COMPARISON OF MOTILITY PATTERNS OF SPERM ASPIRATED FROM AMPLECTANT PAIRS OF *XENOPUS LAEVIS*, BY ELECTRO-EJACULATION AND FROM THE TESTES

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Studies on anuran spermatozoa have been restricted to the use of testicular sperm. Although these studies have been effective in creating baseline data, the motility parameters of ejaculate spermatozoa could present a clearer picture of the reproductive physiology. Methods were devised to aspirate sperm from amplectant males and collect the ejaculate from males. Sperm motion was then studied quantitatively and the various sperm motion parameters compared. Further research is necessary to determine whether Wolffian duct and cloacal secretions play a role in altering sperm motion characteristics of anuran testicular spermatozoa.

INTRODUCTION

Most studies regarding amphibian sperm physiology are concerned with testicular sperm. Anurans do not possess epididymides and previous researchers such as Poirier & Spink (1971) and Reed & Stanley (1972) have assumed that sperm maturation is completed in the testes. Evaluation of the sperm motion characteristics indicated that the anuran spermatozoa are adapted for fertilization in their respective fertilization environments (Wilson, 1994). Studies pertaining to the fertilizing capacity and motility of anuran spermatozoa should be undertaken on ejaculate sperm to effectively evaluate the motility parameters.

Xenopus laevis is widely used as a laboratory model for anatomical and physiological studies and earlier researchers laid the basis for studies of the reproductive biology. *X. laevis* is regarded as one of the more primitive aquatic breeders (Passmore & Carruthers, 1979) and the data assembled from these toads can be used in research of more advanced anurans. Wolf & Hedrick (1971) used *X. laevis* to investigate the viability and *in vitro* fertilization of the gametes. Grey, Wolf & Hedrick (1974) enhanced our knowledge on fertilization in *Xenopus* by investigating the fertilization envelope. Wolf (1974) developed a method to recover cortical granules which prevent polyspermic fertilization from *Xenopus* eggs. Spannhof, Fiedler, Schlisio & Jürss (1976) investigated osmotic and ionic regulation in *X. laevis*. A low osmolality is necessary for the initiation of sperm motility in *X. laevis* (Inoda & Morisawa, 1987).

Anuran reproductive tracts undergo characteristic seasonal changes with regard to the size of the testes and reproductive accessories (Mann & Lutwak-Mann, 1963). In this study we attempted to simulate these changes via peptide injections with human chorionic gonadotrophin (hCG). *X. laevis* toads were injected to go into amplexus and the ejaculate sperm collected. An electro-ejaculation technique was also used to collect sperm from the cloacae of the males.

The purpose of our study was therefore to develop techniques and present data pertaining to the motility characteristics of ejaculate *Xenopus* spermatozoa. The research included the following:

(1) Techniques were devised to aspirate and collect spermatozoa from amplectant males, electro-ejaculated males, or from dissected testicular tissue.

(2) The swimming characteristics and percentage motility of the spermatozoa obtained via the various techniques were then compared.

(3) Determination of the osmolality and pH of the medium in which the spermatozoa are ejaculated. Osmolalities of the cloacal fluid and sperm suspensions were also determined.

MATERIALS AND METHODS

Xenopus laevis adults were obtained from a breeding farm in Cape Town. Twelve females and fifteen males were used. They were kept in a steel tank at temperatures ranging from 18° to 25°C. The water in the tank was changed every second day. The animals were fed every morning between 10.00 and 11.00 hr with fish food pellets.

ASPIRATION OF SPERM

Male and female toads were kept apart in separate fish tanks with the water at a constant 23°C for 24 hours prior to the injections. A disposable syringe with a bent needle, was used to administer the hCG (pregnyl) as described by Brown (1970). Three pairs of toads were used each time for sperm aspiration. The female toads were given a primer injection of 250 i.u. of pregnyl. The males were given a primer of 150 i.u. Males and females were kept apart at a constant temperature and after 48 hrs the booster injections were administered. Females were given a booster of 300 i.u. and males 200

i.u. The injections were administered subcutaneously in the dorsal lymph sacs. The toads were then randomly paired off and placed in the two fish tanks.

Different dosages of pregnyl were also compared. Higher dosages in females (up to 350 i.u. as primer) induced them to deposit eggs within an hour of the primer injections. It was found that with dosages as high as 300 i.u. (booster injection) in males, amplexus was induced, but throughout amplexus no sperm was ejaculated.

