OBSERVATION

Study of Morphological and Metric variation in the testis at different Seasons in three species of *Bufo*

Generally in case of family Bufonidae, the breeding season is June to August, April-May is the prebreeding season and September-October is the postbreeding season. From my observation on testicular morphology and metric studies it is clear that in all the three species of *Bufo* there is a generalized increase in size of the testis with the arrival of breeding season. The testes size reaches the peak in the June-August season, indicating their maximum breeding activity at this season. In this period, the testes are engorged with mature and healthy sperms which are to be utilized for reproduction. Of the three species, maximum morphological and metrological variation is observed in the testes of *Bufo melanostictus*, while the minimum variation is observed in *Bufo stomaticus*. (Figure- 3)

From the above observation one particular feature is to be noted that in every species, in every season the size of the right testicular lobe becomes slightly larger than the left. This is a peculiar characteristic feature, which needs further detailed study in anatomical and endocrinological level.

PLATE – 2:

Individual sperm of three different species under light microscope at a magnification of X6000.

A & B→ *Bufo himalayanus*,

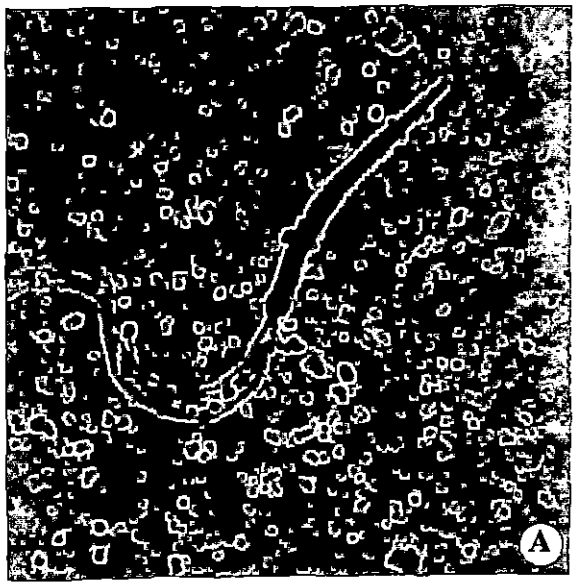
C & D→ *Bufo stomaticus*,

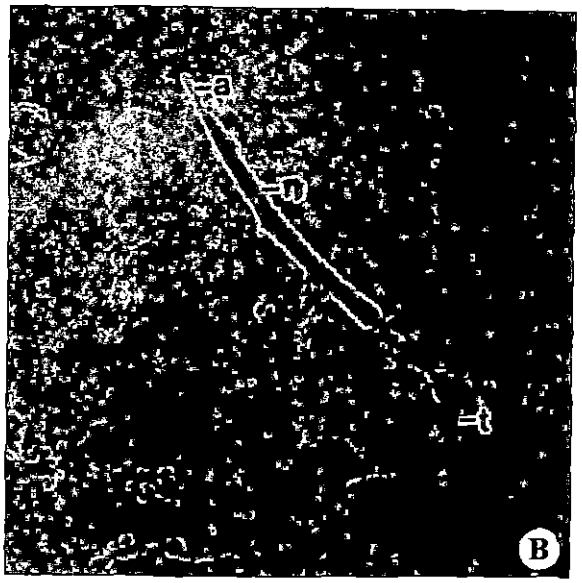
E & F→ *Bufo melanostictus*.

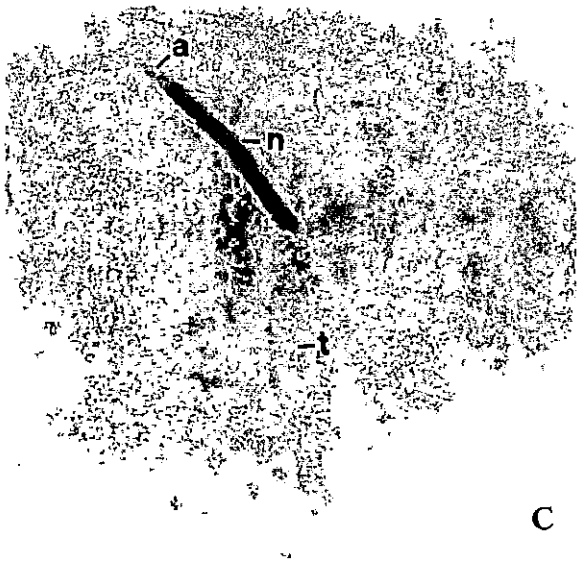
Indication: a- acrosome, n- nucleus, mp- middle piece, t- tail.

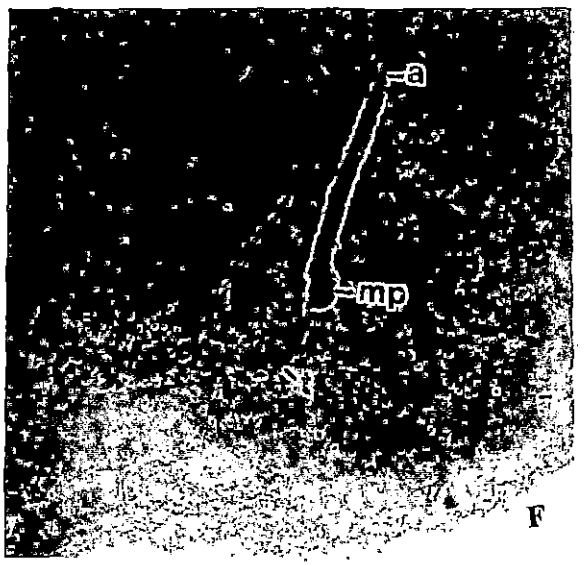
All the species shows biflagellate tail, a distinct acrosome and a flattened post-nuclear middle piece

PLATE – 2









Light Microscopic Observation

The light microscopic observation shows a typical anuran morphology of sperm, i.e. with a slender head, small middle piece and long tail.

B*ufo himalayanus*

The ripe spermatozoon of *Bufo himalayanus* possesses a deeply stained head consisting of a cylindrical nucleus and a pointed needle-like acrosome situated at its anterior tip. The acrosomal tip is slightly curved like a notch (Plate-3, Fig. D). The head of *Bufo himalayanus* is about 29x2µm in size, larger than that of other two species studied and have a sword like appearance. (Plate-2, Fig. A, B ; Plate-3, Fig. A)

Immediately behind the head and in intimate contact with it is a small and equally deeply stained middle-piece where the mitochondria are clumped together (Plate-3, Fig. A).

The tail is biflagellate and about 46µm in length. There are two axial filaments joined together by a rudimentary undulating membrane. Each filament arises from its own centriole, but as both the centrioles side by side at the base of the small deeply staining middle-piece, they cannot always be clearly made out (Plate-2, Fig. A, B ; Plate-3, Fig. A, G).

Bufo stomaticus

The sperm head of Bufo stomaticus is deeply stained nucleus with a pointed less stained needle like acrosome at its anterior tip (Plate-

PLATE – 3:

Light microscopic pictures of individual sperm at X4000

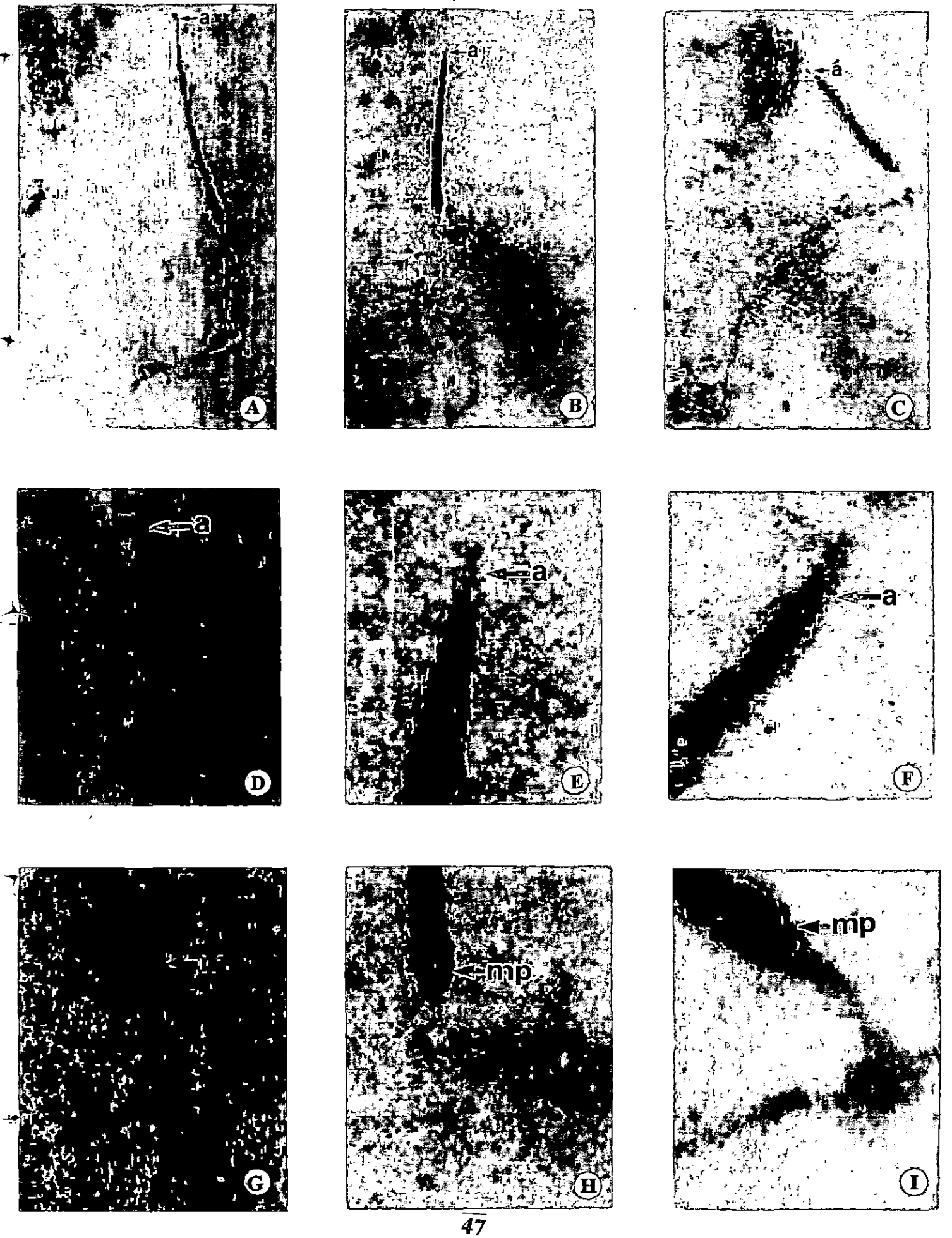
A & B→ *Bufo himalayanus*;

C & D→ *Bufo stomaticus*;

E & F→ *Bufo melanostictus*.

Indication :- a- acrosome; n- nucleus; mp- middle piece; t- tail filament. All the species showing biflagellate tail, a distinct acrosome and a flattened postnuclear middle piece.

PLATE – 3



2, Fig. C, D ; Plate-3, Fig. B, E). It measures 25x3µm in size. The cylindrical head region shows irregular surface morphology.

The deeply stained head is followed by an equally stained slightly flat mid piece from which the tail filaments arises. Mid piece contains irregularly arranged groups of mitochondria which helps in flagellar movement (Plate-3, Fig. B).

The tail of *B. stomaticus* is biflagellar in appearance and 40µm in length. Both the filaments arise as a single fiber and after a distance both the fibers separate distinctly and runs parallel to each other. A fine membrane connects both the fibers throughout the length (Plate-3, Fig. B, H).

Bufo melanostictus

In *Bufo melanostictus* the head is cylindrical elongated having a size of 28x3µm. The head hears a darkly stained nucleus and a pointed acrosome at its tip (Plate-3, Fig. F). Surface morphology of the head is smooth (Plate-2, Fig. E, F ; Plate-3, Fig. C).

The head is followed by an equally stained middle piece from where the tail filament arises (Plate-.3, Fig. C, I).

The tail in *B. melanostictus* if biflagellar in organization and both the filaments arise from a single point in the middle piece. The tail length is 46µm, but the undulating membrane in between the tail filaments is not well visualized (Plate-2, Fig. E, F ; Plate-3, Fig. I).

A wide range of morphological variations are observed in the three species of bufonids (Table- 1,2,3).

Using the detailed metric study, following classes of sperm morphology can be recognized.

In ***Bufo himalayanus,*** (Table-1) about 66% of the sperm head is about 28x3um in size while 28% of the sperm head class has the

TABLE - 1

Metric details of *Bufo himalayanus* sperm (in μm)

SL No	Head Lth.	Head Dtr.	Tail Lth.	SL No	Head Lth.	Head Dtr.	Tail Lth.
1	32	3	40	26	28	2	40
2	32	2	48	27	28	2	24
3	28	2	56	28	32	2	48
4	28	3	56	29	28	2	60
5	32	2	52	30	32	2	56
6	24	2	56	31	32	2	40
7	32	2	60	32	28	2	52
8	32	2	52	33	24	2	32
9	32	2	56	34	28	2	48
10	28	2	48	35	28	2	48
11	28	2	48	36	28	4	40
12	32	2	56	37	28	2	56
13	32	2	40	38	28	2	48
14	28	3	48	39	28	2	56
15	24	2	48	40	28	2	56
16	28	2	56	41	28	2	56
17	28	2	20	42	28	2	48
18	28	2	56	43	28	2	32
19	32	2	64	44	28	2	56
20	28	2	28	45	28	2	56
21	28	2	36	46	28	2	56
22	32	2	24	47	28	2	32
23	28	2	60	48	28	2	56
24	32	2	48	49	28	2	40
25	32	2	48	50	28	2	48

CALCULATION

Head length	Occurance
32	28%
28	66%
24	6%

Tail length	Occurance
I I-20	2%
21-30	6%
31-40	20%
41-50	26%
51-60	44%
61-70	2%

Total length	Occurance
21-40	0%
41-60	14%
61-80	44%
81-100	42%

MEAN HEAD LENGTH- 28.88μ m ± 2.16

MEAN TAIL LENGTH- 46.00μ m ± 10.63

MEAN TOTAL LENGTH- 75.64μ m ±13.88

TABLE - 2

Metric detalis of *Bufo stomaticus* sperm (in μm)

SL No.	Head Lth.	Head Dtr	Tail Lth.	SL No.	Head Lth.	Head Dtr.	Tail Lth.
1	24	8	20	26	28	2	12
2	24	4	40	27	28	2	20
3	8	4	60	28	28	3	40
4	28	3	48	29	28	2	40
5	28	3	16	30	28	2	48
6	28	3	40	31	28	3	20
7	28	3	40	32	28	6	40
8	28	3	44	33	28	2	20
9	12	4	12	34	28	2	40
10	24	3	48	35	28	2	36
11	28	2	32	36	28	2	16
12	24	3	48	37	28	2	40
13	12	4	20	38	28	2	24
14	28	2	32	39	28	2	40
15	24	3	48	40	28	2	36
16	28	3	40	41	24	2	40
17	8	5	32	42	24	2	32
18	28	3	40	43	28	2	60
19	28	3	32	44	24	2	40
20	28	3	40	45	28	2	44
21	28	2	24	46	28	3	40
22	24	3	40	47	20	4	40
23	6	6	28	48	24	2	20
24	24	3	36	49	28	2	40
25	24	3	32	50	28	2	42

CALCULATION

Head length	Occurance	Tail length	Occurance	Total length	Occurance
28	64%	11-20	22%	21-40	10%
24	24%	21-30	06%	41-60	32%
2O	02%	31-40	54%	61-80	56%
12	04%	41-50	14%	81-100	02%
08	02%	51-60	04%		
		61-70	00%		

MEAN HEAD LENGTH-25 μ m ± 5.6 ±
MEAN TAIL LENGTH – 32.2 μ m ±10.78
MEAN TOTAL LENGTH -60.16 μ m ±14.00

TABLE - 3

Metric details of *Bufo melanostictus* sperm (in µm)

SL No	Head Lth.	Head Dtr.	Tail Lth.	SL No.	Head Lth.	Head Dtr.	Tail Lth.
1	32	3	60	26	32	3	48
2	28	3	16	27	28	2	40
3	28	3	40	28	32	3	56
4	28	3	48	29	32	3	48
5	28	3	60	30	32	3	48
6	16	5	56	31	32	4	40
7	24	2	48	32	32	3	32
8	28	3	32	33	32	3	56
9	32	3	40	34	32	3	40
10	32	2	40	35	32	2	32
11	28	2	64	36	28	3	48
12	20	2	44	37	32	4	56
13	32	3	32	38	28	4	56
14	32	3	48	39	32	3	48
15	28	3	32	40	32	4	52
16	32	3	48	41	32	3	56
17	32	3	40	42	28	3	40
18	24	2	44	43	32	3	64
19	32	3	40	44	28	3	48
20	32	3	56	45	28	4	48
21	24	2	40	46	28	3	40
22	32	3	40	47	28	3	56
23	28	4	52	48	32	4	48
24	32	3	56	49	32	3	40
25	32	3	56	50	32	4	40

CALCULATION

Head length	Occurance
32	58%
28	32%
24	6%
20	2%
16	2%

Tail length	Occurance
I I - 20	2%
21-30	0%
31-40	38%
41-50	28%
51-60	28%
61-70	4%

Total length	Occurance
21-40	0%
41-60	6%
61-80	64%
81-100	30%

MEAN HEAD LENGTH - 29.52 µm ± 3.40
MEAN TAIL LENGTH - 44.26 µm ± 9.97
MEAN TOTAL LENGT - 74.84 µm ± 10.99

FIGURE - 4

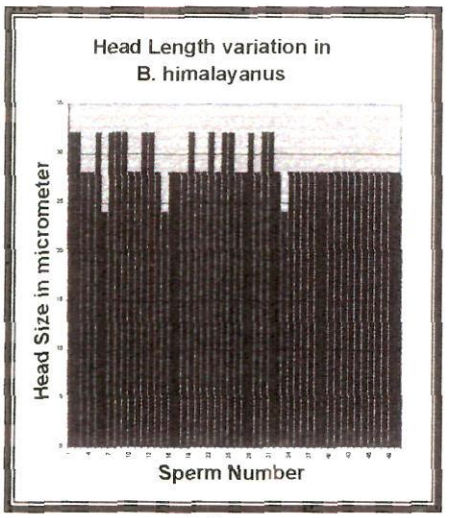
Histogram of the head length variation in the three *Bufo* species.

