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AUGMENTING REPRODUCTIVE EFFICIENCIES IN MITHUN THROUGH BIOTECHNOLOGICAL INTERVENTION

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Background

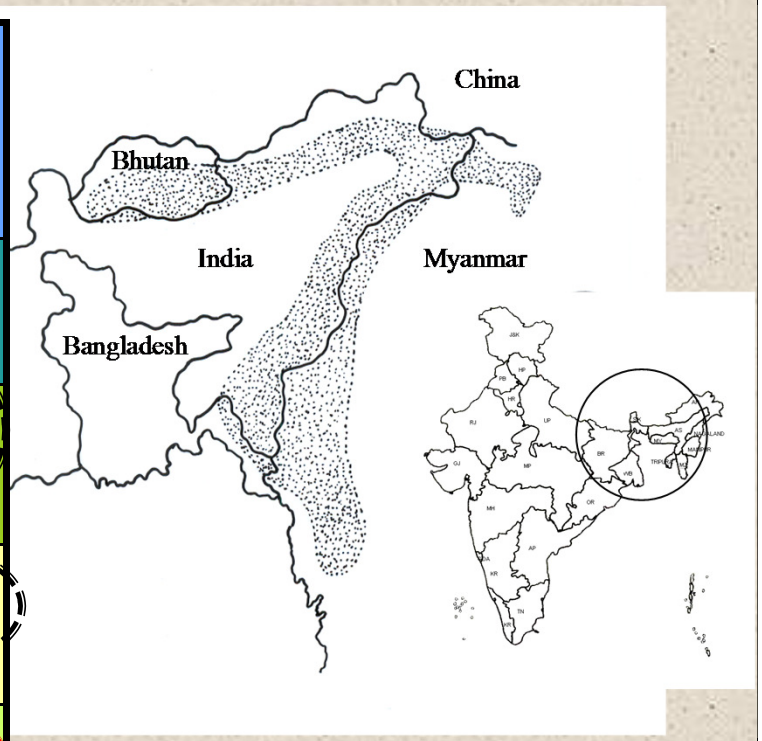
- North-East India - rich in floral and faunal biodiversity
- Nagaland is endowed with rich germplasm of different endemic species including mithun (*Bos frontalis*
- Arunachal Pradesh, Mizoram and Manipur
- It plays an important role in the socio-economic & cultural life of the tribal population of NEH region
- Reared mainly for **meat** production

- There is an immense scope to increase meat production to meet the demand of the fast growing population by exploiting the rate of reproductive potential of mithun through judicious application of assisted reproductive technologies.

- However, understanding of endocrinology and reproductive physiology is of paramount importance for application of assisted reproductive technologies

Recent trend in Mithun population

State	1997	2003	2007
Arunachal	1,24,194	1,84,343	2,18,931
Nagaland	33,445	40,452	33,385
Manipur	16,660	19,737	10,024
Mizoram	2,594	1,783	1,939
Total	1,76,893	2,46,315	2,64,279



MITHUN INHABITED AREA

(NOT TO THE SCALE)

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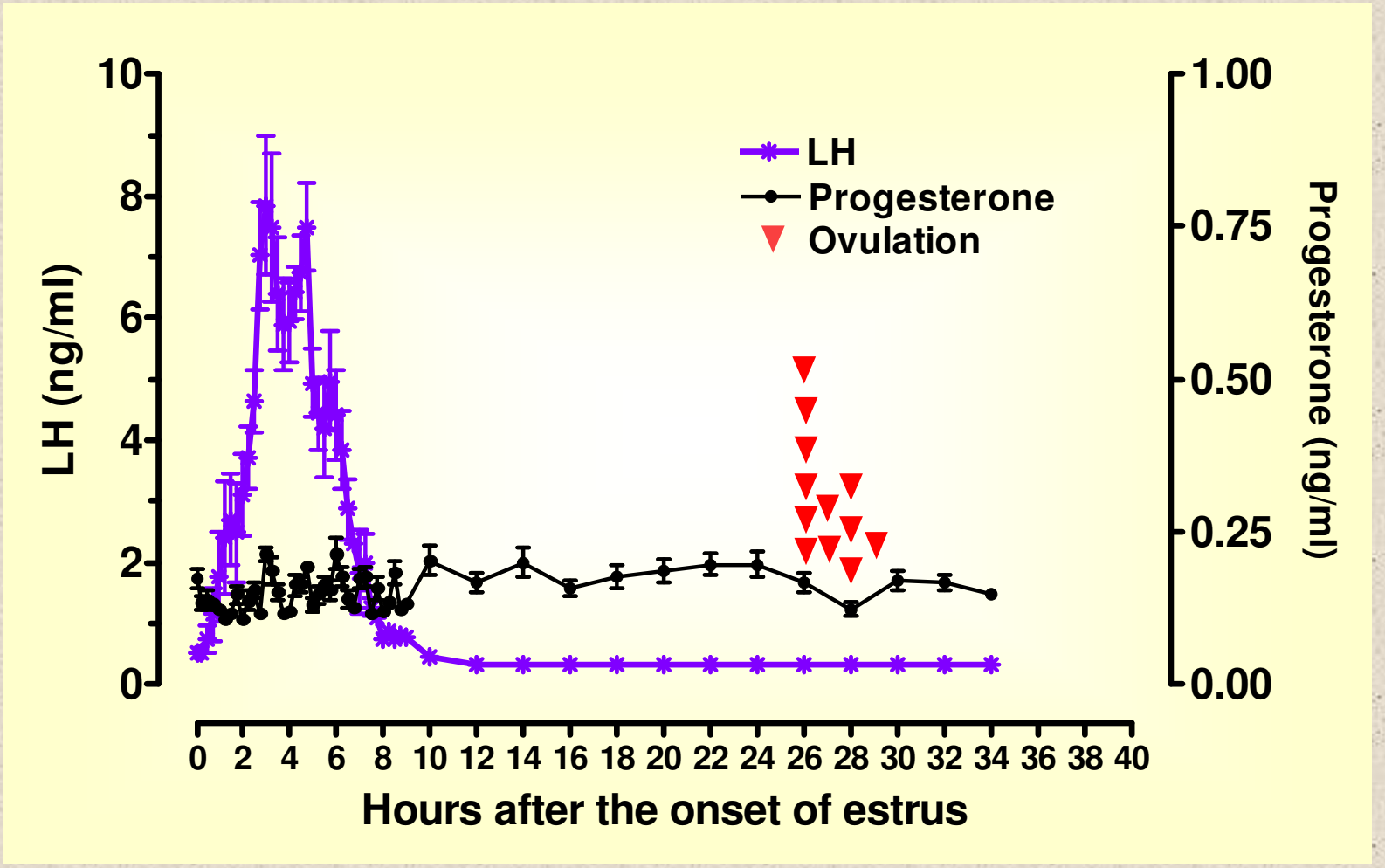
Timing of Ovulation in Relation to Onset of Estrus and LH Peak in Mithun Cows

Fig. Changes in the plasma LH and progesterone profile (mean±SEM) in mithun cows (n=12) after onset of estrus. Blood samples were collected at 15 min intervals after the initial expression of heat symptoms by the mithuns for 9 h period and thereafter at an interval of 2 h till 4h post ovulation. Ovulation was confirmed by rectal palpation at 2 h intervals.



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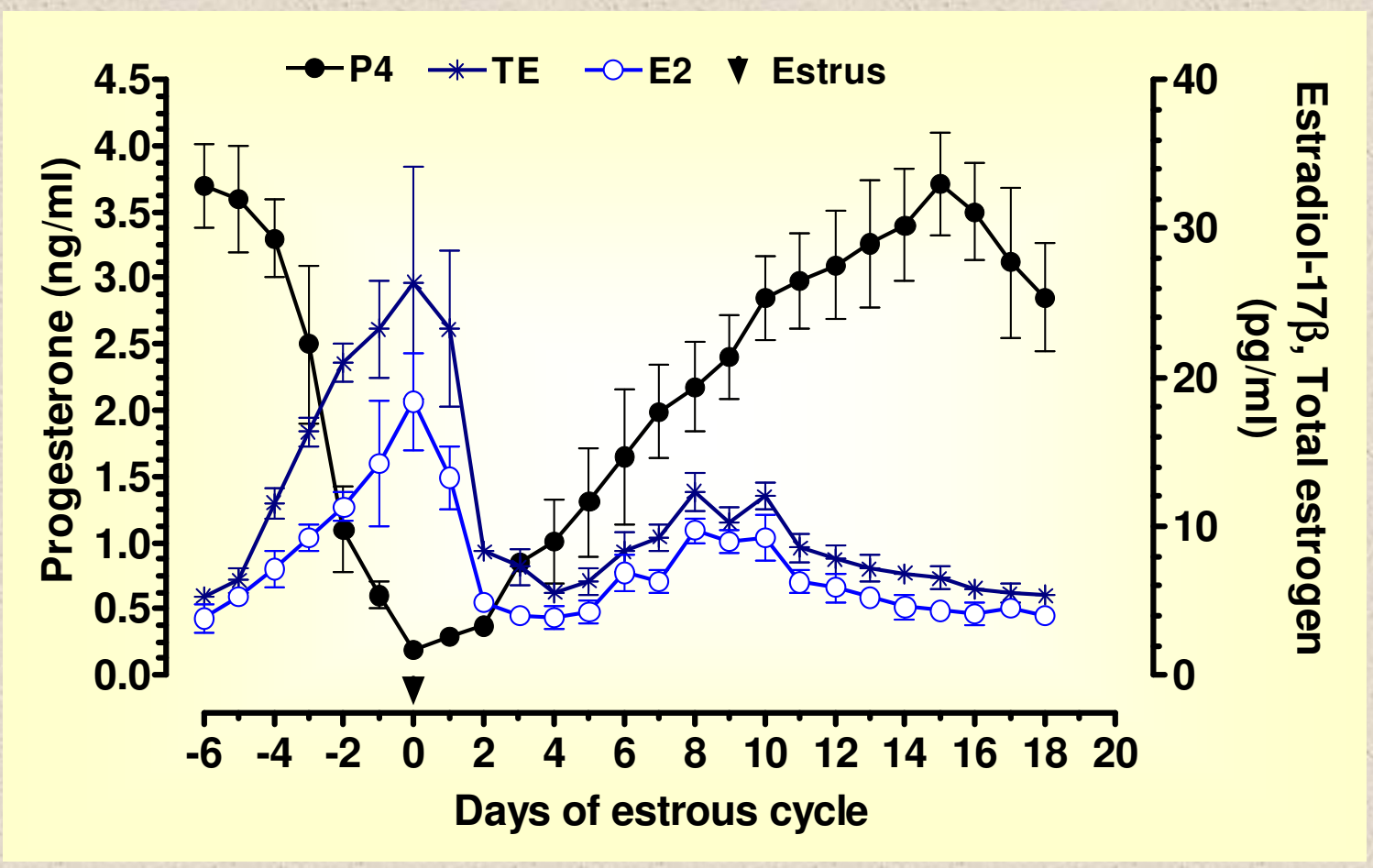
Mean (±SEM) plasma estradiol-17β (E2), total estrogen (TE) and progesterone (P4) profiles during the different days of estrous cycle in mithun cows (n=12). Blood samples were collected daily for the entire cycle.



Estrus synchronization using $PGF_{2\alpha}$

- Two injections of $PGF_{2\alpha}$ were given at 11 days apart
- Animals were observed for signs of estrus after second injection of $PGF_{2\alpha}$
- Estrus was identified by observing behavioural signs of estrus and through rectal examination. It was further confirmed by progesterone profiles
- Time of ovulation was determined by per recta examination of ovaries at every 2h intervals from the onset of estrus

Plasma LH characteristics and timing of ovulation in mithuns subjected to two injection of $PGF_{2\alpha}$ at 11 days apart for estrus synchronization

Parameters	Animals	Mean±SEM	Range
Highest LH peak concentration (ng/ml)	24	12.54±1.37	7.34 to 22.65
Duration of LH surge (h)	24	14.76±1.47	10 to 19
Time from:			
2^{nd} $PGF_{2\alpha}$ injection to onset of estrus	**24**	**43.52±5.93**	**36 to 58**
Onset of estrus to onset of LH surge (h)	24	2.45±0.43	1.5 to 3.25
Onset of estrus to ovulation (h)	23*	33.95±1.41	27 to 39
2^{nd} $PGF_{2\alpha}$ injection to ovulation	**23***	**74.5±5.93**	**66 to 78**
After end of LH surge to ovulation (h)	23*	20.45±0.89	17 to 24

*One animal did not ovulate

All the cows treated with the protocol were inseminated naturally twice at 60 and 72h after second $PGF_{2\alpha}$. Out of 12 cows inseminated, 9 (75%) were conceived.

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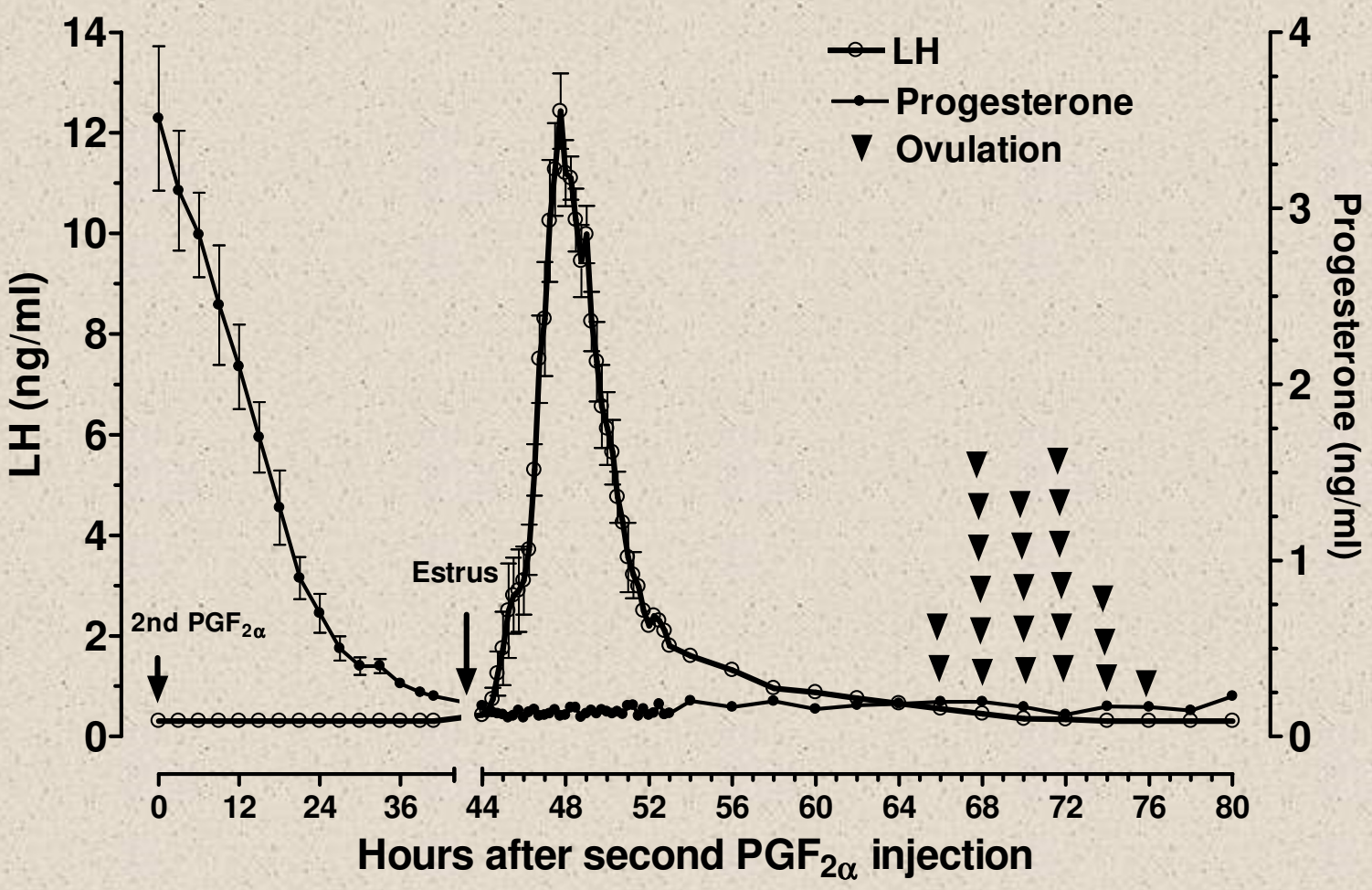
Mean (±SEM) plasma concentrations of LH and progesterone in Mithun cows (n=24) subjected to two injection of $PGF_{2\alpha}$ at 11 days apart for oestrus synchronization.



