In vitro Embryo Production in Calves

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Genetic Improvement

In 1987, the concept of MOET programs was introduced by the University of Guelph

Genetic Change =

$$\frac{\text{Sel. Int. x Acc. x } h^2}{\text{Generation Interval}}$$

*Shortening generation intervals will impact genetic gain

What role will genomics play?

IVF Terminology

OPU - Ovum pick-up, or ultrasound-guided oocyte aspiration

LOPU – Laparoscopic ovum pick-up

IVM - In vitro maturation

IVF - In vitro fertilization

IVC - In vitro culture

IVP/IVEP - In vitro production of embryos

The effect of follicle size on OPU/IVF results

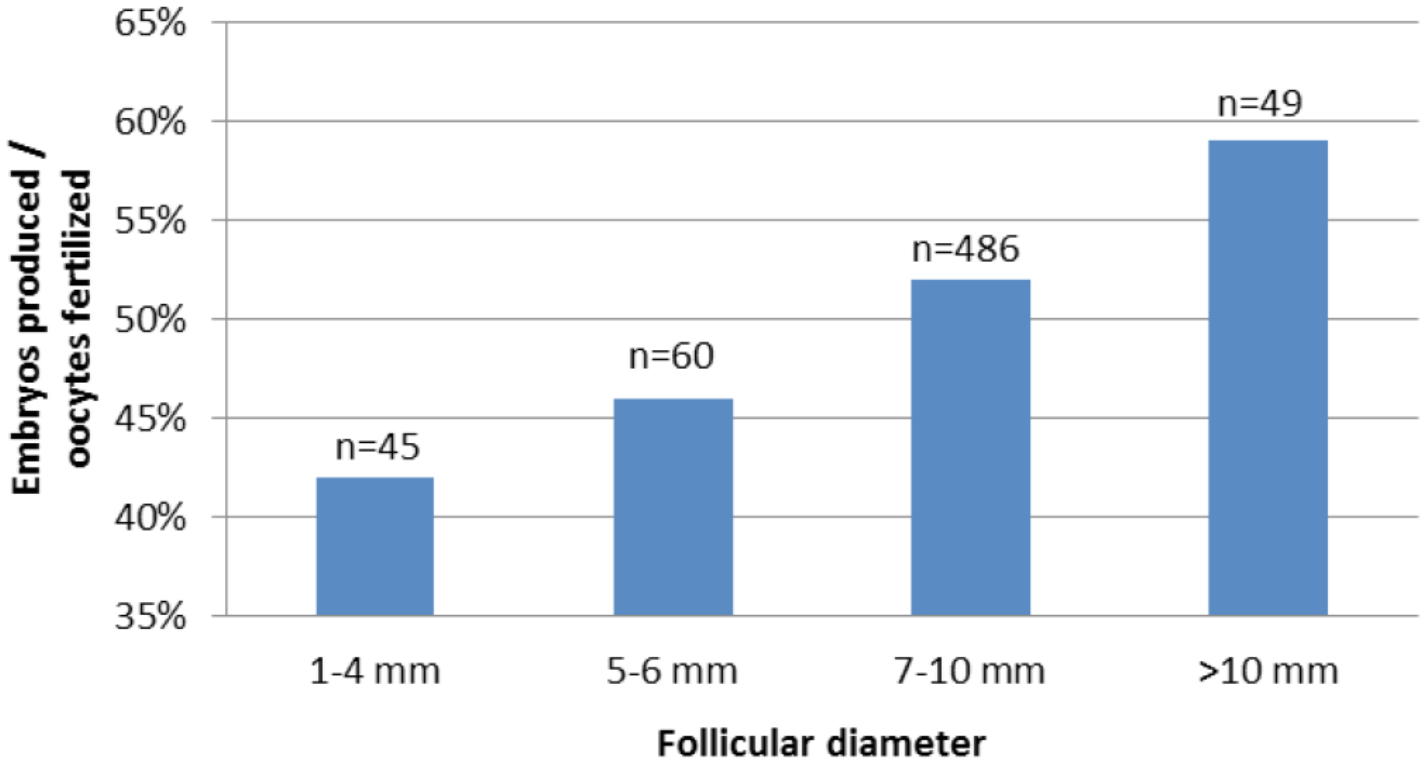
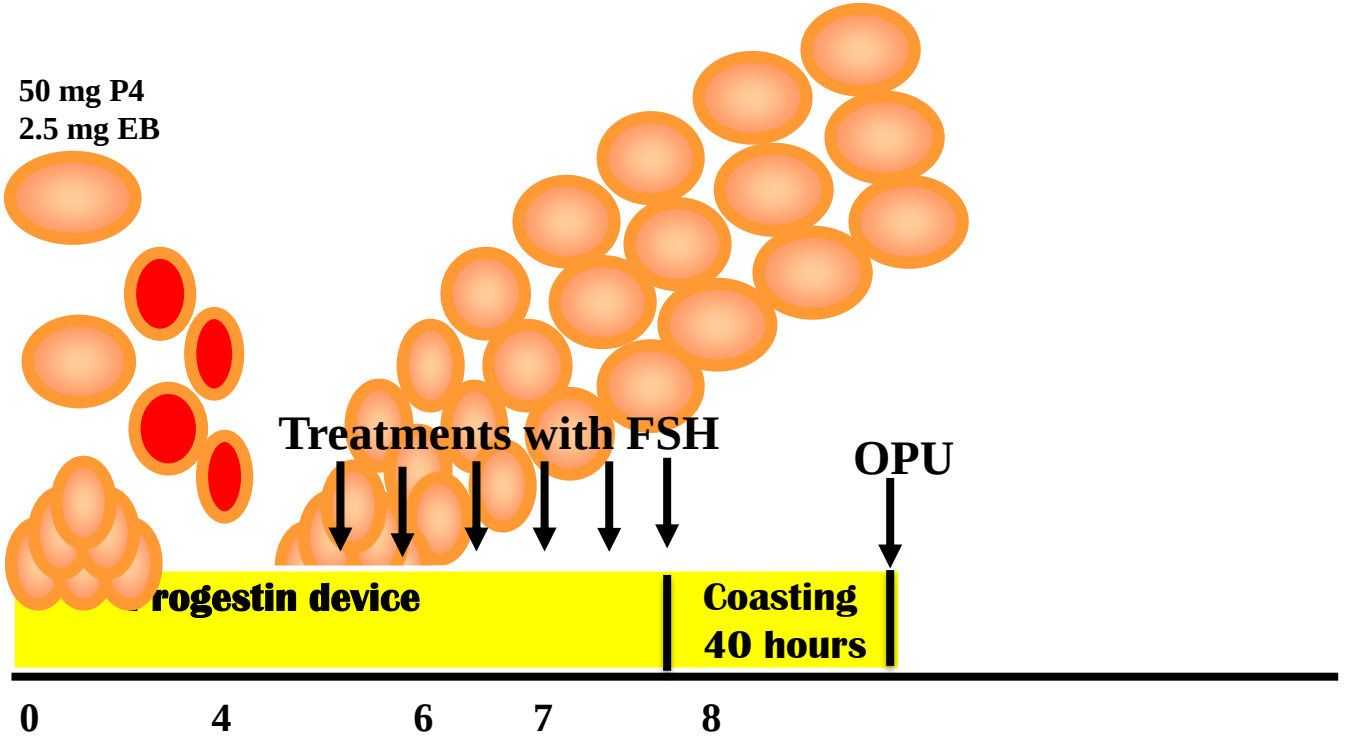


Figure 1. Relation between the percentage of transferable embryos (morula or blastocysts of Quality 1 or 2) produced after 7 or 8 days of in vitro culture vs follicle diameter of the majority of the follicles (> 2/3) at the time of OPU. (n = number of OPU sessions).

Blondin and Vigneault, 2013

Treatment with gonadotrophins for OPU



Blondin et al., 2012 Lonergan and Fair 2008

In vitro embryo production in calves

Background:

1) Attempts to produce “in vivo” embryos from calves have been unsuccessful
   - small percentage of superstimulated follicles ovulated
   - only unfertilized ova were recovered
2) In 1990’s, IVF with calf oocytes was attempted with variable success
   - initial efforts produced rather poor results
   - low developmental capacity (<10%)
   - wasn’t possible to predict breeding value at such a young age
   - technology was abandoned
4) Today OPU/IVF technology and culture conditions has improved greatly
5) Reproducing animals at early ages now possible through genomic testing
   -development of genetic markers that allow the prediction of the production phenotype of calves
6) However, hormonal regimes applied to calves must be tested and improved
   - must recover oocytes that are competent for full development
   - Age and gonadotropin stimulation would seem to be important

General procedures in calves (2 to 6 months of age)

- Insert small ruminant vaginal device
- Initiate FSH treatments after 2 days (dose?)
- Twice daily FSH treatments for 48 – 72 hours
- Coasting periods range from 12 to 36 hours
- LOPU after coasting period
- After LOPU, wash ovaries with warm saline
- Suture or glue holes in abdomen
- Repeat in approximately 2 weeks

Preparation for Laparoscopy (LOPU)

Laparoscopy

1) Fasting
2) Anaesthesia
3) Clipping and surgical prep.
4) Laparoscopic equipment
5) Closure of incisions
6) Recovery from anaesthesia