Collection of sperm from amplectant males in the tanks. Males started their mating calls from the water approximately one hour after the primer injections. The nuptial pads on the hands of the males could be discerned approximately five hours after the primer. Within an hour after the booster injections the toads went into amplexus. *Xenopus* has the primitive inguinal clasp and the toads could stay in amplexus for up to 30 hrs.

During the time of amplexus the toads were closely observed for changes in their movements. The females exhibit a jerky swimming movement when they are ready to deposit their eggs. The male bends his back so as to bring his cloaca nearer to that of the female. The distance from the cloaca of the male to that of the female normally ranges from 19.6 to 27.4 mm. When the female goes into her egg-laying swimming routine, the male brings his cloaca to within 5 mm of that of the female. The eggs were released by the female, passed along the abdomen of the male and induced the male to ejaculate. This ejaculate was collected with a Gilson pipetman pipette.

Collection of sperm from amplectant males in chamber or bag. We devised a chamber, which we called the Lewis chamber, to house an amplectant pair of toads. It was made of perspex with holes in the casing to provide air. The chamber was 30 cm deep, 30cm long and 20 cm wide with a double bottom. The top part of the bottom of the chamber sloped inwards into a funnel-like structure so that the sperm and fluids could run down to the true bottom from where the sperm were collected via a pipette. This was done to collect pure ejaculate without the water as collected in the fish tank. The Lewis chamber yielded poor results as the holes in the casing caused the chamber to have the same effects as a desiccator, dehydrating the toads.

Another method used to obtain pure ejaculate, was to put the amplectant pairs in sealed A4 size plastic bags. This method yielded better results than the Lewis chamber. The ejaculate obtained by this method, however, contained mucous from the toads. It is possible that the effect of the sealed bags cause stress in the animals.

Electro-ejaculation. Male toads were injected with 250 i.u. pregnyl 12 to 24 hrs before sperm collection. They were then anaesthetized with MS222 and the abdominal cavity exposed. A stimulating electrode was connected to a Harvard stimulator using multiple pulses (50 Hz at 6 to 8 Volts). The testes and Wolffian ducts were stimulated for approximately 10 seconds at a time.

The best results, pertaining to amount of sperm aspirated, were obtained when the testes were stimulated. Spermatozoa were subsequently collected with a Gilson pipetman pipette from the cloaca.

All the spermatozoa sampled were suspended in a 10% Ham's F10 solution. This is a nutrient medium with an osmolality of approximately 300 mOsm/kg. Different dilutions of Ham's F10 were used to test the effect of osmotic concentrations on the motility of the spermatozoa (Wilson, 1994). It was found that the vigour of sperm motility and the percentage motile sperm decreased rapidly in suspensions with an osmolality higher than 50 mOsm/kg and no sperm motion could be detected in suspensions higher than 200 mOsm/kg. These results are in line with the findings of Inoda & Morisawa (1987), and Bernardini, Andrietti, Camatini & Cosson (1988), that effective sperm motion of these anurans is initiated by low osmolalities. Ham's F10, diluted in distilled water to 10%, had an osmolality of 30 mOsm/kg and this suspension was found to be ideal for studying anuran sperm motion (Wilson, 1994).

After spermatozoa were collected by electro-ejaculation, the testes were removed and spermatozoa aspirated from them by squashing them between microslides.

ASSESSMENT OF THE MOTILITY PATTERNS

Five µl of sperm were suspended in 5 ml 10% Ham's F10 solution in a petri dish by placing the sperm in the centre of the solution in the petri dish. Sperm were allowed to swim out towards the periphery of the dish. This technique we called the swim out technique. One ml of suspension was then collected from the periphery of the petri dish and placed in a sperm chamber. The sperm chamber is made from perspex and is 8 cm in length and 1 cm thick. It has a cavity in the centre approximately 8 mm deep. The suspension in the cavity is then covered with a coverslip which can be screwed in position.