Estrus synchronization using Ovsynch protocol

- Day 0 : Injection of GnRH
- Day 7 : Injection of PGF_{2a}
- Day 9 : Injection of GnRH
- Day10: Heat detection
- Ovulation time: 2h interval

Plasma LH characteristics and timing of ovulation in mithuns (n=23) subjected to Ovsynch protocol

Parameters	Animals	Mean±SEM	Range
Highest LH peak concentration (ng/ml)	23	12.23±0.66	9.03 to 17.22
Duration of LH surge(h)	23	8.25±1.38	6 to 12
Time from:			
a) Onset of LH surge after 2nd GnRH Injection (h)	23	1.75±0.44	1.25 to 2.75
b) Ovulation after 2nd GnRH injection (h)	**23**	**26.75±2.02**	**19 to 33**
c) Ovulation after end of LH surge (h)	23	18.62±1.69	15 to 27

fixed time artificial insemination 12 to 16 h after the second GnRH injection

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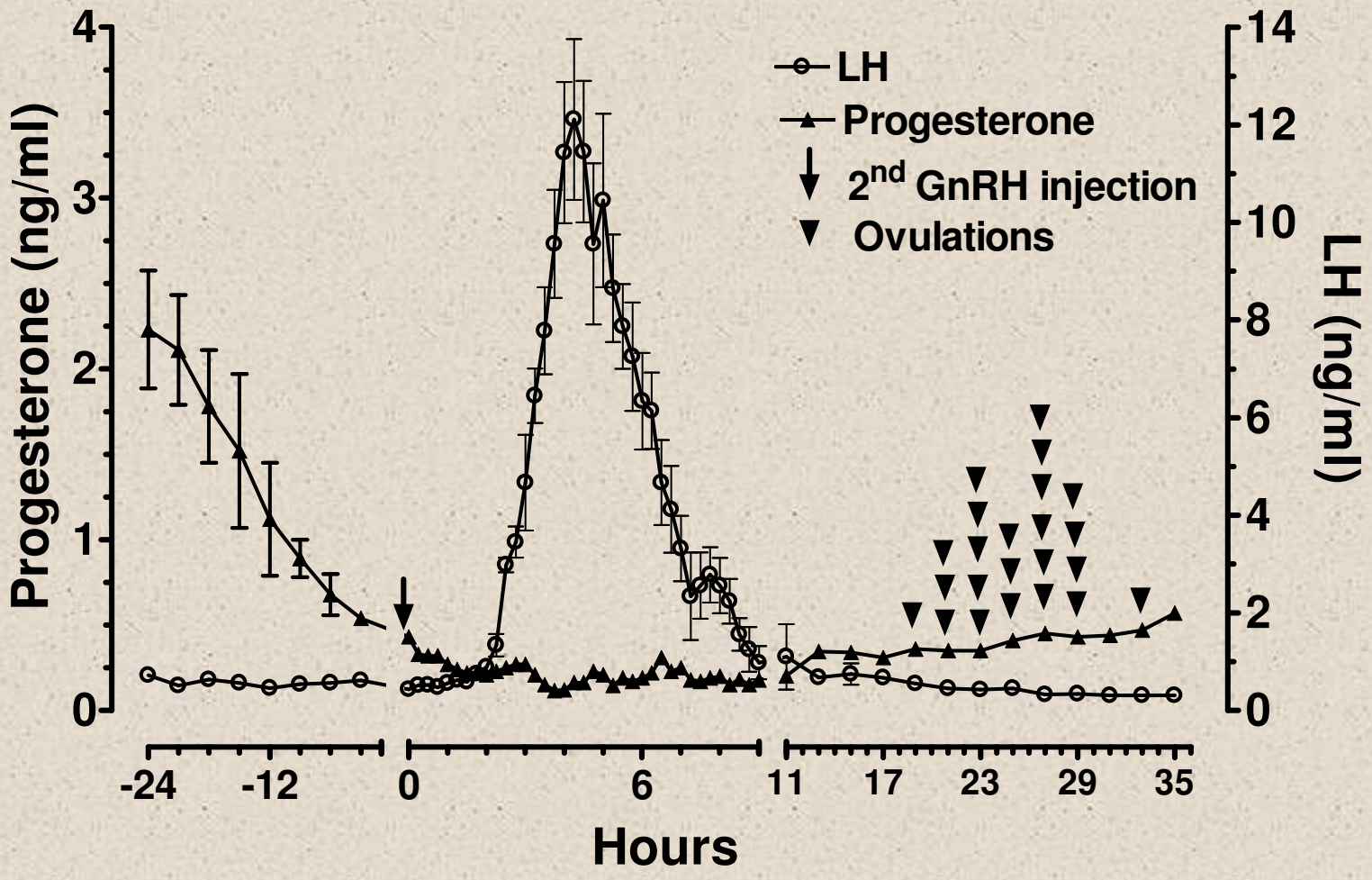
Mean (±SEM) plasma concentrations of LH and progesterone in Mithun cows (n=23) treated with Ovsynch protocol. Receptal (3.5ml; GnRH analogue) was injected and blood samples were collected at 15 min interval one hour prior to and 9-h post GnRH administration (0h) and thereafter at 2h interval till 2h post-ovulation.



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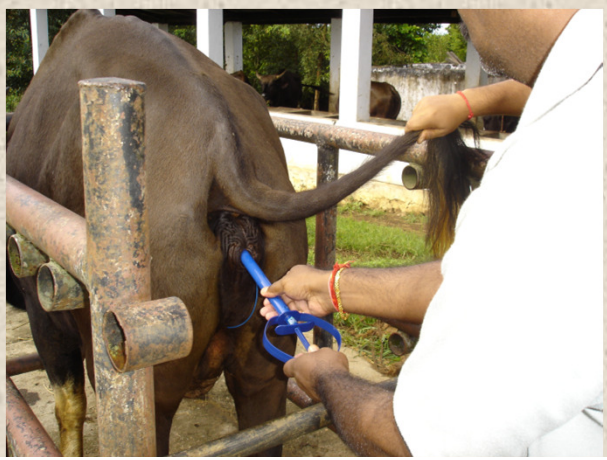
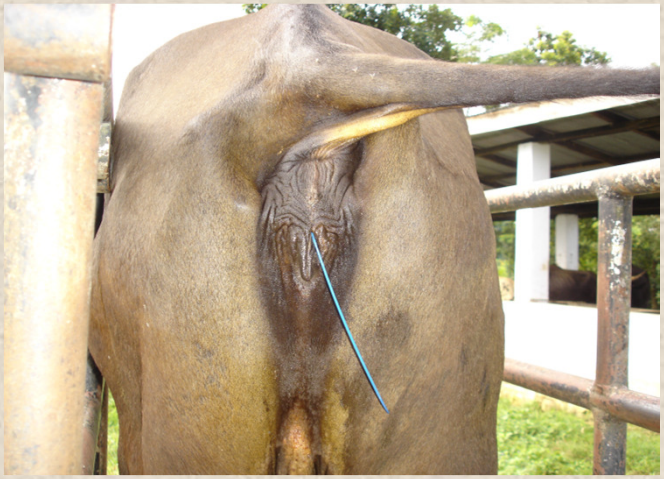
Estrus synchronization using CIDR

- CIDR insert: Day 0
- PGF_{2a} injection -Day 6.
- CIDR removal - Day 7
- Observed for signs of estrus – day 8
- Ovulation time - every 2h interval

CIDR with applicator



CIDR is inserted in a mithun cow



Mithun cow with an inserted CIDR

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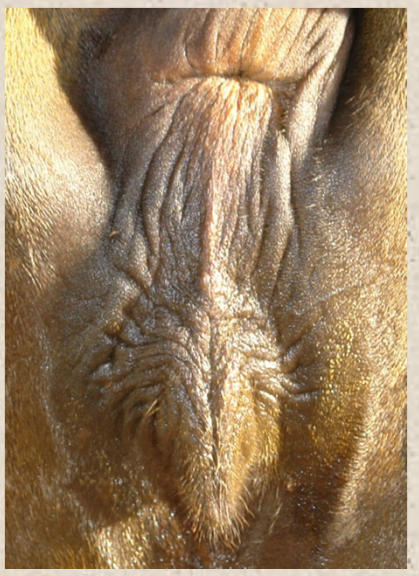
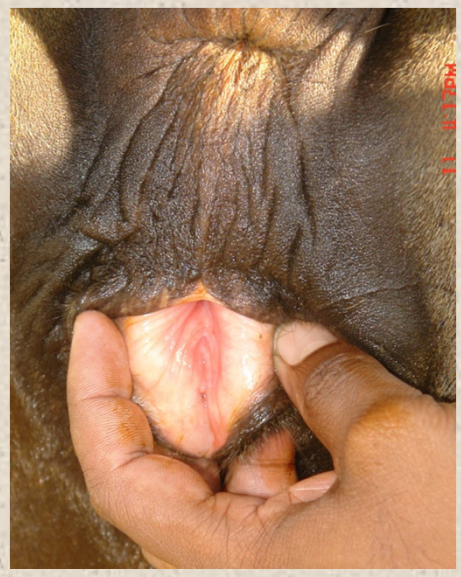
- **Intensity of estrus more prominent than spontaneous estrus**





- **CIDR - Very much useful for induction of postpartum oestrus**

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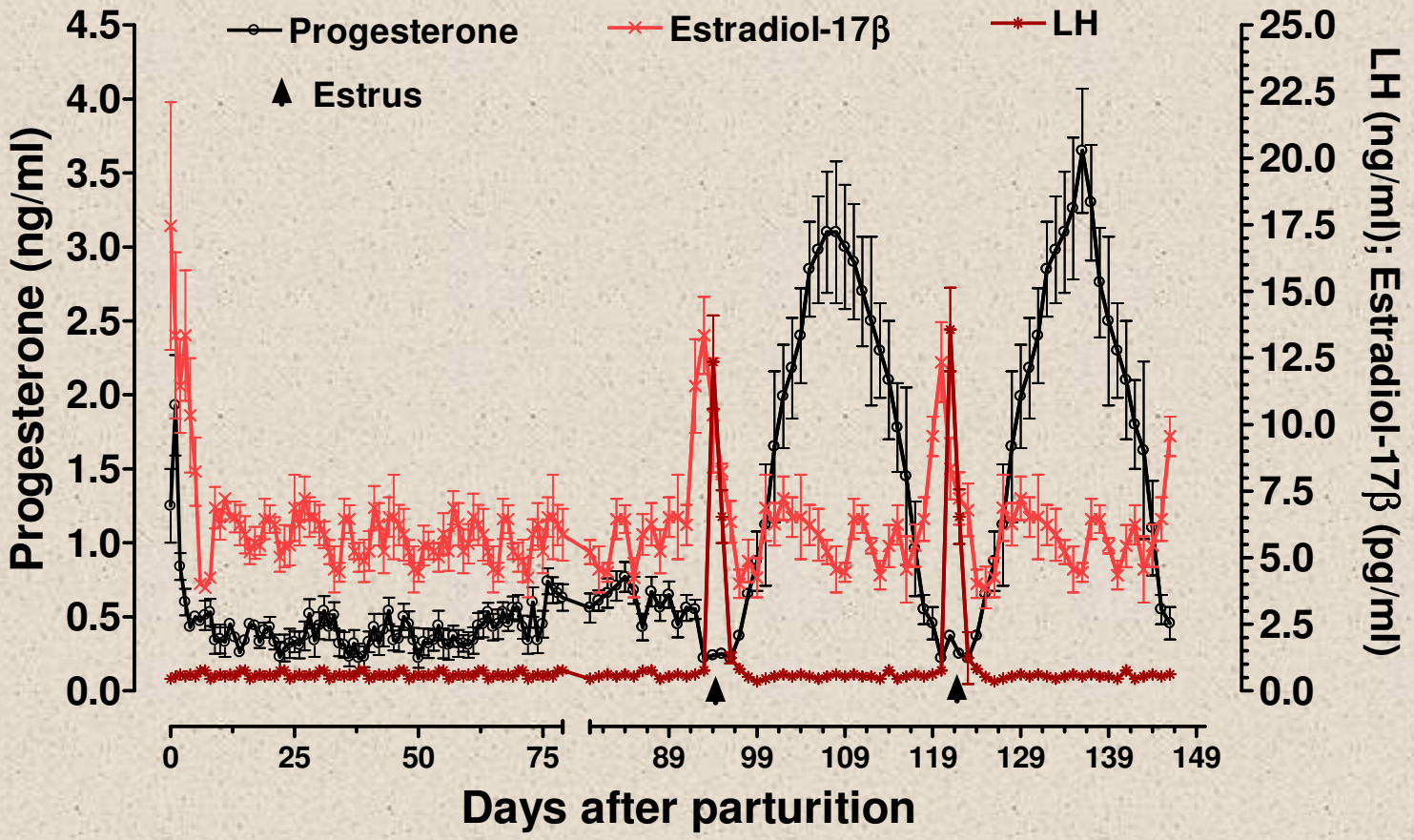
Mean (±SEM) plasma profiles of progesterone, LH and estradiol-17β in post-partum mithun cows (n=37) from parturition (day 0) to onset of first post-partum estrus. Blood samples were collected daily from each animal for plasma progesterone, LH and estradiol-17β. Plasma hormone profiles suggested that the mithun cows exhibited first estrus at day 97±19.6 postpartum (range: day 78 to 113 postpartum). Plasma progesterone and LH concentrations remained basal till first postpartum estrus, which was silent in most of the cows. Plasma estradiol-17β exhibited little fluctuation from the day of parturition till first postpartum estrus where peak concentration was recorded.



Application of CIDR for early induction of estrus in postpartum mithun cows

Plasma LH characteristics and timing of estrus and ovulation in postpartum mithuns (n=24) subjected to CIDR.

Parameters	Animals	Mean±SEM	Range
Highest LH peak concentration (ng/ml)	24	12.11±1.65	8.54 to 15. 9
Duration of LH surge (h)	24	9.15±2.08	8.25 to 11.0
Time from (h):			
Onset of LH peak after $PGF_{2\alpha}$ injection	24	70.60±3.44	66 to 76
Onset of estrus after $PGF_{2\alpha}$ injection	24	67.2±2.30	56 to 72
Onset of estrus after CIDR removal	24	44±3.44	36 to 54
Ovulation after $PGF_{2\alpha}$ injection	24	86.5±4.12	76 to 92
Ovulation after CIDR removal	**24**	**63.52±3.50**	**48 to 60**
Ovulation after end of LH surge (h)	24	20.52±1.69	18 to 24

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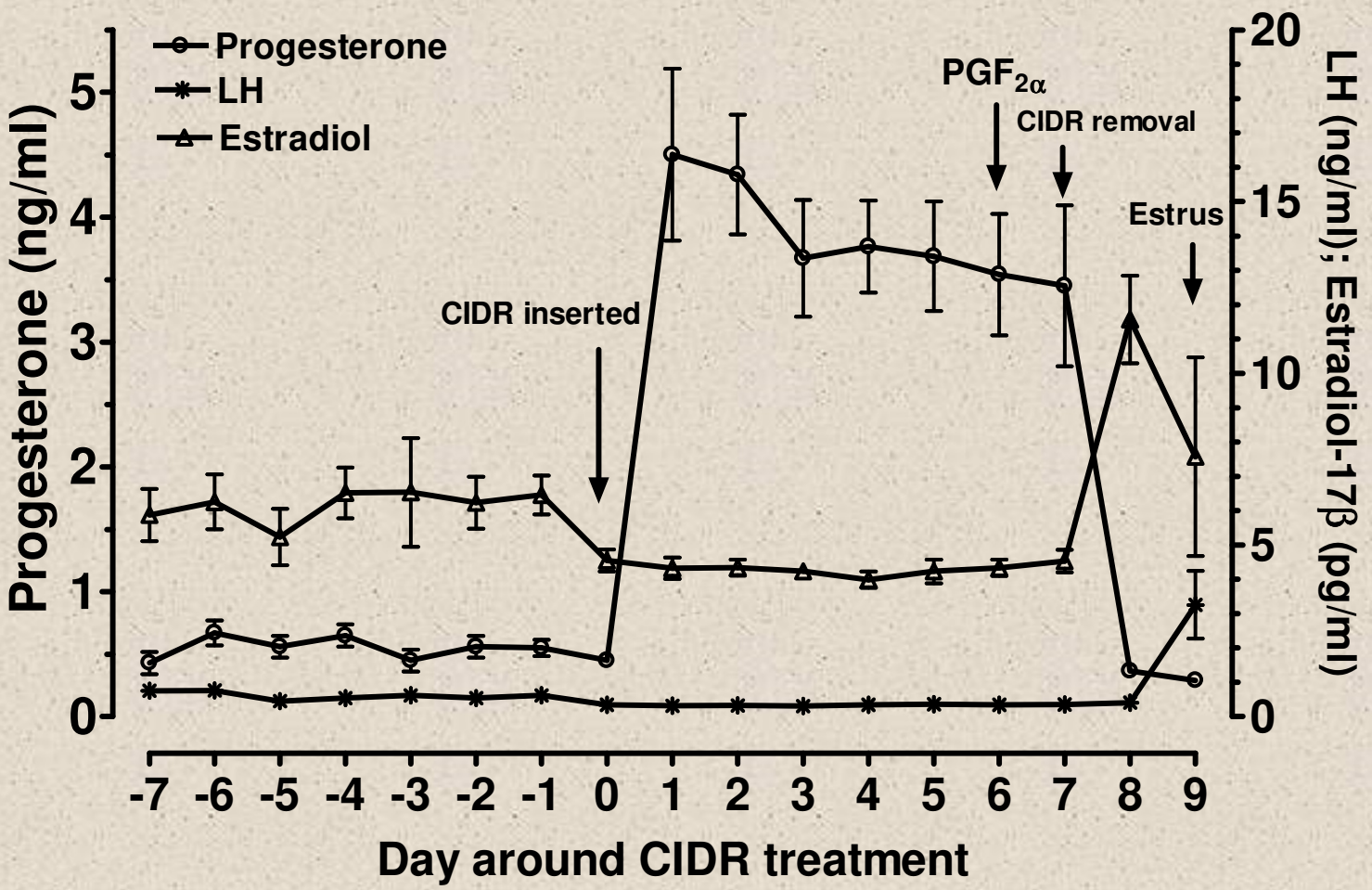
Mean (±SEM) profile of plasma progesterone, LH and estradiol-17β in postpartum anoestrus mithun cows (n=24) treated with CIDR and $PGF_{2\alpha}$. Blood samples were collected 7 days prior to and 9 days after CIDR administration (day 0= day of CIDR administration).



