
<tr>
<th></th>
<th>Dog I</th>
<th>Dog II</th>
<th>Dogs I &amp; II</th>
<th>No. ejaculates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unfractioned ejaculate volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (\times) /week</td>
<td>12.0 (\pm) 0.4</td>
<td>9.6 (\pm) 0.2</td>
<td>10.8 (\pm) 0.2</td>
<td>79, 86, 165</td>
</tr>
<tr>
<td>1 (\times) /week</td>
<td>17.7 (\pm) 0.5</td>
<td>11.5 (\pm) 0.2</td>
<td>14.7 (\pm) 0.5</td>
<td>41, 37, 78</td>
</tr>
<tr>
<td>Collection frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) /week</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 1 (\times) /week</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) &amp; 1 (\times) /week</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td><strong>Sperm concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (\times) /week</td>
<td>46.4 (\pm) 4.0</td>
<td>61.0 (\pm) 6.6</td>
<td>54.2 (\pm) 4.1</td>
<td>21, 24, 45</td>
</tr>
<tr>
<td>1 (\times) /week</td>
<td>60.6 (\pm) 4.6</td>
<td>64.3 (\pm) 3.7</td>
<td>63.0 (\pm) 2.9</td>
<td>10, 18, 28</td>
</tr>
<tr>
<td>Collection frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) /week</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 1 (\times) /week</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) &amp; 1 (\times) /week</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td><strong>Second fraction volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (\times) /week</td>
<td>2.7 (\pm) 0.1</td>
<td>2.2 (\pm) 0.1</td>
<td>2.4 (\pm) 0.1</td>
<td>36, 43, 79</td>
</tr>
<tr>
<td>1 (\times) /week</td>
<td>3.0 (\pm) 0.2</td>
<td>2.3 (\pm) 0.1</td>
<td>2.7 (\pm) 0.1</td>
<td>20, 12, 32</td>
</tr>
<tr>
<td>Collection frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) /week</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 1 (\times) /week</td>
<td>*</td>
<td>*</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) &amp; 1 (\times) /week</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td><strong>Sperm concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (\times) /week</td>
<td>260.4 (\pm) 24.5</td>
<td>222.2 (\pm) 17.1</td>
<td>240.1 (\pm) 14.7</td>
<td>38, 43, 81</td>
</tr>
<tr>
<td>1 (\times) /week</td>
<td>271.6 (\pm) 17.6</td>
<td>280.0 (\pm) 30.2</td>
<td>274.7 (\pm) 15.5</td>
<td>20, 12, 32</td>
</tr>
<tr>
<td>Collection frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) /week</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 1 (\times) /week</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Dogs I &amp; II, 2 (\times) &amp; 1 (\times) /week</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

** Significant difference between collection frequencies or dogs, \(P < 0.01\)

* Significant difference between collection frequencies or dogs, \(P < 0.05\)

n.s. No significant difference between collection frequencies or dogs, \(P > 0.05\)
but only at the 5% level for second-fraction sperm-density values, and there were no significant differences between dogs for unfractionated-ejaculate sperm-density values.

In an analysis of the relation between semen quality and collection frequency, significant differences between the two collection frequencies at the 1% level were only found for unfractionated-ejaculate volume values. Differences between dogs over the 2-year period were significant at the 1% level for ejaculate-volume values, but only at the 5% level for unfractionated-ejaculate sperm-density values, and there were no significant differences between dogs for second-fraction sperm-density values.

The effect of rapid collection on ejaculate volume and sperm density was studied by performing the so-called 'exhaustion test'. The results are shown in Table 5.

**Table 5**

**Effect of Rapid, Successive Collections of Semen on Volume, Sperm Concentration and Motility Rating of Ejaculates Obtained from Dogs I and II**

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate No.</th>
<th>Time of collection (min)</th>
<th>Volume (ml)</th>
<th>Sperm concentration (cells × 10⁹/ml)</th>
<th>Motility rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>0</td>
<td>24-4</td>
<td>61</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30</td>
<td>4-7</td>
<td>16</td>
<td>3½</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>45</td>
<td>0-0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>60</td>
<td>0-0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>0</td>
<td>12-4</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>1-8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30</td>
<td>1-6</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>45</td>
<td>0-5</td>
<td>few spermatozoa</td>
<td>½</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>60</td>
<td>0-3</td>
<td>„</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>75</td>
<td>0-5</td>
<td>„</td>
<td>0</td>
</tr>
</tbody>
</table>

**Sperm Motility**

The results of observations on the motility of spermatozoa from unfractionated-ejaculate and second-fraction samples, diluted with a number of different substrates, are shown in the histogram in Text-fig. 1. This records the percentages of different semen samples incubated both at 37°C and at room temperature, in which the spermatozoa reached 0 motility during the normal observation period of 8 to 9 hr.

