The multiple challenge of horse cloning: production, health, social acceptation, law, genetic application.

Palmer, E., Chavatte-Palmer, P. and Reis, A.P.

CRYOZOOTECH, SONCHAMP, France

e 16 août 2009

2003, the year of equine cloning

A Mule Cloned From Fetal Cells by Nuclear Transfer

Gordon L. Woods,[1*†] Kenneth L. White,[2*] Dirk K. Vanderwall,[1*] Guang-Peng Li,[2] Kenneth I. Aston,[2] Thomas D. Bunch,[2] Lora N. Meerdo,[2] Barry J. Pate[2]

Science, 29 may 2003

brief communications

A cloned horse born to its dam twin

A birth announcement calls for a rethink on the immunological demands of pregnancy.

Cesare Galli, Irina Lagutina, Gabriella Crotti, Silvia Colleoni, Paola Turini, Nunzia Ponderato, Roberto Duchi, Giovanna Lazzari

Laboratorio di Tecnologie della Riproduzione, CIZ srl, Istituto Sperimentale Italiano Lazzaro Spallanzani, 26100 Cremona, Italy

Several animal species, including sheep, mice, cattle, goats, rabbits, cats, pigs and, more recently, mules[1] have been reproduced by somatic cell cloning, with the offspring being a genetic copy of the animal donor of the nuclear material used for transfer into an enucleated oocyte. Here we use this technology to clone an adult horse and show that it is possible to establish a viable, full-term pregnancy in which the surrogate mother is also the nuclear donor. The cloned offspring is therefore genetically identical to the mare who carried it, challenging the idea that maternal immunological recognition of fetal antigens

Our cloning procedure was relatively efficient, as one live foal was produced from four pregnancies, although there was high developmental failure from the cleavage stage to blastocyst (8 of 467 and 14 of 286 developed in the male and female cell lines, respectively) and early implantation. This success was aided by advances in assisted reproduction in the horse[2], particularly at the oocyte-activation stage, when protein synthesis and phosphorylation must both be inhibited[3], and in the refinement of the zona-free manipulation technique[4,5].

The remarkable birth of a live foal from its genetically identical recipient is at odds

Nature, 7 august 2003

One idea: restaure the breeding capacity of castrated champions

Cloning work performed at different partners laboratories

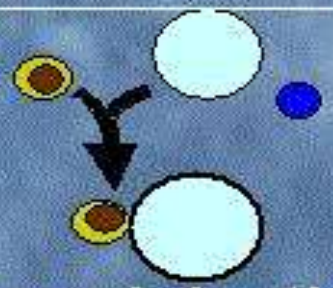
- **CIZ (Galli)**
- **TAMU (Choi & Hinrichs)**
- **VIAGEN (Polejaeva)**