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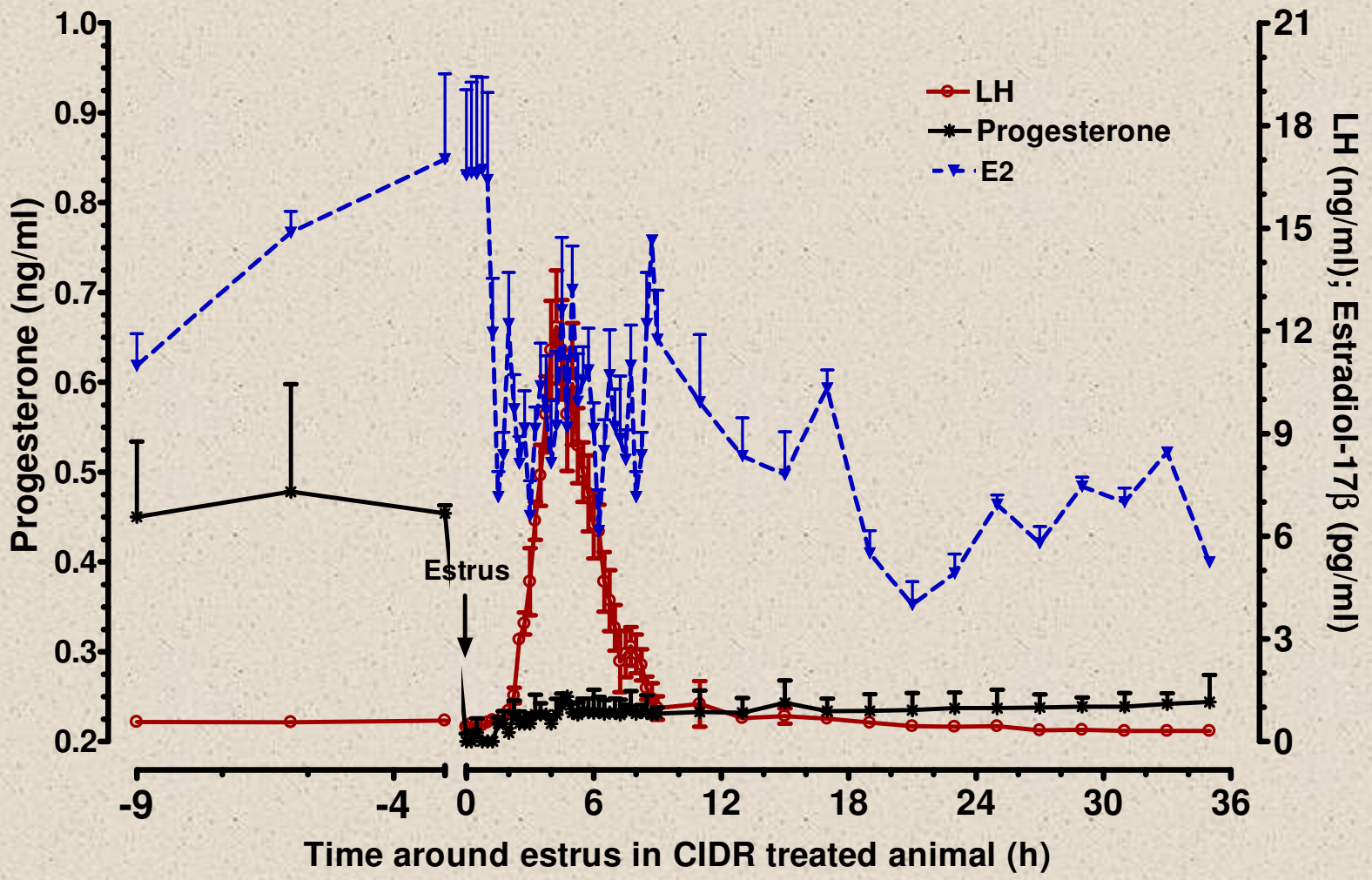
Mean (±SEM) profile of plasma progesterone, LH and estradiol-17β in postpartum anoestrus mithun cows (n=24) treated with CIDR and $PGF_{2\alpha}$. Blood samples were collected 9 h prior to estrus at every 3h interval and then at an interval of 15 min for 9h post-estrus and thereafter at every 2 h interval till 4h post-ovulation



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Application of CIDR for synchronization of estrus in cyclic mithun cows

Parameters	Animals	Mean±SEM	Range
Highest LH peak concentration (ng/ml)	24	19.78±1.89	14.8 to 22.6
Duration of LH surge (h)	24	14.25±2.34	10.50 to 16.25
Time from (h):			
Onset of LH peak after $PGF_{2\alpha}$ injection	24	66.40±2.78	56 to 68
Onset of estrus after $PGF_{2\alpha}$ injection	24	63.50±2.23	54 to 67
Onset of estrus after CIDR removal	24	40±2.56	32 to 49
Ovulation after $PGF_{2\alpha}$ injection	24	81.8±3.05	71 to 87
Ovulation after CIDR removal	24	59.52±3.25	44 to 56
Ovulation after end of LH surge (h)	24	17.37±1.82	15 to 21

Initially, 22 mithun cows were synchronized for estrus with CIDR and inseminated at 48 and 60h following withdrawal of CIDR insert.

A conception rate of 72.7% was achieved.

Application of Heatsynch protocol in mithuns

Protocol:

Day 0: GnRH

Day 7: $PGF_{2\alpha}$ injection

Day 8: ECP injection

Day 8 through 9: Estrus detection

Day 8 through 10: Insemination

Plasma LH characteristics and timing of estrus and ovulation in mithuns (n=12) subjected to Heatsynch protocol

Parameters	Animals	Mean±SEM	Range
Highest LH peak concentration (ng/ml)	12	9.73±1.66	7.23 to 16.19
Duration of LH surge (h)	12	16.25±2.38	12.50 to 19.0
Time from (h):			
Onset of LH peak after ECP injection	12	21.90±1.44	18 to 26
Onset of estrus after ECP injection	12	20.2±1.32	16 to 24
Ovulation after $PGF_{2\alpha}$ injection	12	76.5±2.12	60 to 72
Ovulation after ECP injection	12	51.84±2.25	48 to 60
Ovulation after end of LH surge (h)	12	20.62±1.69	16 to 24

The pregnancy rate of 75% was achieved with natural service on detected estrus

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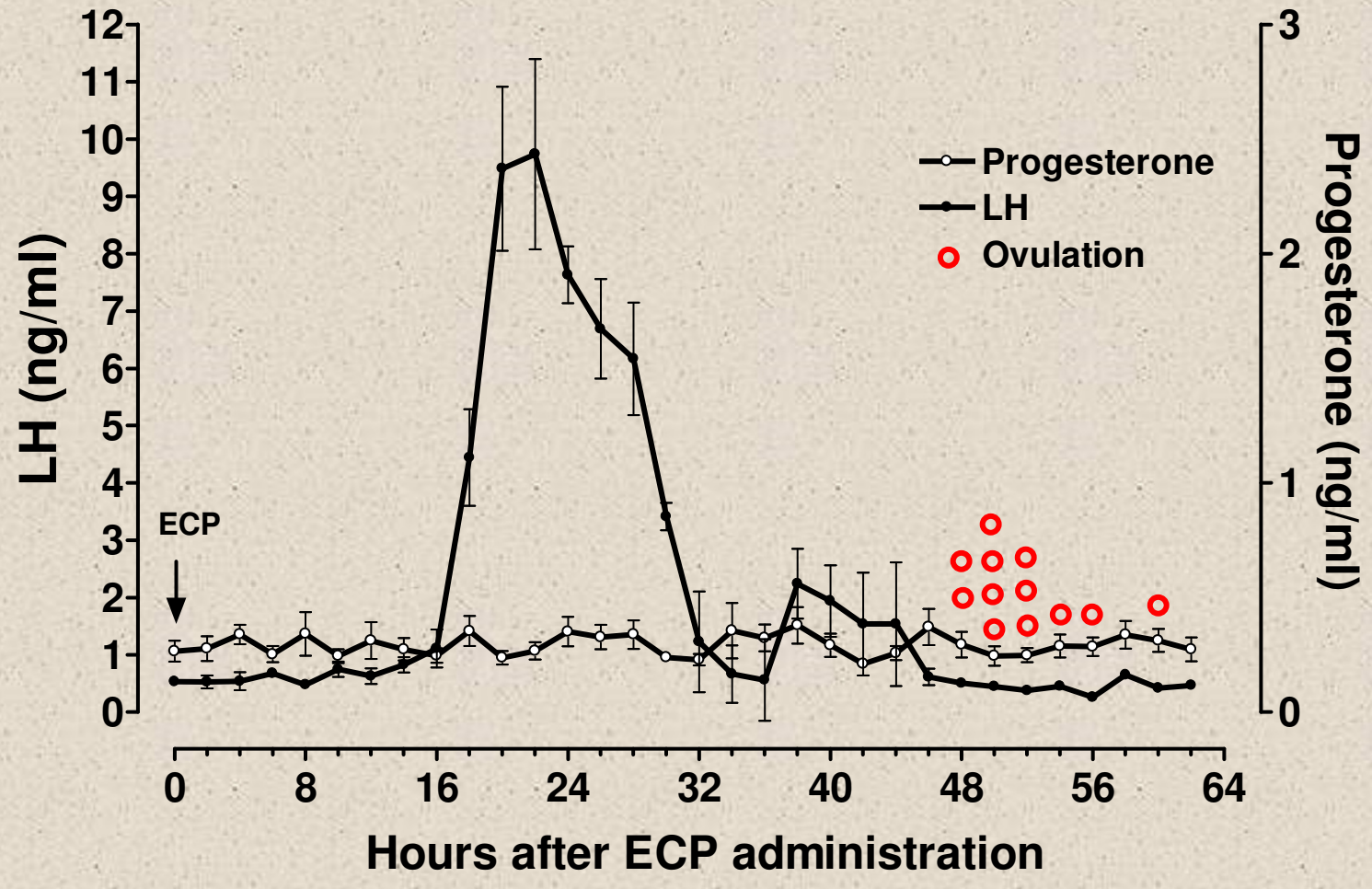
Changes in plasma LH and progesterone (mean±SEM) after the administration of ECP in mithuns (n=12) according to the Heatsynch protocol of estrus synchronization. Blood samples were collected at 2h intervals beginning with the time of ECP administration until 2h post-ovulation



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Standardisation of Innovative method of semen collection in Mithun

It was difficult to tease the mithun bull without estrus animal

Urine from estrus mithun cows were collected and stored at refrigerated temperature

This urine samples were sprinkled over the perineal region of mithun cow not in estrus

Bull reacted as an estrus cow and semen was collected successfully

Preservation of mithun semen

A) Protocols for semen preservation at refrigeration temperature

After collection, semen was diluted in Tris-egg-yolk extender

↓

Preserved at 4°C

↓

Evaluated at 6 hour interval (progressive motility, live & dead count, acrosomal integrity and morphological abnormalities)

Sperm were classified into four categories namely a) live, intact acrosome b) live, damaged acrosome c) dead, intact acrosome and d) dead , damaged acrosome

Different parameters of mithun semen

Particulars	Mean ± SE
Volume (ml)	2.2 ± 0.3
Colour	Creamy white
Mass activity(5+ scale)	3.3 ± 0.2 (5 point scale)
Progressive motility (%) immediately after dilution	66. 7 ± 3.3
Spermatozoa concentration (10^6 ml^{-1})	550 ± 46

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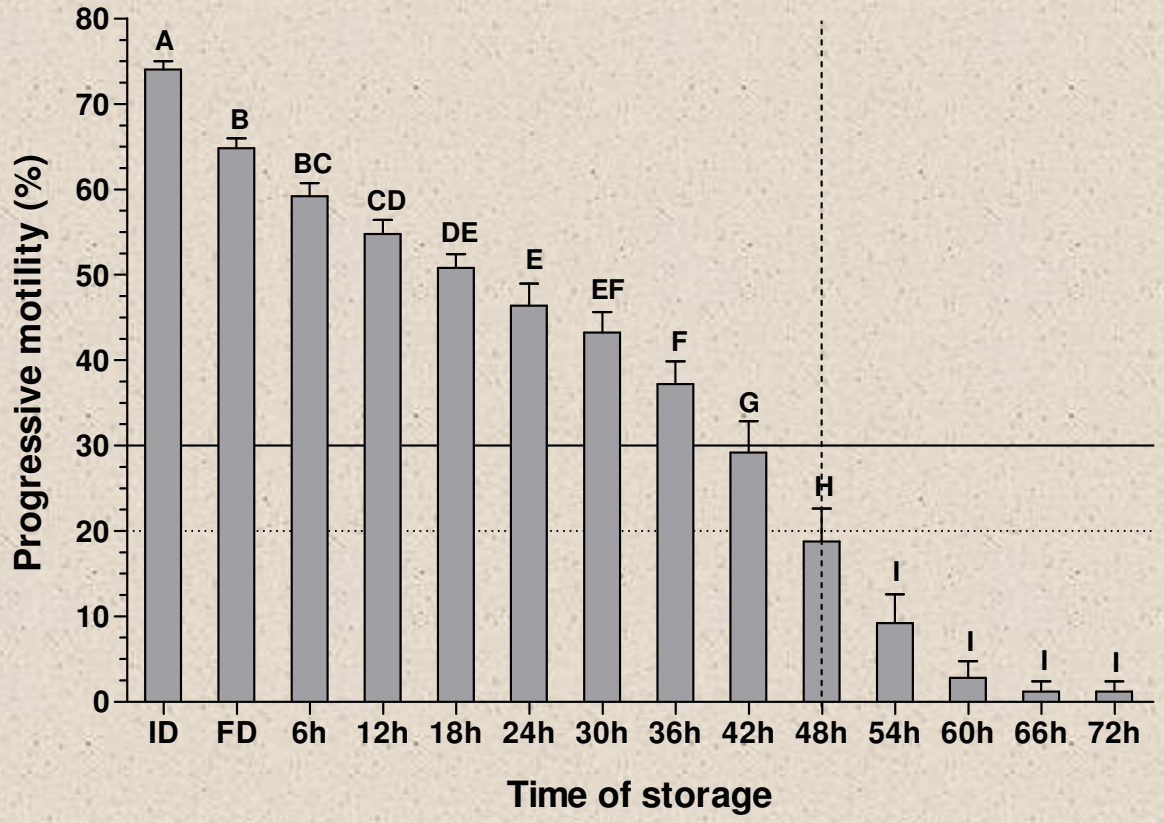
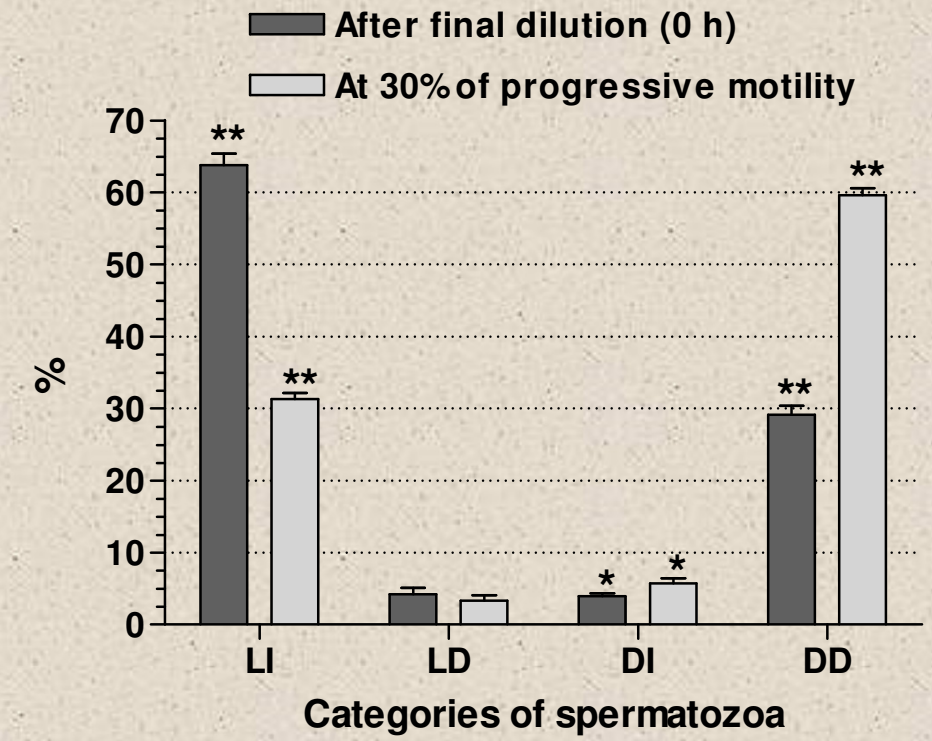


ID: after initial dilution; FD: after final dilution at 0h

Variations (Mean ± SE) in the liveability and acrosomal integrity of mithun spermatozoa after final dilution and at the time of 30% progressive motility following storage at 4°C; ** indicates values within row differ significantly (P<0.01) and * indicates values within row differ significantly (P<0.05)