None of the samples incubated at a temperature of 37°C retained any degree of motility for a period of 24 hr, with the exception of one second-fraction semen sample diluted one in five with egg-yolk citrate which had a motility rating of ½ after this time.

At room temperature (18 to 23°C), none of the spermatozoa in the unfractionated-ejaculate semen samples retained any degree of motility for as long as 24 hr, but in one of the seven samples diluted one in two with Ringer-phosphate solution alone, and in two of the six samples diluted with 10 and 20% concentrations of acetone and ether in Ringer-phosphate solution, the spermatozoa still had a motility rating of ½ after 19 hr. Some degree of motility after
24 hr was most frequently found in the second-fraction semen samples held at room temperature. Three of the forty-nine undiluted semen samples had a motility rating of 1½ (minimal progressive motility) after 24 hr. Two of the five samples diluted one in two with prostatic secretion (third fraction of the ejaculate), five of the thirteen samples diluted one in two with Ringer-phosphate solution alone, and even one of the three samples diluted one in two with 0.9% sodium chloride solution had a motility rating of 1½ after 24 hr. All of the three samples diluted one in two with 1% ammonium phosphate solution retained some degree of motility (½ to 1½) for 24 hr, and one of these samples still had a motility rating of 1 after 43 hr. All of the four samples diluted one in two with 10 and 20% concentrations of acetone and ether in Ringer-phosphate solution had a motility rating of 1½ after 24 hr, and some degree of motility was still discernible in 10% acetone and ether, after 31 hr.

Text-fig. 1. Comparison of the duration of motility of dog spermatozoa from unfractionated ejaculate (U.E.) and second fraction (2nd Fr) semen samples under varying conditions at room temperature (18 to 23°C) and at 37°C. 
- samples at room temperature; □, samples at 37°C.

Experiments were also carried out in order to assess the effect of different concentrations of ethanol and methanol on the duration of motility of the spermatozoa in (a) three unfractionated-ejaculate, and (b) three second-fraction semen samples at room temperature (19 to 22°C), and to compare these effects with (c) those obtained by Ivanov (1913). The average motility ratings obtained in the presence of 2, 5, 10 and 20% concentrations of alcohol, made up by suitable dilution of 95% alcohol with water, were: in 2% alcohol, (a) 3, sluggish progressive motility, after 2½ hr, (b) 3 after 4 hr, (c) 'mouvements progressifs énergiques' after 8 hr; in 5% alcohol; (a) 2½, progressive motility but many stationary cells with vibrating tails, after 1½ hr, (b) 2⅛ after 3 hr,
(c) 'mouvements progressifs' after 2 hr; in 10% alcohol; (a) 1, tails vibrating with only an occasional cell in actual motion, after ½ hr, (b) 1 after 3 hr; (c) 'mouvements progressifs' after ½ hr; in 20% alcohol; (a) ½, only a few cells per field showing any movement, after 10 min, (b) ½ after 1 hr, (c) 'mouvements oscillatoires faibles' after 1 to 5 min.

No difference was detected between the effects of ethanol and methanol on sperm motility. It will be seen that the present findings were comparable with those of Ivanov, though progressive motility appeared to be maintained for longer in his samples. It is difficult, however, to make a direct comparison since no information is available on the temperature at which Ivanov's experiments were performed, or on the number of semen samples on which his results were based, and it must be assumed that he used unfractionated-ejaculate material.

The results of a comparison of the effects of 10 and 20% alcohol concentrations made up either in ion-free water, or in Ringer-phosphate solution, on the duration of sperm motility in the unfractionated-ejaculate and second-fraction semen samples maintained at room temperature are shown in Table 6.

