SPERMATORRHEA IN MARSUPIALS, WITH SPECIAL REFERENCE TO THE ACTION OF SEX HORMONES ON SPERMATOGENESIS OF TRICHOUSURUS VULPECULA.

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Since the observation was made some three years ago\(^1\) that the urine of some male marsupials, e.g. Trichosurus vulpecula, Perameles nasuta, Caloprymnys campestris, contained spermatozoa, over a thousand urine specimens from forty male possums or phalangers (Trichosurus vulpecula) and from other marsupials have been examined under normal and experimental conditions. After some remarks on the method of collecting and examining urine specimens, these findings will be described under the following headings:

1. Spermatorrhoea and spermatozoal motility in normal fully grown phalangers and other marsupials.
2. The action of gonadotropic hormone on spermatogenesis.
3. The action of oestrogens on spermatogenesis.
4. The action of testosterone on spermatogenesis and spermatozoal motility.
5. The action of progesterone on spermatogenesis.

Method of Collecting and Examining Specimens.

In most instances the vestigial cloaca present in marsupials does not interfere with the collection of urine free of faeces. As pointed out before, urine can be obtained by pressing on the lower part of the abdomen or by placing the animal in a metabolism cage.\(^1\) For Trichosurus vulpecula the quickest and most effective method is to have the animal held by an assistant and to grasp the bladder through the abdominal wall. Provided it contains some urine, the bladder is easily located and pressure on it initiates urination. The urine is best collected in a centrifuge tube. Sometimes it is advisable to collect the urine in two or more consecutive tubes because the first few millilitres voided may contain large amounts of phosphate and carbonate which may hide many of the spermatozoa from view. If it is only a question of numbers of spermatozoa present, phosphates and carbonates can be dissolved by adding a trace of acetic acid (20%). In a urine treated in this manner the spermatozoa can be counted in a counting chamber for blood-cells, there being usually no other obstructing materials present except a few so-called prostatic bodies. In the present investigation, however, counting was rarely done and in the first instance the urine was examined for the presence or absence of spermatozoa. For this purpose a drop of the uncentrifuged urine was examined microscopically and the urine was considered to contain a normal amount of spermatozoa if more than five spermatozoa were found to be present in a low power microscopic field. In a urine of average concentration anything below five was considered as probably abnormal and a fresh specimen obtained a few hours later was examined to verify this impression.
The spermatozoa were also examined for motility in fresh unacidified urine kept in a stoppered vessel at room temperature and at 38° C.

The differences in motility have been recorded as:

(a) Strongly motile.
(b) Motile.
(c) Vibrating.

(a) Strongly motile spermatozoa moved rapidly across the field of the microscope.
(b) Motile spermatozoa showed intense movement of the body and the tail but swam about the field only sluggishly or not at all. Strong motility and motility could be readily observed under low power magnification (×70 approximately).
(c) Motility of a vibrating nature could only be seen distinctly under higher power magnification (×370 approximately). This was usually a rapid oscillating movement sometimes more pronounced in the head and body and sometimes more pronounced in the tail of a stationary spermatozoon.

(1) Spermatorrhœa in Normal Fully-grown Phalangers.

On examining the urine of adolescent male possums (Trichosurus vulpecula) kept in captivity, it was noted that spermatozoa began to appear in it only when the animal, judging by its bodyweight and its appearance, such as size of testicles, was fully grown. This was at an age of more than one year, and usually less than two years. For the first few weeks spermatorrhœa was somewhat irregular, i.e. a few spermatozoa were present in one specimen and absent in the next one. But after this initial period, spermatozoa in large amounts were found in any specimen of urine measuring 1 ml. or more, obtained at any time of the day or any time of the year. In the great majority of cases they numbered about from 10-100 in any low power field examined under a microscope, the number depending to a certain extent, on the dilution of the urine and as to whether the specimen was obtained from a very full or only slightly filled bladder. In the case of a full bladder, spermatozoa were much more numerous in the first 10 ml. than in the last 10 ml. out of the total volume of about 50 ml. Most of the spermatozoa voided with the urine were usually of normal appearance. When viewed on the flat they exhibited the characteristic arrow or spade-like head (Fig. 1). Identical findings were obtained from a kangaroo rat (Pseudoxyprymnus rufescens) observed for a period of one year.