A-I and A-II showing the occurrence of head length in *Bufo himalayanus* – The maximum head length is 32μm which occupies only 28% of the total sperm count, but the majority of the sperm (66% of the total sperm count) has the size of 28μm.

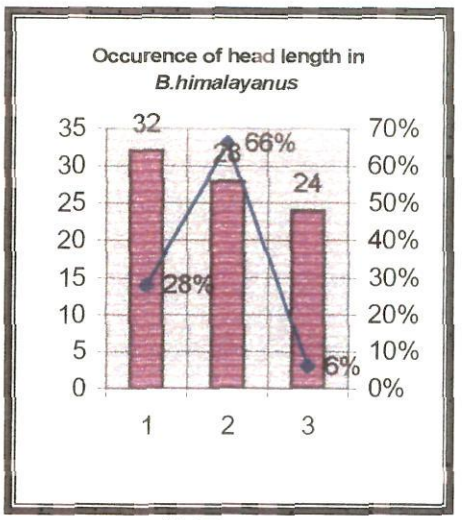
B-I and B-II showing the occurrence of head length in *Bufo stomaticus* - The maximum head length is 28μm which occupies 64% of the total sperm count.

C-I and C-II showing the occurrence of head length in *Bufo melanostictus* – The maximum head length is 32μm which occupies 58% of the total sperm count.

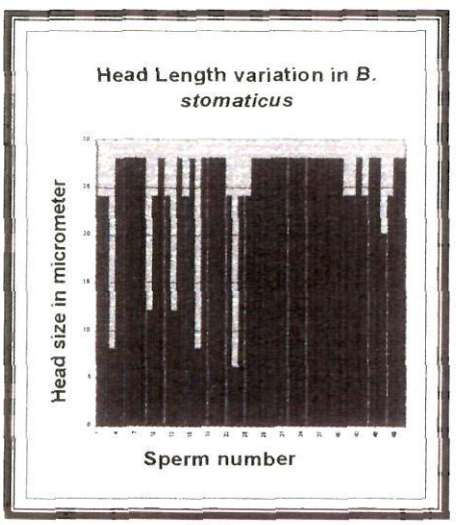
FIGURE-4



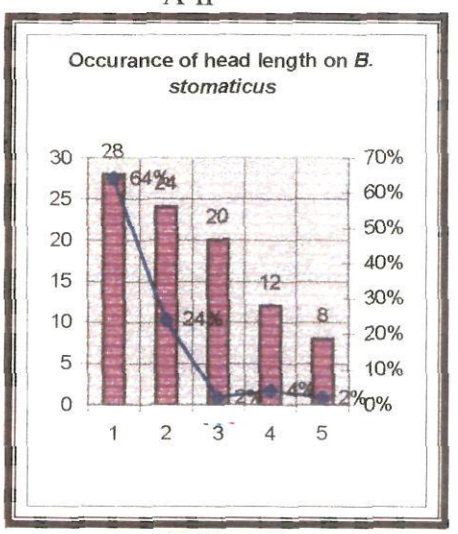
A-I



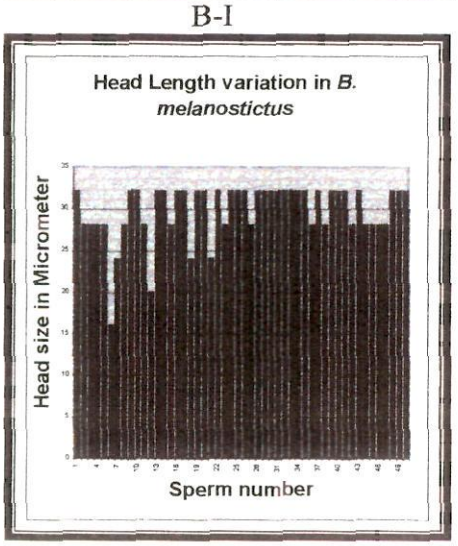
A-II



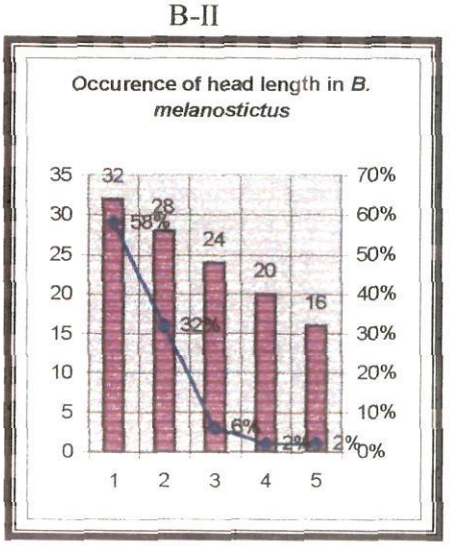
B-I



B-II

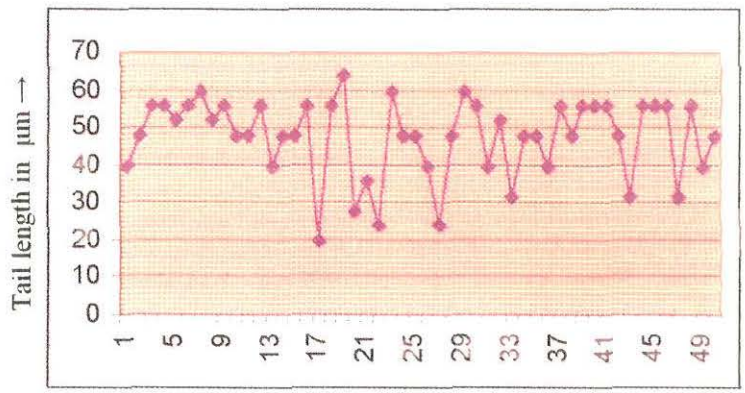


C-I

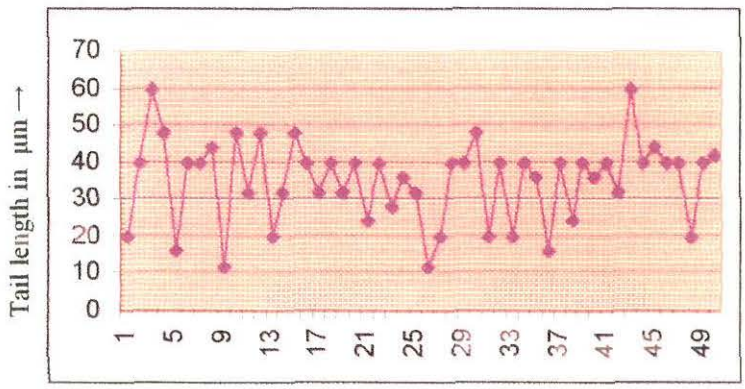


C-II

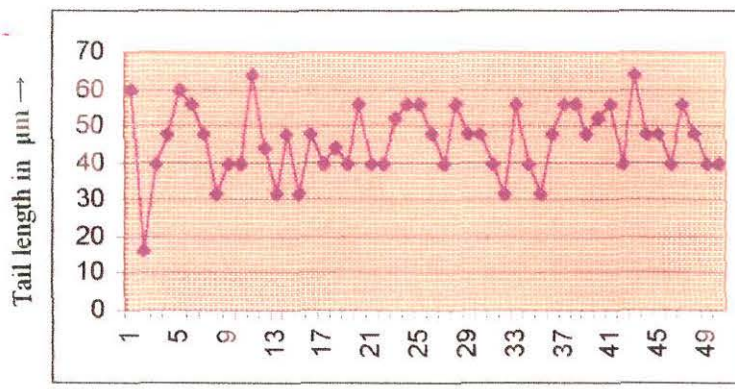
FIGURE-5



B himalayanus tail Sperm number →



B.stomaticus tail Sperm number →



B.melanostictus tail Sperm number →

FIGURE – 5

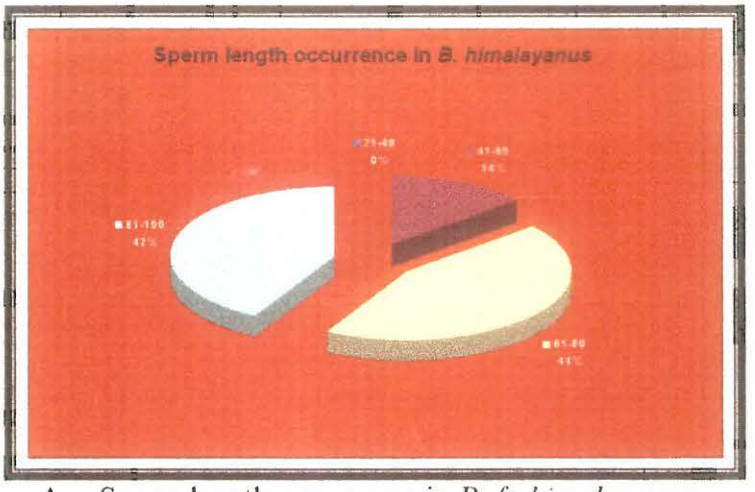
Frequency curve showing the occurance of sperm tail length in µm

A. Showing the tail length variations in 50 different sperms of *Bufo himalayanus.*
B. Showing the tail length variations in 50 different sperms of *Bufo stomaticus.*
C. Showing the tail length variations in 50 different sperms of *Bufo melanostictus.*

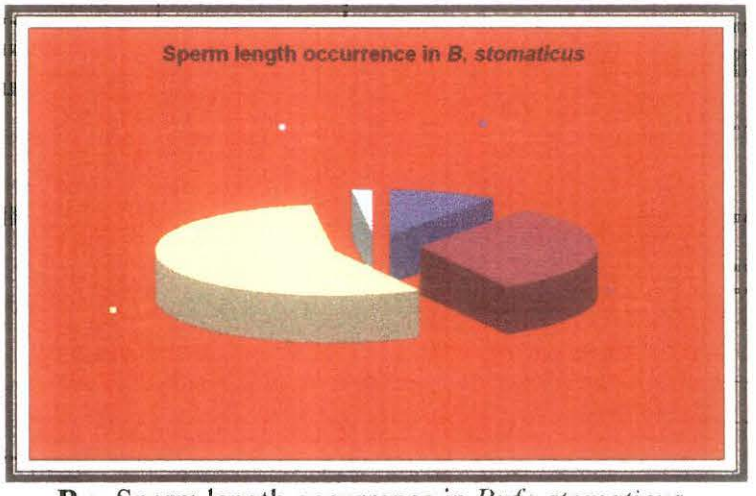
FIGURE – 6

Pie diagram of the occurrence of different sperm length group in the three different *Bufo* species studied.

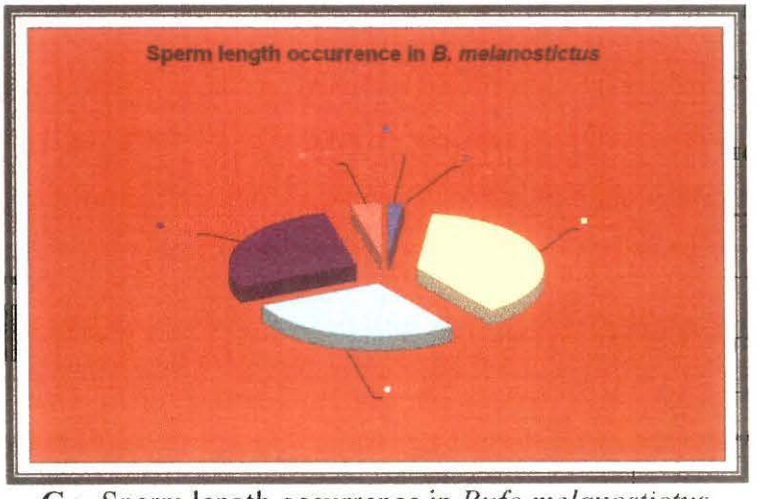
FIGURE- 6



A :- Sperm length occurrence in *Bufo himalayanus*



B :- Sperm length occurrence in *Bufo stomaticus*



C :- Sperm length occurrence in *Bufo melanostictus*

larger dimension (32x2μm). A very few, 6% has the smaller dimension (24x2μm). (Fig.- 4AI, 4AII)

In ***Bufo stomaticus,*** (Table- 2) four distinct head classes are observed. The majority of the sperms (64%) are about 28x2μm in size. About 26% of the sperms of the same species are between 20-24x2μn in size, while about 10% of the sperms are microcephalic and are about 5-8x4μm and almost oval in shape. (Fig.- 4BI, 4BII)

Similarly in ***Bufo melanosticus*** (Table- 3) three sperm classes has been recognized. 80% of the total sperm head are 28-32x2μm in size and rod like in appearance, while about 08% is 22x3μm in size. A very few sperms, 2% are micro-cephalic and heads are about 16x2μm in size. (Fig.- 4CI, 4CII)

The neck region is morphologically not sharply distinct.

Like the head morphology, the tail part exhibits great variations within a species studied.

In ***Bufo himalayanus*** the majority of the sperm (45%) have the tail length of 52-62 μm.(mean -56μm). However the maximum length of the tail is 64μm, while a very few have extremely short tail whose length is about 20μm. An intermediate class having 31-50μm has also been observed.

In ***Bufo stomaticus*** majority (68%) of sperm tails lie between 32μm and 48μm (mean 40μm). However the maximum tail length is 60um, having 4% occurance. A tail class is also observed (28%) which has the smallest tail size with mean value 20μm.

In ***Bufo melanostictus,*** (64%) of the sperm tail lies between 32μm & 48μm (mean 40μm). The largest tail is 64μm in length, which occupies 4% of the total sperm population and the smallest tail size is 16μm, which is 2% of the total cell counted.

The total length variation in all the three species can be clearly observed from the figures 5A,5B and 5C.

Scanning Electron Microscopic Observation

The Scanning Electron Microscopic Observation supports the light microscopic features mentioned earlier. The head, neck, and tail parts of individual species are well documented and easily recognized (Plate-4, Fig. A,B,C).

Bufo himalayanus

In ***Bufo himalayanus,*** the head is elongated, slender with a cup like depression at the acrosomal end. Surface morphology of the head piece is smooth (Plate-5, Fig. A).

The neck region is not morphologically differentiable under SEM, as it is over-flanked by the posterior portion of the head and is only recognizable by a swollen portion at the posterior most part of the head (Plate-6, Fig. A).

The tail is biflagellate and are connected by a membranous sheath medially, however such membranous sheath is not visible at the posterior end of the tail, which terminates in a knob like structure (Plate-7, Fig. A).

Bufo stomaticus

In case of ***Bufo stomaticus,*** the head is slightly curved with a pointed anterior portion as a barb. The head surface is not smooth and bears small wart like projections of variable shape and size along its length (Plate-5, Fig. B).

Like *B. himalayanus* the neck region is not morphologically visible and is over-flanked by the posterior end of the head. Mid piece is slightly flattened than the head (Plate-6, Fig. B).

The tail is biflagellate , but both the flagella are free from one another i.e. not connected by proteinacious sheath as found in *B.*

PLATE – 4:

Scanning electron micrographs of the whole sperm of three different species.