Particulars	After final dilution (0 h)	At 30% of progressive motility
Live spermatozoa with intact acrosome (%)	63.8 ± 1.6**	31.3 ± 0.9**
Live spermatozoa with damaged acrosome (%)	4.2 ± 0.8	3.4 ± 0.8
Dead spermatozoa with intact acrosome (%)	3.9 ± 0.4*	5.7 ± 0.7*
Dead spermatozoa with damaged acrosome (%)	29.1 ± 1.3**	59.6 ± 1.0**

Variations (Mean ± SE) in the liveability and acrosomal integrity of mithun spermatozoa after final dilution and at the time of 30% progressive motility following storage at 4℃; ** indicates P<0.01 and * indicates P<0.05

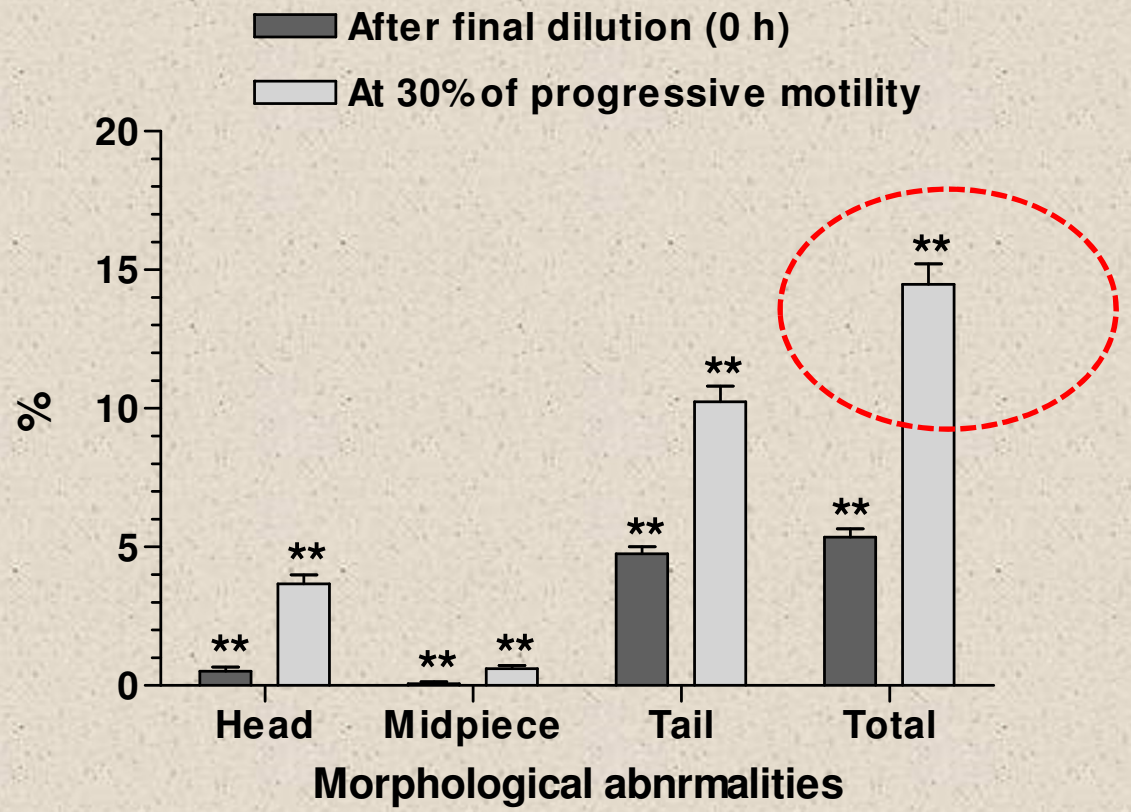


LI: live spermatozoa with intact acrosome
LD: live spermatozoa with damaged acrosome
DI: dead spermatozoa with intact acrosome
DD: dead spermatozoa with damaged acrosome

Variations (Mean ± SE) in the morphological abnormalities of Mithun spermatozoa after initial dilution and at the time of 30% progressive motility following storage at 4℃; ** indicates values within row differ significantly (P<0.01)

Morphological abnormalities	After final dilution (0 h)	At 30% of progressive motility
Head (%)	0.5 ± 0.2**	3.7 ± 0.3**
Mid piece (%)	0.1 ± 0.1**	0.6 ± 0.1**
Tail (%)	4.8 ± 0.3**	10.2 ± 0.6**
Total (%)	5.4 ± 0.3**	14.5 ± 0.8**

Variations (Mean ± SE) in the morphological abnormalities of mithun spermatozoa after final dilution and at the time of 30% progressive motility following storage at 4℃; ** indicates P<0.01



Seasonal variation in the quality of Mithun semen

Variations (Mean±SE) in seminal parameters during different seasons in Mithun

Parameters	Winter (Nov-Feb)	Pre-monsoon (Mar-Jun)
Semen Volume(ml)	1.90±0.15	1.80±0.10
Sperm concentration($X10^6$/ml)	497.50±25.69	469.41±17.62
Mass activity (0-5 point scale)	3.3±0.2	3.4±0.1

Variations (Mean±S.E.) in progressive mortality of Mithun sperm after different h of preservation at 4^0C; 0 h indicates immediately before cooling at 4^0C

Time of preservation	Winter (Nov-Feb)	Pre-monsoon (Mar-Jun)
0h	65.0±2.1	64.7±1.4
12h	52.5±2.9	55.9±1.9
24h	45.0±3.6	48.2±2.5
36h	37.5±3.7	38.8±2.5

Variations (Mean±S.E.) in total morphological abnormalities of Mithun sperm after different h of presentation at 4^0C; 0 h indicates immediately before cooling at 4^0C

Time of preservation	Winter (Nov-Feb)	Pre-monsoon (Mar-Jun)
0h	5.2±0.5	5.3±0.3
12h	7.4±0.8	7.5±0.6
24h	9.6±1.2	10.6±0.8
36h	12.1±1.4	13.5±0.9

B) Protocol for cryopreservation of semen

a) Tris-egg yolk diluent with glycerol **(Single dose (3,4,5, 6 or 7% glycerol and split doses(5,6 or 7% glycerol)**

b) Citrate-egg yolk diluent with glycerol **(Single dose (5, 6 or 7% glycerol)**

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After collection , semen was diluted in Tris-egg-yolk/Citrate-egg yolk extender

↓

Following the determination of spermatozoa concentration, semen sample was diluted with Tris–egg yolk extender and glycerol in single dose (3,4,5, 6 or 7% glycerol (v/v)) and split doses (5,6 or 7% glycerol)/Citrate-egg yolk extender and glycerol in single dose (5, 6 or 7% glycerol (v/v)) .

↓

The diluted semen sample was loaded into 0.5 ml straws, which were sealed with polyvinyl alcohol (PVA) powder. The straws were then equilibrated at 5°C for 4 h .After equilibration, straws were frozen in liquid nitrogen vapour, 5 cm above the liquid nitrogen level for 10 min and then plunged into liquid nitrogen for storage.

↓

Evaluation of progressive motility, live & dead count, acrosomal integrity and morphological abnormalities were done in fresh samples, in diluted samples after cooling (at 5°C) and in cryopreserved (7 days of storage) samples

Sperm were classified into four categories namely a) live, intact acrosome b) live, damaged acrosome c) dead, intact acrosome and d) dead , damage dacrosome.

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Cryopreservation of mithun semen

Glycerol added in a single dose

Variations (Mean ± SE) in progressive motility during different stages of freezing. Semen samples were cryopreserved with 3, 4, 5, 6 or 7% glycerol. [A, B] indicates values with different superscript within column differ significantly (P<0.01)

Glycerol concentration (%)	Progressive motility (%)		
	After initial dilution	After 4 h equilibration	After freezing and thawing
3	‡67.8±2.8	†43.2±2.3	ŏ23.9±2.0^{A}
4	‡67.2±2.3	†44.2±2.4	ŏ26.5±2.7^{AB}
5	‡65.3±4.1	†49.8±2.2	ŏ40.6±1.7^{C}
6	‡66.1±2.9	†45.2±2.5	ŏ31.7±1.1^{B}
7	‡66.3±3.7	†45.1±3.0	ŏ17.4±1.3^{D}

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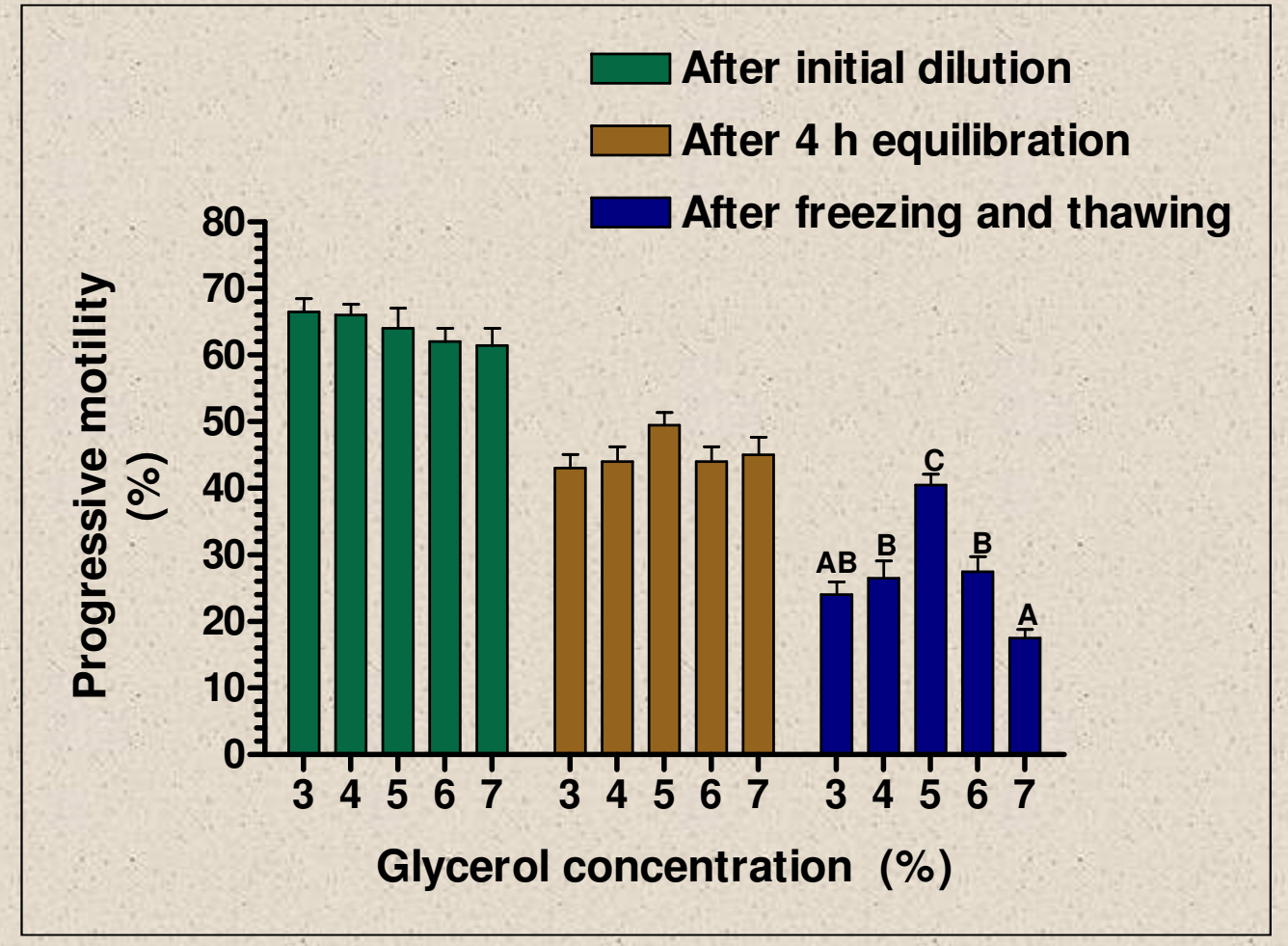
Variations (Mean ± SE) in progressive motility during different stages of freezing. Semen samples were cryopreserved with 3, 4, 5, 6 or 7% glycerol. A,B on error bar indicates a significant difference (P<0.01)



Variations (Mean ± SE) in liveability and acrosomal integrity of mithun spermatozoa during different stages of freezing. Semen samples were cryopreserved with 3, 4, 5, 6 or 7% glycerol. $^{A, B, C}$ indicates values with different superscript within column under a particular freezing stage differ significantly (P<0.01)

Stage and glycerol concentration (%)	Status of spermatozoa			
	L-I	L-D	D-I	D-D
After initial dilution				
3%	‡70.9±2.7	‡2.9±0.3	‡6.2±0.8	‡21.3±1.8
4%	‡71.3±2.4	‡3.0±0.3	‡6.5+0.9	‡20.6±1.9
5%	‡69.2±4.0	3.0±0.6	‡6.9±0.9	‡22.5±2.8
6%	‡70.3±3.3	‡1.2±0.5	‡8.0±0.7	‡22.1±2.3
7%	‡71.5±4.1	‡2.0±0.5	‡7.6±0.8	‡20.8±2.1
After 4 h equilibration				
3%	†46.7±2.6	†4.7±0.5	†14.4±1.2	†34.6±2.0
4%	†45.6±2.1	†5.0±0.7	†15.1±1.3	†34.4±1.5
5%	†52.8±2.2	5.2±0.8	ŏ11.1±1.1	†31.9±2.3
6%	†49.4±2.4	†5.2±0.7	†13.0±1.1	†33.3±3.2
7%	†49.9±3.2	†5.6±0.4	†12.1±1.0	†33.4±1.9
After freezing thawing				
3%	ŏ18.0±1.7^{A}	ŏ10.7±0.5^{A}	ŏ20.8±2.3^{A}	ŏ50.8±2.5^{A}
4%	ŏ20.2±2.2^{A}	ŏ10.5±0.5^{A}	ŏ20.6±2.3^{A}	ŏ49.5±2.4^{A}
5%	ŏ43.4±1.8^{B}	4.8±1.2^{B}	ŏ10.3±1.^{2B}	ŏ40.8±2.7^{B}
6%	ŏ35.1±1.0^{C}	ŏ7.0±0.5^{C}	ŏ16.5±1.^{0A}	ŏ41.6±0.9^{B}
7%	ŏ21.2±1.4^{A}	ŏ11.0±0.6^{A}	ŏ17.9±0.9^{A}	ŏ50.0±2.4^{A}

L-I: Live sperm with intact acrosome; L-D: Live sperm with damaged acrosome;
D-I: Dead sperm with intact acrosome ; D-D: Dead sperm with damaged acrosome