**Table 6**

<table>
<thead>
<tr>
<th></th>
<th>Unfractionated ejaculate</th>
<th>Second fraction</th>
<th>No. ejaculates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer-phosphate solution alone</td>
<td>9 ± 1.3</td>
<td>31±0.6</td>
<td>9.3</td>
</tr>
<tr>
<td>10% alcohol in ion-free water</td>
<td>1±0.2</td>
<td>6±0.2</td>
<td>4.2</td>
</tr>
<tr>
<td>10% alcohol in Ringer-phosphate solution</td>
<td>8±0.1</td>
<td>32±0.5</td>
<td>4.2</td>
</tr>
<tr>
<td>20% alcohol in ion-free water</td>
<td>½±0.2</td>
<td>1±0.0</td>
<td>12.4</td>
</tr>
<tr>
<td>20% alcohol in Ringer-phosphate solution</td>
<td>5±1.4</td>
<td>30±0.0</td>
<td>5.2</td>
</tr>
</tbody>
</table>

In a comparison of egg-yolk-phosphate, egg-yolk-citrate and sterile-milk diluents, it was observed that when spermatozoa had become completely immotile in the egg-yolk-phosphate diluent, sperm-cells from the same semen sample still retained their initial motility in the egg-yolk-citrate and sterile-milk diluents. Of seventeen second fraction semen samples diluted one in five with egg-yolk citrate and held at room temperature, four samples still retained their initial motility (3½ to 4) after 24 hr, and only two samples had reached 0 motility in that time. At 37°C, however, all except one of fourteen second fraction samples were completely immotile after 24 hr. The longest preservation of some degree of progressive motility (1½) in this study, 32 hr, was observed in one second-fraction semen sample diluted with egg-yolk citrate.

**DISCUSSION**

Most of the observations on the different characteristics of the canine ejaculate obtained in the present study corresponded well with those of other authors,
in particular with regard to the appearance of the ejaculate (Ivanov, 1917),
the ejaculate volume for medium-sized dogs (Amantea, 1914b; Coulomb, 1952;
Garcia, 1957), sperm concentration (Miovanov, 1934; Alvarez, 1947; Garcia,
1957), the percentage of ‘dead’ spermatozoa (Hancock & Rowlands, 1949;
Garcia, 1957; Davoine, 1958), normal sperm dimensions (Miotti, 1942;
Laurans et al., 1947-48; Antoine, 1952; Coulomb, 1952; Harrop, 1955; Garcia,
1957; White, 1958), sperm abnormalities (Peters, 1943; Laurans et al., 1947-48;
Antoine, 1952; Coulomb, 1952; Letard et al., 1957; Davoine, 1958), sperm
survival times, at room temperature and at 37° C (Bederke, 1933; Freiberg,
1934; Dubincik, 1934; Laurans et al., 1947-48; Asher & Kaemmerer, 1950),
in the presence of glucose, fructose, and mannose (Bernstein, 1933; Bederke,
1933), in the presence of alcohol (Ivanov, 1913; Farrell, 1938), in the presence
of egg-yolk ‘extenders’ (Wilczynska & Laurans, 1947; Laurans et al., 1947-48;
Coulomb, 1952; Brochart & Coulomb, 1952; Nales, 1957; Bendorf & Chung,
1958), and in the presence of milk diluents (Bendorf & Chung, 1958).

Amantea (1914b) and Antoine (1952) both described a sperm-rich first
fraction for the canine ejaculate, and Antoine concluded that the sperm-free
first fraction described by other authors either did not exist, or occurred
in such small quantities that it could not be detected. In the course of the
present study, it was noted that 3 to 4 months after the initial collection of
semen from Dogs I and II, while the volumes of the ejaculate fractions were
in no way abnormal, the sperm densities of the first fractions (97 to 416 × 10⁶
cells per millilitre) did exceed those of the second fractions (45 to 171 × 10⁶
cells per millilitre) of these ejaculates. Examination of fractionated ejaculates
from the same dogs a few weeks later showed the first fractions to contain very
few spermatozoa. A sperm-rich first fraction appeared to be atypical, and was
attributed to a diminution or absence of the secretions of the urethral glands,
which have been regarded as the source of this portion of the ejaculate (Freiberg,
1934).

The possible contribution of the third fraction to the sperm content of the
total ejaculate seems to have been largely ignored in the literature. In this
study, ejaculates were obtained with second fractions of average sperm density
associated with third-fraction sperm concentrations of up to 58 × 10⁶ cells per
millilitre. This value is comparable to that found for the average sperm density
of the unfractionated ejaculate, 54 × 10⁶ cells per millilitre.