A→ *Bufo himalayanus*

B→ *Bufo stomaticus*

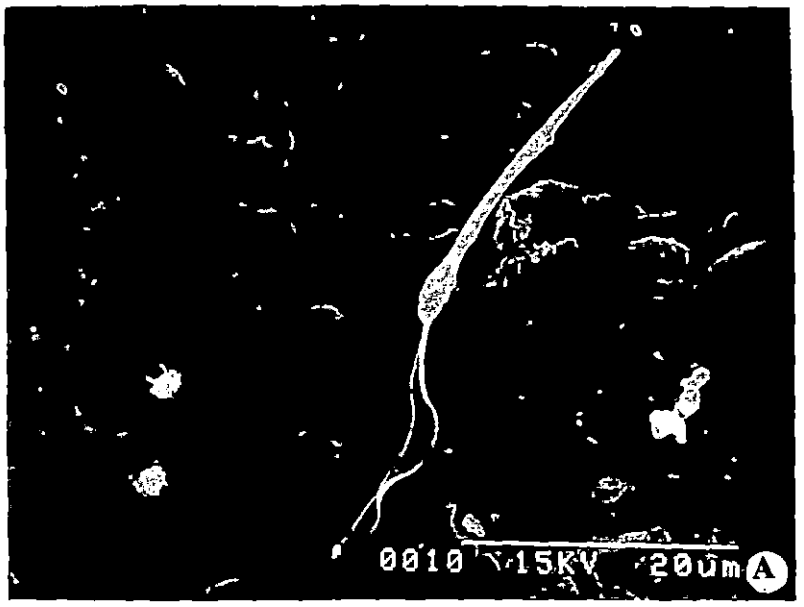
C→ *Bufo melanostictus*

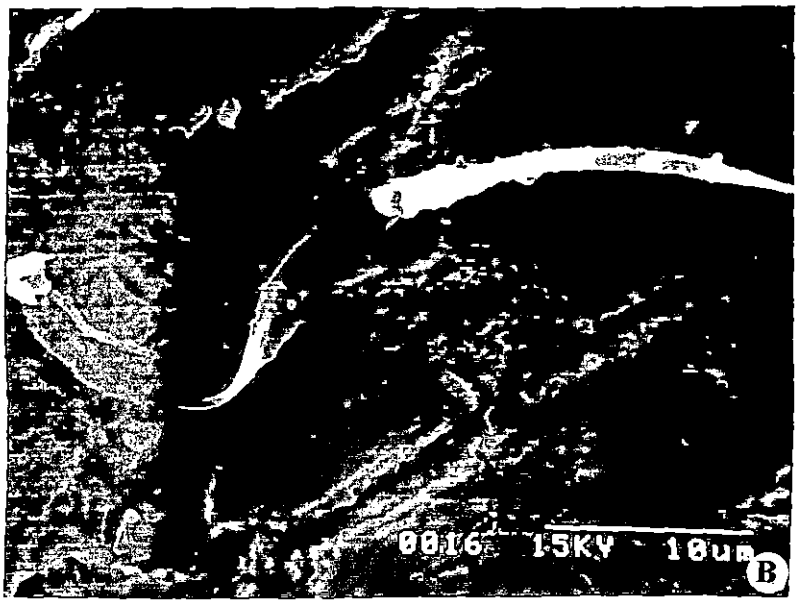
A→ Sperm of *B.himalayanus* showing an anterior bulb like acrosomal tip, swollen middle piece and distinct tail filaments attached at the lower extreme tip by end bulb.

B → Sperm of *B.stomaticus* showing pointed tip and short tail filament with end bulb. Rough head surface morphology is a distinctive feature.

C→ Sperm of *B.melanostictus* showing enlarged, curved head with niddle like pointed acrosomal tip and distinct middle piece.

PLATE – 4





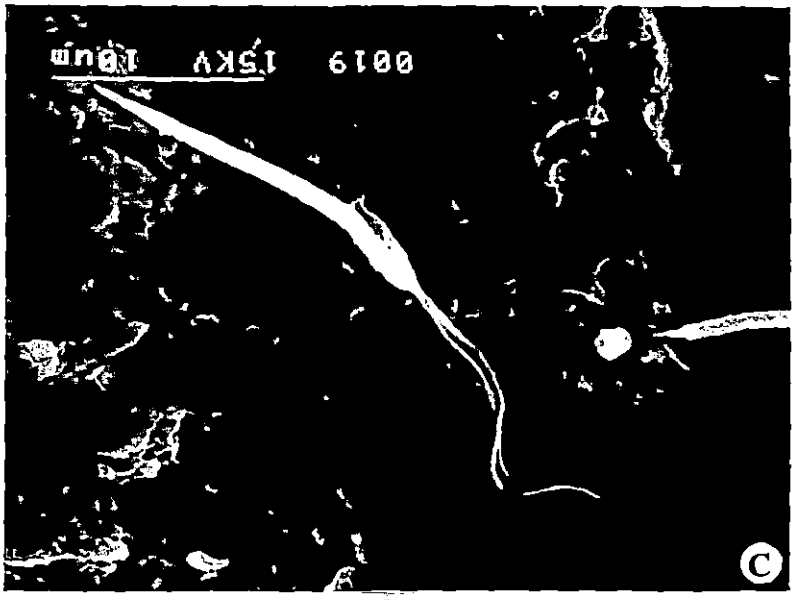


PLATE – 5:

Scanning Electron micrographs of the head region of sperm of the three different species studied.

A→ Sperm head of *Bufo himalayanus*

B→ Sperm head of *Bufo stomaticus*

C→ Sperm head of *Bufo melanostictus*

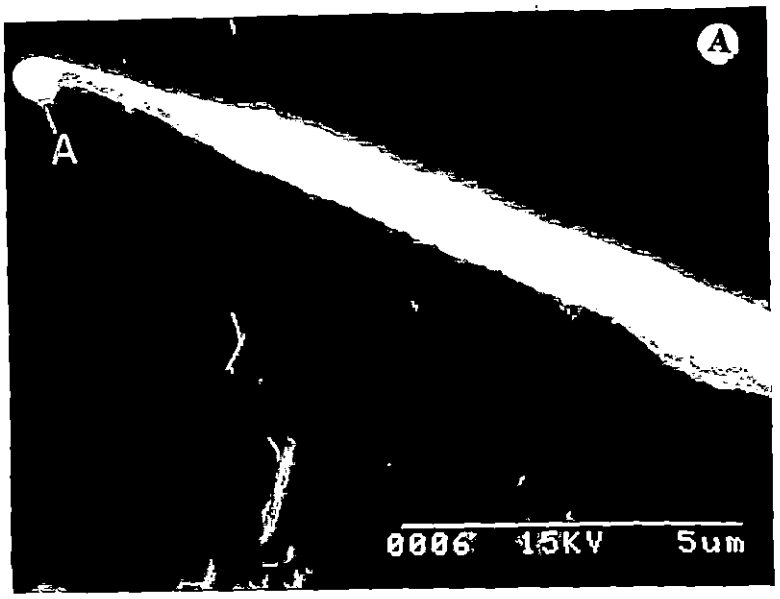
Figure A. shows the swollen cup like acrosomal tip with smooth head surface morphology.

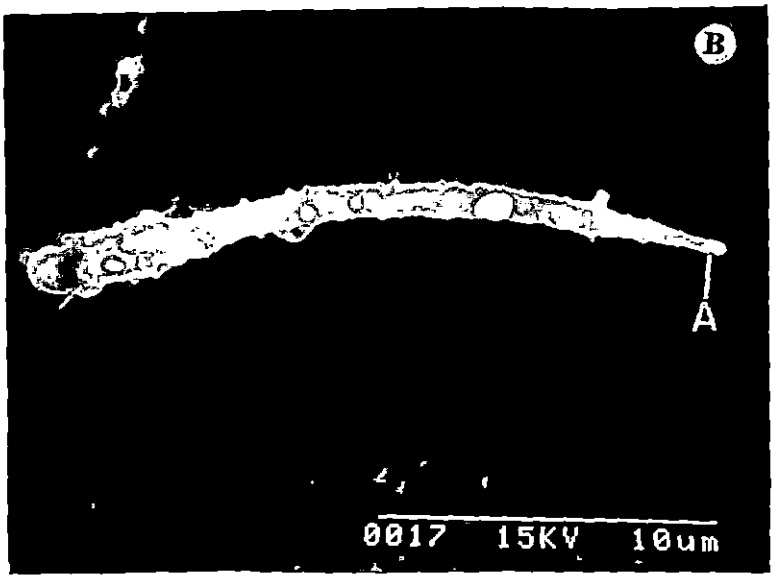
Figure B. shows the rough surface morphology of the *B. stomaticus* head with a pointed acrosomal tip.

Figure C. shows the pin pointed acrosomal tip of *Bufo melanostictus* head with a smooth surface morphology.

A- indicates the acrosome in all the figures.

PLATE – 5





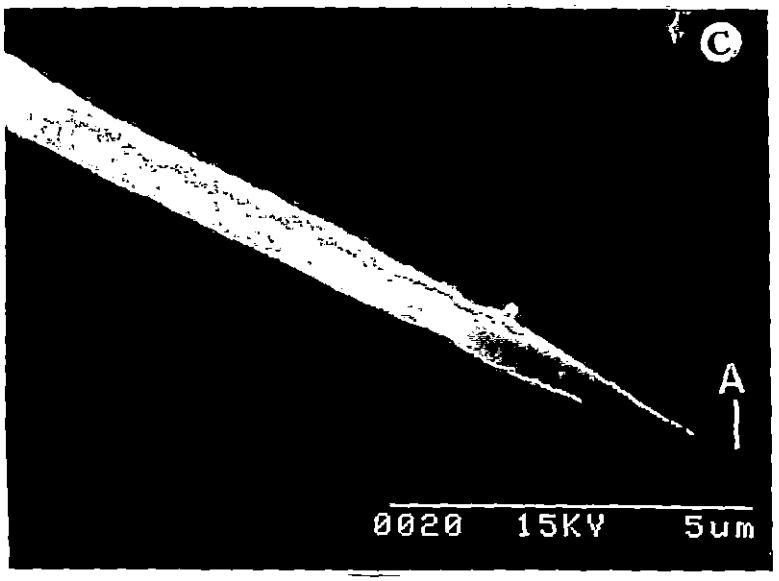


PLATE – 6:

Scanning Electron micrographs of the sperm mid-piece and the arising tail filament of the three different species studied.

A→ Sperm head of *Bufo himalayanus*

B→ Sperm head of *Bufo stomaticus*

C→ Sperm head of *Bufo melanostictus*

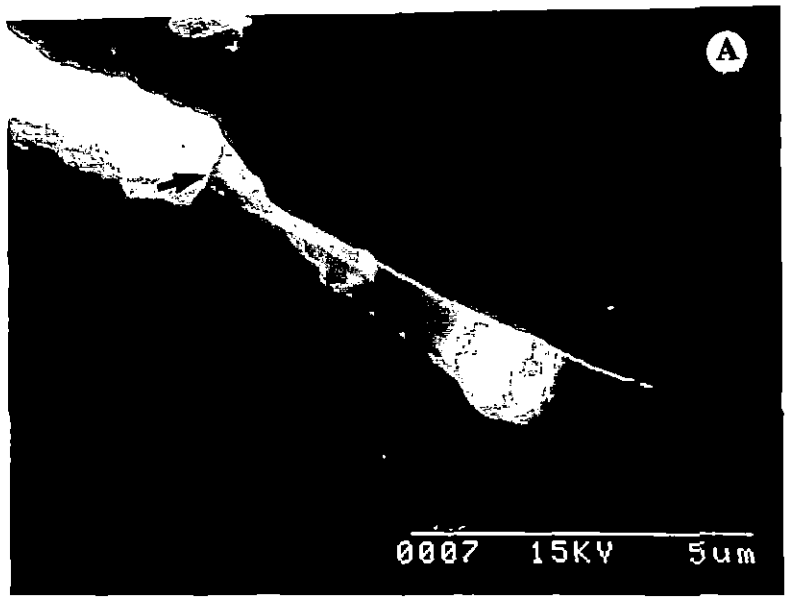
Figure A. shows that in *B. himalayanus* the tail filament arise as a flat process from the middle piece.

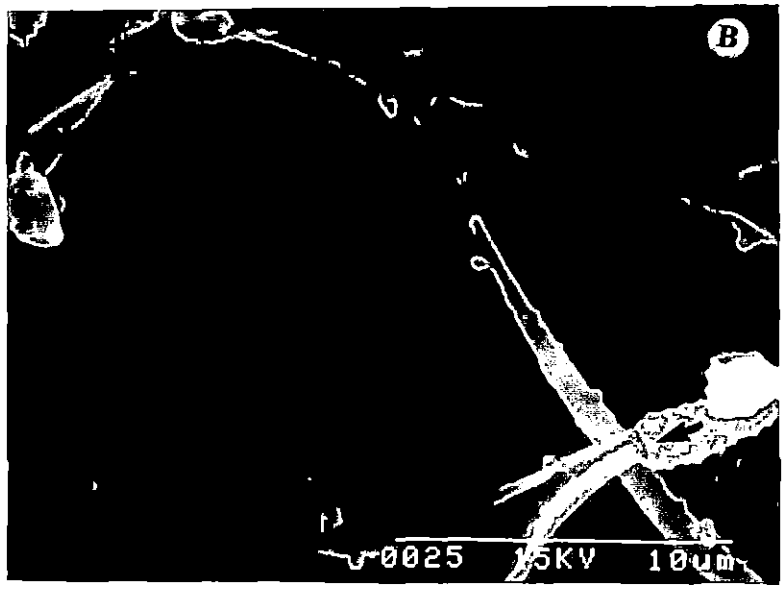
Figure B. shows that in *B. stomaticus* the tail filament and the undulating membrane arise separately from the mid-piece.

Figure C. shows that the tail filament of *B. melanostictus* arises as a slender process from the mid-piece, after a certain distance the undulating membrane projects out from the tail filament.

The black arrow in all the figures indicates the junctional region of the mid-piece and the tail filament.

PLATE – 6





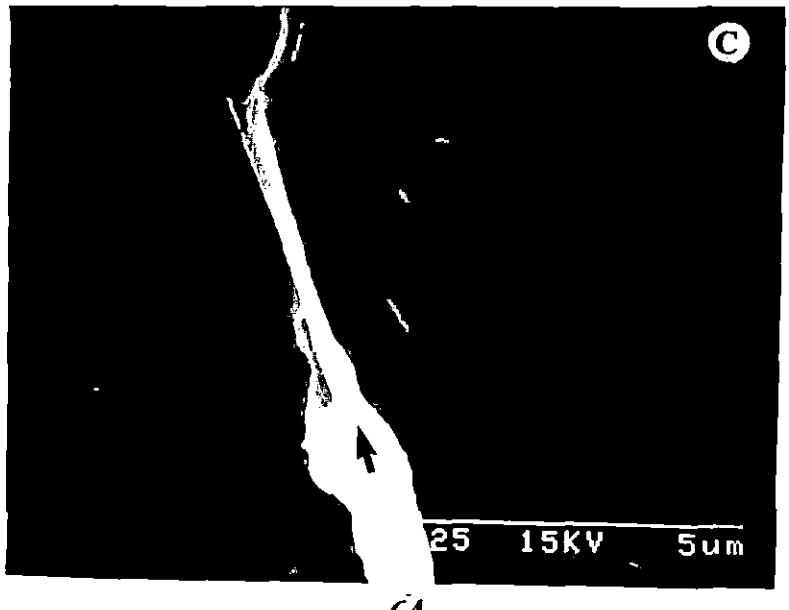


PLATE – 7:

Scanning Electron micrographs of the sperm tail of the three different species studied.

A→ Sperm head of *Bufo himalayanus*

B→ Sperm head of *Bufo stomaticus*

C→ Sperm head of *Bufo melanostictus*

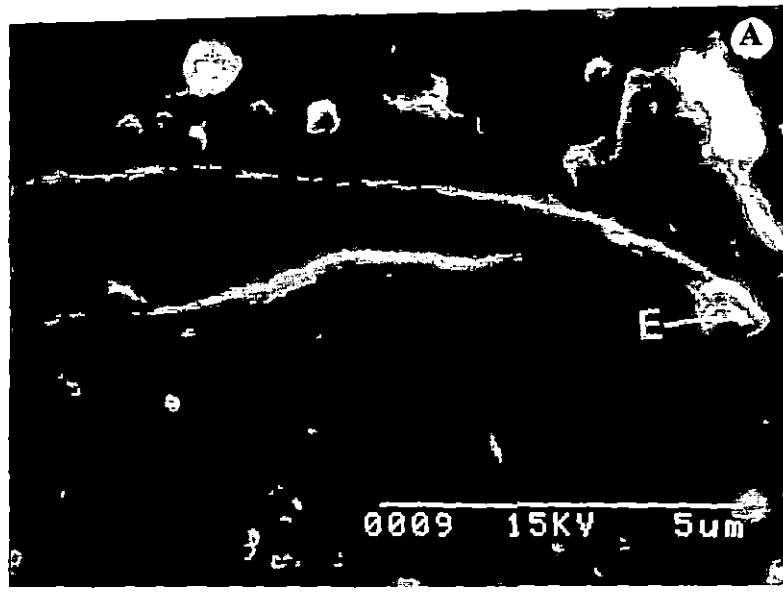
Figure A. shows that in *B. himalayanus* the sperm tail ends in a button like knob.