The sperm chamber was placed on an inverted Zeiss microscope (ICM 405) using the 16X phase contrast objective. Every 15 min for up to 5 hrs the swimming patterns of the spermatozoa were videotaped. At each videotaping session at least four different fields were taped. The swimming patterns of at least 75 spermatozoa of each specimen were videotaped at the different time intervals. Davis & Katz (1989) proposed that between 50 to 200 spermatozoa be used for computer-aided sperm analysis. Analysis of the sperm swimming patterns was done with the Sperm Motility Quantifier (SMQ) (Wirsam Scientific, Pty LTD).

The SMQ is a Computer Assisted Sperm Motility Analysis (CASMA) system, developed by Van der Horst (1992). Seventeen motility parameters as well as sperm density measurements can be assessed by this system in both the automated and manual modes. The videotaped swimming patterns of the spermatozoa were played back via a VCR through the SMQ card in a 486 computer, to a TV monitor.

TABLE 1. Interpretation of the sperm motility parameters (Van der Horst, 1992).

Parameter	Abbreviation	Explanation
Curvilinear velocity	VCL	The time average velocity of the sperm head on its actual (precise) path. Expressed in μm/s.
Straightline velocity	VSL	The time average velocity of the sperm head projected along the straight line between its first and final detected positions. Expressed in μm/s.
Average Path velocity	VAP	The time average velocity of the sperm head projected along its spatial average trajectory. Expressed in μm/s.
Linearity	LIN	Ratio of projected length to total length of the curvilinear trajectory. Expressed as %.
Dance	DNC	Space occupied by the sperm head path during 1 sec. Product of VCL and mnALH. Expressed in μm²/s.
Percentage Motile Sperm	PM	Percent motility of sperm population. Represents all forms of motility.

Video images were captured at 3 Hz (Frameskip 4). This means that the image was captured every tenth of a second. Sperm motion was taped for 8 seconds at a time (As *Xenopus* sperm are slow-moving it was possible to keep individual sperm in focus over this time interval - about 12 spermatozoa were visible per field). Automatic evaluation of the motility parameters was then performed. Data on the motility patterns were stored as text files (.DXP and .PXP) and imported to a spreadsheet for further analysis.

OSMOLALITY AND PH MEASUREMENTS

Osmolality measurements were made on a Wescor 5500 Vapor Pressure Osmometer using Wescor sample discs type SS-033 and only required a sample of 7 to 8 il. The pH readings were done on a Beckman Zeromatic IV pH meter and on a pH M80 portable pH meter No. 64R53N46.

Measurements were done on the following suspensions to determine the osmolalities and pH of the micro-fertilization environment into which the spermatozoa are deposited: (1) eggs and mucous in the plastic bag; (2) fluid extracted from the cloaca of the female; (3) eggs and tank water; (4) tank water; (5) eggs and fluid in the Lewis chamber

RESULTS

The motility characteristics of the spermatozoa aspirated by the methods described were analysed and statistically compared. For the purposes of this study the following parameters were statistically compared: VCL; VSL; LIN; VAP; DNC; and PM (Abbreviations

as described in Table 1). Table 2 and Figs. 1 and 2 detail the differences and similarities of the various motility parameters of the sperm aspirated via the different methods.

Fig. 1 indicates the high velocities of sperm from the amplectant males. It is clear from the graph that these values differ significantly from values obtained from testicular sperm. The velocities of the sperm aspirated via electro-ejaculation and sperm from the testes after electro-ejaculation, are more or less similar.

Multivariate analyses were performed using star symbol plot analyses (Fig. 2). This method analyses data by presenting them as rays in a star symbol. The lowest value of a particular parameter is calculated and expressed as a ray 10% the length of the highest value. All the data present in a single set of star symbols are
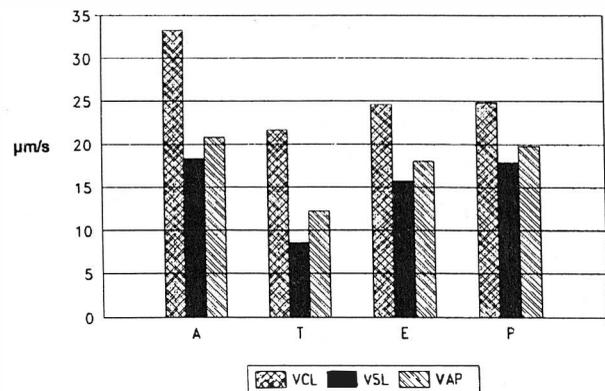

FIG. 1. Comparison of the velocities of the sperm aspirated by different methods.