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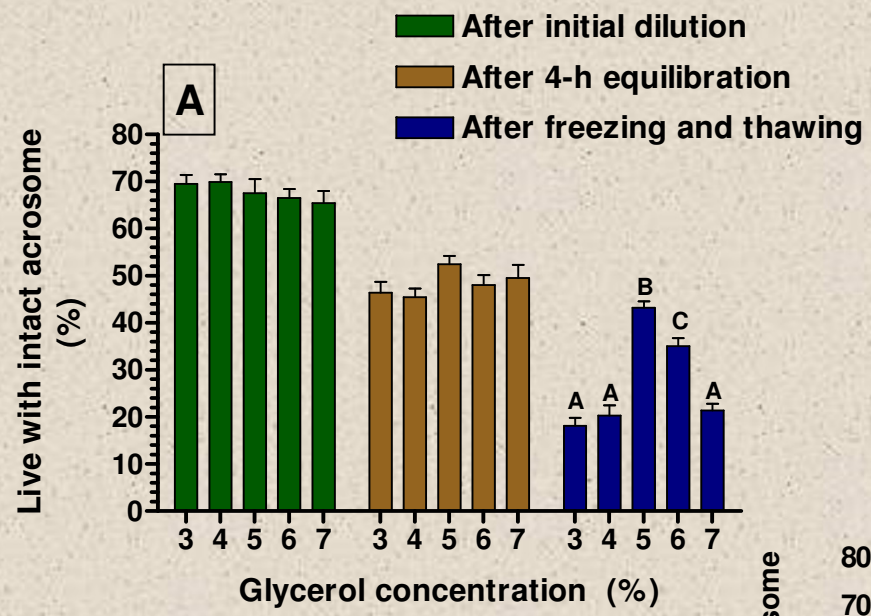
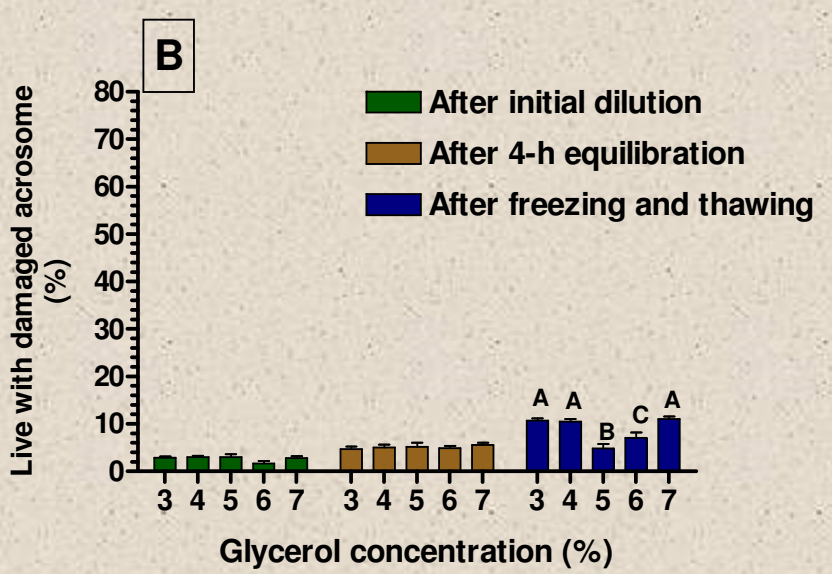
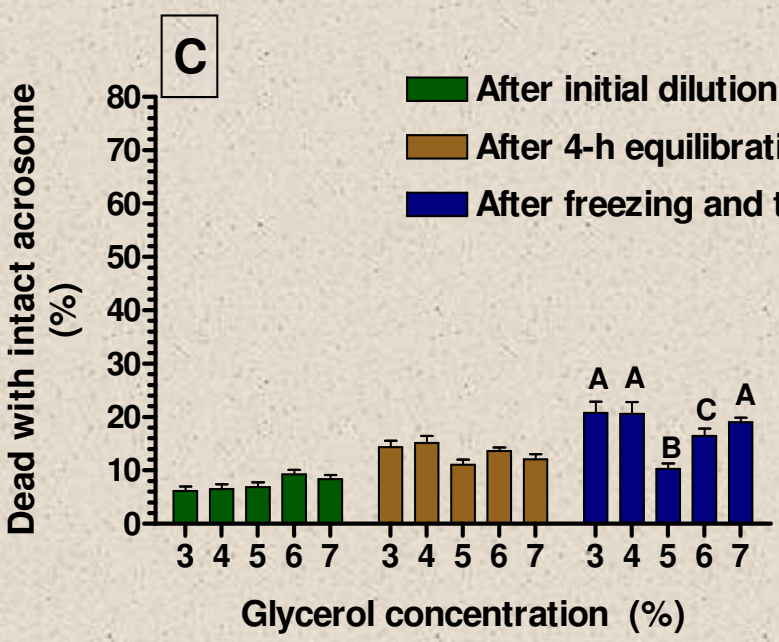
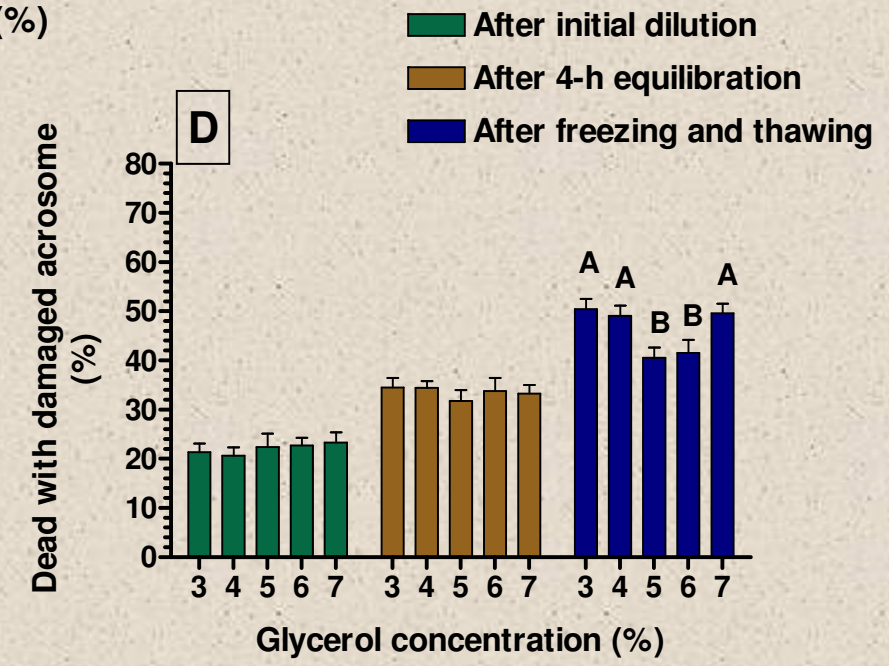
Variations (Mean ± SE) in live spermatozoa with intact acrosome (Panel A), live spermatozoa with damaged acrosome (Panel B), dead spermatozoa with intact acrosome (Panel C) and dead spermatozoa with damaged acrosome (Panel D) during different stages of freezing. Semen samples were cryopreserved with 3, 4, 5, 6 or 7% glycerol. A, B, C on error bar indicates P<0.01





N R C Mithun


C
After initial dilution
After 4-h equilibration
After freezing and thawing
Dead with intact acrosome (%)
0
10
20
30
40
50
60
70
80
3 4 5 6 7 3 4 5 6 7 3 4 5 6 7
A A B C A
Glycerol concentration (%)

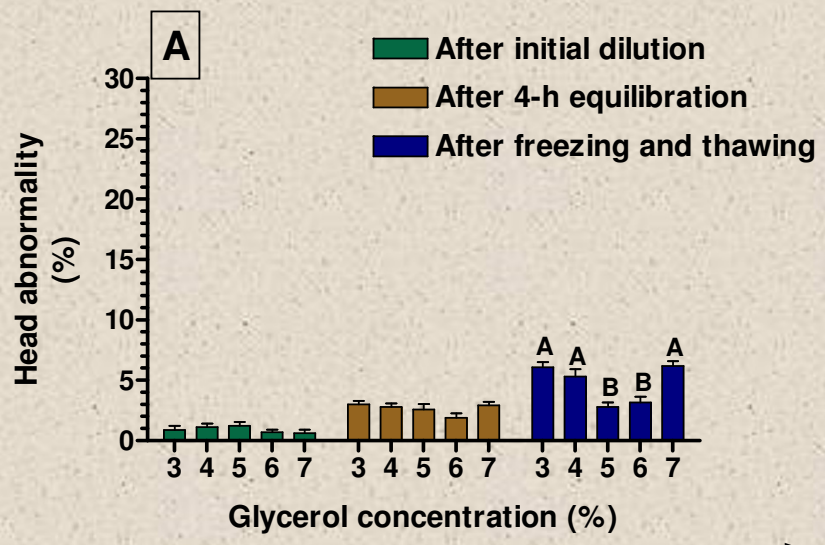

After initial dilution
After 4-h equilibration
After freezing and thawing
D
Dead with damaged acrosome (%)
0
10
20
30
40
50
60
70
80
3 4 5 6 7 3 4 5 6 7 3 4 5 6 7
A A B B A
Glycerol concentration (%)

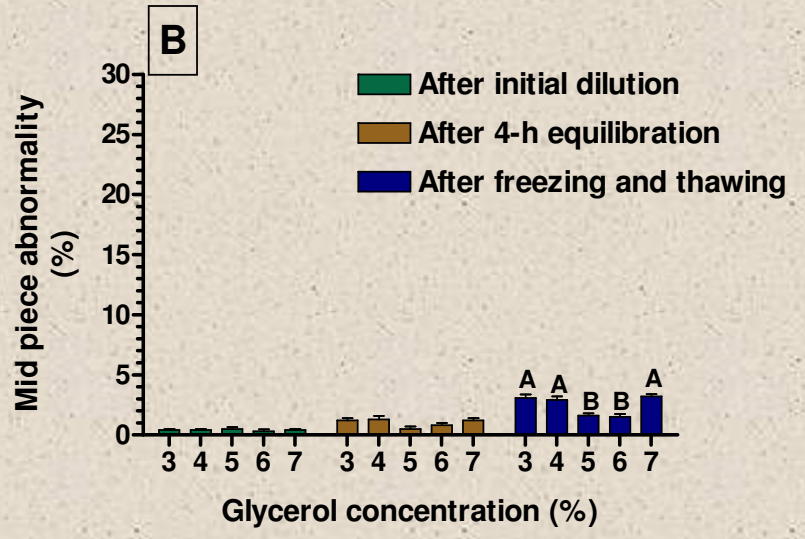
Variations (Mean ± SE) in head, mid piece, tail and total abnormalities of spermatozoa during different stages of freezing. Semen samples were cryopreserved with 3, 4, 5, 6 or 7% glycerol. $^{A, B}$ indicates values with different superscript within column under a particular freezing stage differ significantly (P<0.01)

Stage and glycerol concentration (%)	Spermatozoa abnormalities			
	Head	Mid piece	Tail	Total
After initial dilution				
3%	‡0.9 ± 0.3	‡0.2 ± 0.2	‡4.7 ± 0.3	‡5.8 ± 0.5
4%	‡1.1 ± 0.3	‡0.4 ± 0.2	‡3.4 ± 0.5	‡4.9 ± 0.5
5%	‡1.2 ± 0.4	‡0.5 ± 0.2	‡4.1 ± 0.3	‡5.8 ± 0.3
6%	‡0.8 ± 0.3	‡0.2 ± 0.2	‡4.7 ± 0.5	‡5.7 ± 0.6
7%	‡0.6 ± 0.2	‡0.4 ± 0.2	‡4.6 ± 0.3	‡5.6 ± 0.4
After 4 h equilibration				
3%	†3.0 ± 0.3	†1.2 ± 0.3	†9.4 ± 0.5^{A}	†13.6 ± 0.8^{A}
4%	†2.8 ± 0.3	†1.3 ± 0.3	†8.6 ± 0.8^{AB}	†12.7 ± 1.1^{A}
5%	♂2.6 ± 0.4	‡0.5 ± 0.2	†6.5 ± 0.3^{C}	†9.6 ± 0.6^{B}
6%	†2.0 ± 0.5	‡0.7 ± 0.2	†7.0 ± 0.7^{BC}	†9.5 ± 0.9^{B}
7%	†2.9 ± 0.3	†1.2 ± 0.3	†9.5 ± 0.4^{A}	†13.6 ± 0.8^{A}
After freezing thawing				
3%	♂6.1 ± 0.4^{A}	♂3.1 ± 0.3^{A}	♂16.3 ± 0.9^{A}	♂25.3 ± 1.0^{A}
4%	♂5.3 ± 0.6^{A}	♂2.9 ± 0.3^{A}	♂16.1 ± 0.8^{A}	♂24.2 ± 1.0^{A}
5%	♂2.8 ± 0.4^{B}	♂1.6 ± 0.2^{B}	♂9.7 ± 0.5^{B}	♂14.1 ± 0.8^{B}
6%	♂3.2 ± 0.4^{B}	♂1.5 ± 0.2^{B}	♂9.0 ± 0.6^{B}	♂13.7 ± 1.0^{B}
7%	♂6.2 ± 0.5^{A}	♂3.2 ± 0.3^{A}	♂16.6 ± 0.9^{A}	♂25.8 ± 1.2^{A}

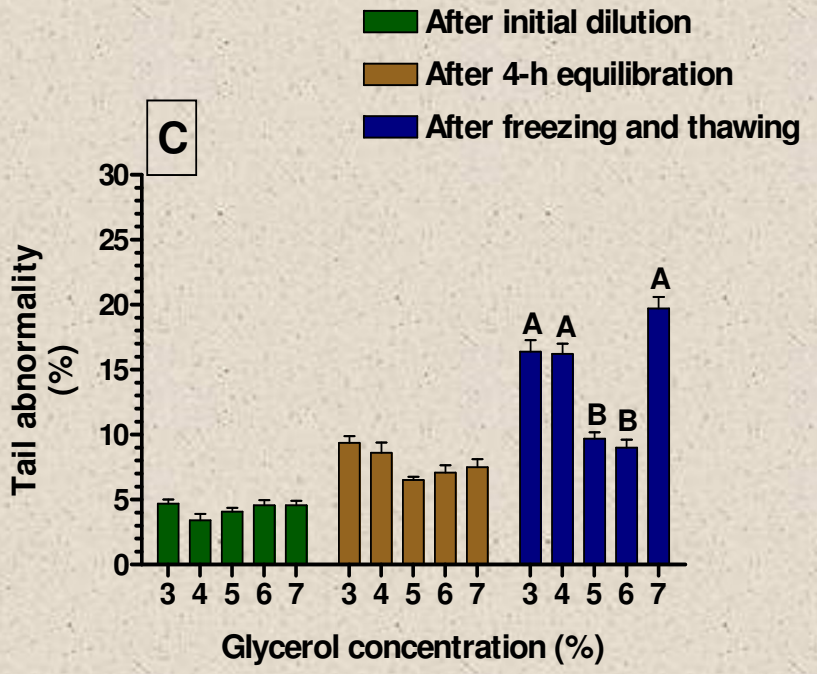
N R C Mithun

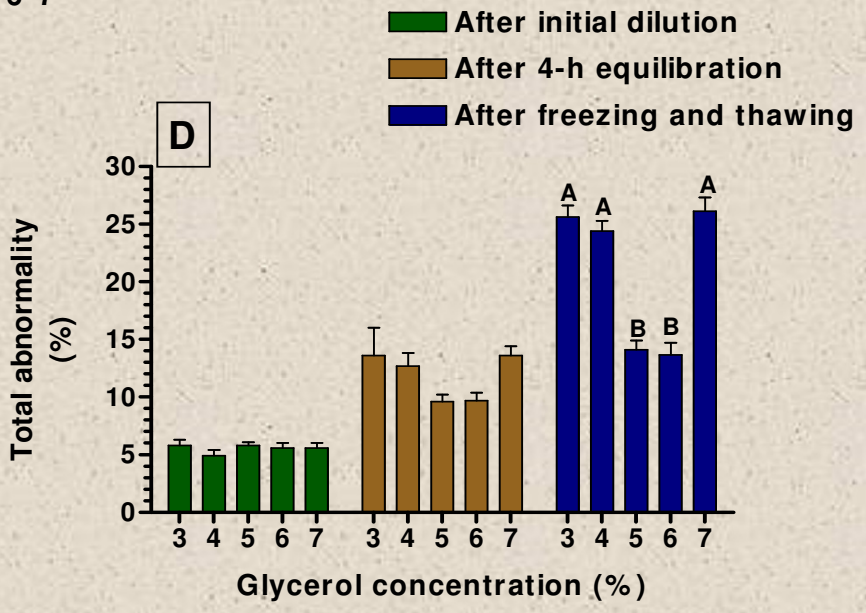
Variations (Mean ± SE) in head (Panel A), mid piece (Panel B), tail (Panel C) and total abnormalities (Panel D) of spermatozoa during different stages of freezing. The semen samples were cryopreserved with 3, 4, 5, 6 or 7% glycerol. A, B on error bar indicates P<0.01





N R C Mithun

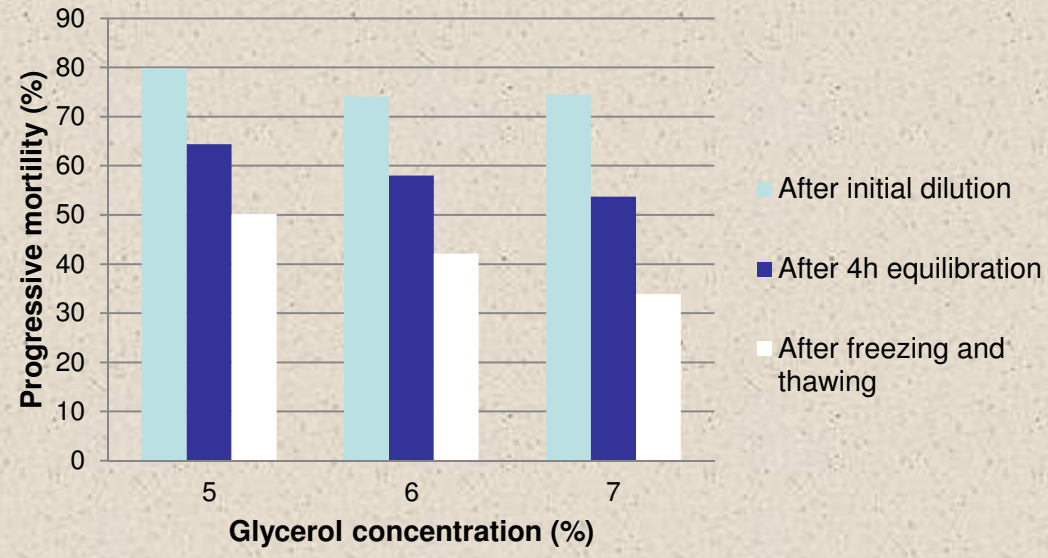

After initial dilution
After 4-h equilibration
After freezing and thawing
C
Tail abnormality (%)
0
5
10
15
20
25
30
3 4 5 6 7 3 4 5 6 7 3 4 5 6 7
A A B B A
Glycerol concentration (%)


After initial dilution
After 4-h equilibration
After freezing and thawing
D
Total abnormality (%)
0
5
10
15
20
25
30
3 4 5 6 7 3 4 5 6 7 3 4 5 6 7
A A B B A
Glycerol concentration (%)

N R C Mithun

Cryopreservation of mithun semen

Glycerol added in split doses



Glycerol concentration (%)	Progressive motility (%)		
	After initial dilution	After 4 h equilibration	After freezing and thawing
5	‡79.8±2.3	†64.4±1.5^{A}	ŏ50.2±1.9^{A}
6	‡74.1±2.9	†58.0±2.5^{B}	ŏ42.1±1.5^{B}
7	‡74.5±2.7	†53.7±2.0^{B}	ŏ33.9±1.4^{C}