CRYOZOOTECH CLONING PROCESS

Biopsy

Cell culture

Oocyte production



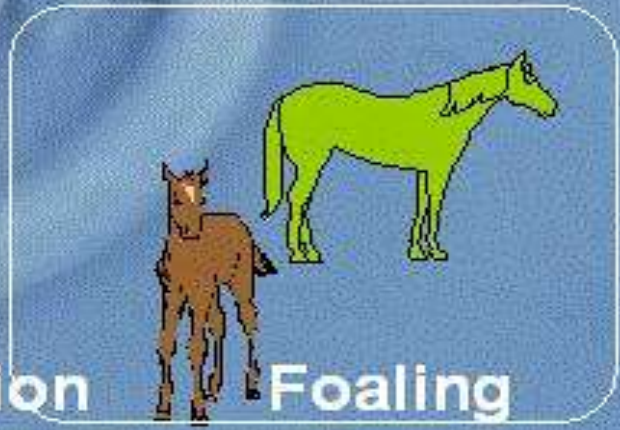
Micro-injection

Embryo culture

Cryo-Bank

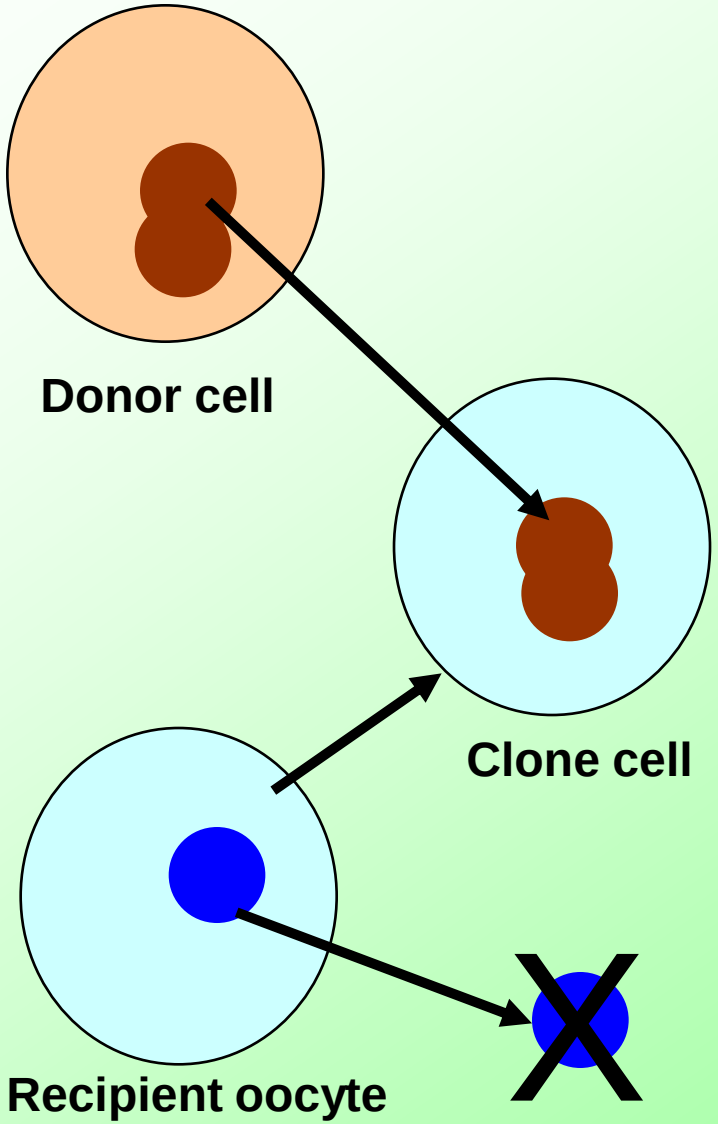
Embryo transfer

Gestation



Foaling

transmission de l'AND nucléaire et cytoplasmique au clone



A long process from slaughter house to delivering a foal

- Ovary
- Follicle
- Oocyte collection
- In vitro maturation
- Enucleation of oocyte
- Fusion or injection of donor cell
- Activation
- 2 cell stage
- In vitro blastocyst
- G15 intra uterine vesicle
- G30 embryo + heart
- Term (foaling)
- Healthy foal

Few papers give efficiency estimations along the process and will serve as references

- **Lagutina et al. 2005 étude A**
- **Lagutina et al. 2005 étude B**
- **Hinrichs et al. 2007 étude A**
- **Hinrichs et al. 2007 étude B**
- **Choi et al (2003) étude A**
- **Choi et al (2002) études A et B**

Follicles per Ovary and oocytes per follicle

● Hinrichs, Choi	6	0.75
● Galli and Lagutina	5.3	0.75
● Cryozootech	4.5	0.75

Maturation rate (1st P.B.)

	CP	Exp	total
Choi et al	25%	62%	
Lagutina et al	51%	60%	
Cryozootech			53%

Enucleation rate

- **Choi et al 2002** **81%**
- **Hinrichs 2007** **94%**

Fusion or injection

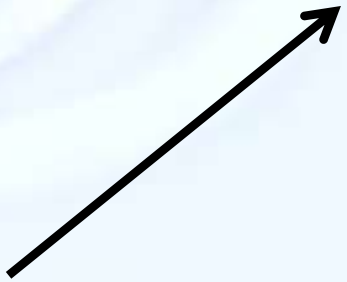
- **Galli, intact Zona 67%**
- **Galli, Zona free 98%**
- **Choi, Hinrichs Piezo injection 95%**

Activation

- **Choi et al 2003 95%**
- **Hinrichs 2007 95%**

Production of 2cell embryos

- **Lagutina** **Z intact** **68%**
- **Lagutina** **Zona free** **85%**
- **Hinrichs 2007** **78%**
- **Choi 2003** **51%**



2cell to Blastocysts

- **Lagutina** **5.5%**
- **Hinrichs 2007** **7.1%**
- **(Cryozootech cell lines 4.5%)**