TABLE 2. Data on the motility parameters of sperm aspirated by different methods. A, sperm obtained from amplectant males; T, testicular sperm (toads not subjected to pregnyl injections); E, sperm obtained via electro-ejaculation technique; P, sperm obtained from testes after pregnyl injections and electro-ejaculation. Values shown are means±SD.

	A	T	E	P
VCL (μm/s)	33.2±12.6	21.7±7.7	24.6±10.2	24.8±6.6
VSL (μm/s)	18.3±13.2	8.6±3.5	15.7±9.9	17.9±6.7
LIN (%)	49.7±21.3	41.3±14.3	60.6±19.4	70.7±16.5
VAP (μm/s)	20.8±12.7	12.2±3.7	18.0±9.8	19.8±6.1
DNC (μm²/s)	61.8±40.8	62.4±63.5	30.0±23.4	28.9±16.7
PM (%)	27.5±2.3	12.9±4.6	48.3±3.4	15.1±2.8

FIG. 2. Star symbol plot analysis of motion parameters of sperm aspirated by different methods.

therefore compared. These star symbols were used as a multivariate test to graphically compare patterns in sperm motility parameters. The star symbol plots indicate similar patterns of the electro-ejaculate and testicular sperm after hCG (pregnyl) injections. The star symbol plot reflecting the values for sperm from amplectant males displays the high values of these spermatozoa, whilst the plot for testicular sperm reflects their low overall values.

The osmolalities of the different solutions and pH values are recorded in Table 3.

DISCUSSION

It is problematic to obtain an ejaculate from submammalian vertebrates and invertebrates. Anuran spermatozoa are thought to mature in the testes (Poirier & Spink, 1971; Reed & Stanley, 1972) because of the lack of epididymides. To date, the reproductive biology of frogs and toads has primarily involved investigations on testicular sperm. This study was undertaken to determine whether viable sperm can be obtained by using different techniques to aspirate sperm via an ejaculate. Sperm motility parameters were used as criteria to ascertain the viability of ejaculate sperm.
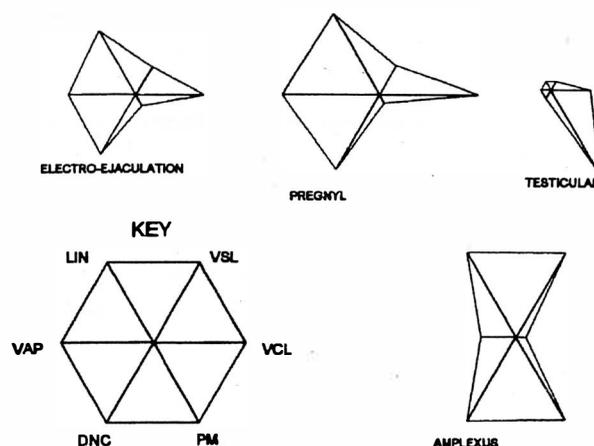
Ham's F10 as medium to assess sperm motility was found to be ideal (Wilson, 1994). Bernardini, Andrietti et al. (1988) found the duration of *Xenopus* sperm motility to be less than 10 mins. We still found motile sperm in the 10% Ham's F10 suspension after 5 hrs of swim out (Wilson, 1994).

Xenopus has a very low percentage of motile testicular sperm. This is in accord with the findings of Van der Horst (1976). The percentage motile sperm aspirated from the testes after pregnyl injections and electro-ejaculation showed no significant difference with "normally aspirated" testicular sperm. The higher percentages of motile sperm in the electro-ejaculate and ejaculate from the amplectant males (Table 2) differed significantly from testicular sperm ($P < 0.01$). These differences may indicate that maturation changes take place in the reproductive ducts during ejaculation. In the testes, the spermatozoa are neatly packaged and embedded in Sertoli cells (Wilson, 1994). Maturation is not yet completed and this may possibly be one of the reasons for the low percentage of motile testicular spermatozoa. Another factor may be the low percentage of normal spermatozoa in the testis of the toad (Wilson, 1994).