Spermatozoal Motility.—Originally it was stated that the majority of spermatozoa obtained in the urine are non-motile and that only a very few may be still moving about in a sluggish fashion. However, after studying the spermatozoa carefully under high power magnification (×370 approximately) it was noted in addition to the rare motility observed under low power magnification that in the majority of specimens many more and sometimes as many as half of the spermatozoa present showed a form of motility which has been designated as "vibration". These spermatozoa were stationary, but their bodies showed fast vibrating movements which sometimes were pronounced in only a part of the spermatozoon, such as the region of the head or the tail. If the urine was kept in a stoppered vessel these oscillations persisted for about 1 to 4 hours at room temperature. Cooling the urine down to 4° C. and warming it up again to 38° C. did not materially influence longevity.

Finally "strong motility" and widespread "motility" were also observed when a special attempt was made to study spermatorrhœa during the main breeding period in autumn. On fine days following a week of rain late in March, 1942, it was observed that all the five male possums kept in the laboratory exhibited widespread spermatozoal motility. Varying numbers of spermatozoa
exhibited strong motility, i.e. they moved about at great speed in the drop of urine examined. The longevity of these spermatozoa in urine was remarkable because in some instances motility was still observed nine hours after voiding the urine.

Fig. 1. Spermatozoon and some débris from fresh urine of Trichosurus vulpecula, unstained. Magnification ×1,000 approx.

This qualitatively and quantitatively increased motility persisted for about 1-2 weeks. During this period the possums in the laboratory mated and those obtained from the bush were pregnant, as evidenced by the appearance of pouch young in the following week.

In this connexion it may also be mentioned that males may be fertile at least twice or probably several times during the year. For example, it was observed in our laboratories that one male sired two successive young in the same female in the same year. Furthermore, pregnancies were observed which did not refer to the general breeding seasons in autumn and spring. This would indicate that at least some males must be capable of producing fertilisation in between breeding seasons.

Phalangers and other marsupials possess comparatively large testicles which on histological examination appear to be producing large numbers of spermatozoa throughout the year. A great number of them can be stored in the epididymis but only few in the vas deferens, which in Trichosurus vulpecula and other marsupials is a narrow tube without an ampulla. There is, however, a definite enlargement in the lumen of the prostatic urethra beginning only a few mm. below the bladder neck at the point where the vas deferens enters the prostatic urethra and extending through the greater part of this organ. On examining the contents of this enlarged section of the urethra numerous spermatozoa were found to be present, and it may be quite probable that spermatozoa emerging in a more or less even flow from the vasa deferentia are stored in this widening and are flushed out on micturition. However, even the bladder may under certain conditions act as a storehouse for spermatozoa, since they were always found in considerable amounts in the urine contained in this organ in animals which had been killed. The entry of spermatozoa into
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the bladder could be explained by the very close position of the openings of the vasa deferentia in relation to the bladder opening. In addition to this flushing out effect, it is also quite probable that on micturition larger numbers of spermatozoa are expelled from the vasa deferentia into the uro-genital canal, where they would mix with the urine coming from the bladder.

(2) The Action of Gonadotropic Hormone on Spermatogenesis.

Two immature male possums of six and ten months of age respectively were given 4 injections of 250 international units of chorionic gonadotropic hormone obtained from human pregnancy urine (Gonan B.D.H.) over a period of three weeks. The younger animal (bodyweight 0.73 kg.) being too immature, showed no definite response and did not develop spermatorrhoea within the next four months.

The older animal (body weight 1.5 kg.) was still a typically immature specimen as indicated by its bodyweight, size of testes and general appearance. The testicles were about the size of a large pea (approximate diameter 0.7 cm.) and the urine contained no spermatozoa. Six weeks after beginning the injections a few spermatozoa were observed in the urine. At this stage the animal was still typically immature in appearance (body weight 1.6 kg.) but the scrotum was elongated and its vessels engorged. The testes were very firm and had increased in size (diam. 1.1 cm.). During the next fortnight approximately 1-6 spermatozoa were observed per low power microscopic field, and a few specimens contained none, but after this spermatorrhoea became as marked and as consistent as in fully grown animals. Increased numbers of them were motile and many vibrating. Simultaneously the animal showed rapid general development, and two months after beginning the injections it was found to weigh 2.4 kg. Controls of similar age did not exhibit spermatorrhoea at any stage of the experiment. Therefore it was concluded that gonadotropic hormone brought on precocious spermatorrhoea.

Similar amounts of gonadotropic hormone were injected into fully grown animals. No definite response was noted, but it was felt that the number of spermatozoa had increased on account of the injections.

(3) The Action of Estrogens on Spermatogenesis.