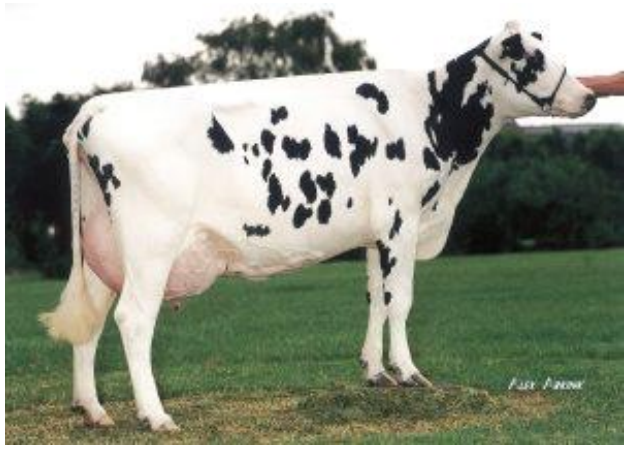
Transvaginal OPU in prepubertal calves

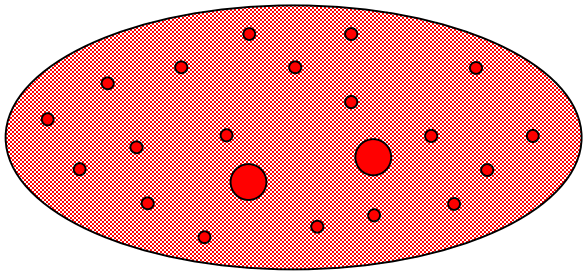
- Standing position:
- Infrastructure – squeeze chute - very important
- Epidural! – good epidural
- Operator – arm and hand size – smaller is better!
- Little manipulation as possible – little space in the rectum for manipulation
- Needle, tubing, flow rate… same as for adult animals!
- Handle for ultrasound probe and needle guide - special size for calves

Adult animals →

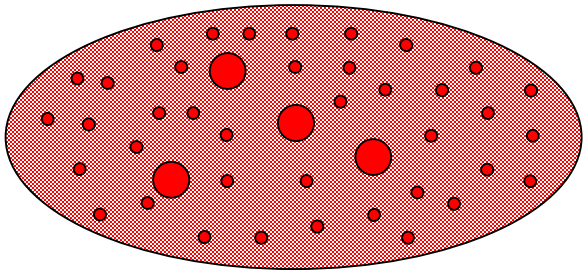
Calves →

Quantity and quality of follicles in *Bos taurus* and *Bos indicus*





Ovary



Ovary

AFC and ovarian response in prepubertal calves

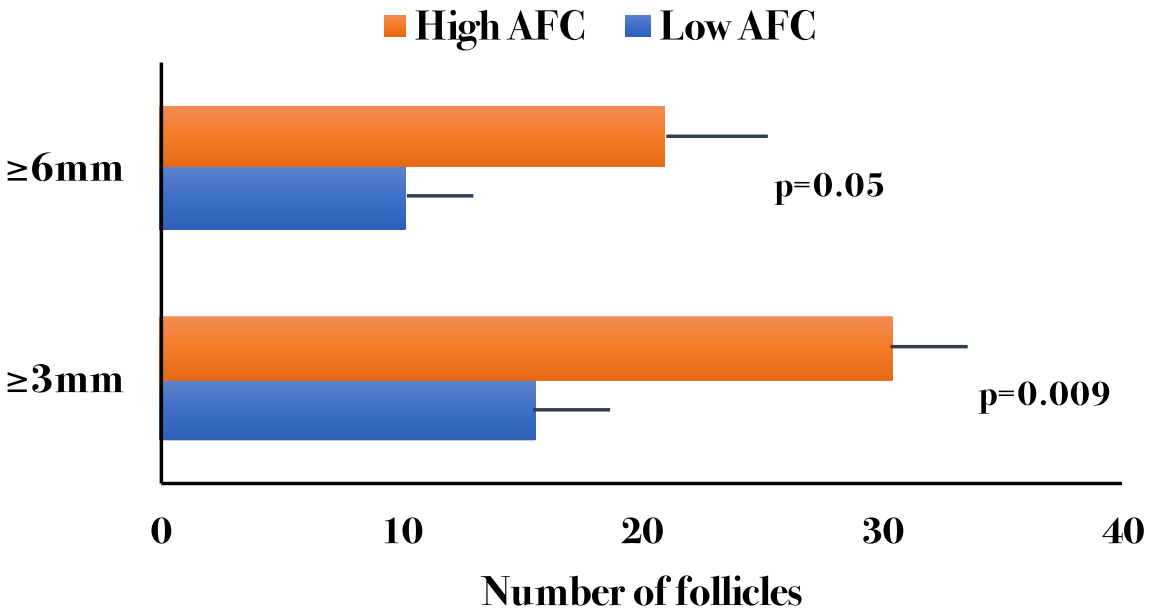
In adult cattle, the number of follicles recruited into successive follicular waves is consistent within individuals;

Among individuals, however, the ovarian response to gonadotropin stimulation is highly variable

Objective: to compare the ovarian response to superstimulation in calves with high vs low AFC at wave emergence

Krause ART, Dias FCF, Adams GP, Mapletoft RJ, Huanca WF, Zwiefelhofer EM, Singh J. - IETS 2017

Numbers of follicles by antral follicle counts



- ✓ Total number of follicles ≥3 and ≥6 mm were greater in High than Low AFC group. High AFC group had a greater number of 6-8 mm follicles at oocyte collection than Low AFC group.

Krause ART, Dias FCF, Adams GP, Mapletoft RJ, Huanca WF, Zwiefelhofer EM, Singh J. - IETS 2017

Contents lists available at ScienceDirect

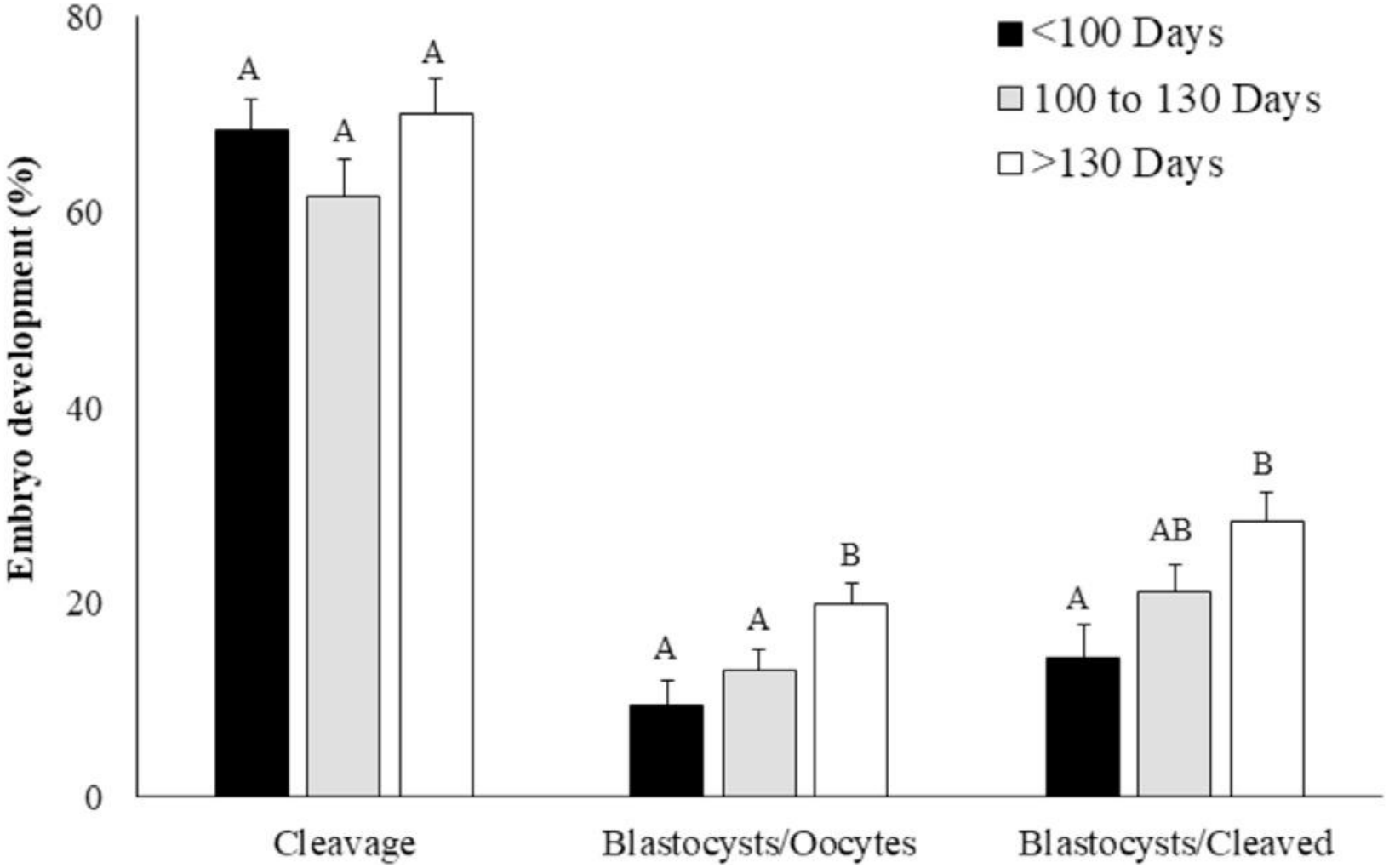
Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

Plasma anti-Müllerian hormone as a predictive endocrine marker to select *Bos taurus* (Holstein) and *Bos indicus* (Nelore) calves for in vitro embryo production

E.O.S. Batista [a,*], B.M. Guerreiro [a], B.G. Freitas [a], J.C.B. Silva [b], L.M. Vieira [a], R.M. Ferreira [c], R.G. Rezende [a], A.C. Basso [d], R.N.V.R. Lopes [e], F.P. Rennó [f], A.H. Souza [g], P.S. Baruselli [a]

Impact of age on cleavage and development to the blastocyst stage of oocytes collected from calves of 2-6 months of age