TABLE 3. Data on the osmolalities and pH values of the suspensions (Mean±SD; range)

SUSPENSION	OSMOLALITY (mOsm)	pH
Tank water	0±1; 39 - 41	7.85±0.13; 7.62 - 7.96
Eggs and tank water	45±6; 41 - 63	7.15±0.06; 7.07 - 7.23
Fluid in plastic bag	112±6; 99 - 126	7.94±0.19; 7.72 - 8.21
Fluid in Lewis chamber	72±1; 71 - 73	7.10±0.08; 7.01 - 7.22
Female cloacal fluid	49±7; 38 - 56	7.94±0.07; 7.82 - 7.99

More significant, however, were the differences in the motility parameters. Analysis of these parameters indicated that the spermatozoa obtained from the amplectant pairs had significantly higher velocities (VCL, VSL and VAP) than sperm obtained from the testes ($P < 0.01$). Bernardini, Belgiojoso & Camatini (1988) concluded that the respiration rate of *Xenopus* spermatozoa is not affected by their status of motility. They used testicular spermatozoa and a comparative study of spermatozoa obtained from males in amplexus would be extremely valuable.

It is clear from Table 2 and Fig. 1 that the spermatozoa obtained from the amplectant males differed significantly from those obtained from the testes for most of the motion parameters. A high value of DNC may be an indication of the viability of the sperm to fertilize ($P < 0.01$). Penetration of the jelly layers surrounding the eggs involves chemical and mechanical means. Spermatozoa normally exhibit a star-spin movement (lashing about of the sperm heads) in the vicinity of the eggs (Yanagimachi, 1988). This is a mechanical movement which helps with the penetration of the egg capsules. A high DNC value could be indicative of this hyperactivation. Normally aspirated testicular spermatozoa and spermatozoa from amplectant males exhibited the highest DNC values (Table 2). The high DNC value of the spermatozoa from the amplectant males could be explained in terms of the mechanisms necessary to penetrate the egg capsules.

Spermatozoa obtained via electro-ejaculation and from the testes of the electro-ejaculated toads displayed significantly higher LIN than those obtained via other means ($P < 0.01$). The stimulation of the testes may be instrumental in the sperm having a high LIN. It did not seem that the pregnyl injections played a role in the spermatozoon linearity as the amplectant males were given pregnyl injections as well. The linearity of testicular spermatozoa and spermatozoa aspirated from amplectant males differed significantly from those of the electro-ejaculated sperm and sperm from testes after pregnyl injections and electro-ejaculation.

It was clear that the motility parameters of the electro-ejaculate spermatozoa and spermatozoa from testes after pregnyl injections, exhibited more similarities. Their pattern of motion also showed similarities. This is portrayed in the star symbol plot (Fig. 2). This could be ascribed to the fact that the spermatozoa analysed were obtained from the same specimens. The small star symbol representing motility parameters of testicular sperm is an indication that these parameters were almost consistently lower than those of the others.

Inoda & Morisawa (1987) in comparing freshwater fishes and anurans in relation to the environment in which reproduction takes place, found that hypo-osmolality stimulated sperm motility in *Xenopus*. Bernardini, Andrietti *et al.* (1988) found anuran sperm motility to be inhibited at osmolarities higher than 200 mOsm/l. The results as reflected in Table 3 indicate the low osmolality of the aqueous environment in which the spermatozoa are released. Suspensions with a low osmolality, such as Ham's F10 (10%) and Toad Zwartkops solutions (Van der Horst, 1986) were found to be ideal solutions in which to initiate sperm motility.

A pH ranging from 7.1 to 7.9 is crucial for fertilization in anurans (Miceli, Fernández, Mansilla & Cabada, 1987; Bernardini, Andrietti *et al.*, 1988; Díaz Fontdevila, Bloj & Cabada, 1991). We found the pH values of the different media in which the spermatozoa were released, to fall within this range.

The results of our study indicated that motile sperm can be obtained from testes, electro-ejaculate, and from amplectant males. Differences in the various sperm motility parameters may reflect physiological differences between sperm from the testes and the ejaculate. It may be an indication that the Wolffian ducts and cloacal secretions play a part in the final maturation phases of the spermatozoa. The higher velocities of the sperm from amplectant males suggested that amplexus is a major stimulus in bringing the spermatozoa to maximum fertilizing capacity. Further analyses regarding the physiology of ejaculate sperm in the anurans are required in order to ascertain the functions of the reproductive tracts and cloacal secretions in the maturation of spermatozoa.

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