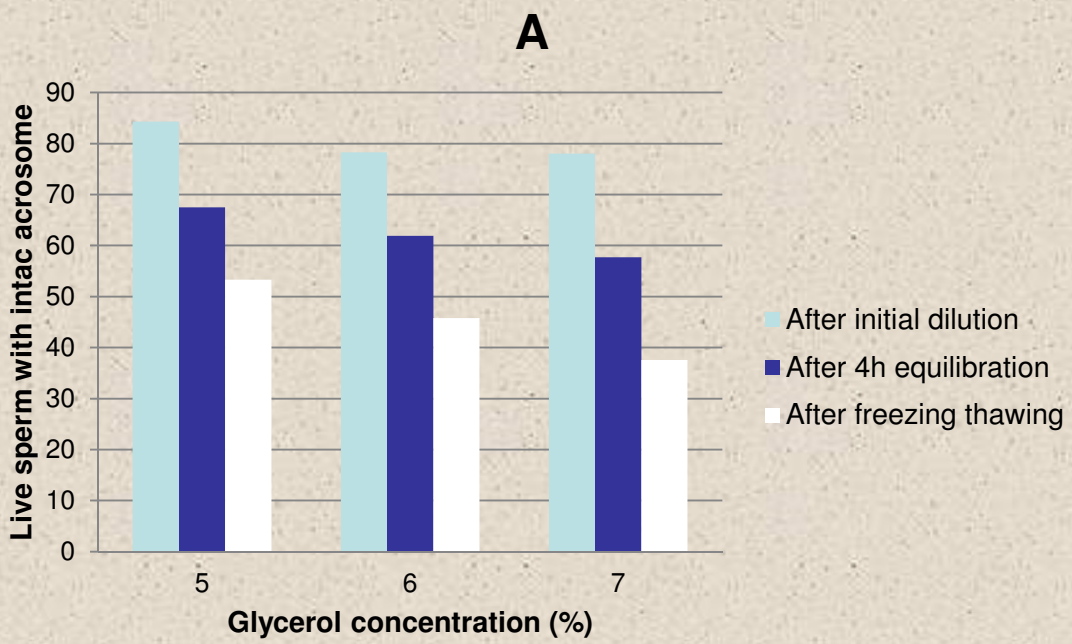
Variations (Mean ± SE) in progressive motility during different stages of freezing. Semen samples were cryopreserved with 5, 6 or 7% glycerol. $^{A, B}$ indicates values with different superscript within column differ significantly (P<0.01)

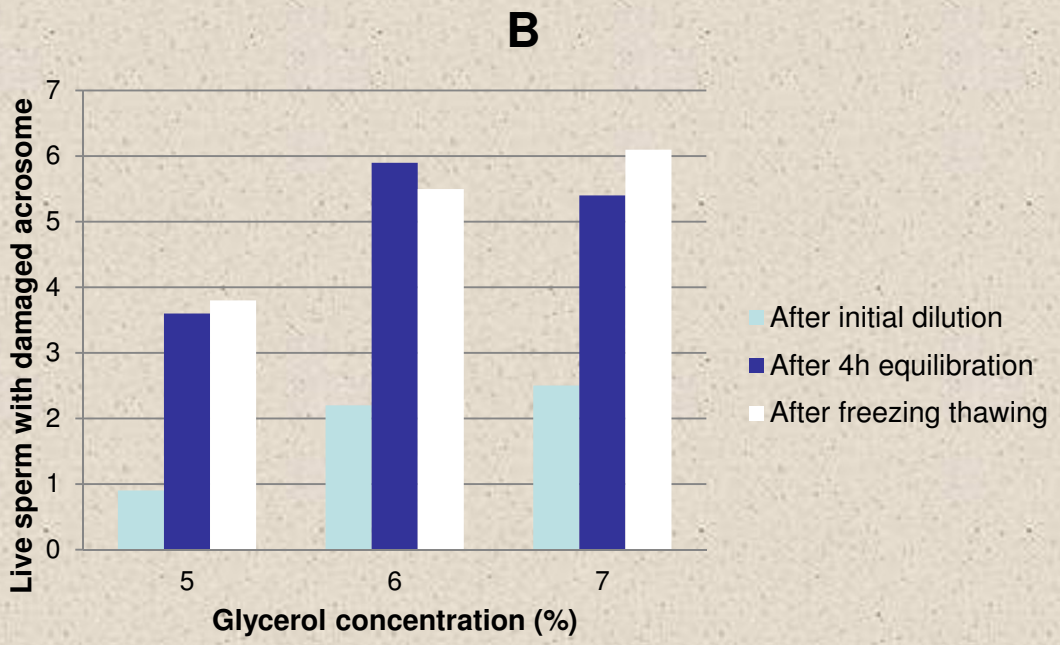
Table 5. Variations (Mean ± SE) in liveability and acrosomal integrity of mithun spermatozoa during different stages of freezing. Semen samples were cryopreserved with 5, 6 or 7% glycerol. $^{A, B, C}$ (P<0.01) or $^{a, b}$ (P<0.05) indicates values with different superscript within column under a particular freezing stage differ significantly

Stage and glycerol concentration (%)	Status of spermatozoa			
	L-I	L-D	D-I	D-D
After initial dilution				
5%	‡84.3±3.1	‡0.9±0.3	‡4.7±0.6	‡13.6±1.7
6%	‡78.3±3.4	‡2.2±0..6	‡5.5±0.5	‡16.4±1.6
7%	‡78.0±2.9	‡2.5±0.5	‡5.7±0.6	‡16.3±1.4
After 4 h equilibration				
5%	†67.5±1.2^{A}	♂3.6±0.5^{A}	†9.3±0.5^{A}	†20.5±0.9^{A}
6%	†61.9±2.2^{B}	♂5.9±0.3^{B}	†12.0±0.8^{B}	†20.9±2.0^{A}
7%	†57.7±2.2^{B}	♂5.4±0.3^{B}	†11.7±0.9^{B}	†25.6±1.3^{B}
After freezing thawing				
5%	♂53.3±1.8^{A}	♂3.8±0.5^{A}	♂13.3±0.6^{A}	♂30.0±1.9^{A}
6%	♂45.8±1.6^{B}	♂5.5±0.3^{B}	♂16.2±0.7^{B}	♂32.7±1.3^{A}
7%	♂37.6±1.2^{C}	♂6.1±0.4^{B}	♂19.3±0.6^{C}	♂37.2±1.1^{B}

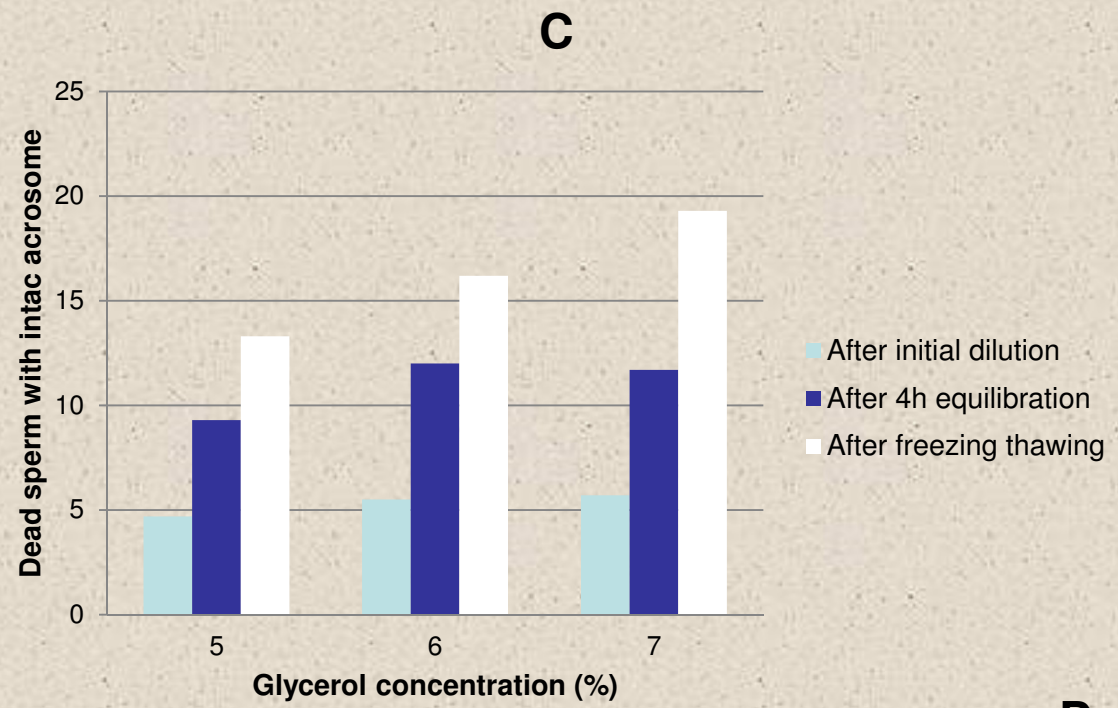
L-I: Live sperm with intact acrosome; L-D: Live sperm with damaged acrosome;
D-I: Dead sperm with intact acrosome ; D-D: Dead sperm with damaged acrosome

N R C Mithun


A
Live sperm with intac acrosome
90
80
70
60
50
40
30
20
10
0
5
6
7
Glycerol concentration (%)
After initial dilution
After 4h equilibration
After freezing thawing


B
Live sperm with damaged acrosome
7
6
5
4
3
2
1
0
5
6
7
Glycerol concentration (%)
After initial dilution
After 4h equilibration
After freezing thawing

N R C Mithun


C
Dead sperm with intac acrosome
25
20
15
10
5
0
5
6
7
Glycerol concentration (%)
After initial dilution
After 4h equilibration
After freezing thawing

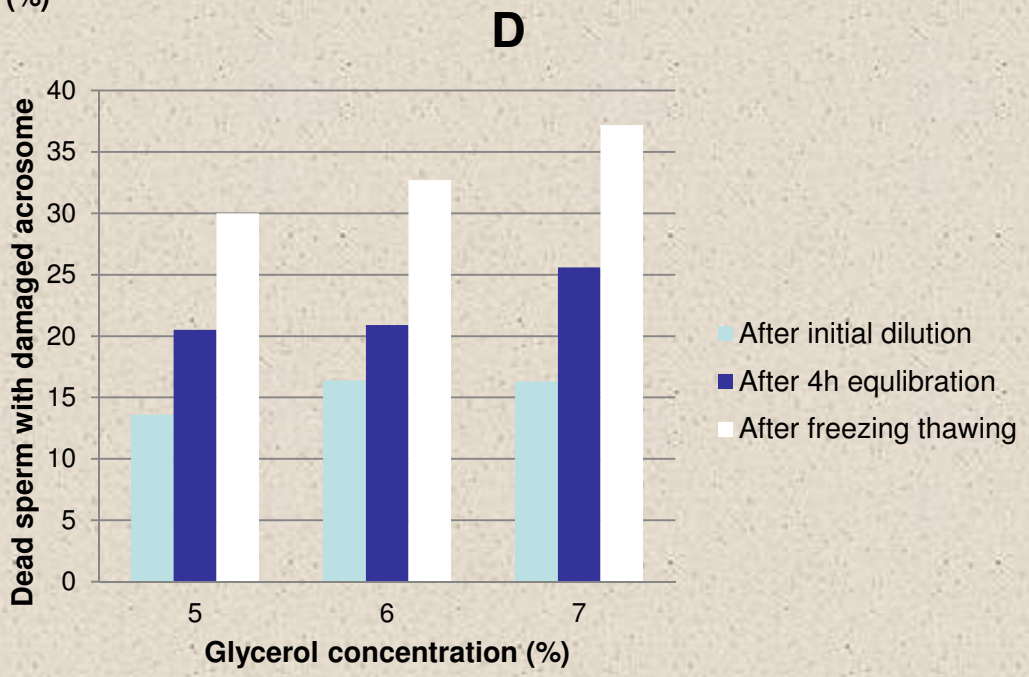
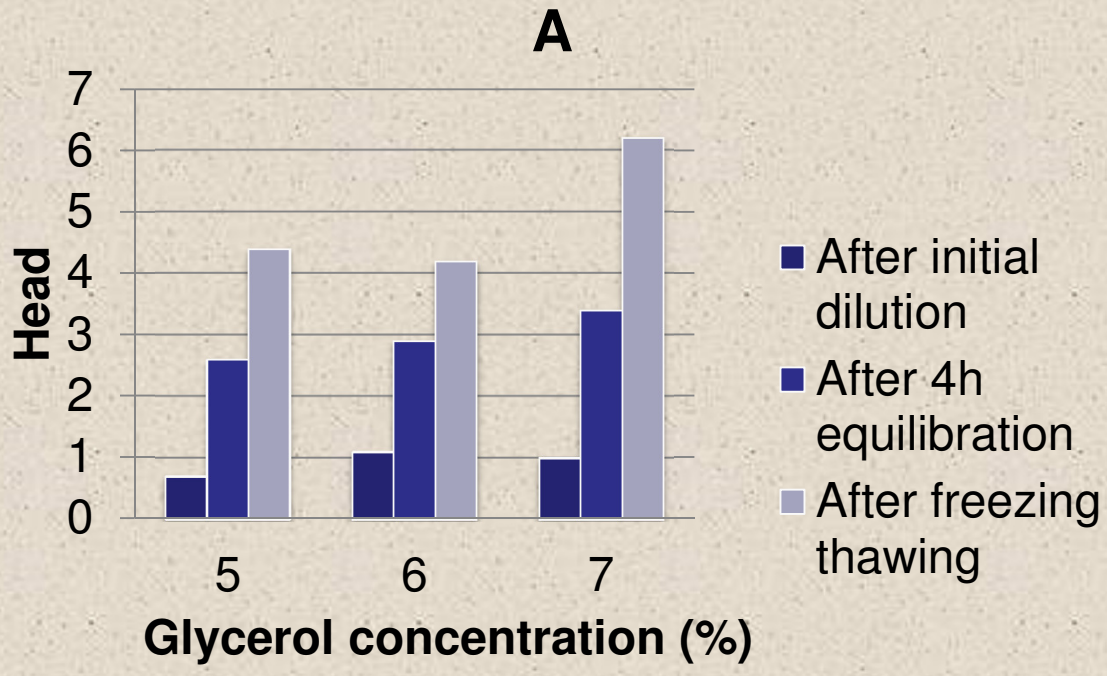
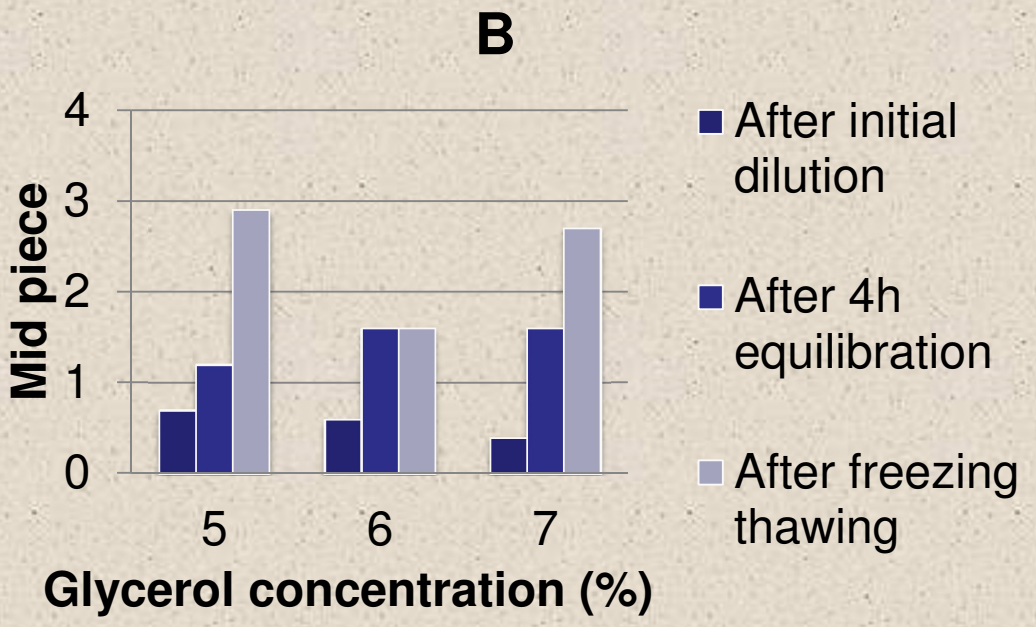

D
Dead sperm with damaged acrosome
40
35
30
25
20
15
10
5
0
5
6
7
Glycerol concentration (%)
After initial dilution
After 4h equlibration
After freezing thawing

Table 6. Variations (Mean ± SE) in head, mid piece, tail and total abnormalities of spermatozoa during different stages of freezing. Semen samples were cryopreserved with 5, 6 or 7% glycerol. $^{A, B, C}$ (P<0.01) or $^{a, b}$ (P<0.05) indicates values with different superscript within column under a particular freezing stage differ significantly