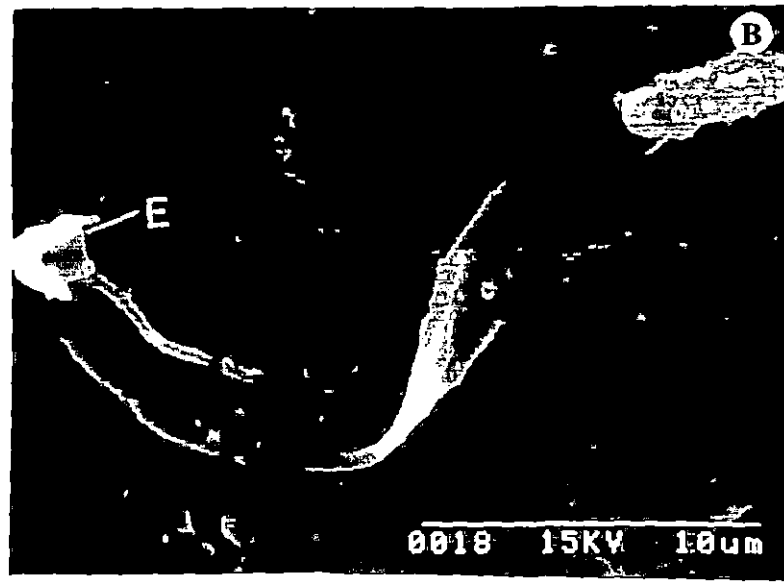
Figure B. shows that in *B. stomaticus* the end bulb of the sperm tail is somewhat flat with a bi-lobed appearance.

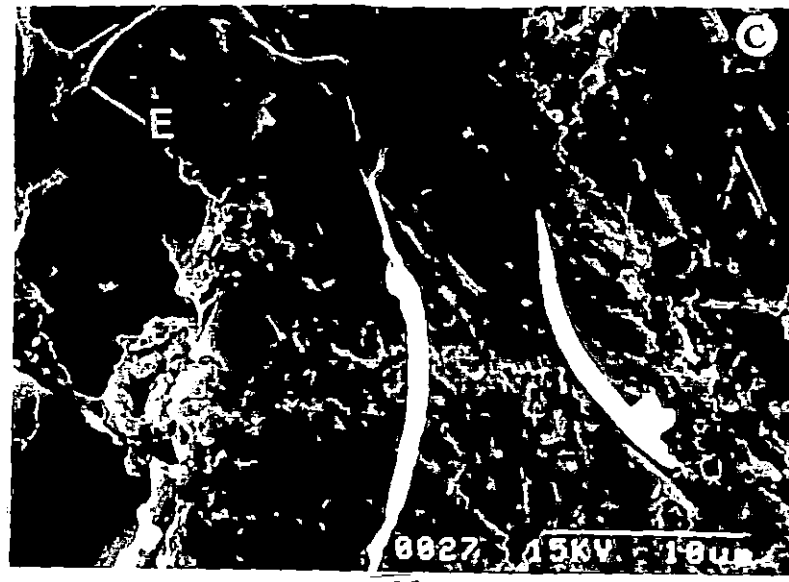
Figure C. shows that in *B. melanostictus* the sperm tail also ends in a flattened extreme end but it is not so distinct.

The tail end is marked as E in all the figures.

PLATE – 7







himalayanus. However at the posterior end of the tail filament, both the flagellum unite as a bi-lobed knob (Plate-7, Fig. B).

Bufo melanostictus

The head morphology of *Bufo melanostictus* strongly resembles with that of *B. stomaticus*. The head is elongated, with anterior barb like projection but without warts on the head surface. It is cylindrical in shape and pointed at its anterior end (Plate-5, Fig. C).

The neck, unlike other two species studied is morphologically visible as elongated rod like region. The tail filament appears from this thread like middle piece (Plate-6, Fig. C).

Tail is bi-flagellate, the flagella are free from one another throughout the entire length and each terminates as small knob at the posterior end (Plate-7, Fig. C).

As observed under the Scanning Electron Microscope, the sperm morphology exhibits a wide range of variation in all the three species studied.

The spermatogenetic pattern and the release of is almost similar in all the three species.

Unlike other vertebrates, in amphibians, sperm matures in groups occupying numerous pockets in the testis. From the scanning electron micrographs it is observed that the inner morphology of the testes are not smooth. Instead, pouch like pockets appear throughout the testes body. These pockets are mainly of two types; one, the empty pockets and the other, the sperm pockets. The sperm pockets are filled with mature sperms, which are ready to be released. The empty pockets are those which have released their mature sperms and contain only underdeveloped gonial cells and developing spermatocytic cells.

It is also observed that majority of the empty nests are lying in the periphery and the sperm pockets towards the center indicating the gradual maturation of sperms from the center to the periphery.

Transmission Electron Microscopic Observation

Bufo himalayanus

Under TEM the anterior and middle portion of the *Bufo himalayanus* sperm is well differentiated with conical sharp head, tapering towards the anterior end (Plate-11, Fig. a). The nucleus is elongated and with electron dense granules as seen in transverse and longitudinal sections (Plate-11, Fig. B, C; Plate-13, Fig. A; Plate-12, Fig. D). However, the concentration of electron dense granules is not uniform throughout the length of the nucleus. At the proximal end of the nucleus the concentration of electron dense material is less than the distal end. The middle portion of the head exhibits a maximum concentration of electron dense granules (Plate-12, Fig.-D).

The anterior tip of the head terminates in an electron lucent acrosomal cap. The acrosomal cap sends off acrosomal barb at the anterior end (Plate-12, Fig. C). The acrosomal barb is wooly in appearance. In light magnification it takes a cup shaped rigid flattened structure. However in higher magnification such rigidity is not observed (Plate-12, Fig. B).

The middle piece is elongated with moderately electron lucent vacuoles surrounded by heavily packed multi-layers of mitochondria. In longitudinal section mitochondria takes circular shapes. However mitochondria are not arranged in any ordered fashion, and as a result, appears to be randomly arranged in longitudinal and transverse section (Plate- 12, Fig. D; Plate-13, Fig. B).

Two distinctly visible centrioles are present - proximal and distal. The proximal centriole is located in close proximity to the nuclear notch at the basal end of the head. It lies in a parallel fashion to the head nucleus (Plate- 12, Fig. D). The distal centriole lies perpendicular to the nuclear axis, slightly away from the proximal centriole. Microfilaments of

PLATE – 8:

Scanning Electron micrographs of the longitudinal section of *Bufo himalayanus* testis.

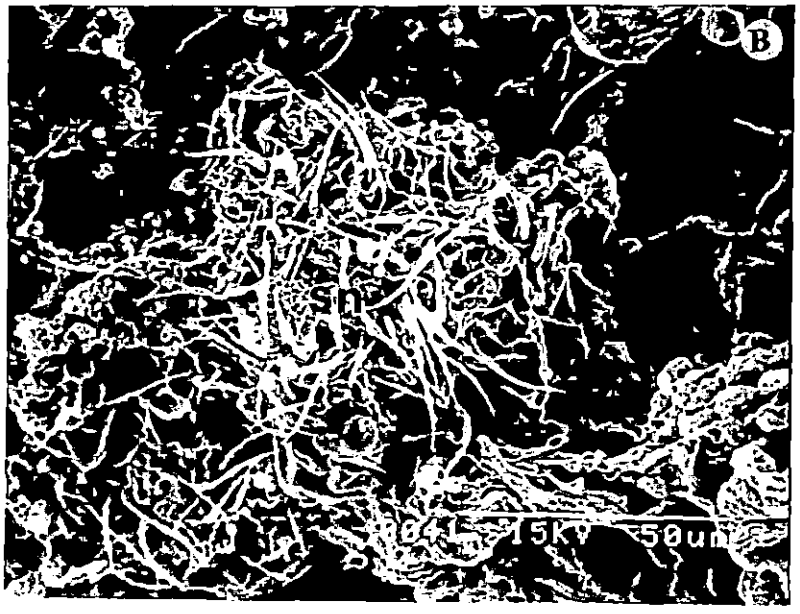
Figure A. Longitudinal section of *B. himalayanus* testis at low magnification showing the inner surface morphology; sperm cluster and the empty nests are well visualized.

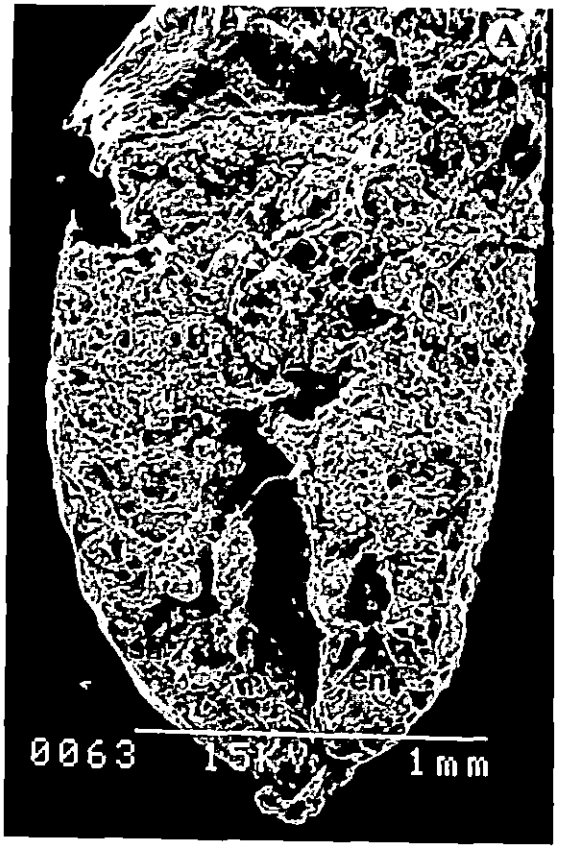
Figure B. Sperm nest, showing cluster of mature sperm.

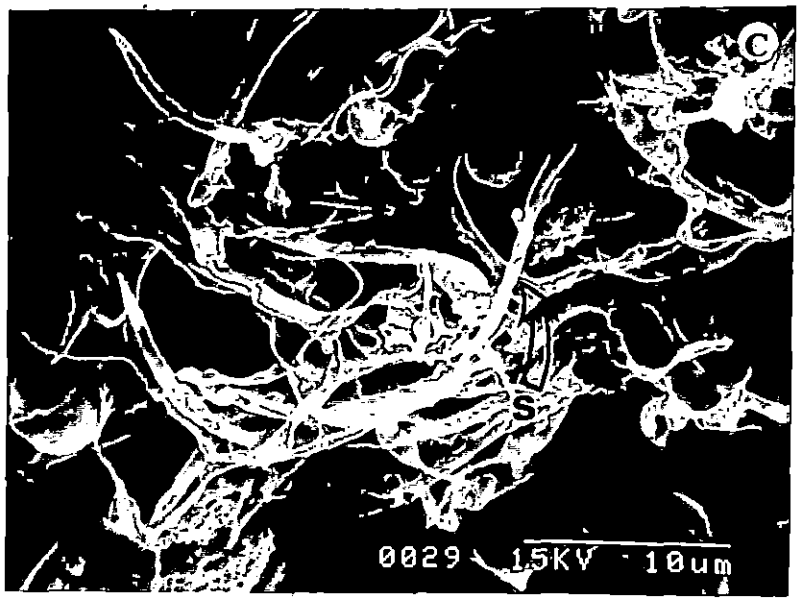
Figure C. Magnified view of sperm nest.

Figure D. Empty nest indicating clearly that the mature sperms has been released.

Indication: SN - sperm nest; S – mature sperm; EN – empty nest.


B
0041 15KV 50um


A
0063 15KV 1mm


C
S
0029 15KV 10um

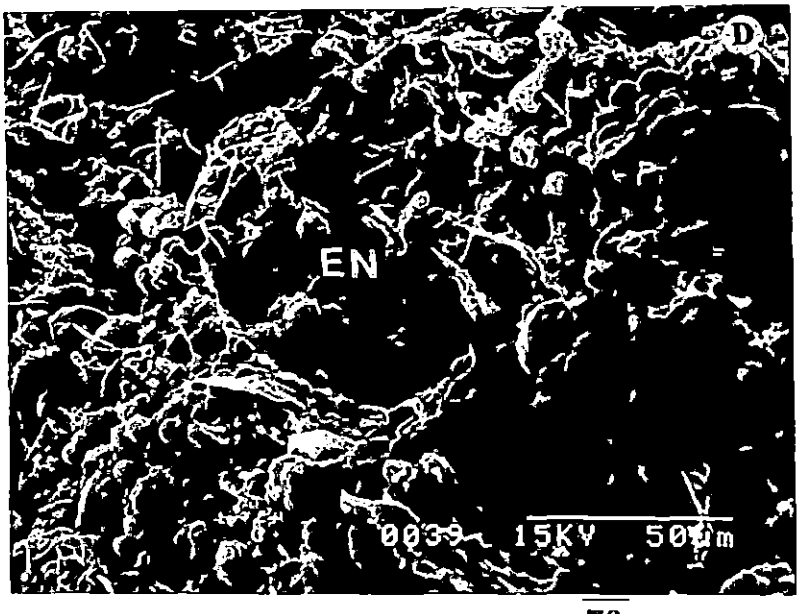

D
EN
0039 15KV 50um

PLATE – 9:

Scanning Electron micrographs of the longitudinal section of the testis of *Bufo stomaticus.*

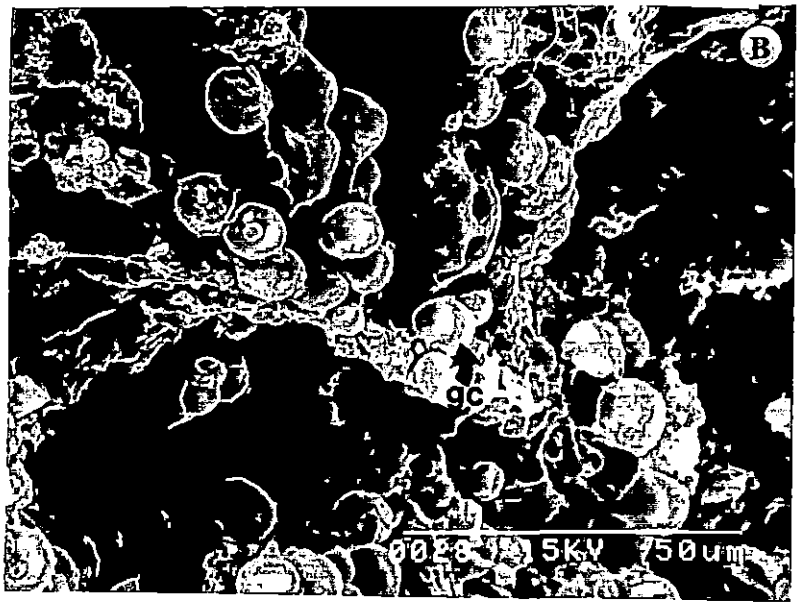
Figure A. Longitudinal section of *B. stomaticus* testis at low magnification showing the inner surface morphology; sperm cluster and the empty nests are well visualized.

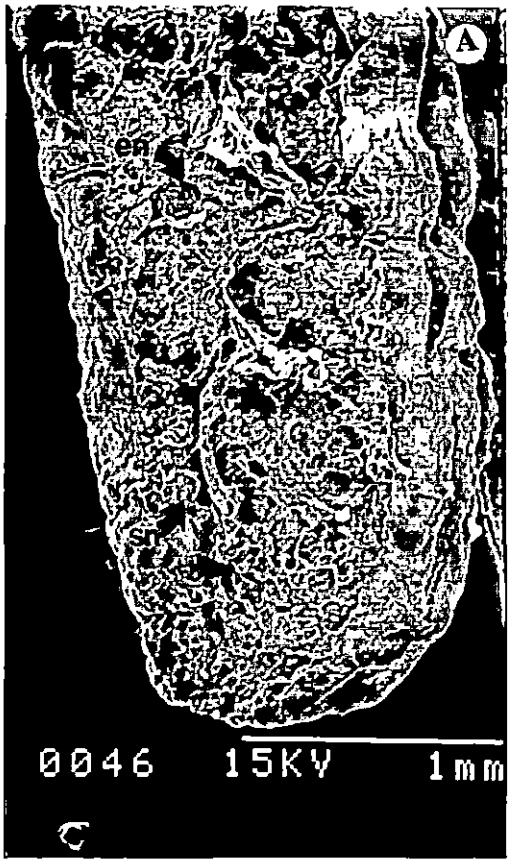
Figure B. Cluster of goneal cells in a developing nest.