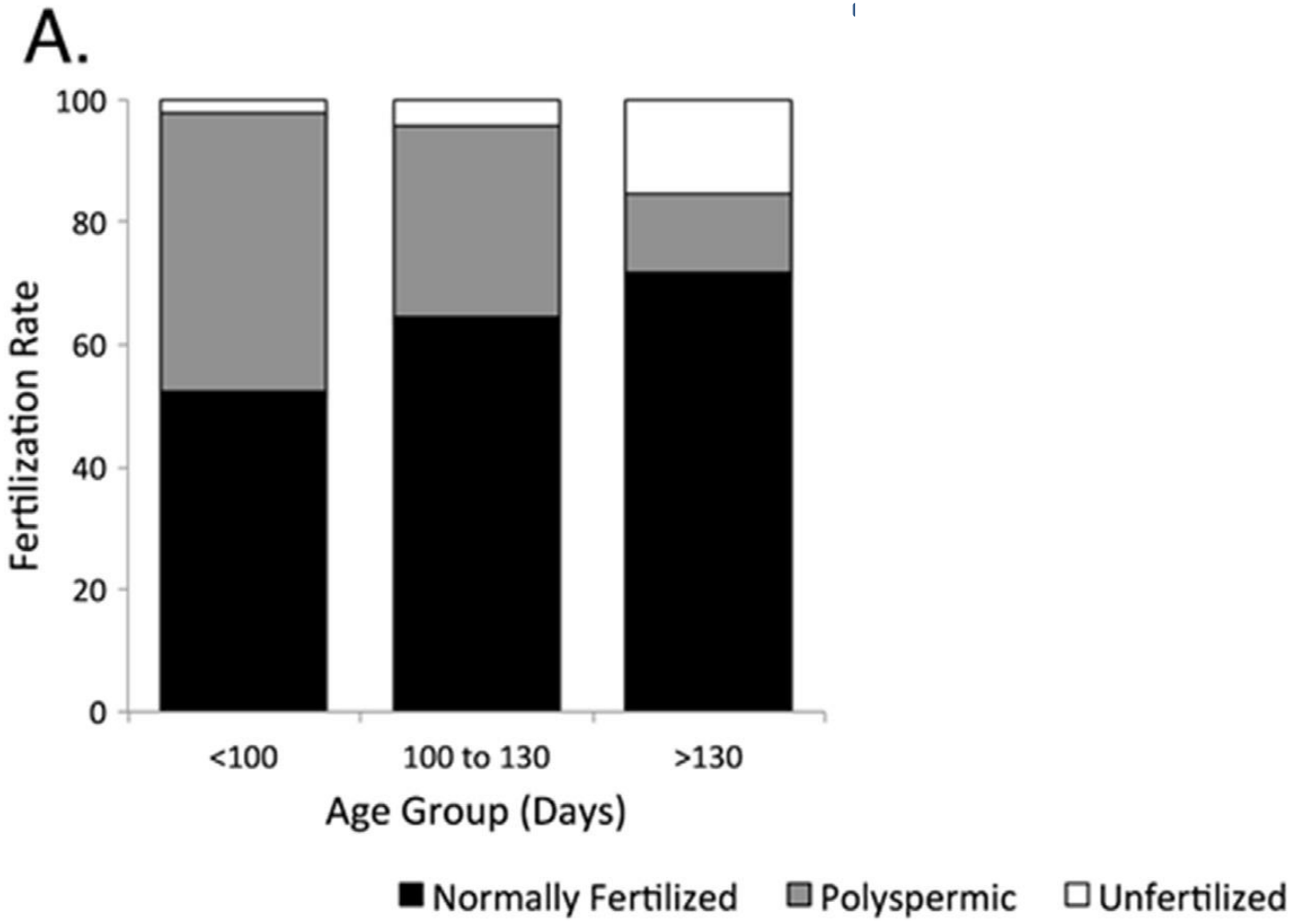


Baldassarre et al., 2018

AB - P<0.01

Normal fertilization and polyspermy rates among groups

Normally Fertilized = 2 polar bodies and 2 pronuclei. Polyspermic = 3 or more pronuclei. Unfertilized = Metaphase II. A,B - $P<0.05$.



Currin et al., 2017

Pregnancy rate of calf embryos produced in vitro (2015)

Cycling Heifers (2 year)	29/56	51.8%
Heifer Calves (2 to 4 months)	13/30	43.3%

IN VITRO BRASIL

CV NELORE MOCHO

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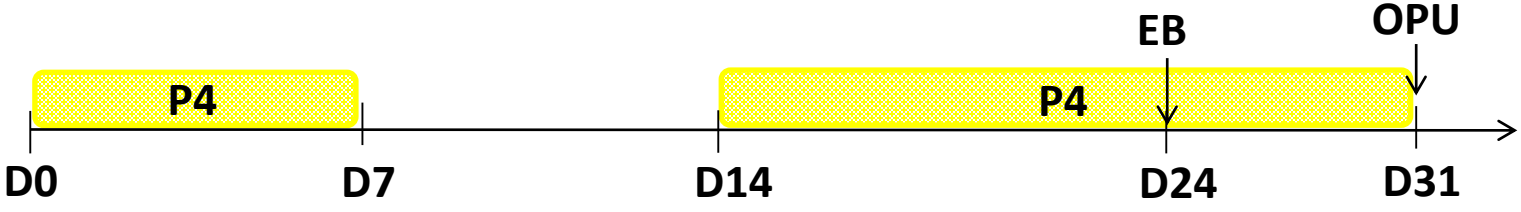
Progesterone priming effect on oocyte quality and in vitro embryo production of prepubertal Nelore heifers

EXPERIMENTAL DESIGN

1) Group Negative Control (prepubertal heifers; n=10)

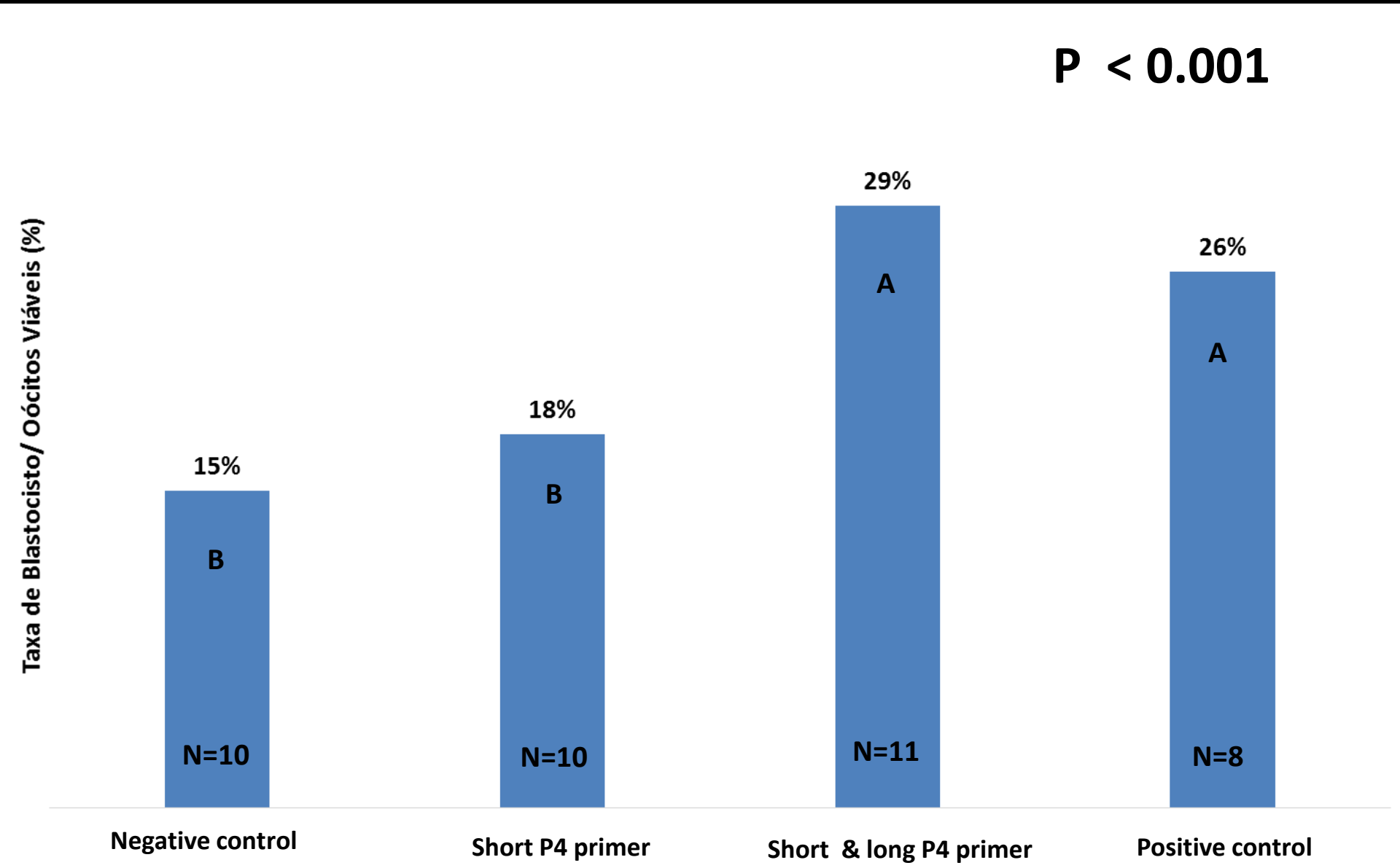
2) Group Short P4 primer (n=11)

3) Group Short and Long P4 primer (n=11)