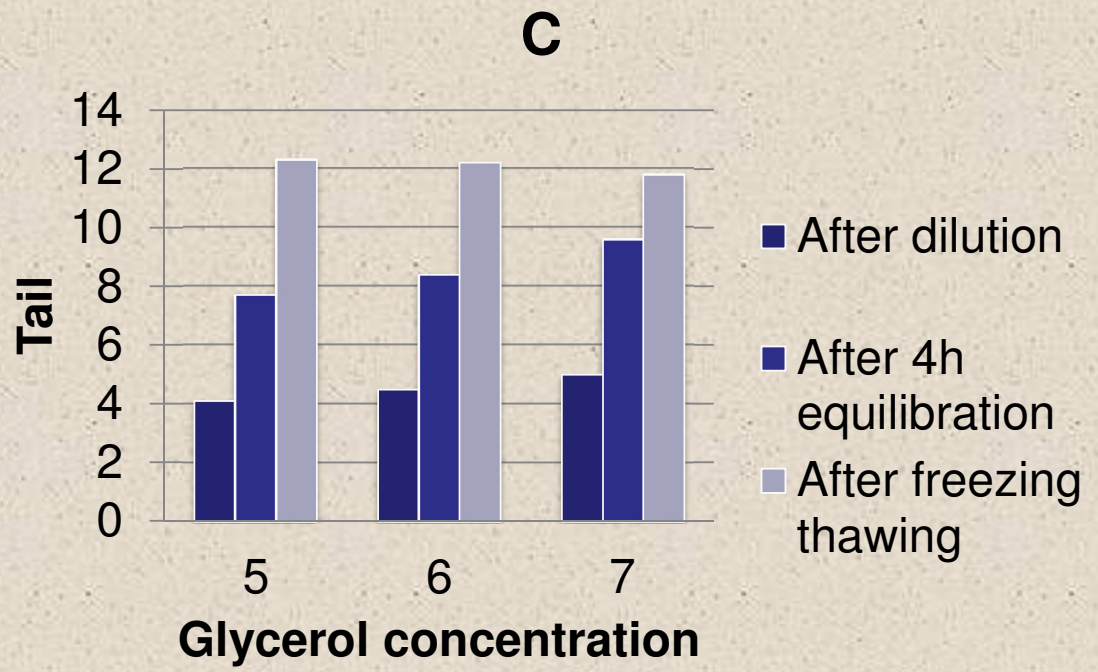
Stage and glycerol concentration (%)	Spermatozoa abnormalities			
	Head	Mid piece	Tail	Total
After initial dilution				
5%	‡0.7±0.3	‡0.7±0.2	‡4.1 ± 0.3	‡5.5 ± 0.5
6%	‡1.1±0.3	‡0.6±0.2	‡4.5 ± 0.3	‡6.2±0.4
7%	‡1.0±0.3	‡0.4±0.2	‡5.0±0.3	‡6.4±0.5
After 4 h equilibration				
5%	ϭ2.6 ± 0.2	‡1.2±0.2	†7.7±0.7	†11.5±0.8^{A}
6%	†2.9 ± 0.2	‡1.6±0.3	†8.4±0.5	†12.9±0.8^{AB}
7%	†3.4±0.4	†1.6 ± 0.2	†9.6±0.5	†14.5±0.5^{B}
After freezing thawing				
5%	ϭ4.4 ± 0.3^{A}	ϭ2.9±0.3^{A}	ϭ12.3±0.7	ϭ19.5 ± 1.0
6%	ϭ4.2 ± 0.4^{A}	ϭ1.6 ± 0.2^{B}	ϭ12.2 ± 0.8	ϭ17.9 ± 1.1
7%	ϭ6.2 ± 0.6^{B}	ϭ 2.7 ± 0.3^{A}	ϭ11.8 ± 0.6	ϭ20.6 ± 0.5

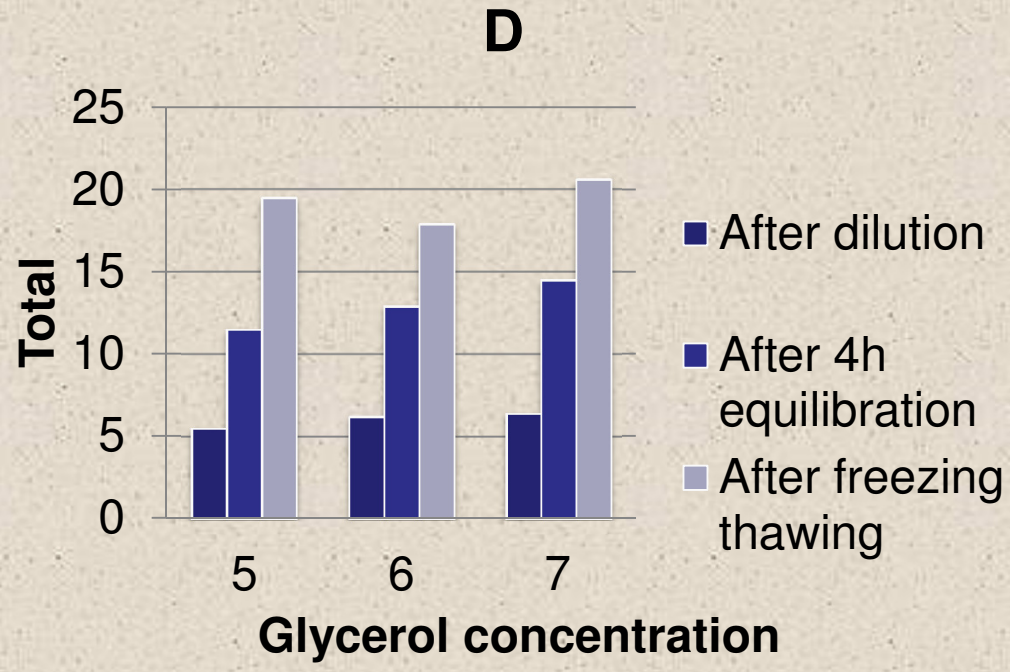
N R C Mithun


A
Head
7
6
5
4
3
2
1
0
5
6
7
Glycerol concentration (%)
After initial dilution
After 4h equilibration
After freezing thawing


B
Mid piece
4
3
2
1
0
5
6
7
Glycerol concentration (%)
After initial dilution
After 4h equilibration
After freezing thawing

N R C Mithun


C
Tail
14
12
10
8
6
4
2
0
5
6
7
Glycerol concentration
After dilution
After 4h equilibration
After freezing thawing

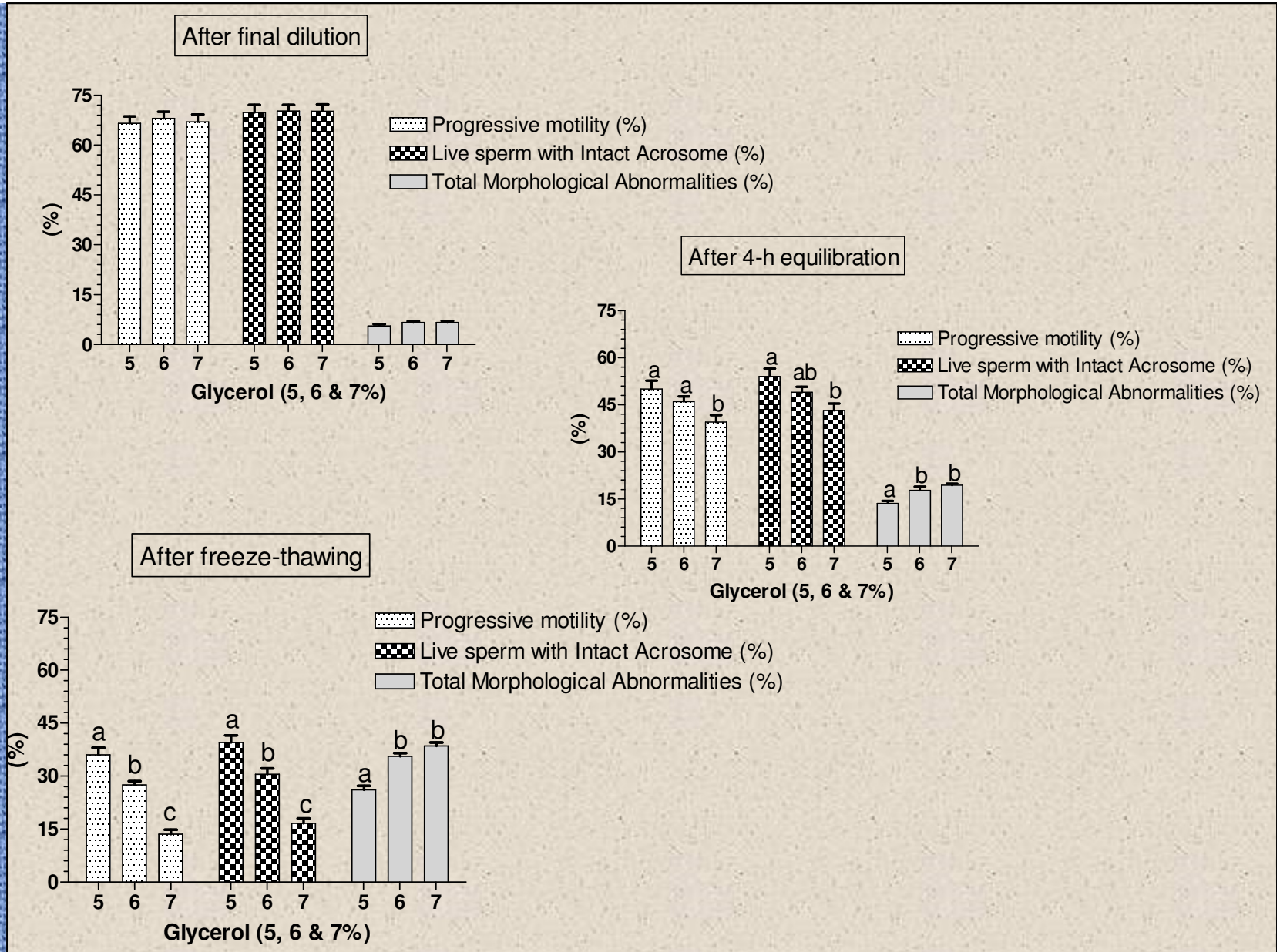

D
Total
25
20
15
10
5
0
5
6
7
Glycerol concentration
After dilution
After 4h equilibration
After freezing thawing

Citrate-egg yolk diluent with glycerol

Variations (Mean±S.E.) in sperm qualitative parameters at different stages of cryopreservation. Mithun semen was cryopreserved in citrate-egg yolk-glycerol diluent and glycerol was added into diluent in a single dose.

Particulars	**Glycerol concentration (%)**		
	5	**6**	**7**
After final dilution			
Progressive motility (%)	66.5±2.1	68.0±2.0	67.0±2.2
Live sperm with intact acrosome (%)	69.8±2.3	70.3±1.8	70.2±2.1
Total morphological abnormalities (%)	5.6±0.5	6.6.±0.4	6.6±0.5
After 4-h equilibration			
Progressive motility (%)	50.0 ± 2.7^{a}	46.0 ± 1.7^{a}	39.5 ± 2.2^{b}
Live sperm with intact acrosome (%)	54.0 ± 2.6^{a}	49.0 ± 1.7^{ab}	43.2 ± 2.2^{b}
Total morphological abnormalities (%)	13.6 ± 0.8^{a}	17.8 ± 1.1^{b}	19.4 ± 0.5^{b}
After freeze-thawing			
Progressive motility (%)	36.0 ± 2.1^{a}	27.5 ± 1.1^{b}	13.5 ± 1.3^{c}
Live sperm with intact acrosome (%)	39.5 ± 2.0^{a}	30.5 ± 1.7^{b}	16.6 ± 1.4^{c}
Total morphological abnormalities (%)	26.1 ± 1.1^{a}	35.6 ± 0.9^{b}	38.5 ± 1.0^{b}

N R C Mithun



Variations (Mean±S.E.) in sperm qualitative parameters at different stages of cryopreservation. Mithun semen was cryopreserved in citrate-egg yolk-glycerol diluent and glycerol was added into diluent in a single dose.

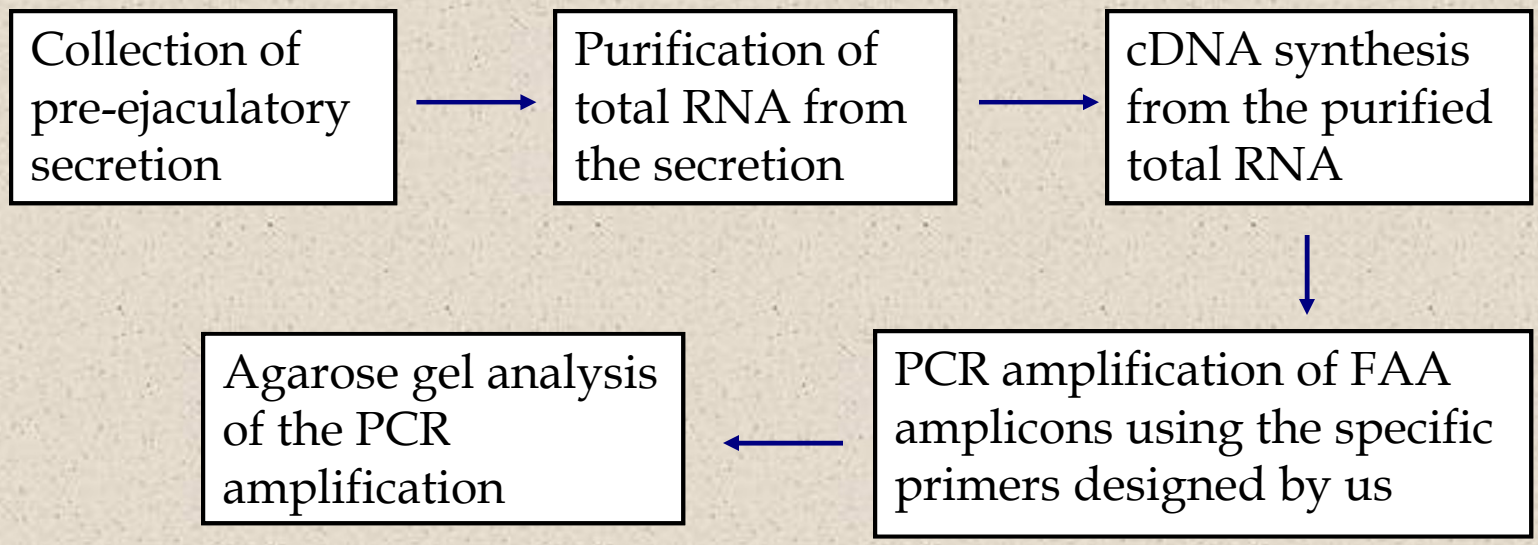
ICAR Mithun

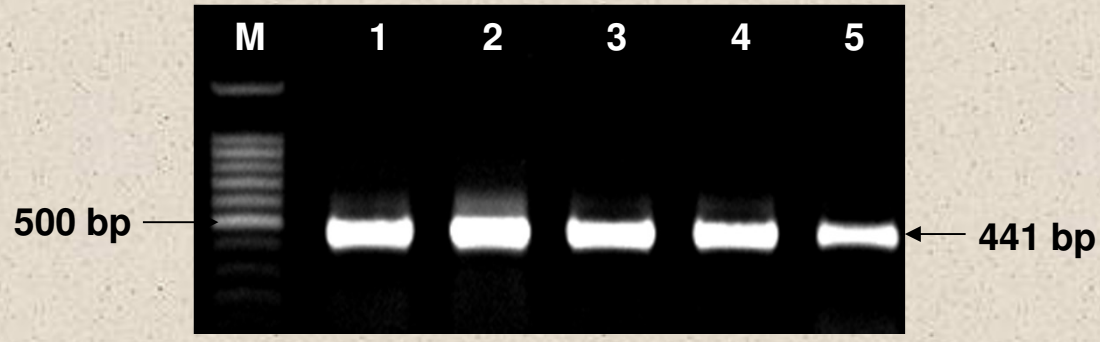
Molecular method for detecting fertility associated antigen in Mithun seminal plasma

- **Detecting transcripts of the fertility associated antigen (FAA) in mithun semen through PCR technique.**
- **Osteopontin and Heparin binding protein transcripts (partial coding sequence) have been amplified and sequenced (published in gene bank - Osteopontin- 423 bp cds, Accession no- GU451284 GU451285 GU451286, Heparin binding protein-156bp cds, Accession no- GU451281 GU451282 GU451283).**

N R C Mithun

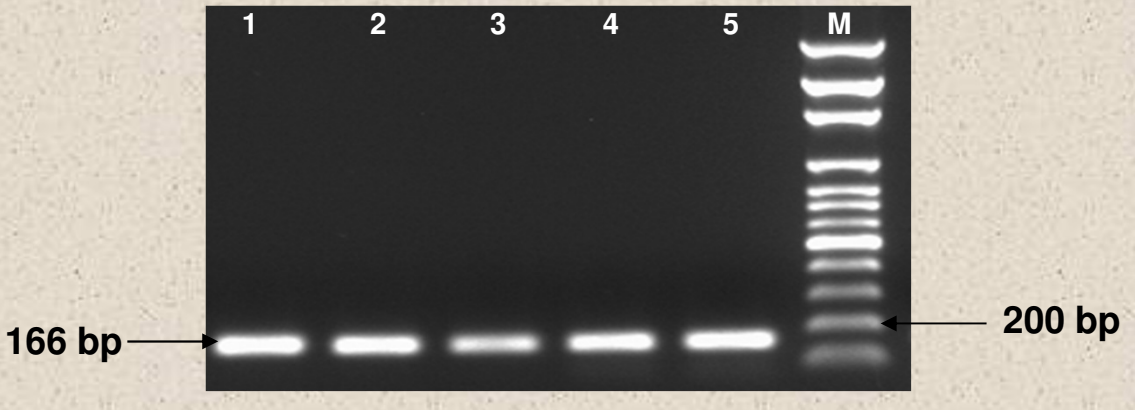
Detection of FAA transcripts



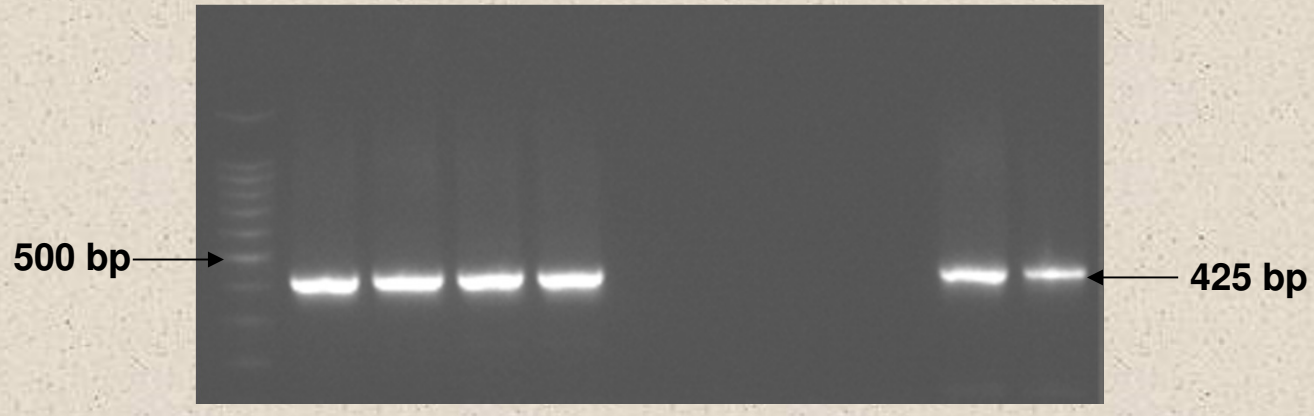


β-actin
(M: 100 bp marker; 1: animal-1; 2: animal-2; 3: animal-3; 4: animal-4; 5: animal-5)

N R C Mithun



Heparin binding protein
(M: 100 bp marker; 1: animal-1; 2: animal-2; 3: animal-3; 4: animal-4; 5: animal-5)



Osteopontin
(M: 100 bp marker; 1-2: animal-1; 3-4: animal-2; 5-6: animal-3; 7-8: animal-4; 9-10: animal 5)

N R C Mithun

The First Mithun Calf Born Through AI at Farm

N R C Mithun

AI at Khonoma village

N R C Mithun

AI CALF BORN AT VILLAGE LEVEL

Superovulation and Embryo Transfer (ETT)

Although AI permits partial improvement in genetic makeup through superior germplasm.