Figure C. Mature sperm cluster in a sperm nest, which is ready for being released.

Figure D. Empty nest showing no mature sperm. All the sperm of that nest has been released.

Indication: SN - sperm nest; EN – empty nest.


B
gc
15KV 750um


A
0046 15KV 1mm


C

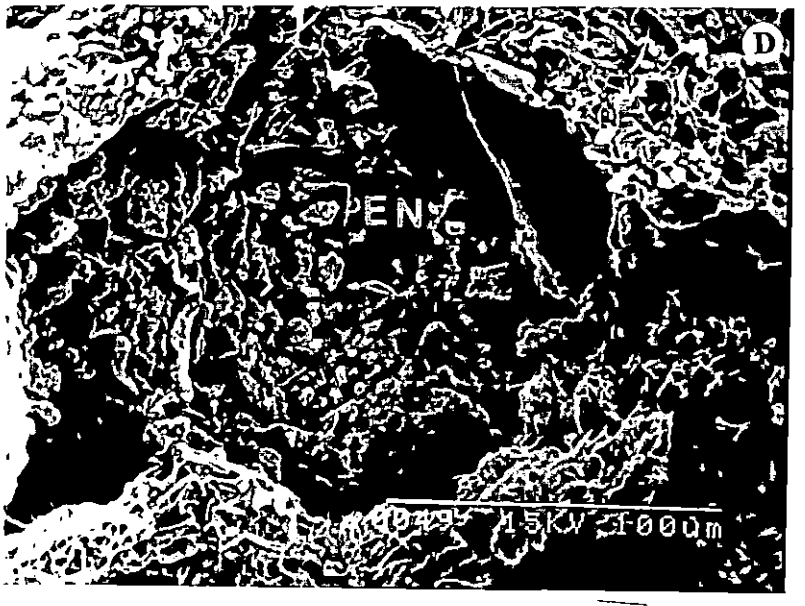

D
OPEN

PLATE – 10:

Scanning Electron micrographs of the longitudinal section of the testis of *Bufo melanostictus*.

Figure A. Longitudinal section of *B. melanostictus* testis at low magnification showing the inner surface morphology. Distinct patches of sperm nests and empty nests are well visualized.

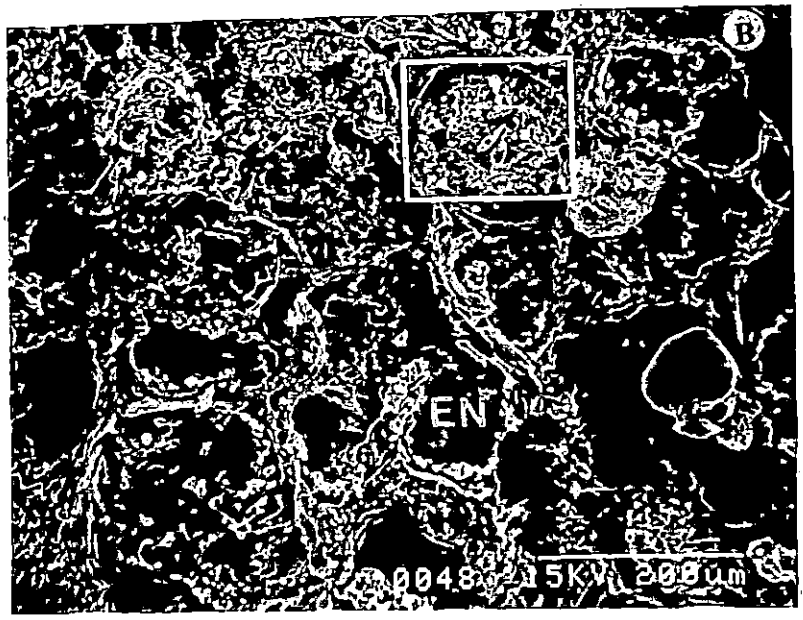
Figure B. Distinct empty and sperm nest; a sperm nest has been highlighted by white rectangular block.

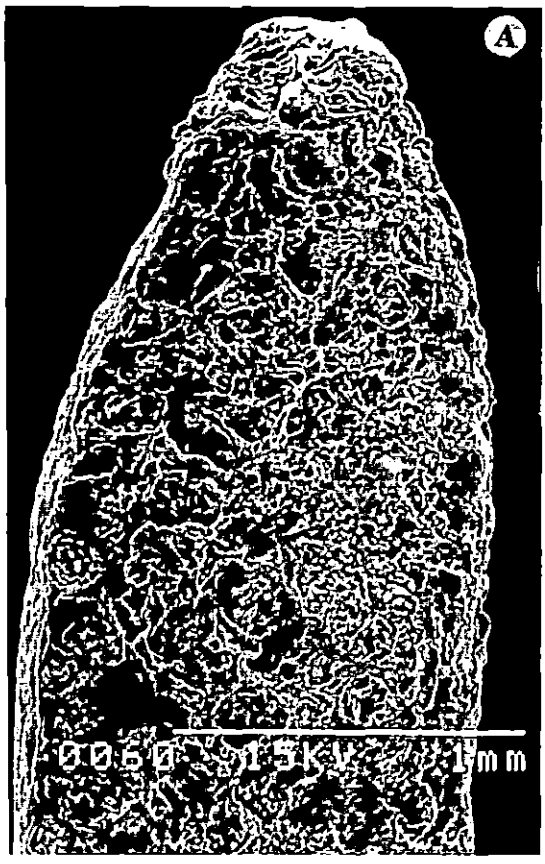
Figure C. Magnified view of the white block in figure A. showing the sperm nest where the mature sperm are well documented.

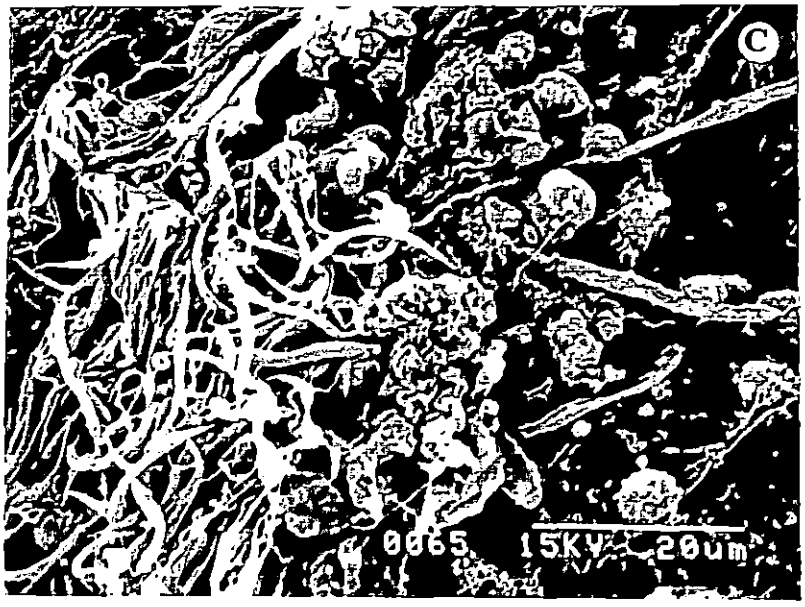
Figure D. Empty nest showing the presence of no mature sperm. All the sperm of that nest has been released.

Indication: SN - sperm nest; EN – empty nest.

PLATE – 10







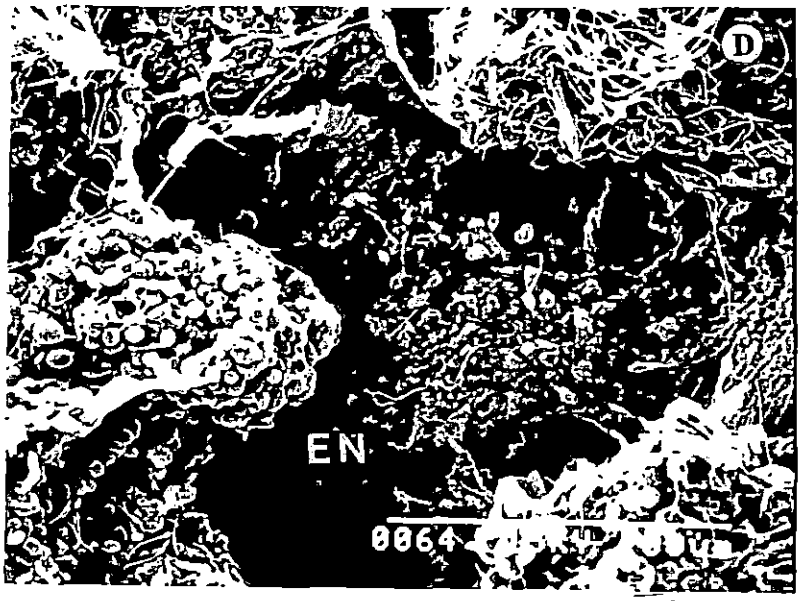


PLATE – 11:

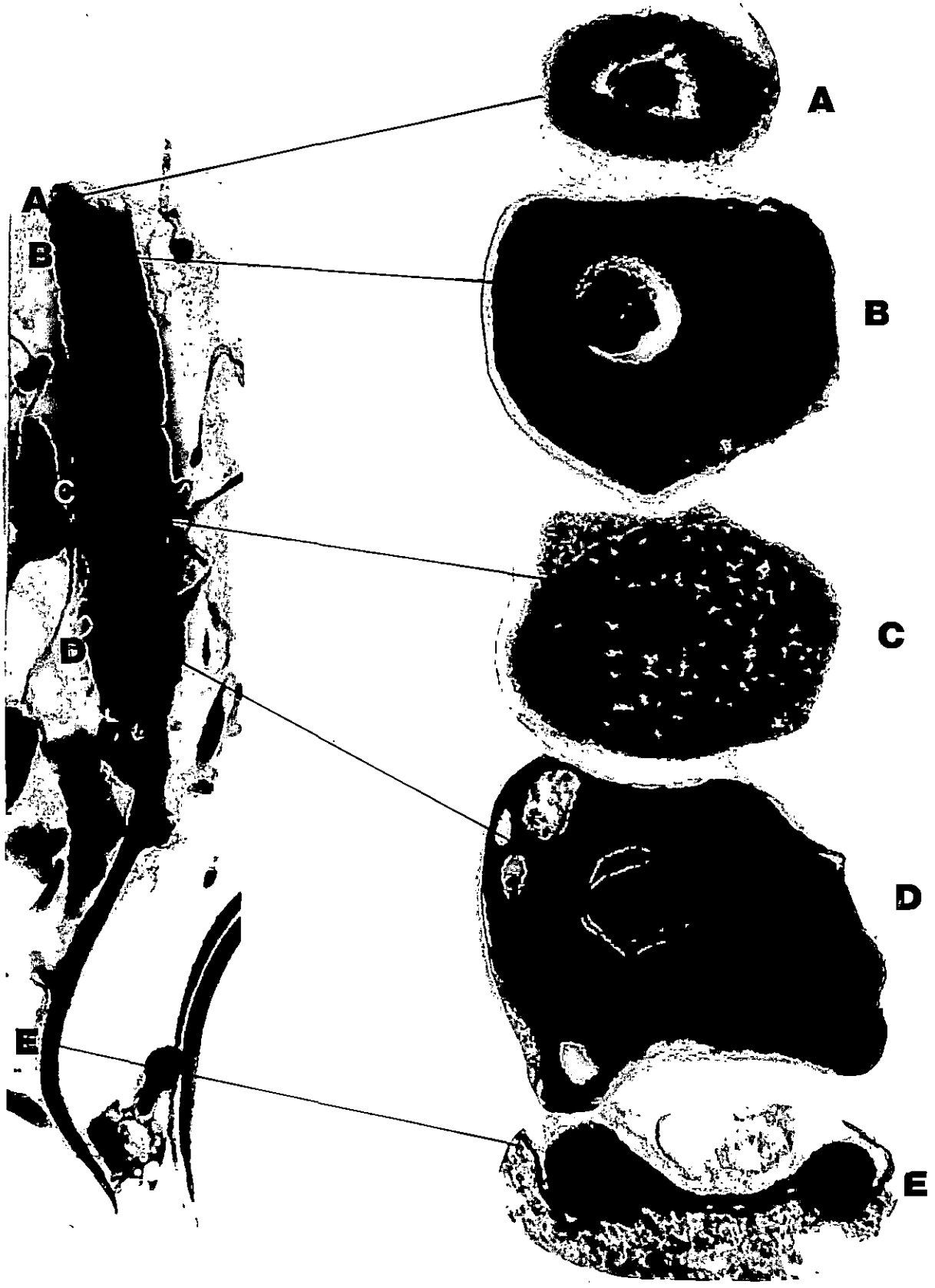
Transmission Electron micrographs of the longitudinal (a) and transverse (b) sections of the *Bufo himalayanus* sperm at different levels, showing the cellular organelles and structural organization.

Figure a. Longitudinal section of the sperm of *B. himalayanus* whose different parts have been marked as A- terminal tip, B- sub-terminal end, C- head nucleus, D- middle piece, and E- tail filament.

Figure b. Transverse section of different parts, which have been marked in the longitudinal section.

A. Transverse section through the acrosomal tip.
B. Grains in the middle circle indicating the presence of nuclear mass encircled by acrosomal part.
C. The middle portion of the nucleus indicated by the presence of pigmented granules throughout the diameter.
D. The middle piece with the peripheral mitochondria region and a central region from where the tail filament arises.
E. Transverse section of tail showing the axial filament with peripheral and central microtubules; an undulating membrane arises from the periphery of the axial filament and extend rightwards to form a button like knob.

PLATE – 11



(a) LONGITUDINAL SECTION (b) TRANSVERSE SECTIONS

PLATE – 12:

Transmission Electron micrographs of different parts of *Bufo himalayanus* sperm.