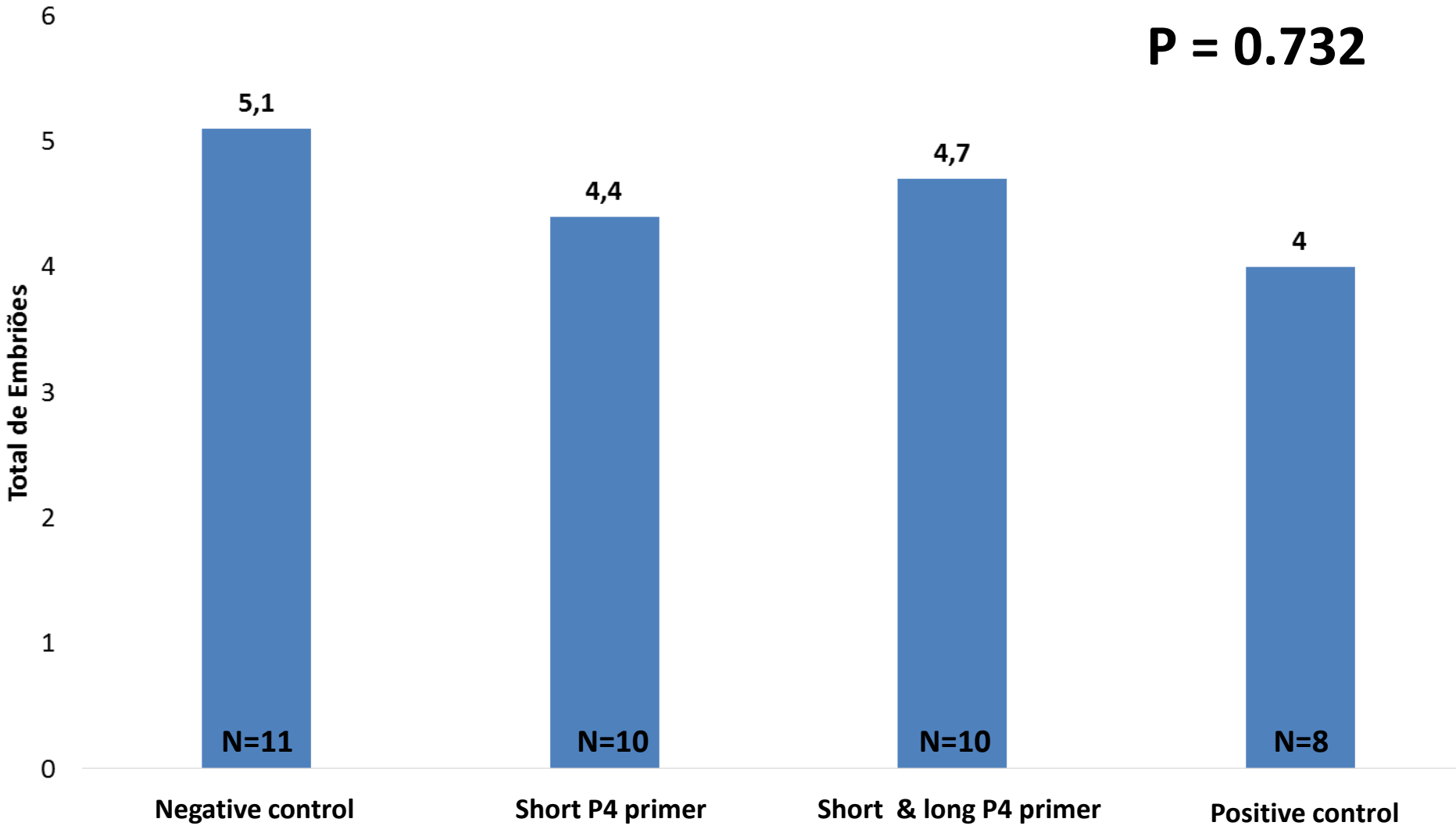


4) Group Positive Control (cycling cows; n=10)

BLASTOCYST RATE/VIABLE OOCYTE (%)



NUMBER OF BLASTOCYSTS/ OPU



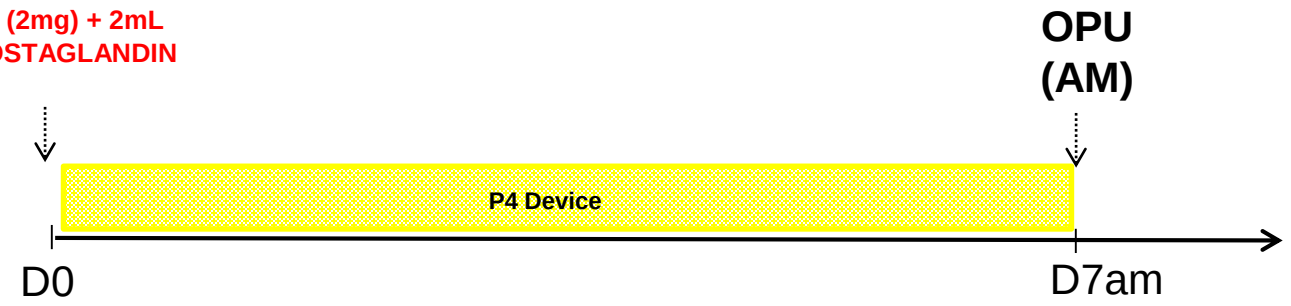
Effect of FSH treatment in OPU programs in prepubertal Holstein heifers

Bruna Martin s Guerreiro
Pietro Sampaio Baruselli

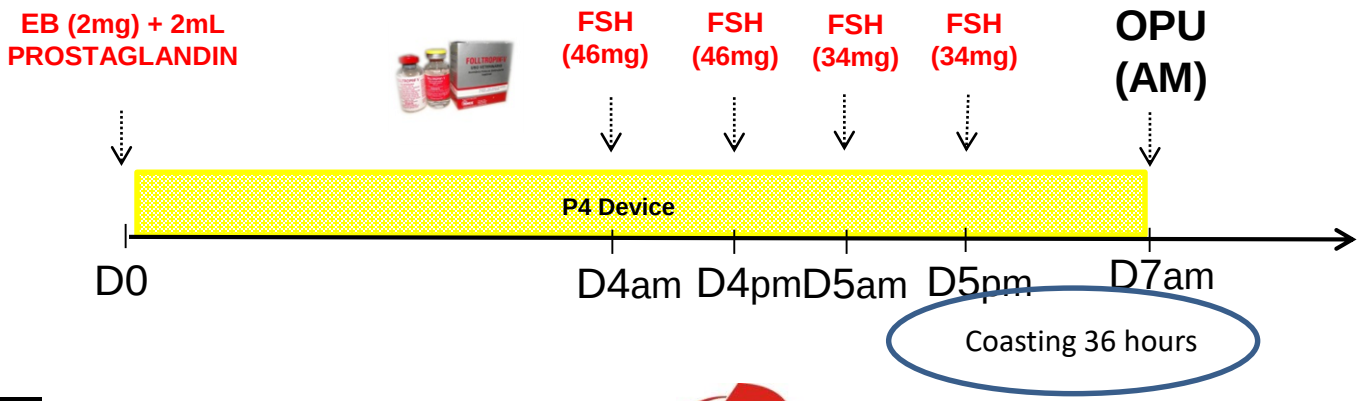
2015

Experimental Design

- **CONTROL** (n = 20 puberal heifers)



- **FSH 160 mg** (n = 20 puberal heifers)



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Effect of FSH treatment in OPU programs in prepubertal Holstein heifers

	CONTROL	FSH
n	**6**	**6**
Follicles aspirated,n	9,0	25,6
Recovery rate, n [1]	70,0	40,0
Total oocytes recovered, n	6,5	10,2
Viable oocytes, n	3,3	5,8
Percentage viable, % [2]	51,3	57,4
Cleaved embryos, n	2,5	5,2
Cleavage rate, % [3]	55.1	84.4
Percentage blastocysts, % [4]	33.3	58.6
Blastocysts, n	1.3	3.5

[1] (No. Oócitos totais/folículos aspirados)*100
[2] (No. oócitos viáveis/oócitos totais)*100
[3] (No. oócitos clivados/Oócitos viáveis)*100
[4] (No. blastocistos/Oócitos viáveis)*100

Quality of oocytes and in vitro embryo production from Nelore heifers

3 mo old heifers - P4 (n=8)

3 mo old heifers - P4+FSH (n=8)

Puberal heifers Positive control (n=8)

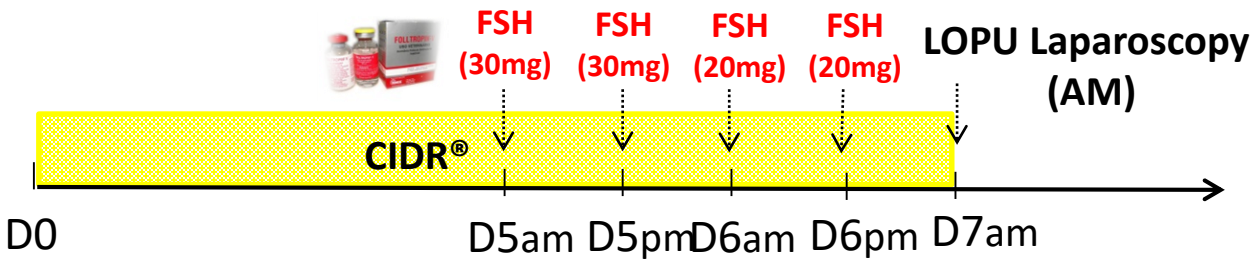
LOPU (laparoscopy)

Evaluation of oocyte quality, IVEP

Treatment groups and experimental design

1. Group heifers with **P4** (n = 8)

2. Group heifers with **P4 + FSH** (n = 8)



3. Group puberal heifers **Positive Control** (n = 8)

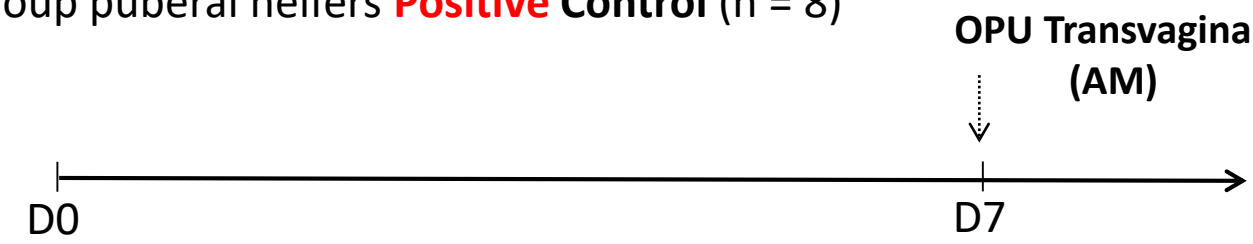


Table 1. Number of visualized follicles, COCs and blastocysts (mean ± SEM) after LOPU-IVP in *Bos indicus* (Nelore) donor calves and after OPU - IVP in *Bos indicus* (Nelore) cycling heifers.