The analysis of canine sperm abnormalities revealed a marked difference
between the association of the majority of abnormalities with ‘dead’ spermatozoa,
and of cytoplasmic droplets with ‘live’ spermatozoa. While the presence
of the greater number of abnormalities may contribute to the premature death
of the affected cells, it appears that for cytoplasmic droplets it is their presence
that is associated with cell survival, and their absence that precedes cell
death, or the absorption of such stains as eosin or congo red. From the appear-
ance of attached and even of shed droplets in nigrosin-eosin preparations of
canine spermatozoa, the cytoplasmic material is resistant to staining with
eosin, and thus the same material, when in contact with the sperm-cell, might
have a protective function. In this connexion, it is of interest that Gatenby &
Collery (1943) observed that the argentophil cytoplasmic droplet material
derived from the epididymal cells was sufficiently fluid to cover the entire surface of the midpiece of human spermatozoa. The same authors, however, considered that the cytoplasmic material produced by the canine epididymal cells was too viscous to produce a similar effect when in contact with dog spermatozoa.

No changes comparable to the seasonal variations that occur in the body weight of the raccoon dog (Schneider, 1950, 1951), and in the reproductive organs and spermatogenesis of the fox (Starkov, 1937) have been detected in the dog (Antoine, 1952). The variations in ejaculate volume and sperm density noted here, cannot, with the possible exception of the unfractionated-ejaculate volume, be regarded as indicating that there was a detectable seasonal variation in semen quality. It must, however, be emphasized that such a conclusion is not based on the findings of an experiment specifically designed to elucidate this problem, but is rather an inference from an analysis of the available data. Similarly, the improvement in semen quality associated with the change in collection frequency from twice weekly to once weekly, which was significant in the case of the unfractionated-ejaculate volume, cannot be regarded as a definite indication of the optimal collection frequency. The results may have been influenced by a gradual process of habituation of the dogs to the artificial method of semen collection over an extended period.

The failure to obtain more than one or two successive ejaculates from a dog allowing brief rest periods of 5 to 10 min between collections was attributed to the loss of libido, which increasingly accompanied the re-presentation of the artificial vagina, rather than to exhaustion of the genital tract secretions. This may be compared with the finding of Huggins, Masina, Eichelberger & Wharton (1939), who showed that while the canine prostate does not become refractory to pilocarpine, signs of ‘prostatic fatigue’ occur after repeated stimulation.

The beneficial effect of phosphate in prolonging the survival times of dog spermatozoa was apparent, not only from a comparison of the survival times of spermatozoa in the presence and absence of Ringer-phosphate solution with or without additional substances such as alcohol, acetone, ether, and glycerol, but also from the results obtained with an ammonium phosphate diluent. It was, therefore, of interest that the motility of dog spermatozoa at room temperature was better preserved in egg-yolk-citrate than in egg-yolk-phosphate diluent, more particularly since Dubinčik (1934) had ascribed an injurious effect on the spermatozoa of a number of different species, including the dog, to the citrate ion.

ACKNOWLEDGMENTS

Part of this study was carried out while the author was supported by a Scholarship from the Medical Research Council.

The author wishes to thank Dr T. Mann, F.R.S., and Dr R. V. Short, for reading and discussing the manuscript, and Mr A. V. Guntrip for the preparation of the micrographs.
Studies on dog semen I

REFERENCES


Studies on dog semen I

EXPLANATION OF PLATE 1

Abnormalities affecting head and neck regions of dog spermatozoa

- Fig. 1. Macrocephaly.
- Fig. 2. Microcephaly.
- Fig. 3. Double head.
- Fig. 4. Pointed head.
- Fig. 5. Indented head.
- Fig. 6. Opaque head.
- Fig. 7. Free head.
- Fig. 8. Bent head.
- Fig. 9. Swollen acrosome.
- Fig. 10. Thickened neck.
- Fig. 11. Eccentric insertion.
- Fig. 12. Disintegration of neck region.
- Fig. 13. Neck cytoplasmic droplet.

Abnormalities affecting midpiece and tail regions of dog spermatozoa

- Fig. 14. Thickened midpiece.
- Fig. 15. Thinned midpiece.
- Fig. 16. Coiled midpiece.
- Fig. 17. Kinked midpiece.
- Fig. 18. Double midpiece.
- Fig. 19. Bent midpiece.
- Fig. 20. Extraneous material surrounding midpiece.
- Fig. 21. Proximal cytoplasmic droplet.
- Fig. 22. Mid cytoplasmic droplet.
- Fig. 23. Distal cytoplasmic droplet.
- Fig. 24. 'Live' and 'dead' spermatozoa showing grooving of the midpiece of the 'dead' spermatozoon.
- Fig. 25. Thinned tail.
- Fig. 26. Double tail.
- Fig. 27. Treble tail.
- Fig. 28. Coiled tail.
- Fig. 29. Looped tail.
- Fig. 30. Kinked tail.
- Fig. 31. Folded tail.
- Fig. 32. Detached tail.
- Fig. 33. Tail cytoplasmic droplet.