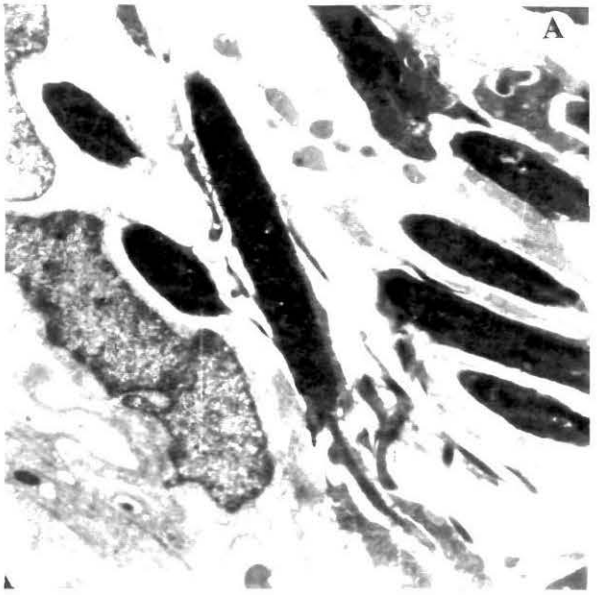
Figure A. Whole sperm of *Bufo himalayanus*. x2040

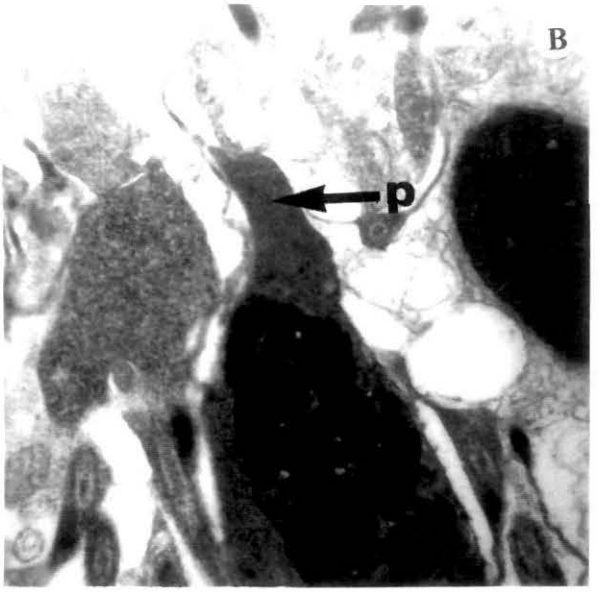
Figure B. Lightly pigmented distinct conical perforatorium (p). x8200

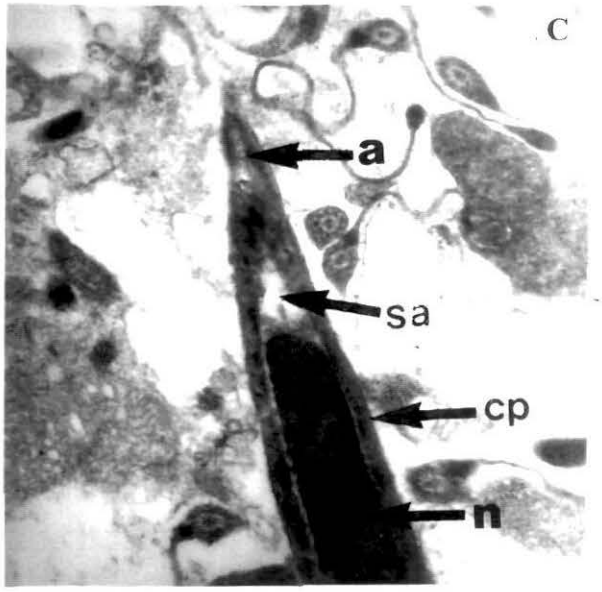
Figure C. Sperm head showing lightly pigmented acrosome (a), a distinct subacrosomal space (sa), a darkly pigmented nucleus (n) and an outer cytoplasmic layer (cp). x8200

Figure D. Darkly pigmented granules throughout the nucleus (n). At the subnuclear notch lies both the centrioles (c), proximal centriole (pc) and the distal centriole (dc) are located perpendicular to each other. x8200

PLATE – 12







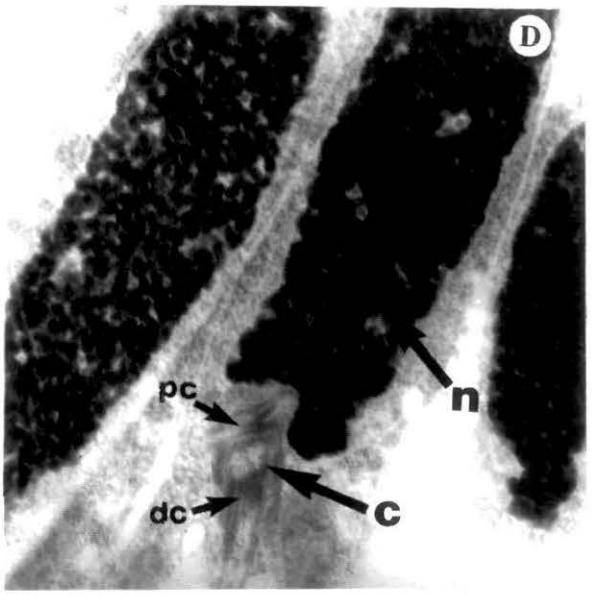


PLATE – 13:

Transmission Electron micrographs of different parts of *Bufo himalayanus* sperm.

Figure A. Transverse section of head region showing lightly pigmented cytoplasm and deeply pigmented nucleus. Pigmented granules (PG) in the nucleus are well documented. x8200

Figure B. Transverse section of middle piece showing peripheral layers of mitochondria (mc) and the central axial filament (af). x13500

Figure C. Transverse section of flagella showing distinct 18 peripheral and 2 central microtubule (mt). x21500

Figure D. *Transverse section of flagella showing the projecting* undulating membrane (u) from axial filament. x31000

PLATE – 13

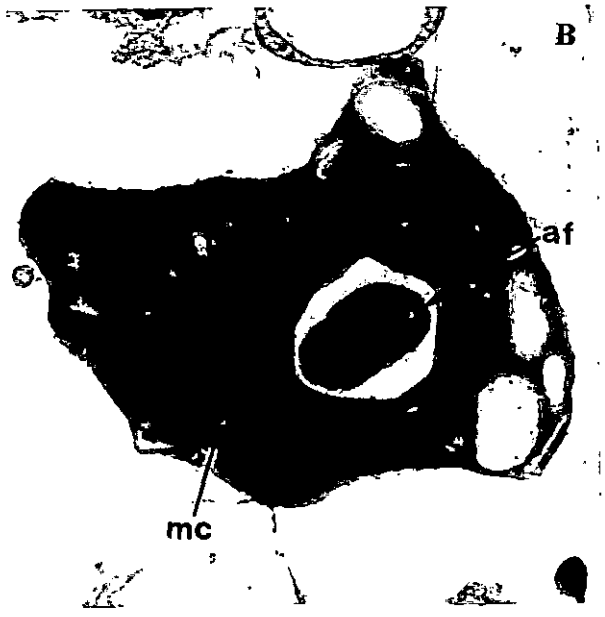


PLATE – 14:

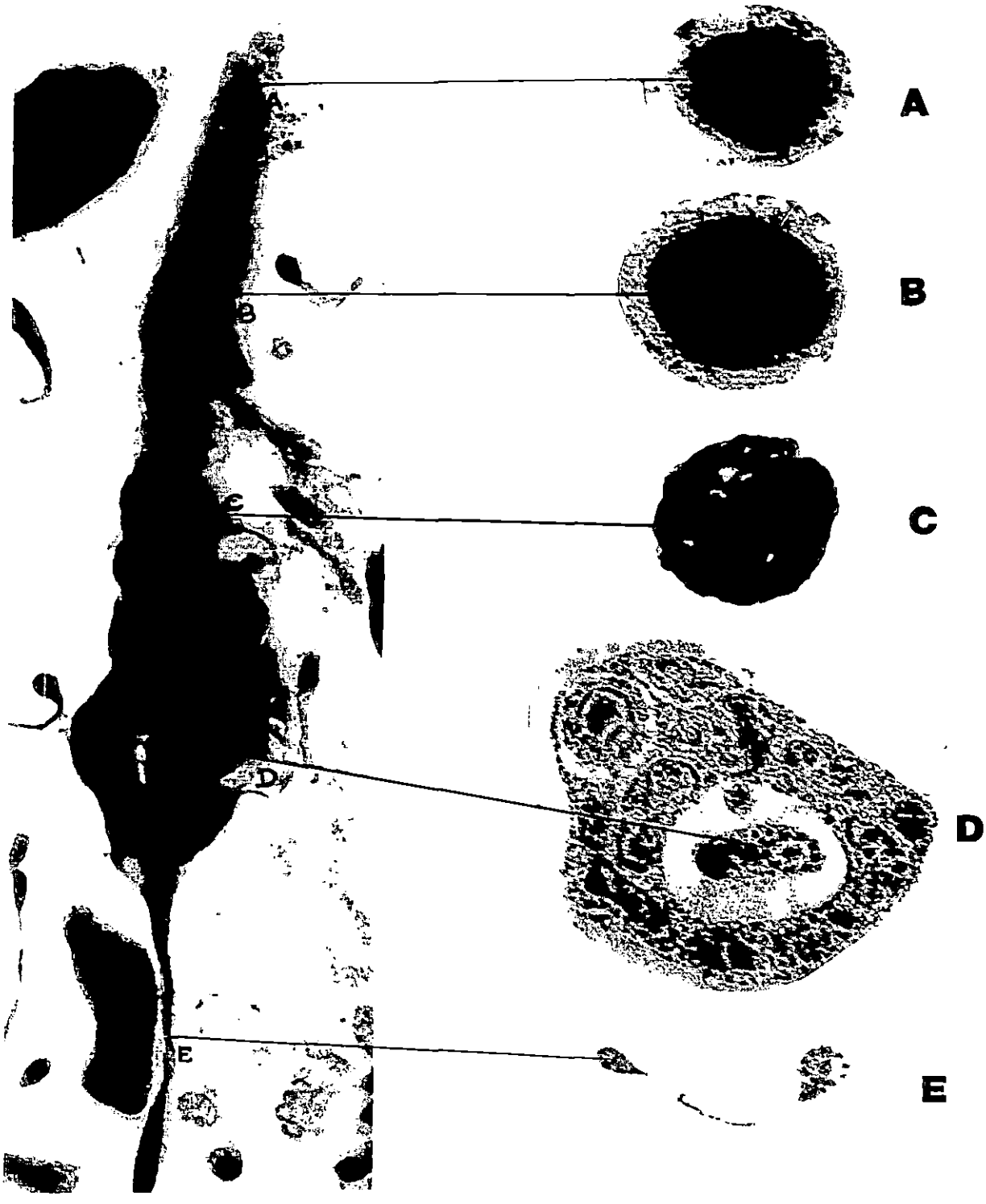
Transmission Electron micrographs of the longitudinal (a) and transverse (b) sections of the *Bufo stomaticus* sperm at different levels, showing the cellular organelles and structural organization.

Figure a. Longitudinal section of the sperm of *B. stomaticus* whose different parts have been marked as A- terminal tip, B- sub-terminal end, C- head nucleus, D- middle piece, and E- tail filament.

Figure b. Transverse section of different parts, which have been marked in the longitudinal section.

A. Transverse section through the acrosomal tip.
B. Showing deeply stained central nucleus encircled by lightly pigmented peripheral cytoplasmic material.
C. The middle portion of the nucleus indicated by the presence of pigmented granules throughout the diameter.
D. The middle piece with the peripheral mitochondria region and a central region from where the tail filament arises.
E. Tail consisting of a distinct axial filament containing 18+2 microtubules and an undulating membrane, which form a bulb like swollen part at the extreme tip.

PLATE – 14



(a) LONGITUDINAL SECTION (b) TRANSVERSE SECTIONS

PLATE – 15:

Transmission Electron micrographs of different parts of *Bufo stomaticus* sperm.

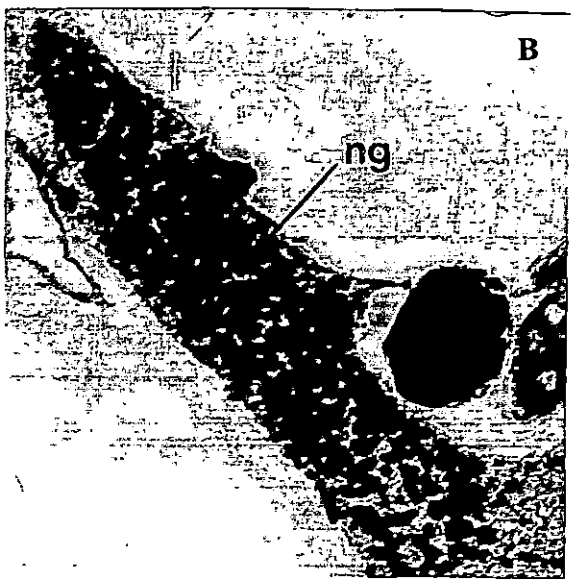
Figure A. Longitudinal section of the whole sperm. x2900

Figure B. Head nucleus showing distinct nuclear granules (ng). x5400

Figure C. Middle piece showing the axial filament (af) arising from the centriole; elongated mitochondria (m) are arranged haphazardly on both the sides of the middle piece. x8200

Figure D. Distinct proximal centriole (pc) located at the subumbrellar nuclear notch, containing 18 peripheral microtubules but no central microtubule. x10500

PLATE – 15



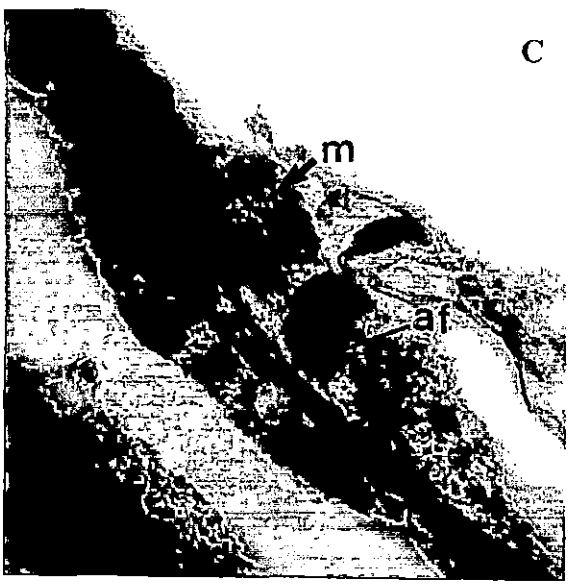


PLATE – 16:

Transmission Electron micrographs of different parts of Bufo stomaticus sperm.

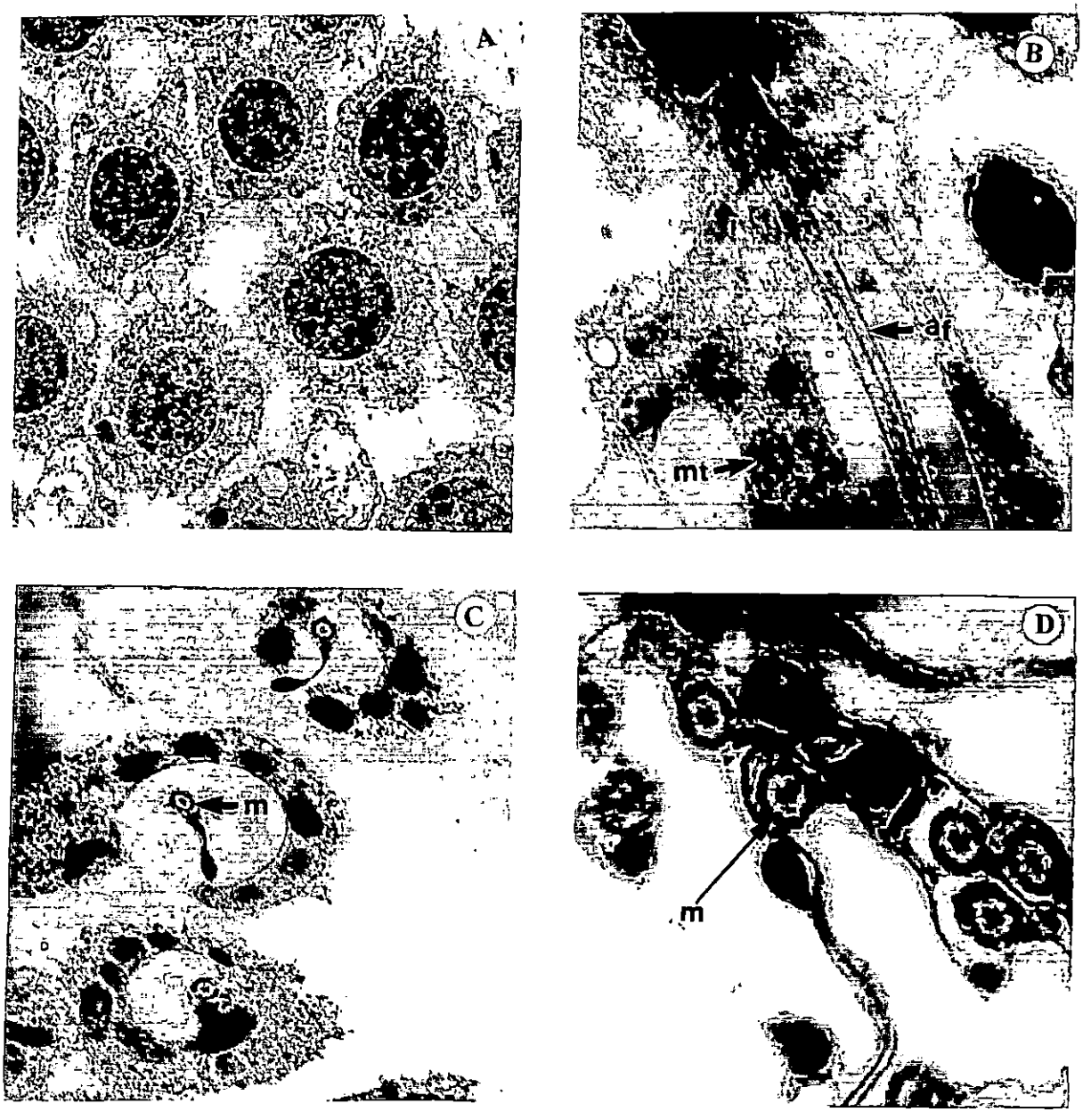
Figure A. Transverse section of the head region of the sperm showing numerous nuclear granules (ng). x2900

Figure B. Axial filament arising from the centriole of the middle piece; numerous oval mitochondria (mc) are located throughout the middle piece. x8200

Figure C. Transverse section of midpiece showing central microtubule encircled by peripheral mitochondrial rim. x5400

Figure D. Transverse section of the axial filament showing distinct 18 peripheral and 2 central microtubules (m). x21500

PLATE – 16



the centrioles are not very distinct as found in other two species described.