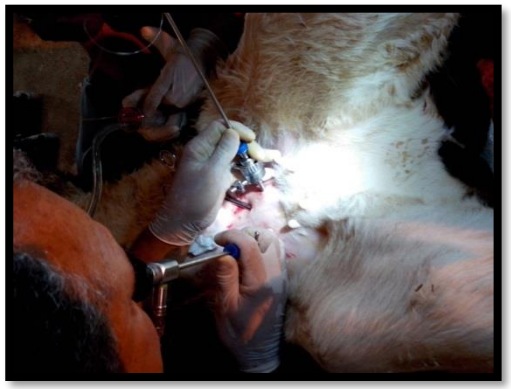
Item	*Bos indicus*			
	Calves without FSH	Calves with FSH	Cycling heifers	P value[d]
Total follicles visualized	19.7 ± 4^{z}	32.3 ± 5.9^{y}	47.1 ± 6.3^{x}	0.003
Total COCs retrieved	13.5 ± 3.6^{b}	20.9 ± 5.1^{ab}	29.9 ± 5.3^{a}	0.04
Recovery rate (%)[e]	68.5^{a}	64.7^{b}	63.6^{b}	0.02
COCs cultured	4.7 ± 1.4^{c}	11.3 ± 4.0^{b}	18.1 ± 4.0^{a}	<0.0001
COCs cultured rate (%)[f]	35.1^{b}	54.3^{a}	60.6^{a}	0.01
Cleavage rate (%)[g]	47.0	52.2	50.3	0.41
Blastocysts produced	1.7 ± 0.7^{b}	2.3 ± 0.8^{b}	9.3 ± 2.0^{a}	<0.0001
Blastocyst rate (%)[h]	12.9^{b}	11.3^{b}	30.9^{a}	<0.0001

[d]Data with different superscripts in the same line differ with $P \leq 0.05$ ($a \neq b \neq c$) or $P \leq 0.06$ ($x \neq y \neq z$). [e]Total number of COCs/number of follicles aspirated. [f]Number of COCs cultured/number of follicles aspirated. [g]Number of cleaved zygotes/ number of COCs. [h]Number of blastocyst/number of COCs.

In vitro embryo production of Holstein calves

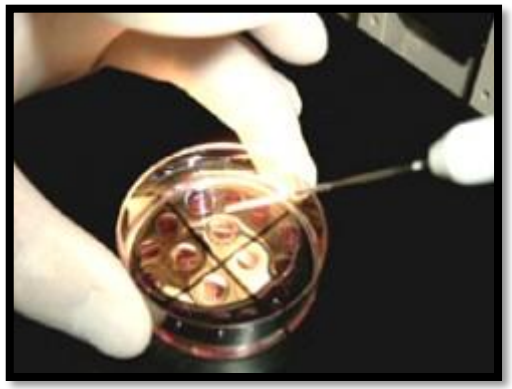
Heifers 2-4 mo (n=24)

Puberal heifers 13-16 mo (n=8)



LOPU - Laparoscopy (calves)

OPU - Transvaginal guided OPU (puberal heifers)

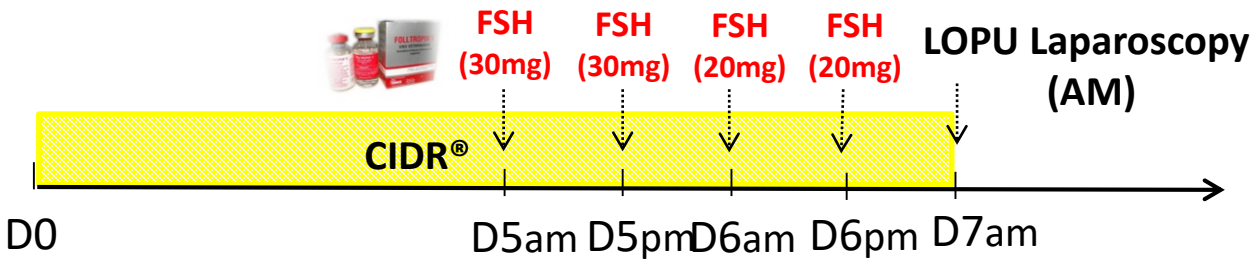


IVEP

EXPERIMENTAL DESIGN

1. Group heifers with **P4** (n = 8)

2. Group heifers with **P4 + FSH** (n = 8)



3. Group puberal heifers **Positive Control** (n = 8)

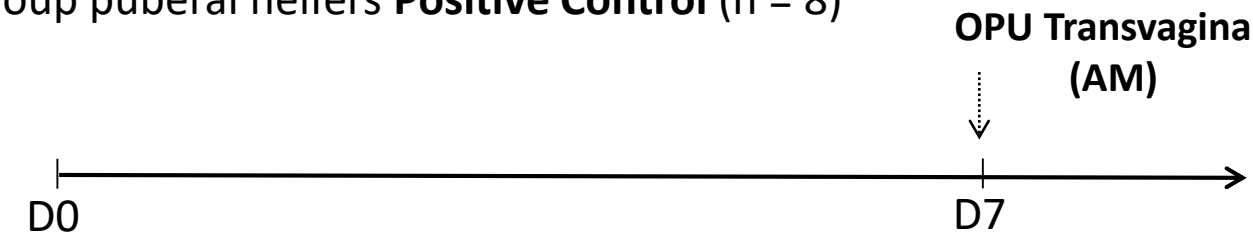


Table 2. Number of visualized follicles, COCs and blastocysts (mean ± SEM) after LOPU-IVP in *Bos taurus* (Holstein) donor calves and after OPU - IVP in *Bos taurus* (Holstein) cycling heifers.

	Bos taurus			
	Calves without FSH	Calves with FSH	Cycling heifers	P-values
Total follicles visualized	22.7 ± 4.2^{b}	54.3 ± 9.5^{a}	24.9 ± 3.6^{b}	0.01
Total COCs retrieved	11.7 ± 2.4^{bx}	22.4 ± 5.4^{a}	9.2 ± 1.7^{cy}	<0.0001
Recovery rate (%)[1]	51.3^{a}	41.3^{a}	36.9^{b}	0.01
COCs cultured	3.6 ± 1.0^{b}	12.3 ± 3.5^{a}	4.7 ± 1.3^{b}	<0.0001
COCs cultured rate (%)[2]	30.7^{b}	37.7^{a}	51.1^{a}	0.02
Cleavage rate (%)[3]	17.8	30.5	26.1	0.47
Blastocyst produced	0.4 ± 0.2	0.7 ± 0.4	0.5 ± 0.3	0.78
Blastocyst rate (%)[4]	2.9	2.0	4.3	0.60

[d]Data with different superscripts in the same line differ with $P \leq 0.05$ ($a \neq b \neq c$) or $P \leq 0.06$ ($x \neq y \neq z$). [e]Total number of COCs/number of follicles aspirated. [f]Number of COCs cultured/number of follicles aspirated. [g]Number of cleaved zygotes/ number of COCs. [h]Number of blastocyst/number of COCs.

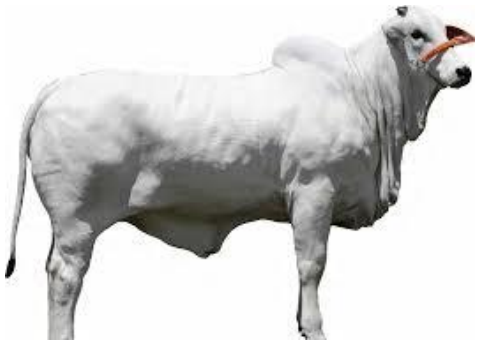
Oocyte quality and IVEP in prepubertal and pubertal heifers

Experimental design

- **Nelore heifers**

Prepubertal
(8 to 12 mo)
n=24

Prepubertal
(18 to 22 mo)
n=20



Pubertal
(22 to 26 mo)
n=25

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Table 4. Number of visualized follicles, COCs and blastocysts (mean ± SEM) after OPU - IVP in *Bos indicus* (Nelore) prepubertal and pubertal and heifers.

Item	Prepubertal heifers (8 - 12 month) (n = 24)	Prepubertal heifers (18 - 22 month) (n = 20)	Pubertal heifers (22 - 26 month) (n = 25)	P value[a]	
				Age[b]	Ciclicality[c]
Total follicles visualized	19.7 ± 2.1	41.3 ± 5.28	34.0 ± 3.3	<0.0001	0.0002
Total COCs retrieved	13.4 ± 1.7	30.8 ± 5.8	22.6 ± 3.2	<0.0001	<0.0001
Total COCs cleaved	5.6 ± 0.8	14.8 ± 2.5	13.3 ± 1.9	<0.0001	<0.0001
COCs cultured	7.6 ± 1.0	16.8 ± 2.7	15.1 ± 2.2	<0.0001	<0.0001
COCs cultured rate (%)[d]	57.0	54.0	60.0	0.13	0.45
Cleavage rate (%)[e]	73.0	88.0	84.0	<0.0001	0.25
Blastocysts produced	1.5 ± 0.3	4.7 ± 0.9	7.2 ± 1.2	<0.0001	<0.0001
Blastocyst rate (%)[f]	20.2	28.1	47.0	0.05	<0.0001

[a]Effect of evaluated group. [b]Effect of age in the prepubertal group (8-12 month *vs*. 18-24 month). [c]Effect of cyclicity (cyclic *vs*. non cyclic). [d]Number of viable oocytes/number of total oocytes. [e]Number of cleaved oocytes/number of cultured oocytes. [f]Number of blastocysts/number of cultured oocytes.