Blastocyst to G15 uterine vesicle

- **Galli** **7.3%**
- **Hinrichs 2007 a&b** **61%**
- **(Cryozootech cell lines** **23% n=138)**

G15 to term (Foaling)

- **Galli** **2/10 = 20%**
- **Hinrichs** **8/13 = 62%**
- **(Cryozootech cell lines 10/32 = 30%**

Details of losses during pregnancy (Cryozootech cell lines)

● G15 to G30	32% loss n=138
● G30 to G90	13% loss n=32
● G90 to G150	35% loss n=40
● G150 to foaling	23% loss n=35

Healthy foal at one month

- **Galli** **1 / 2** **=** **50%**
- **Hinrichs** **7 / 8** **=** **87%**
- **Cryozootech births** **6 / 10** **=** **60%**

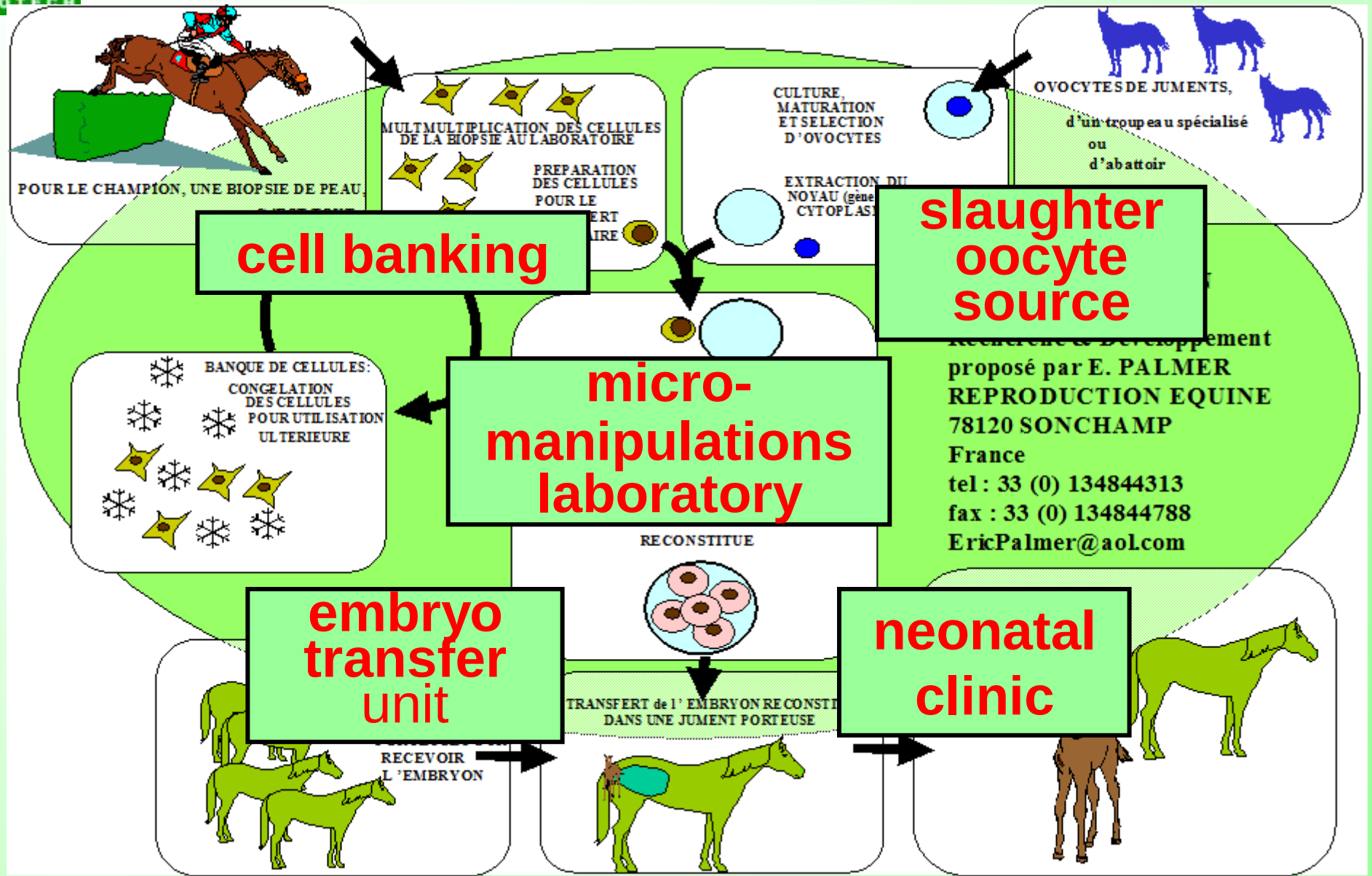
Great differences between cell lines

Horse	Healthy foals	Dead foals	Aborted pregnancies
Pieraz	1	1	
Quidam de Revel	2		
E.T.	1	1	
Poetin	2		