The tail is filamentous in appearance with a central flagellum (Plate-13, Fig. B). Each flagellum consists of an axial filament made up of 20(18+2) submicroscopic longitudinal fibrils arranged in typical outer circlet of nine doublets and inner core of two singlets, which are situated distantly from each other. Thin proteinous filaments connect the central filaments with the peripheral filaments and giving a cart-wheel appearance (Plate-13, Fig. C). Well visible undulating membrane present, which terminates in a thick broad end (Plate-13, Fig. D).

Bufo stomaticus

In *Bufo stomaticus,* the head and middle piece are clearly demarcated (Plate-14, Fig. a). The head is elongated, slightly curved and circular in cross section (plate-14, Fig. b.B, bC) with rounded base and tapering tip. However the nucleus exhibits irregular thickening resulting in a rough surface morphology (Plate-15, Fig. A). Electron dense particles are well visualized throughout the head length in transverse and longitudinal section (Plate-16, Fig. A; Plate-15, Fig. B), but the granules are of smaller size. The concentration of such electron dense granules are lower in the posterior end than in the middle and anterior end (Plate-15, Fig. B).

Well formed acrosome is visible with external acrosomal vesicle and internal subacrosomal cone (Plate-15, Fig. A, B). The acrosomal surface is almost smooth with no surface projections. Acrosomal barb is present but with no distinct conformation.

In *Bufo stomaticus* the middle piece is broad with extended flap like projections on both the sides. Mitochondria are layered on the flaps (Plate-14, Fig. b.D; Plate15, Fig. C). Two types of mitochondria are visible *i.e.* they appear elongated and circular in cross section. The mitochondria are arranged in dispersed manner. Two centrioles are

present- proximal is round and distal is elongated. Both of them lie perpendicular to each other (Plate-15, Fig. C, D). Proximal centriole is situated within the posterior notch of the nucleus. It is round in appearance with nine peripheral submicroscopic tubules (Plate-15, Fig. D). In longitudinal section the posterior centriole is elongated in appearance (Plate-15, Fig. C; Plate-16, Fig. B).

The tail is filamentous in appearance with a central core made of flagella (Plate-16, Fig. B, C). Each flagellum is made up of microtubules, which are arranged in usual 9+2 fashion. There are nine peripheral doublets and two distinctly separated central singlet filaments. Both types of filaments are interconnected by protein fibers giving the appearance of a wheel (Plate-16, Fig-D). Undulating membrane originates from the axial filaments but the extend of the membrane is variable. The undulating membrane terminates in an end bulb of medium size, superficially resembling the axial filament (Plate-16, Fig-C, D). This type of organization gives a biflagellar appearance of the tail (Plate-14, Fig.-b.D). (However, it may be mentioned that under light and electron microscopic observation the sperm tail has been described as biflagellate).

Bufo melanostictus

In *Bufo melanostictus* the head nucleus is elongated, slightly curved and circular in cross section (Plate-17, Fig. a, b). The posterior end of the head nucleus is almost round and anterior tip is pointed. Numerous electron dense granules are present throughout the entire length of the nucleus). The anterior portion of the head bears electron lucent acrosome cap (Plate-18, Fig. A).

The acrosome cap is smooth in morphology with distinct acrosome and subacrosomal cone over the pointed head (Plate-18. Fig. C). Distinct niddle shaped acrosomal barb is visible in the extreme tip of the head (Plate-18, Fig. B).

PLATE – 17:

Transmission Electron micrographs of the longitudinal (a) and transverse (b) sections of the *Bufo melanostictus* sperm at different levels, showing the cellular organelles and structural organization.

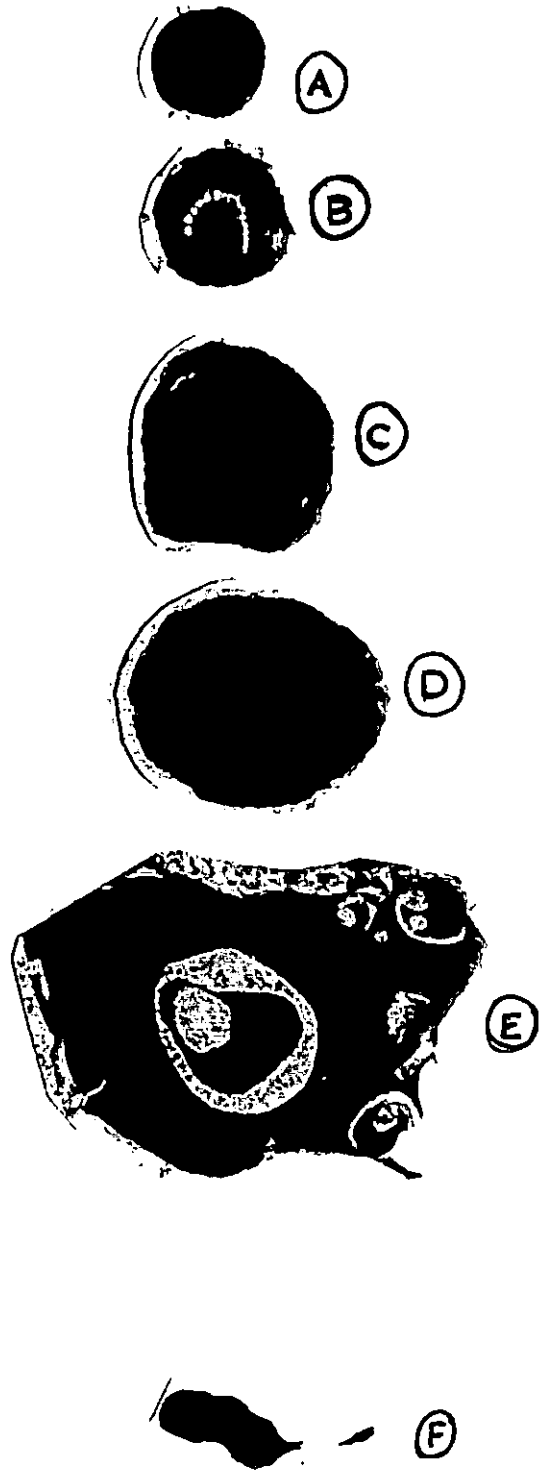
Figure a. Longitudinal section of the sperm of *B. melanostictus* whose different parts have been marked as A- terminal tip, B- sub-terminal end, C- anterior head nucleus, D- Posterior head nucleus, E. middle piece, and F- tail filament.

Figure b. Transverse section of different parts, which have been marked in the longitudinal section.

A. Transverse section through the acrosomal tip.
B. Grains in the middle circle indicating the presence of nuclear mass encircled by acrosomal part.
C. The deeply pigmented central nuclear region gradually increase in diameter indicating the anterior region of the nucleus.
D. The posterior portion of the nucleus indicated by the presence of pigmented granules throughout the diameter.
E. The middle piece with the peripheral mitochondria region and a central region from where the tail filament arises.
F. Transverse section of tail showing the axial filament with peripheral and central microtubules; an undulating membrane arises from the periphery of the axial filament and extend rightwards to form a button like knob.

PLATE – 17

(a) LONGITUDINAL SECTION



(b) TRANSVERSE SECTIONS

PLATE – 18:

Transmission Electron micrographs of different parts of *Bufo melanostictus* sperm.

Figure A. Longitudinal section of the whole sperm showing the proximal (PC) and distal centriole (DC). x3400

Figure B. Head portion showing distinct pinpointed perforatorium (P). x13500

Figure C. Distinct acrosome (A) and subacrosomal space is well visualized. The acrosomal membrane is continuous with the nuclear membrane. x8200

Figure D. Numerous sperms remail embedded in the Sertoli cell. x2050

PLATE – 18

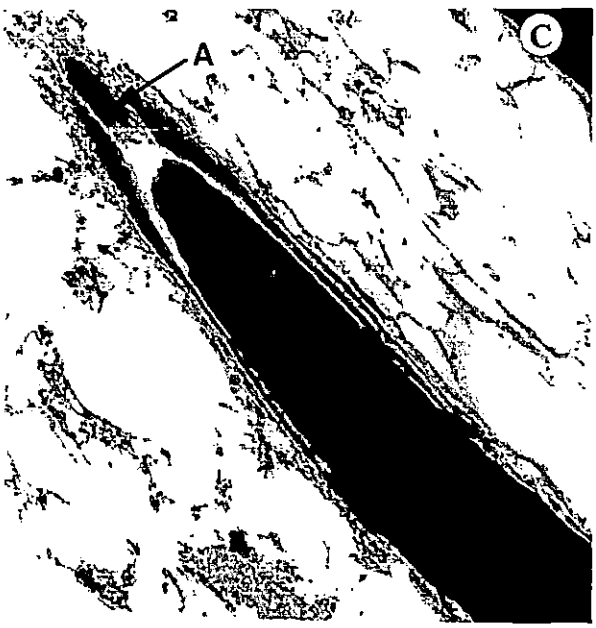


PLATE – 19:

Transmission Electron micrographs of different parts of *Bufo melanostictus* sperm.

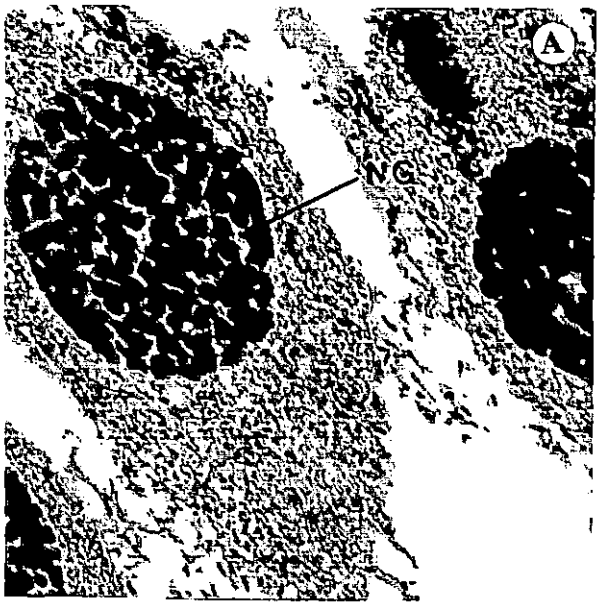
Figure A. Transverse section of nucleus showing numerous electron dense granules (NG). x10500

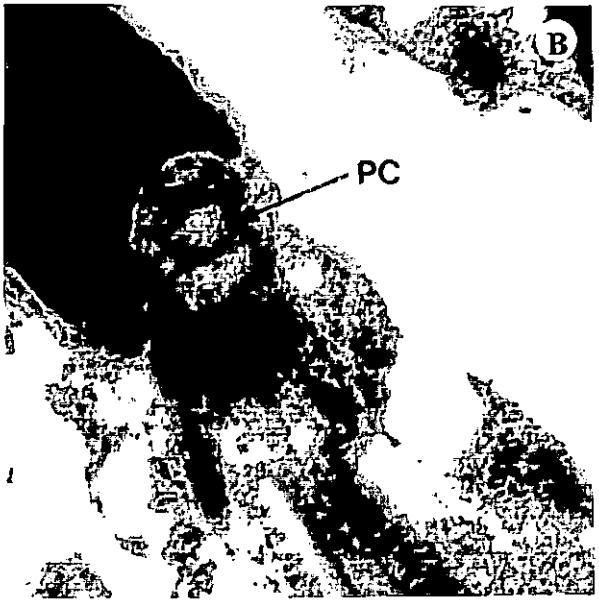
Figure B. Tail filament arising from the distal centriole; proximal centriole (pc) distinct and located in the subumbrellar nuclear notch. x13500

Figure C. Middle piece showing central axial filament (af) surrounded by numerous mitochondria. x5400

Figure D. Distinct axial filament with 18 peripheral and 2 central microtubule; undulating membrane arising from this axial filament. x21500

PLATE – 19





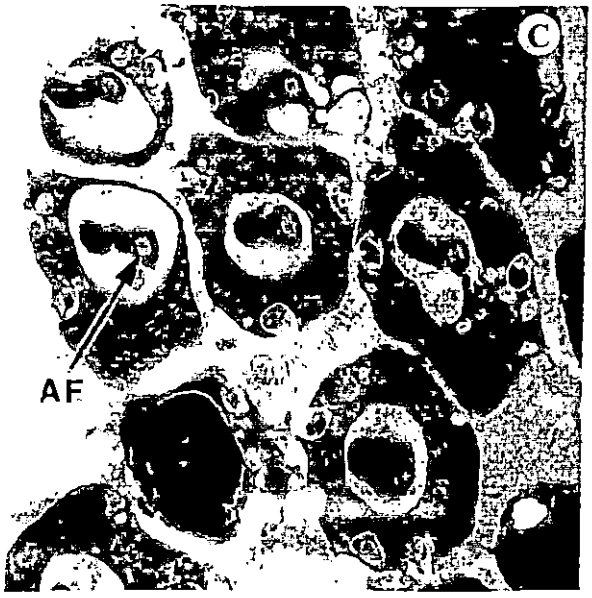


PLATE – 20:

Light microscopic picture of sperm of *Rana cyanophlyctis* at x3000 magnification.

A, B, C shows normal sperm with elongated nucleus and single tail filament.

D- Megacephalic sperm

E- Interstitial cells of Leydig

F- Sperms hanging from the Sertoli cell

PLATE – 20

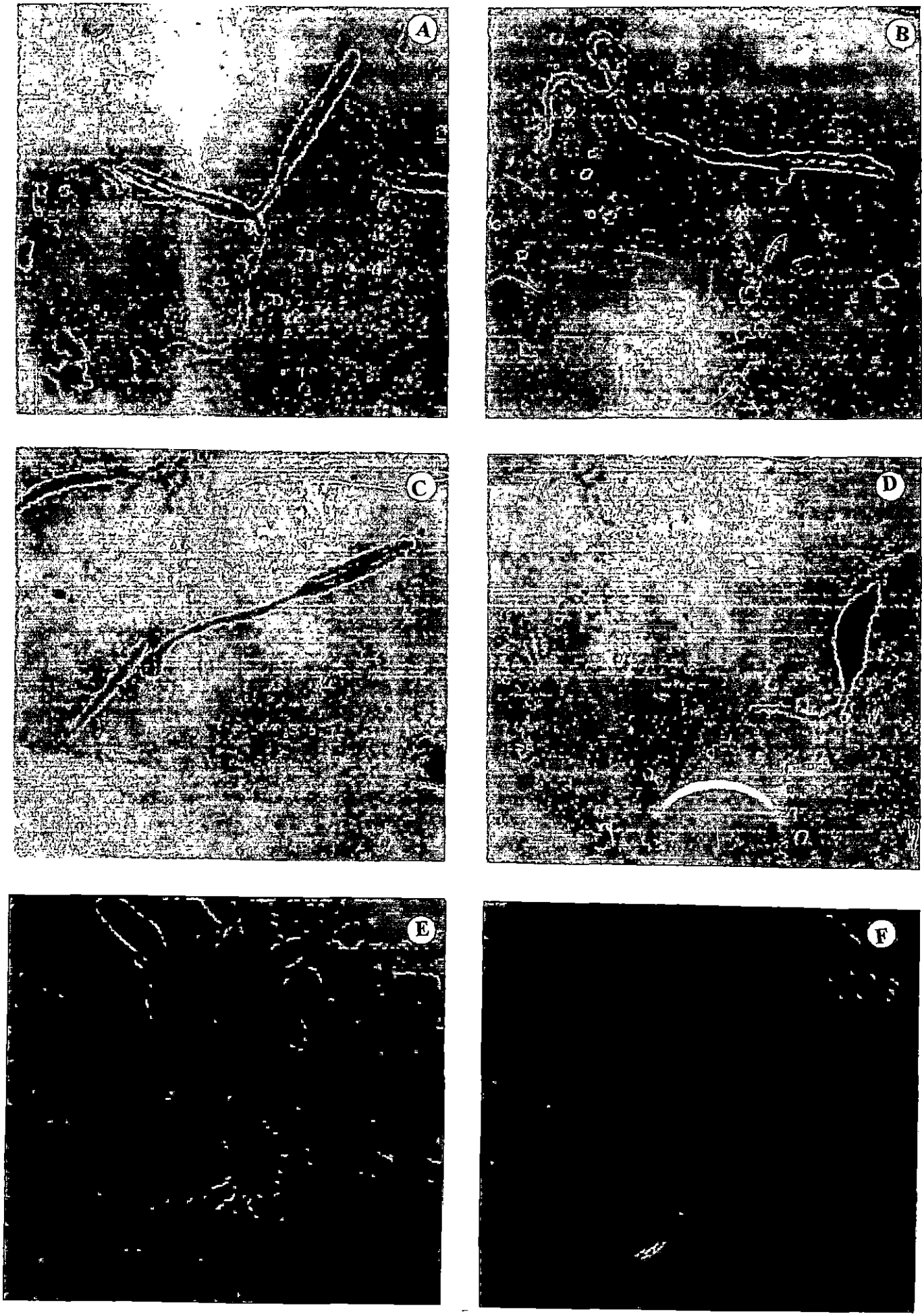


PLATE – 21:

Scanning and Transmission Electron micrographs of sperm of *Rana cyanophlyctis*.