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In vitro embryo production of prepubertal Holstein donors

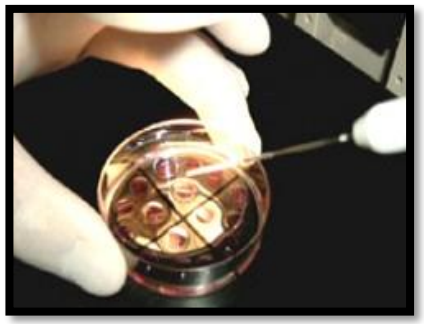
Prepubertal heifers 8-10 m (n=32)

Pubertal heifers 12-14 m (n=32)

Lactating cows (n=32)

Non-lactating cows (n=32)

OPU +US



Oocyte quality and IVF

SAMVET
PRODUTOS E REPRODUÇÃO BOVINA E EQUINA

bioembryo

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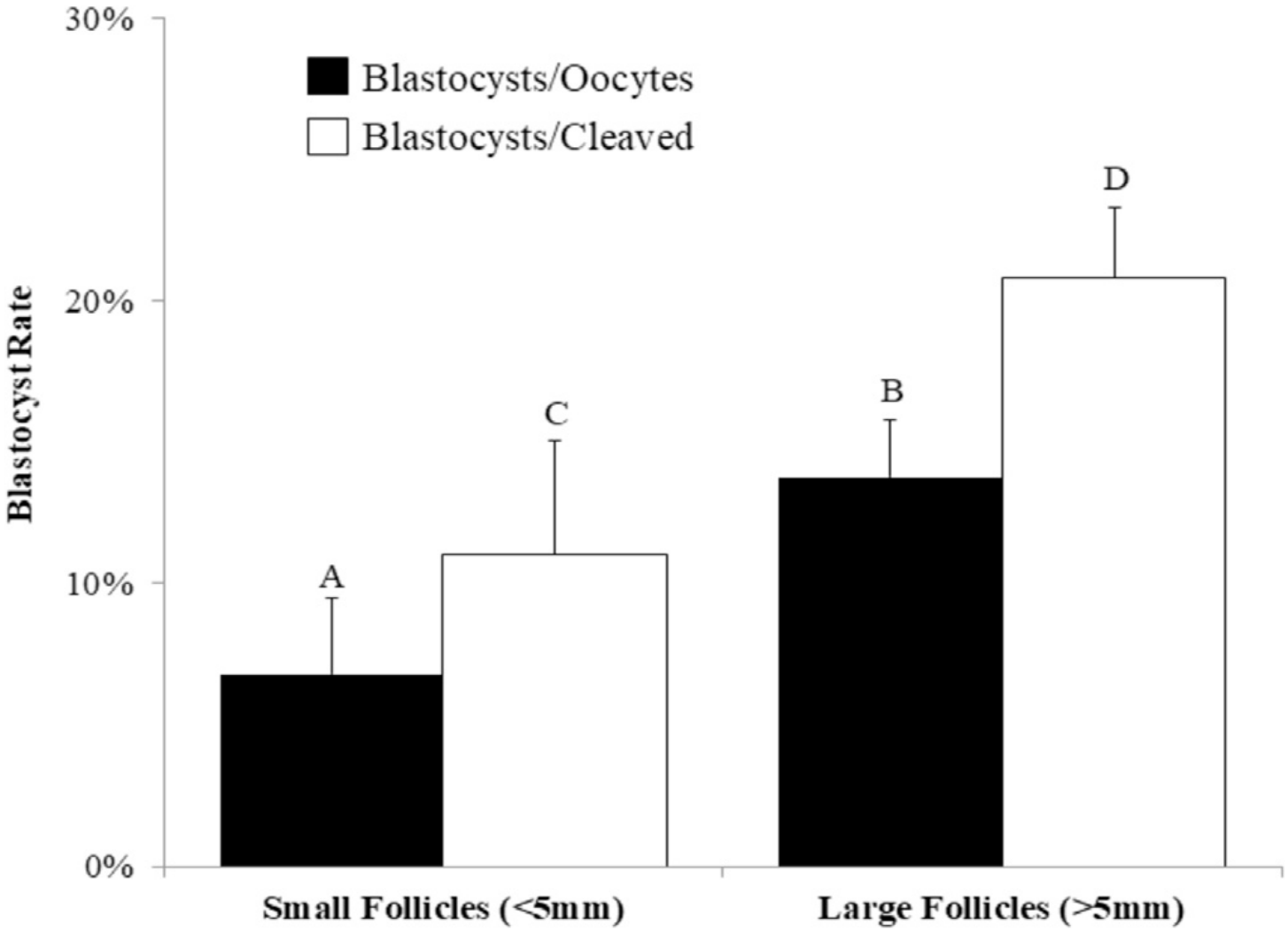
Table 3. Number of aspirated follicles, oocytes and embryos produced after OPU-IVP in prepubertal and pubertal heifers, and in lactating and non-lactating cows from the Holstein breed. Data is presented as mean ± standard error mean.

Item	Heifer		Cows		
	Prepubertal	Pubertal	Lactating	Non-Lactating	P value
N	30	30	30	30	
Total follicles aspirated	18.3 ± 2.1	17.3 ± 1.2	14.0 ± 1.0	17.7 ± 1.7	0.08
Total COC retrieved[1]	14.2 ± 2.2	13.1 ± 1.1	9.8 ± 1.1	14.6 ± 1.7	0.12
COCs cultured	10.5 ± 1.8^{ab}	8.3 ± 0.8^{ab}	6.5 ± 0.9^{b}	11.5 ± 1.4^{a}	0.03
Cleavage rate (%)[2]	68.6^{b}	98.8^{a}	87.6^{a}	90.1^{a}	<0.0001
Blastocysts produced	0.5 ± 0.2^{b}	1.1 ± 0.2^{b}	1.2 ± 0.4^{b}	4.2 ± 0.6^{a}	<0.0001
Blastocysts rate (%)[3]	4.8^{c}	12.7^{b}	18.0^{b}	36.5^{a}	<0.0001

[1]COC - cumulus oocyte complex. [2]Number of cleaved embryos/viable COCs. [3]Number of blastocysts/viable COCs.
[a,b,c]Different letters within rows indicate statistical difference ($P < 0.05$).

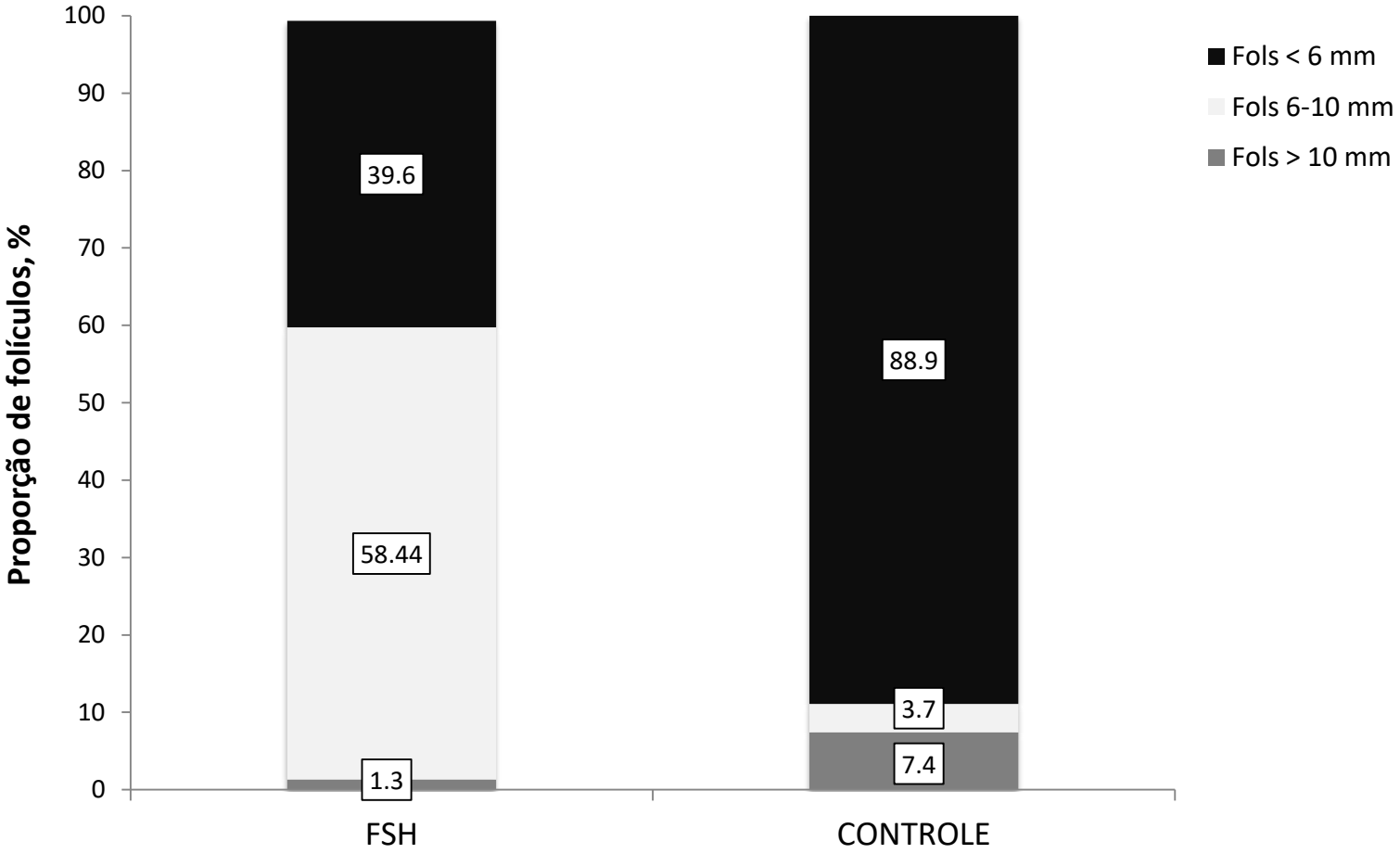
Impact of follicle size in calves on the developmental capacity of oocytes collected from follicles larger and smaller than 5 mm diameter



AB, CD - P< 0.05

Baldassarre et al., 2018

Effect of FSH treatment on follicle sizes in prepubertal Holstein heifers



Effect of length of FSH treatment in heifer calves on the number and size of follicles available for aspiration