CA.		1	
RZ		1	
DK			1
RU			2
GD			2
TG			2
LZ			2
CHZ	1?	1?	2

Resulting prediction model

stage	FOR ONE HEALTHY FOAL			FOR ONE SUCCESSFUL LINE
	Galli	Hinrichs	Cryozootech lines	Cryozootech
ovaries	1253	57	387	576
follicles	6642	342	1730	2505
oocytes	4981	137	1298	1946
M II	2989	55	779	1168
Fused	2490	42	530	795
Blastocysts	136	3	23	35
G15	10	1.85	5.5	8.2
Foaling	2	1.14	1.7	2.5
Healthy foal	1	1	1	1.5

Organisation of production



A long process from birth of the first foal to use in breeding…

- Law and horse cloning
- Social acceptation
- Health of the clones
- Fertility of cloned horses
- registration of clones
- Approval as breeding animal
- Participation in competition

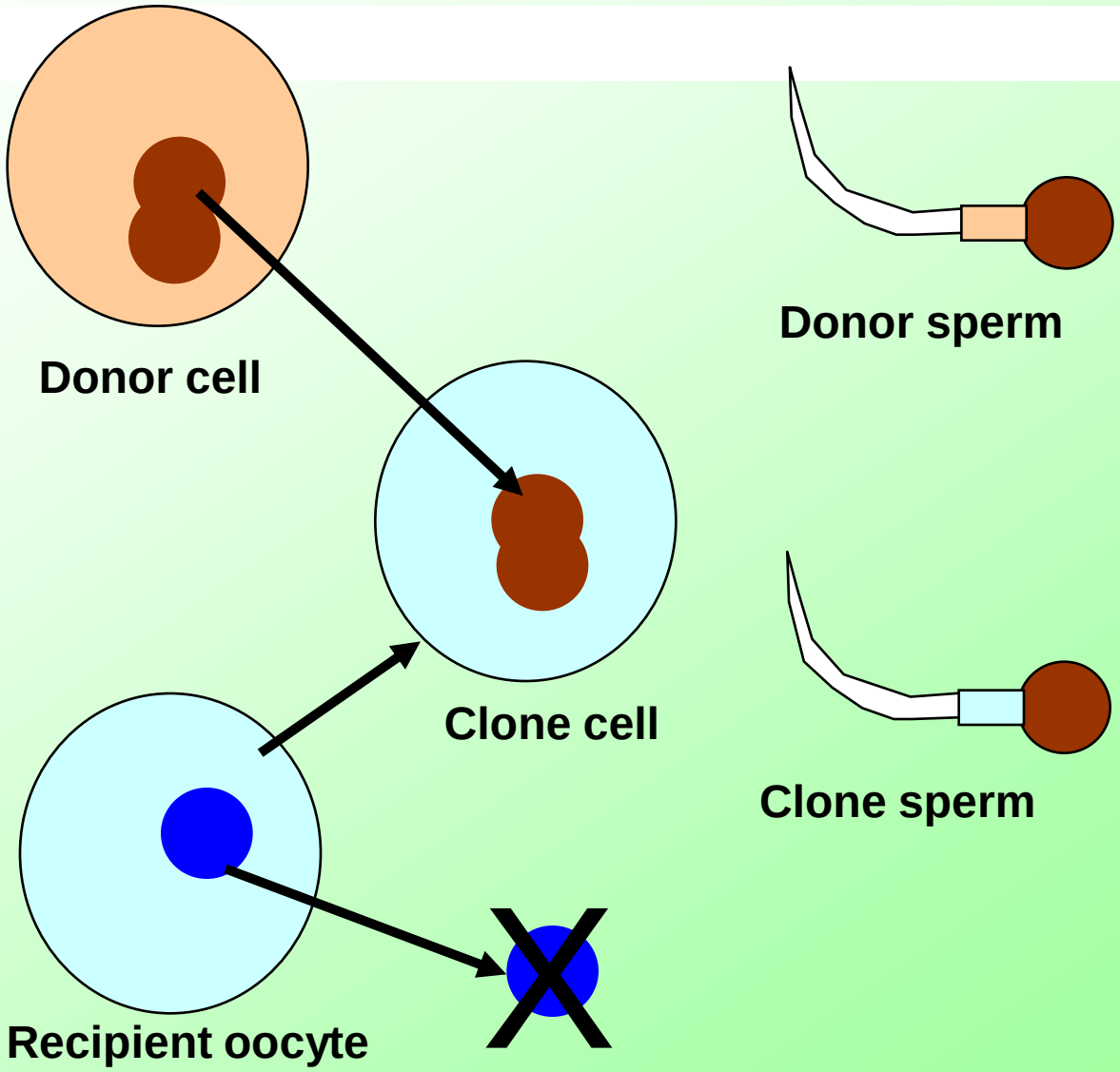
Law and social acceptation of animal cloning