On previous occasions it has been shown that oestrogens, natural (oestrone, oestradiol esters) or synthetic (stilboestrol, hexoestrol) inhibit spermatogenesis. However, to bring on this result large doses are required which seriously interfere with the general health of the animal and frequently lead to its death. For example, the weekly injection of 0.5 mgm. of stilboestrol over a period of 5 weeks (total amount injected 2.5 mgms.) did not diminish spermatorrhoea while the total injection of 7 mgms. administered over a period of 3 weeks led to complete suppression of spermatorrhoea. This animal, however, died from the toxic effects of the stilboestrol and on microscopic examination of testes no spermatozoa were seen. A further experiment (S51) where 4.5 mgms. were given over a period of 3 weeks resulted in only a temporary loss of spermatogenesis. This and another experiment (H50), although mentioned in a previous paper will be described individually because they represent features not yet sufficiently emphasised.

Experiment S51.—The course of this experiment has been described for a period of seven months. The injection of stilboestrol (1.5 mgm. per week for three weeks) brought on testicular ascent. However, in the sixth month of the experiment the external signs of the action of the hormone such as loss of weight and scrotal and testicular atrophy had mostly disappeared and spermatozoa were produced as evidenced by spermatorrhoea. At first they were not numerous and frequently they appeared to be deformed. Within a week, however, they
were as numerous as in controls, but in contrast to the controls, motility and markedly increased vibration were observed. To test the fertility of the animal it was mated with a female which shortly afterwards was found to be pregnant.

Experiment H50.—This animal received injections of 2 mgm. of hexaestrol twice a week over a period of four weeks. Spermatorrhoea was present before the injections were begun and persisted during the first two weeks. For a period of three months after this no spermatozoa were observed in the urine; then spermatozoa began to reappear in the urine, though in small numbers and frequently abnormal in size and shape. Within a fortnight after their reappearance, however, the spermatozoa present in the urine were of approximately normal number and appearance. With regard to motility it was observed in some specimens that for a period of about three weeks, actively motile spermatozoa were present, as well as an exceptionally large number of vibrating spermatozoa in comparison with controls. This temporarily increased, motility subsided again, but spermatorrhoea remained a constant feature as long as the animal was observed, which was a period of 12 months.

These experiments demonstrated that the restoration of spermatogenesis after its inhibition by oestrogens was a permanent one. After the re-establishment of spermatorrhoea, fertility was not interfered with and a temporarily increased motility of the spermatozoa might even indicate increased activity of the gonads.

(4) The Action of Testosterone Propionate on Spermatogenesis and Spermatozoal Motility.

As pointed out before(4) testosterone propionate, when given in large doses over a period of several weeks, may diminish or abolish spermatorrhoea.

However, when only two or three weekly injections of 4-8 mgms. of testosterone propionate had been given, increased motility of the spermatozoa was frequently noted. These findings were made in between breeding seasons on a group of five males and increased vibration lasting for several hours was present in the majority of the urine specimens examined. Many samples showed motility which, in some cases, was observed in practically every spermatozoon. A small number of specimens showed strong motility, i.e. both at room temperature and 38° C. many of the spermatozoa moved about at great speed in the drop examined. In a few cases this strong motility was observed for hours and in one case motility was still observed ten hours after voiding the urine. Generally, in these urine specimens, the spermatozoa behaved as they did during a breeding period described in an earlier part of this paper.

However, as already pointed out the majority of specimens examined after the administration of testosterone propionate, showed only increased vibration or no increase in spermatozoal activity at all. Notwithstanding this fact it was concluded that the markedly increased motility, although only sporadic, was due to the administration of testosterone propionate.

(5) The Action of Progesterone on Spermatogenesis.

On a previous occasion(5) it has been reported that progesterone produces a typically androgenic reaction on the adolescent but almost mature male phalanger. After the weekly injection of 2 mg. of progesterone over a period exceeding two months the penis of these animals was found to be of increased size and protruding from the cloaca for a distance of 3 to 4 cm., while in controls of similar age the copulatory organ is hidden retracted in a preputial fold within the cloacal hillock. As soon as this precocious behaviour was noted in the treated animals, their urine was searched for spermatozoa but with negative results. However, spermatozoa began to appear in the urine at about the same time as they did in untreated controls of similar age; viz. about three months
after the onset of the penial protrusion and as in the untreated animals most of the spermatozoa were not motile. The injections of progesterone were kept up for a period of six months and at frequent intervals the urine was examined. Spermatozoa were always found to be present in approximately the usual amount and predominantly in a non-motile state as in controls. Finally the animals were killed and at post-mortem examination the prostate was found to be about 50% larger than in normal controls.