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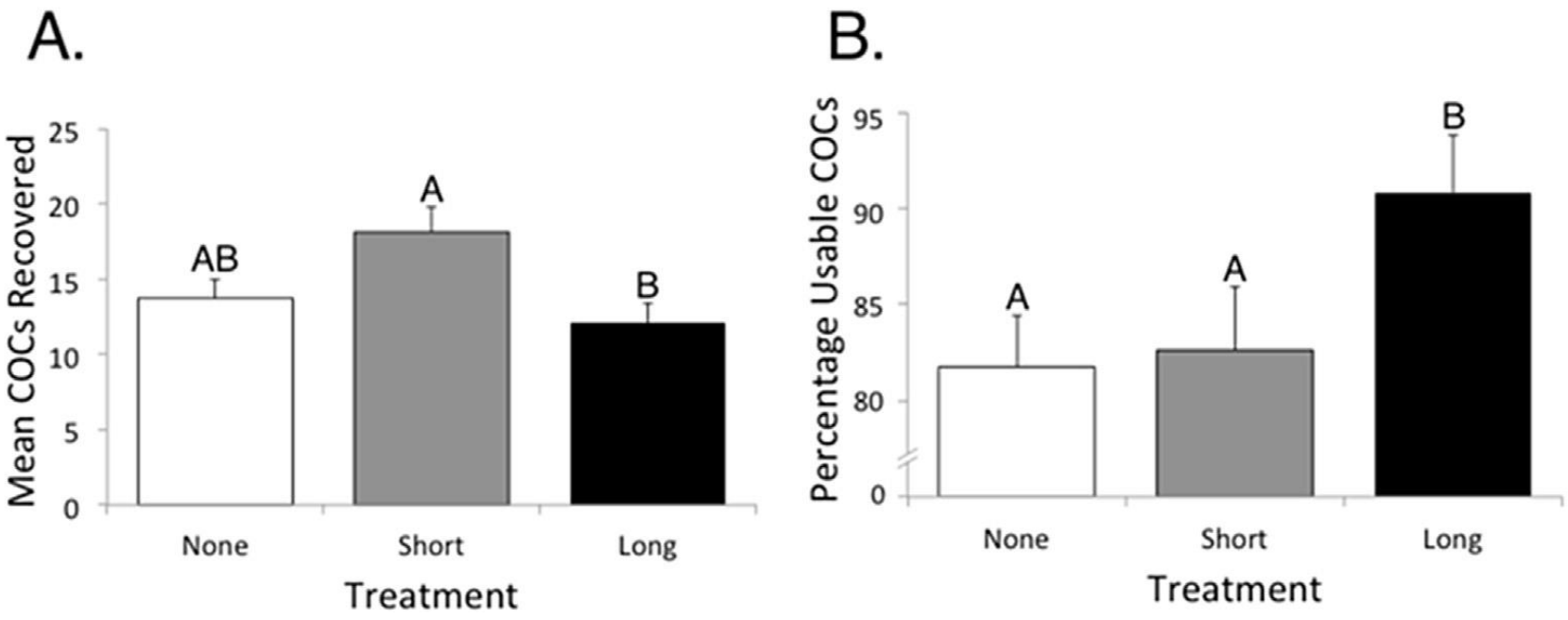
FSH Treatment	Average Follicles Aspirated per donor/per LOPU session			
	Total	>5 mm (%)	3-5 mm (%)	<3 mm (%)
No Treatment	19.8±9[a]	2.4[a]	6.9[a]	90.6[a]
Short Treatment **(36 h)**	20.7±11[a]	11.2[b]	35.2[b]	53.7[b]
Long Treatment **(72 h)**	16.9±10[a]	34.0[b]	42.8[b]	23.2[b]

Results in the same column with different superscript differ significantly ($P < 0.05$).

Currin et al., 2017

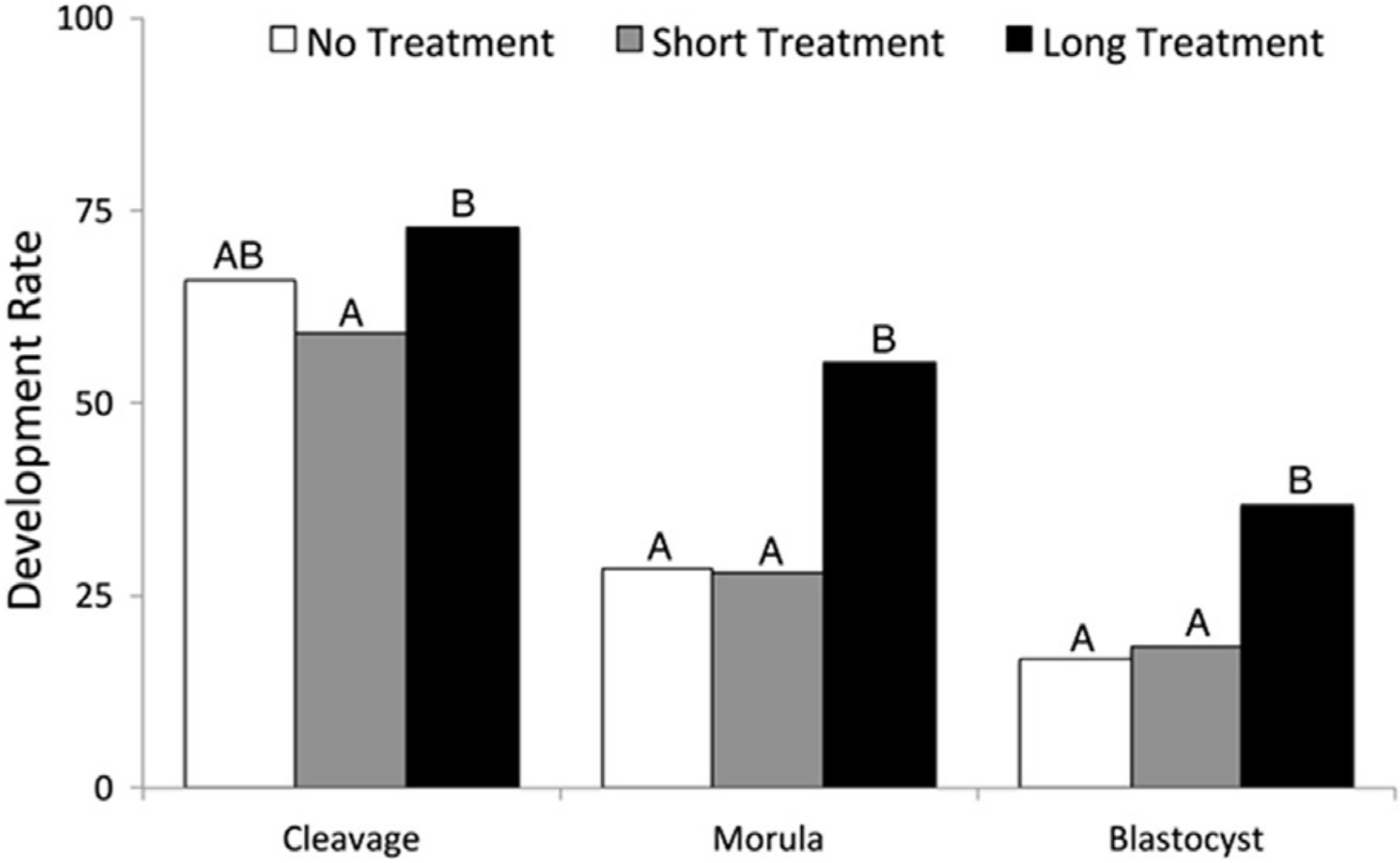
Effect of length of gonadotropin stimulation on the number of oocytes recovered



A,B - $P < 0.05$

Currin et al., 2017

Cleavage and in vitro embryo development by treatment. Morula and Blastocyst rates calculated as a function of total number of oocytes in IVC



A,B - P<0.05

Currin et al., 2017

Short (4 days) vs Long (7 days) protocol

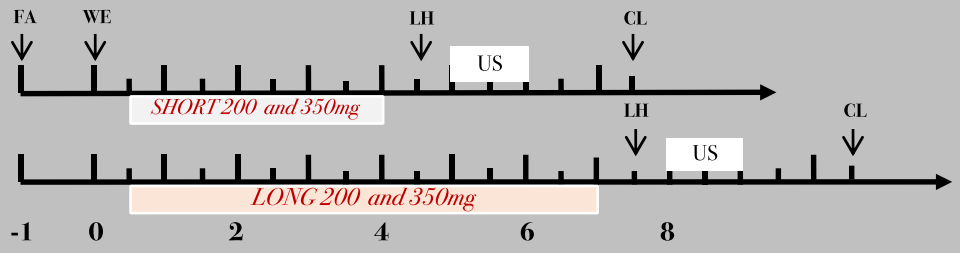
Krause et al., ICAR 2016

- Extending the gonadotropin treatment from 4 to 7 days in **pubertal** cattle resulted in:
  - Greater number of follicles
  - 2.5 times more transferable embryos per animal

  Dias et al., 2013

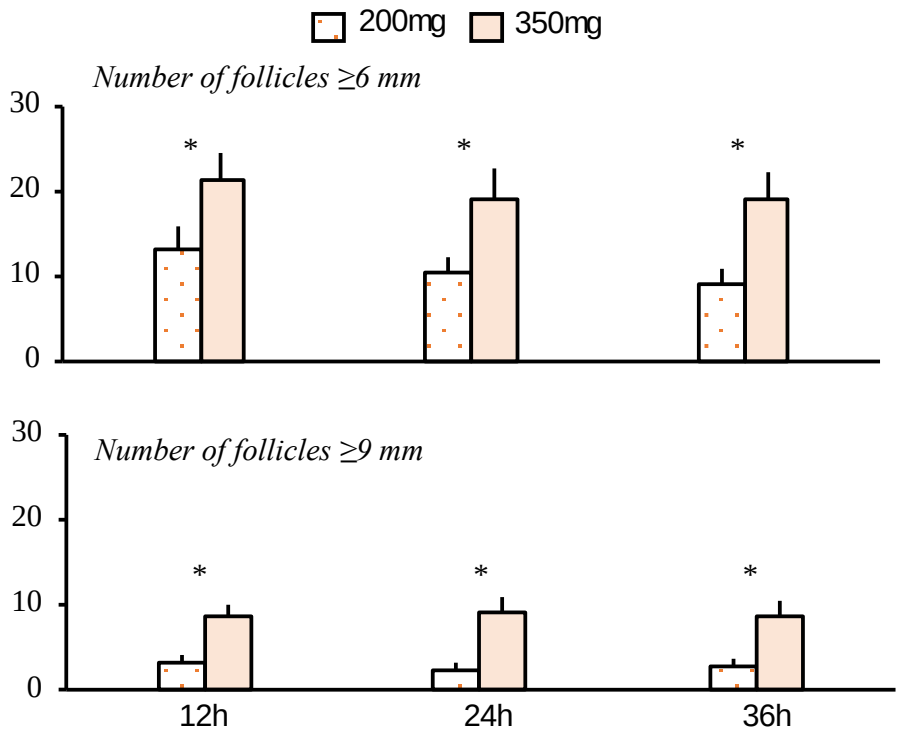
Objective: to examine dose and duration of superstimulatory treatment in 6-month-old prepubertal calves.