- **Law:**
  - **Laws on animal experiments (see GB, DK)**
  - **Laws on animal welfare, vetrinary ethics**
  - **Laws on safety of human consumption**
- **Breeding rules**
  - **Internal rules of different studbooks**
  - **EU rules for recognised studbooks**
- **Rules for competition and races**

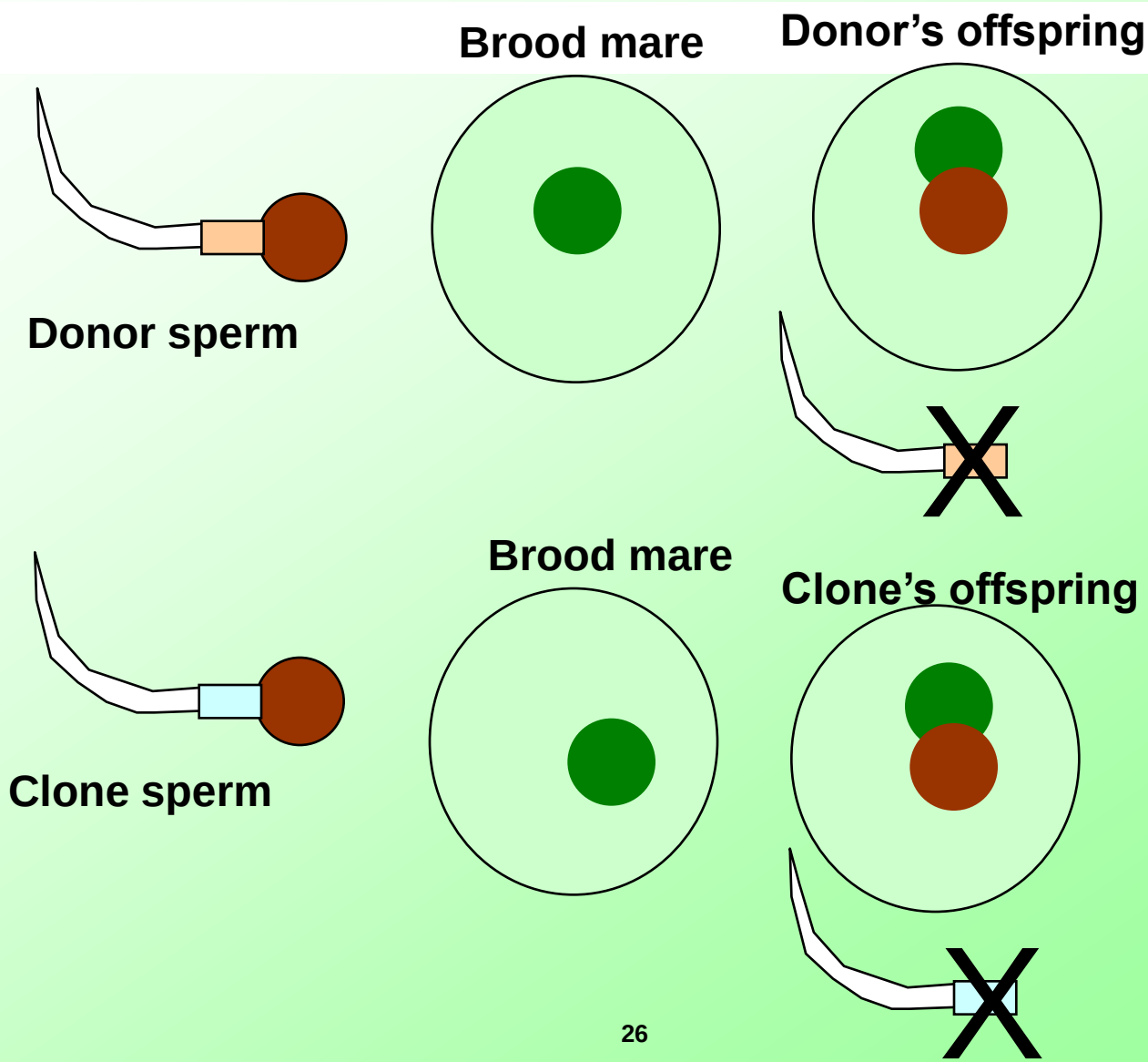
Health of the clones

- **At birth,**
  - **some foals are weak**
  - **of 24 foals born, 9 (37%) died in the first days .**
  - **Of 15 foals surviving at 21 days:**
    - **3/3 four years old are healthy**
    - **1/1 three years old is healthy**
    - **2/2 two years old are healthy**
    - **3/3 yearlings are healthy**
    - **5/5 five months old are healthy**
    - **1/1 two months old is healthy**

transmission de l'AND nucléaire et mitochondrial au spermatozoide