**Discussion.**

In the order Marsupialia it has been established for at least two species of its sub-order Diprotodontia, e.g. *Trichosurus vulpecula* and *Euprymna rufescens*, that spermatozoa are excreted in the urine throughout the year. Many of these still show signs of life though suspended in normal urine, and they can be obtained very easily in any amount without ill effects on the donor. This constant voiding of spermatozoa is in marked contrast to the short period of ejaculation of spermatozoa in higher mammals where those spermatozoa which are not ejaculated disintegrate and are ultimately reabsorbed. On the other hand, the release of spermatozoa as observed in the marsupial relates to non-mammals such as reptiles, where the testes discharge their products to a part of the original kidney. The original kidney duct (Wolffian duct) is employed as a vas deferens. In reptiles, therefore, spermatozoa are transported in the urinary flow and must arrive in the cloaca mixed with urine.

In marsupials the vasa deferentia connecting with the extraabdominally situated testicles link up with the urinary tract below the bladder as in higher mammals. Nevertheless the spermatozoa, with a possible exception of the short period of copulation, still depend on the urine as a medium of transport and excretion as in reptiles. It is not known yet how spermatozoa are transported during the act of copulation.

The study of the action of sex hormones on spermatorrhoea of *Trichosurus vulpecula* supports the contention that spermatorrhoea gives a true picture of the state of spermatogenesis of the animal examined, because these findings are essentially similar to those reported on spermatogenesis in non-marsupials, where usually the testes have been examined histologically in order to investigate the production of spermatozoa.

For example, after the injection of chorionic gonadotropin in adolescent *Trichosurus vulpecula*, spermatozoa were observed about three months earlier than they appeared in controls. This undoubtedly was due to precocious spermatogenesis which also has been reported to occur after the administration of gonadotropic substances, e.g. in certain lizards as well as in ground squirrel. The atrophic actions of large doses of oestrogen on the testes are well recognised, and in rodents the cessation of spermatogenesis has been reported after their administration. This has been confirmed in numerous experiments on possums. The outstanding finding in the present report is the observation that complete recovery of spermatogenesis together with proven fertility occurs within one month after the reappearance of spermatorrhoea. The temporary increased motility observed shortly after recovery of spermatogenesis is noteworthy and in view of the findings after the administration of testosterone, one might assume a late androgenic manifestation of oestrogens.

Repeated injections of testosterone propionate impair the function of the testes as pointed out by Moore and Price, as is the case with oestrogens. In our marsupial, *Trichosurus vulpecula*, spermatorrhoea sometimes ceased after the repeated injection of large doses of testosterone propionate, doses which in no way affected the general health of the animal.

The increased motility observed after a few testosterone injections, though marked, was only sporadic. This was interpreted to mean that testosterone
represents only one factor in several necessary to maintain motility and that on comparatively rare occasions all the factors, as for example the correct concentration of the hormone, the correct pH, the optimum amount of prostatic secretion, etc., are present to bring on and maintain the strong motility. The question as to whether such motile spermatozoa suspended in urine as well as lesser motile ones may be fertile, could only be solved by experiments with artificial insemination. Such experiments, however, have not yet been performed.

Summary.

1. In fully grown healthy male marsupials such as Trichosurus vulpecula and Epyprymnus rufescens, spermatorrhœa is constantly found throughout the year and is directly related to spermatogenesis. Usually the majority of the spermatozoa voided in the urine are non-motile, but a large percentage of them, though stationary, may show a fine vibratory movement under high power magnification. Actual motility visible under low power magnification is rarely seen except during breeding season, when strong motility is also observed. In such urine specimens a number of spermatozoa remained motile "in vitro" for more than nine hours.

2. Gonadotropic hormone obtained from pregnancy urine when administered to immature animals hastens spermatogenesis.

3. Large doses of oestrogen abolish spermatogenesis as shown by the loss of spermatorrhœa and absence of spermatozoa in the testes. If the animal survives the ill effects due to the administration of oestrogen, spermatogenesis, as well as fertility, are re-established in about 3-5 months.

4. The injection of male sex hormone may confer widespread and sometimes even strong motility on the spermatozoa voided in the urine. The injection of frequently repeated doses of testosterone may diminish or even abolish spermatogenesis.

5. Progesterone does not seem to have any effect on spermatogenesis.

6. These findings have been discussed in brief and marsupial and reptilian spermatorrhœa have been compared.

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