TREATMENTS		Dose	
		200mg	***350mg***
Duration	*4 days (Short)*	n=6 25mg/ inj	n=6 44mg/ inj
	7 days (Long)	n=6 14mg/ inj	n=5 25mg/ inj



FA:follicular ablation; WE: wave emergence; US: ovarian ultrassonography; CL: corpora lutea counts.

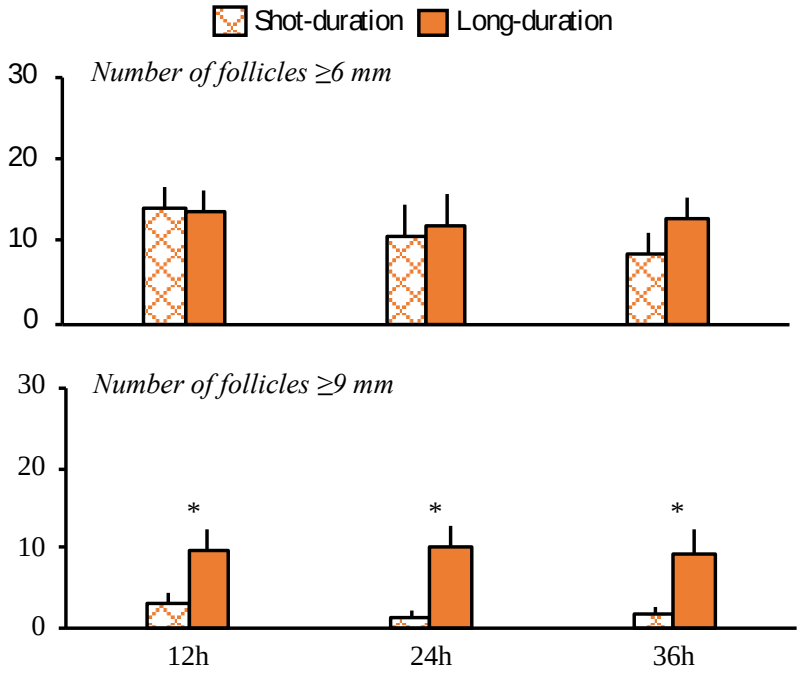
Number of follicles according to FSH dose

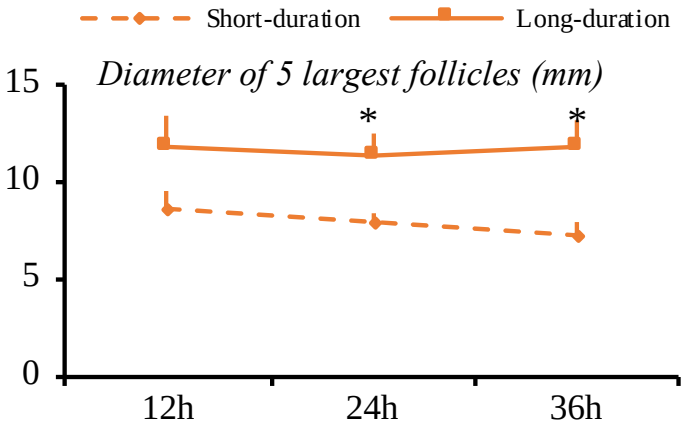


✓ Higher FSH dose resulted in a greater number of follicles ≥6 and ≥9 mm

Krause et al., ICAR 2016

Number of follicles and diameter of the five largest follicles by duration of FSH treatment

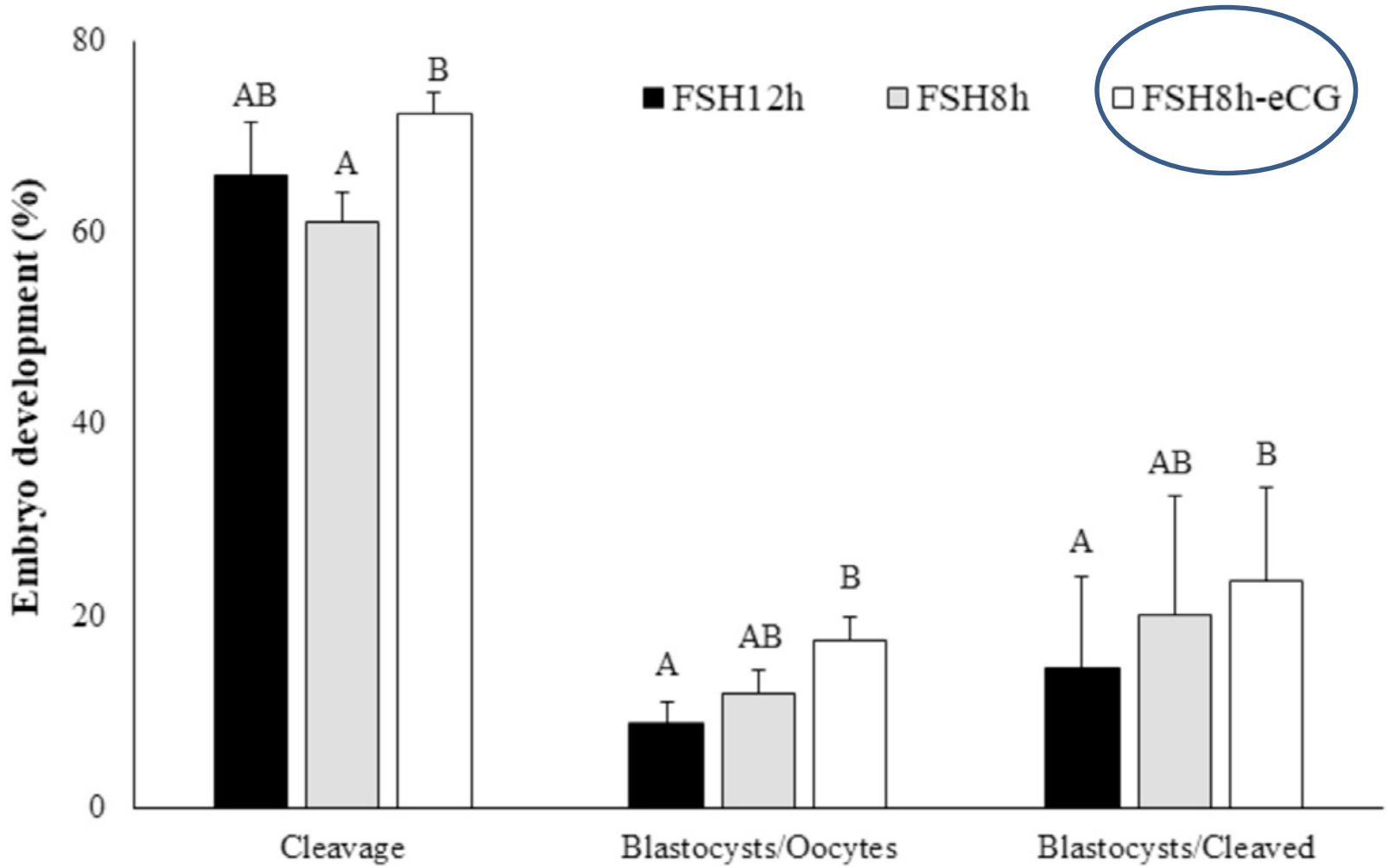




✓ Longer duration of gonadotropin treatment resulted in a greater number of follicles and larger mean follicle diameter

Conclusion: **Extending the period of treatment from 4 to 7 days with a total dose of 350mg FSH was able to induce a greater number of large follicles at the end of the superstimulatory treatment.**

Impact of eCG in a gonadotropin treatment protocol on cleavage and development to blastocyst of oocytes collected from 2 to 6 months old calves



AB - P<0.05

Baldassarre et al., 2018

Summary

- In at least one study the LOPU procedure was safe after repeating 8 times before females were size-suitable for ultrasound-guided OPU
- However, transvaginal ultrasounded-guided OPU is possible in calves
- AFC are repeatable in calves and correlate with AMH circulating levels
- The number of follicles at the beginning of a wave is predictive of the number recruited into subsequent waves in prepubertal calves
- Calves with a high AFC had more follicles available for oocyte collection after superstimulation than calves with a low AFC
- Cyclicity increases COC recovery and blastocyst production in heifers
- Transfer of embryos derived from calf oocytes to adult recipients resulted in pregnancies and development to term comparable with those from adults, but there are still exceptions
- Progesterone priming improves blastocyst production in prepubertal heifers

Summary (continued)

- Longer gonadotropin stimulation of 2-6 month old Holstein calves resulted in a higher proportion of larger follicles available for aspiration, a larger proportion of usable oocytes and greater blastocyst rates.
- Higher FSH doses increase numbers of follicles 6-10 mm in diameter, cleavage rates, blastocyst rates and number of blastocysts
- The addition of eCG improved ovarian superstimulation for COC recovery and blastocyst production
- These protocols repeated every 2 weeks could result in 10-30 transferable embryos before donors are 6 months of age.
- This could translate into a potential for production of 5-15 offspring that will be born before the donor calf reaches breeding age

Thank you

Obrigado