For the rapid multiplication of superior germplasm ETT may be adopted as one of the measures.

The technique provides unlimited scope of producing genetically superior animals within a short period of time.

MOET may be used for genetic enhancement of mithun and *ex situ* conservation of this valuable species

N R C Mithun

Superovulation & ETT protocol

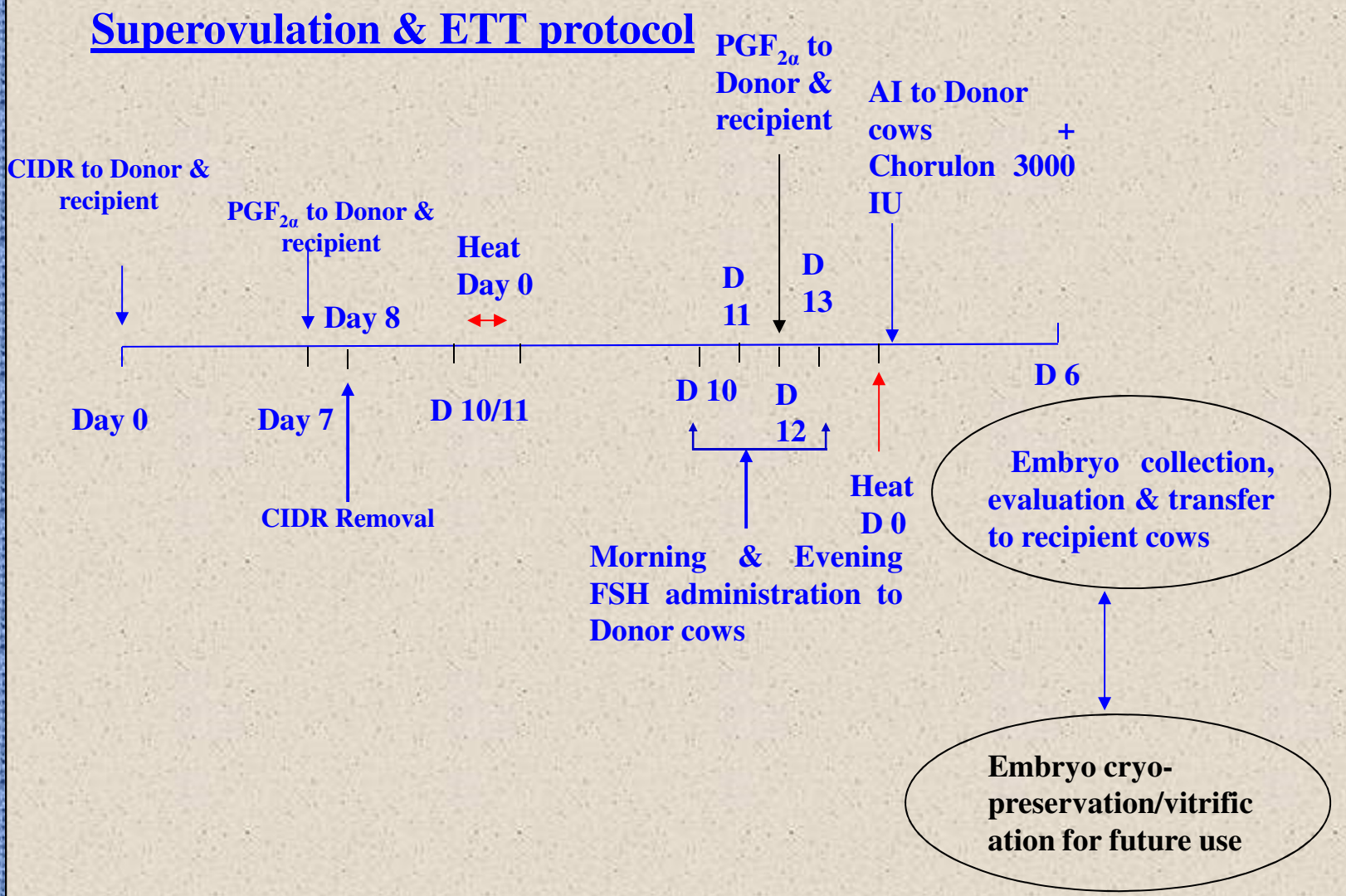


Table 1. Superovulatory regimen for the mithun

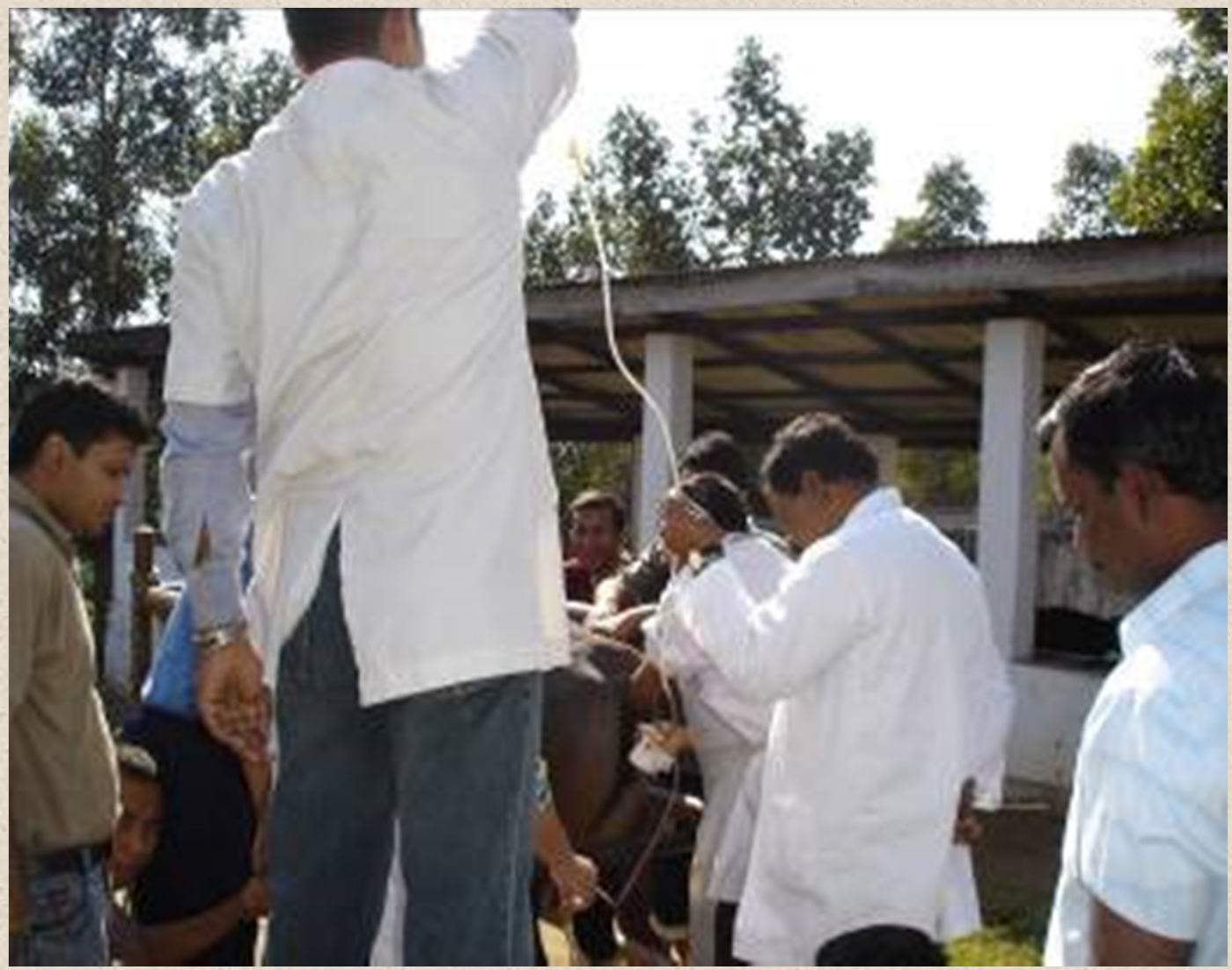
Day of cycle	Folltropin treatment (mg)	
	Morning	Evening
Day 10	50	50
Day 11	50	50
Day 12	50+0.45mg Tiaprost ($PGF_{2\alpha}$ analogue)	50+0.45mg Tiaprost ($PGF_{2\alpha}$ analogue)
Day 13	50	50

N R C Mithun

- Flushing on day 6 of the Oestrus cycle (Day 0- Day of estrus)
- Evaluation of Embryos.
- Transferred to recipients.

N R C Mithun

Standardization of superovulation and embryo transfer protocols in Mithun (*Bos frontalis*)



N R C Mithun

Protocol for Embryo Vitrification

Evaluated Embryo

↓

Embryo washing in DPBS medium

↓

Embryo equilibration in Solution-1 for 5min.(10% glycerol + 0.125M Sucrose + 0.125M Dextrose +10 % FCS in PBS)

↓

Embryo equilibration in Solution-2 for 5min.(10% glycerol + 10% Ethylene glycol + 0.25M Sucrose + 0.25M Dextrose +10 % FCS in PBS)

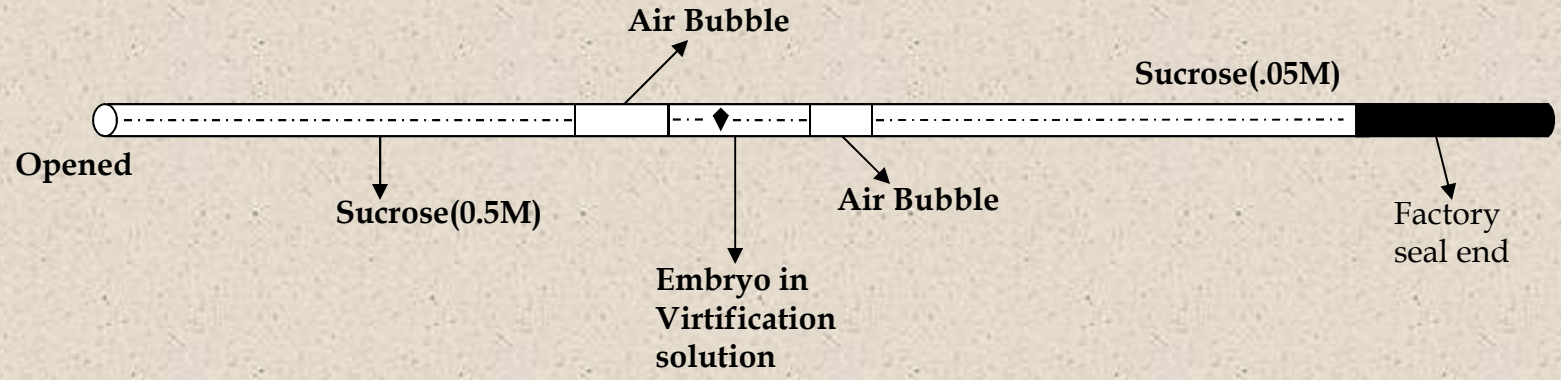
↓

Embryo placed in pre-cooled (4°C) virtification solution(20% glycerol + 20% EG + 0.375M Sucrose + 0.375M Dextrose +10 % FCS in PBS).

↓

Immediately aspiration of embryos (within 60sec) in a 0.5ml French straw loaded with 0.5M sucrose

N R C Mithun



Straw sealing with PVA powder

↓

Kept on liquid nitrogen vapour for 1min

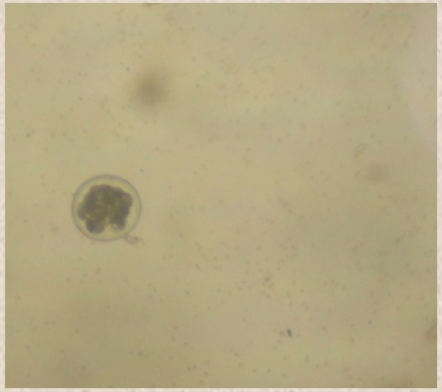
↓

Plunged into LN2(-196°C)

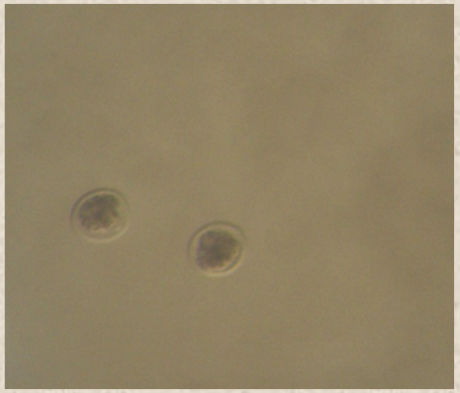
Onset of estrus and duration in mithun cows following $PGF_{2\alpha}$ treatment

No. of animal	No. of animal in oestrus	Interval between PGF_2 α treatment and onset of oestrus (h)	Duration of oestrus(h)
9	9	55.0±3.0	22.5±.2.5

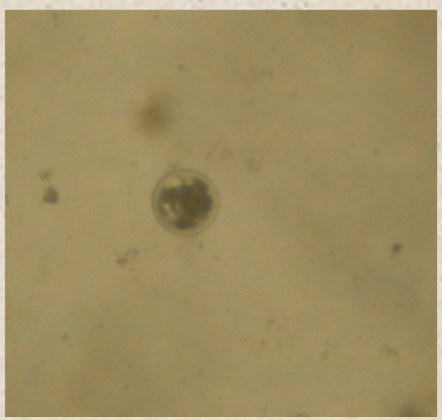
Morula recovered from mithun following superovulation and flushing



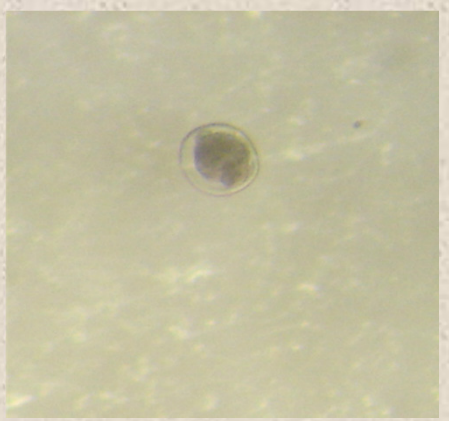
1st Animal



2nd Animal



3rd Animal



4th Animal

Estrus synchronization/superovulation/ETT

BHARAT, first Mithun calf

born through ETT

MOHAN, world's first ETT born mithun calf from 100-day cryopreserved embryo

ICAR-NRC Mithun

Ovum pick-up for in vitro embryo production

A powerful technology for increasing productivity in animals

Instead of superovulation and embryo flushing, a non surgical transvaginal ultrasound guided ovum pick-up (OPU) has been developed followed by in vitro maturation (IVM) and fertilisation (IVF) and embryo culture up to blastocyst stage

OPU can be applied to high- value female calves for breeding even before they reach their normal breeding age.

Transgenesis

In transgenic individual, a foreign desirable gene is deliberately inserted into its genome. This foreign gene is constructed using recombinant DNA methodology. Research is underway to improve the disease resistance, increase the growth and carcass quality of pigs by introducing gene controlling growth

Cloning

✓ Cloning is a process by which animals are reproduced asexually.

✓The process involves removing the chromosomal DNA from mature oocytes and replacing it with a cell from the donor animal to be cloned.

✓ The donor cell is then fused with the enucleated oocyte and activated either chemically or with an electrical pulse to induce activation and reprogramming of the somatic cell genome to that of an embryonic genome.

✓Reconstructed cloned embryos are then cultured for 6 to 9 d and viable embryos are transferred to synchronized recipients and carried to term to produce live cloned offspring

N R C Mithun