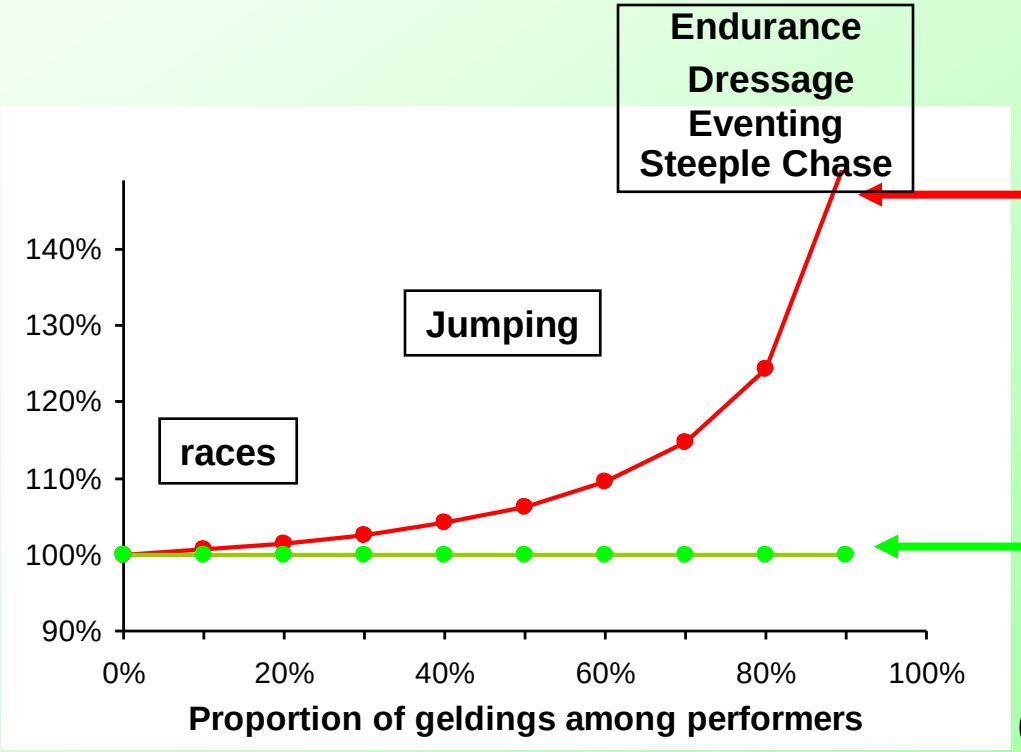


As a breeding animal, the clone is 100% equal to donor



Cloning, a way to accelerate genetic progress

- selecting stallions in a wider population of performers will allow a higher selection rate, and consequently more rapid genetic progress



Genetic progress obtained with the Help of cloning

100 = now genetic progress without the help of cloning

(after Anne Ricard)

Fertility of the clones

- **Prometea, first cloned mare foaled in 2008 at the age of 5**

- **Pieraz, first cloned male**
  - **Mated sucessfuly at 2 years old and produced Pierazade**
  - **Bred 35 mares in 2009, of which 25 are pregnant**

- **ET Cryozootech Stallion**
  - **Bred 6 mares in 2009, of which 4 are pregnant.**

However:

there may be a tendency for delayed puberty and late testis descent.