A. Longitudinal section of *R. cyanophlyctis* testis. (SEM)

B. Sperm nest. (SEM)

C. Single normal sperm. (SEM)

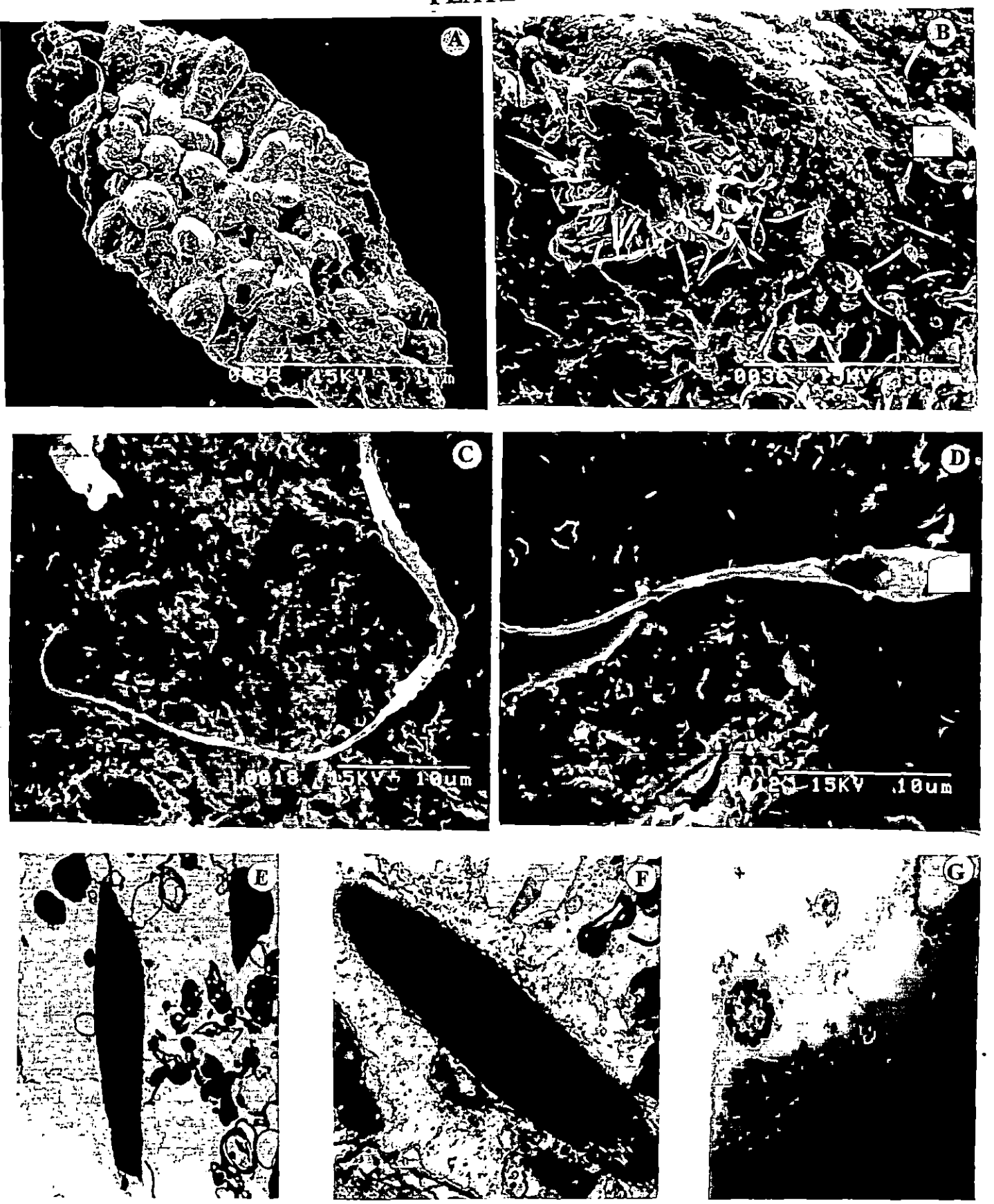
D. Megacephalic sperm (SEM)

E. Pointed acrosomal tip. x3400

F. Electron dense head nucleus. x4600

G. Axial filament of the tail with 18 peripheral and 2 central microtubule but without undulating membrane. x21500

PLATE – 21



Distinct middle piece is visible which starts from the 'n' shaped subumbrellar notch of the nucleus. Numerous mitochondria are packed in this region, which are almost circular in longitudinal section and arranged in a regular fashion on both sides of the anterior end of the tail (Plate-17, Fig. a). Proximal and distal centrioles are well documented and lie perpendicular to one another with a shorter in-between distance. The proximal centriole is located at the base of the head. It is circular in appearance with nine peripheral filaments distinctly visible. The distal centriole is elongated in longitudinal section and continues with the tail filament (Plate18, Fig. A; Plate-19, Fig. B).

The tail has a flagellar structure with 9+2 microtubular arrangement in the axial filament. In cross section the axial filament of the tail contains nine doublets of microtubules encircling closely situated but separate two singlets of microtubules (Plate-19, Fig. D). The central microtubules are connected to the peripheral microtubules by transverse protein fibres. The diameter formed by the peripheral microtubules, are quite large and no distinct membrane is visible on the outer surface of the axial filament. Undulating membrane is present but it is short and stumpy. The extreme end of the undulating membrane is rolled on itself giving a hollow tubular appearance (Plate-19, Fig. C, D).

DNA Analysis Observation

Through Gel Electrophoresis

The isolated DNA samples from individual specimens were analyzed under UV- Spectrophotometer and their purity along with concentration was observed by using the formula -DNA Content = (50 μg / ml) x 1.0 A_{260} .

The obtained results are detailed in Table-4.

TABLE - 4

Spectrophotometric results of isolated DNA

Serial Number	Sample Number	OD at 260 nm	OD at 280 nm	OD260/OD280		DNA Content
01.	Bh1a	0.26	0.14	1.86	✓	**13.0 µg / ml**
02.	Bh1b	0.17	0.09	1.88	✓	**08.5 µg / ml**
03.	Bh1c	0.28	0.18	1.55	✗	14.0 µg / ml
04.	Bh2a	0.22	0.12	1.83	✓	**11.0 µg / ml**
05.	Bh2b	0.24	0.13	1.85	✓	**12.0 µg / ml**
06.	Bh2c	0.22	0.12	1.83	✓	**11.0 µg / ml**
07.	Bh3a	0.16	0.13	1.23	✗	08.0 µg / ml
08.	Bh3b	0.25	0.14	1.78	✗	12.5 µg / ml
09.	Bh3c	0.17	0.10	1.70	✗	08.5 µg / ml
10.	Bs1a	0.15	0.08	1.87	✓	**17.5 µg / ml**
11.	Bs1b	0.22	0.16	1.37	✗	14.5 µg / ml
12.	Bs1c	0.17	0.10	1.70	✗	11.0 µg / ml
13.	Bs2a	0.16	0.09	1.77	✗	08.0 µg / ml
14.	Bs2b	0.16	0.08	2.00	✓	**08.0 µg / ml**
15.	Bs2c	0.21	0.14	1.50	✗	10.5 µg / ml
16.	Bs3a	0.16	0.08	2.00	✓	**08.0 µg / ml**
17.	Bs3b	0.15	0.08	1.87	✓	**07.5 µg / ml**
18.	Bs3c	0.22	0.12	1.83	✓	**11.0 µg / ml**
19.	Bm1a	0.37	0.18	2.05	✓	**18.5 µg / ml**
20.	Bm1b	0.32	0.18	1.77	✗	16.0 µg / ml
21.	Bm1c	0.35	0.15	2.33	✓	**17.5 µg / ml**
22.	Bm2a	0.28	0.18	1.55	✗	14.0 µg / ml
23.	Bm2b	0.28	0.16	1.75	✗	14.0 µg / ml
24.	Bm2c	0.35	0.22	1.59	✗	17.5 µg / ml
25.	Bm3a	0.32	0.18	1.80	✓	**16.0 µg / ml**
26.	Bm3b	0.32	0.17	1.88	✓	**16.0 µg / ml**
27.	Bm3c	0.35	0.21	1.67	✗	17.5 µg / ml

PLATE – 23:

Photographs of DNA after gel electrophoresis-

A. Genomic DNA of three different species of family Bufonidae ;

Lane 1- *Bufo himalayanus*

Lane 2- *Bufo stomaticus*

Lane 3- *Bufo melanostictus*

B. Bam H_1 digested DNA of three different species of family Bufonidae ;

Lane 4- *Bufo himalayanus*

Lane 5- *Bufo stomaticus*

Lane 6- *Bufo melanostictus*

C. Eco R I digested DNA of three different species of family Bufonidae ;

Lane 4- *Bufo himalayanus*

Lane 5- *Bufo stomaticus*

Lane 6- *Bufo melanostictus*

D. Hind II digested DNA of three different species of family Bufonidae ;

Lane 4- *Bufo himalayanus*

Lane 5- *Bufo stomaticus*

Lane 6- *Bufo melanostictus*

PLATE – 23

A. Genomic DNA

B. Bam H_1 digest

C. EcoR I digest

D. Hind II digest

Of these estimated DNA samples, the pure forms were isolated for further electrophoretic analysis and the rest discarded.

Equal amount of DNA from three different species were separated by gel electrophoresis and the banding patterns of the genomic DNA was noted.

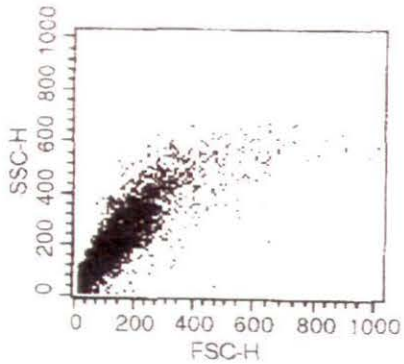
After that, equal amount of DNA from three different species were again taken and digested separately by three different restriction endonuclease enzyme. Finally they were passed through gel for electrophoresis and the results noted.

From the gel pictures (Plate-23) it can be analysed as follows :-
In Gel A (genonic DNA) it can be seen that all the genomic DNA bands were almost in the same level with the *B.himalayaus* is at slightly upper level than the other two (*B. stomaticus and B. melanostictus).* This indicates that the genomic DNA of *B.himalayanus* is slightly greater in size than *B. stomaticus and B. melanostictus.*

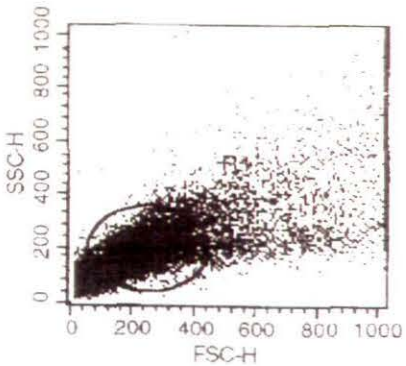
Analysing Gel A we can see that after Bam H_1 digest all the DNA samples in the gel has moved down indicating the restriction cut of the DNA and fragmentation has taken place. Here also we can see that the DNA of *B. himalayanus* is running behind the other two, while that of *B. stomaticus* and *B. melanostictus* are running neck to neck indicating quantative similarity in the genomic DNA if the two (*B. stomaticus* and *B. melanostictus*).

Lane 7, 8, & 9 of Gel C is showing the restriction digest of genomic DNA of all the three species by EcoR I. and it is seen that this particular enzyme is more sensitive to *B. stomaticus* than the other two. But a different situation arises in case of Hind Iiof Gel D . After Hind II digestion it is observed that the DNA of *B.himalayanus* remains relatively intact but that of other two smeared off after Hind II digestion indicating a massive digestion rate.

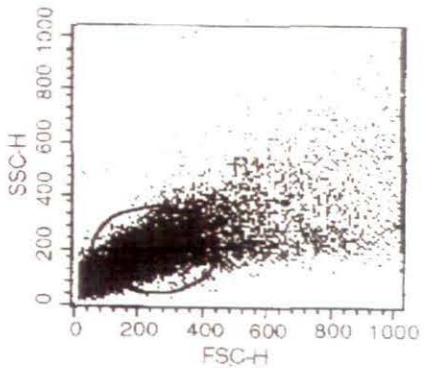
PLATE – 24



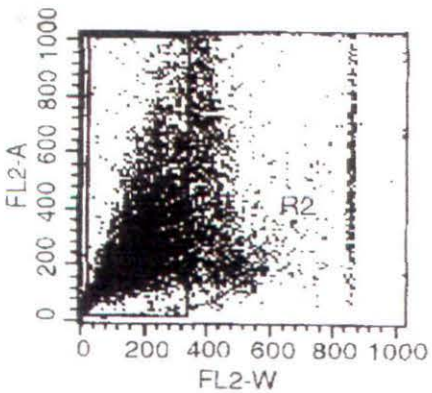
Bufo himalayanus



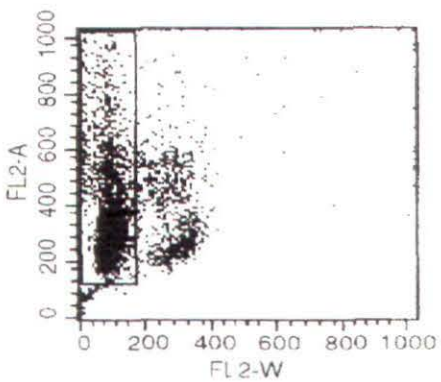
Bufo stomaticus



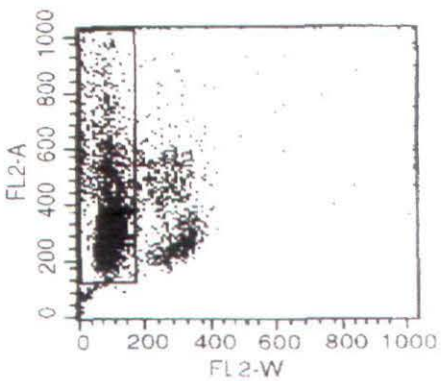
Bufo melanostictus



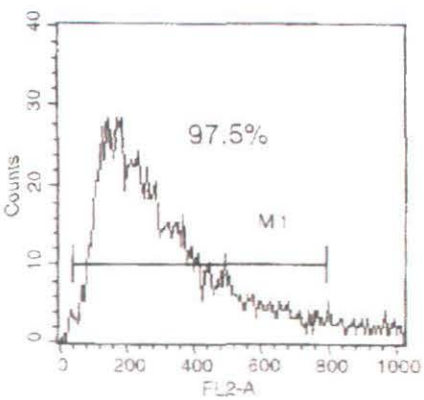
Bufo himalayanus



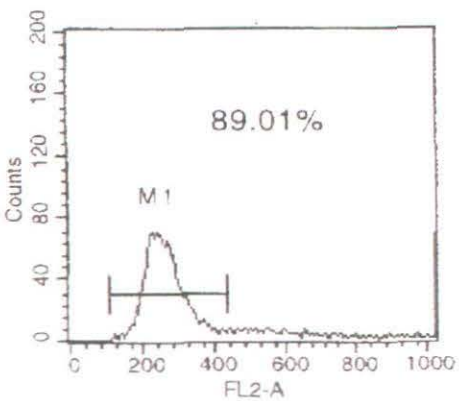
Bufo stomaticus



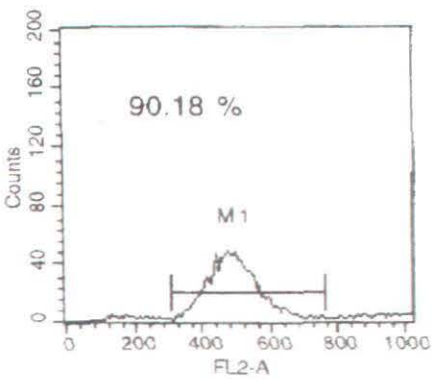
Bufo melanostictus



Bufo himalayanus



Bufo stomaticus



Bufo melanostictus

Result of FACS study for measuring DNA content of sperm of three different species of the family Bufonidae.

Through Fluorescence Activated Cell Shorting

From the graph result (Plate-24), it is observed that the peak value for *Bufo stomaticus* is 309.8, for *Bufo melanostictus* it is 523.34 and for *Bufo himalayanus* it is 280.92. As a single curve is obtained in every case it can be said that the examined cells were containing a same quantity of DNA in every case. From these two type of observation it can be concluded that all the three specimens analyzed under FACS were containing different amount of DNA as the genetic material.