Clones are registered by ZANGERSHEIDE and ANGLO-EUROPEAN STUD BOOK


STUDBOOK ZANGERSHEIDE V.Z.W.

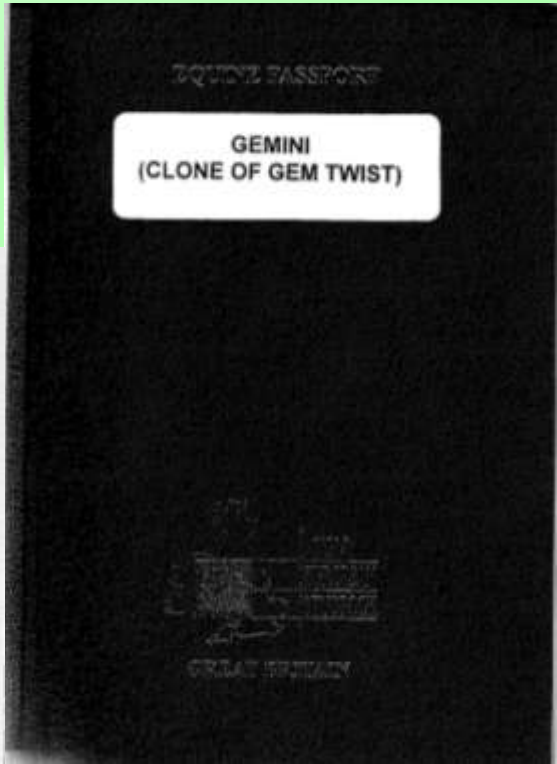
QUIDAM DE REVEL I Z (CL)

PASPOORT
PASSEPORT
PASSPORT
PASS

16/09/2005

Z-711005

ZANGERSHEIDE (Z)



Approval of the clones for breeding were deliverd By Zangersheide and AES studbooks

Studbook Zangersheide V.Z.W.

ATTESTATION

Je soussigné Jean Jacques Vandenberghe, Domein Zangersheide B-3620 Lanaken, agissant en qualité de Vice-président de l'ASBL Studbook Zangersheide certifie que l'étalon:

E.T. Cryozootech Z (CI) – Z6567.06- par Espri FRh et Gracia est admis à la monte publique pour trente juments.

Fait à Lanaken le 10 mars 2009.

J.J.Vandenberghe
Vice-président

Participation of clones to competitions and/or to public tests

- **Race organizations have banned the clones and their offspring from races**
- **Some countries are lobbying to ban clones from FEI…**
- **The breeding organizations (WBFSH, KWPN, say that they want to see the clones in public tests before approval as breeding animals)**
- **The Geneticists say that there is no need to show the clones in competitions before breeding as this will increase the interval between generations, and because the genotype has already been proven by the model.**

Orders may be different from expectation...

More mares and stallions have been ordered than geldings

	Stallions	Mares	Geldings	Total
Jumping	**4**	**4**	**3** *+2 CRZT*	**12** *+2CRZT*
Endurance			*1 CRZT*	*1 CRZT*
Dressage		**1**	*1 CRZT*	**1** *+1 CRZT*
TOTAL	**4**	**5**	**3** *+ 4 CRZT*	**13** *+4CRZT*

The long story of Pieraz Cryozootech Stallion

1996

2001

2001

2003

2005

2006

2007

2008

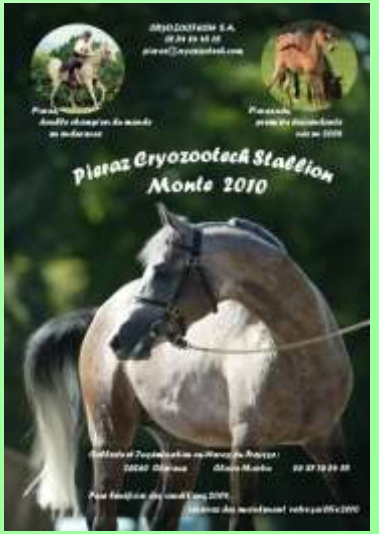
2008

2009 breeding season,

35 mares inseminated

25 pregnant mares

2009



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