Ram semen cryopreservation in Portuguese native breeds:season and breed effects on semen quality variation

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Introduction

- Portugal is a country with meditteranean climate considered a hotspot of animal biodiversity which have 16 native ram breeds with 6 threatened breeds (**Saloia, Churra da Terra Quente, Churra do Minho, Churra Badana, Churra Galega Mirandesa** e **Merina da Beira Baixa)** are considered threatened (<6500) and 1 are endangered (≤1000 ewes) (Churra do Campo).
- Majority of native breeds are explored for dual purpose and in extensive production system based on pasture being supplemented dure some periods (winter, late gestation, lactation) with hay and other roughages (Hay silages, grass silage…).
- Our native breeds have low or moderate seasonality, (February/April) with anual libido variation and semen quality varying according to individual and season of semen collection.
- However in our native sheep breeds natural mating is possible aroung the year without significant decreases in flock fertility with one anual lambing which is the main reproduction schedule in extensive systems.

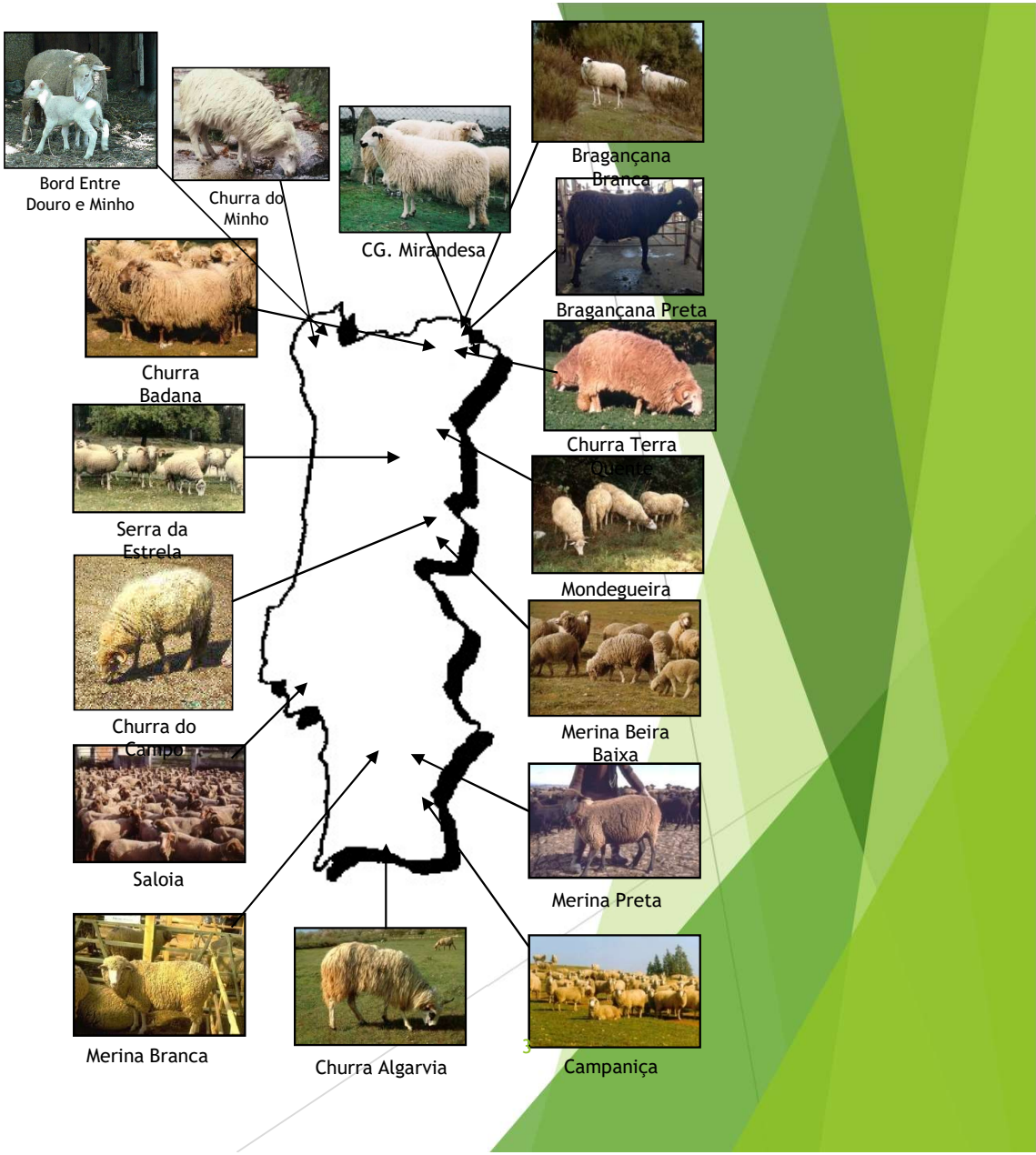
16 native sheep breeds

11 endangered breeds

< 10.000 Ewe (Reg UE nº455/2002)

Bordaleira Entre Douro Minho
Campaniça
Churra Algarvia
Churra Badana
Churra do Minho
Churra do Campo
Churra Galega Mirandesa
Churra Galega Bragançana Preta
Merina da Beira Baixa
Mondegueira
Saloia

Churra da Terra Quente
Churra Galega Bragançana Branca
Merino Branco
Merino Preto
Serra da Estrela



Sheep Native Portuguese Breeds groups based on fleece wool characteristics (dimensions, length, width thickness, smoothness of wool fiber).

Churros

Bordaleiros

Merinos

Objectives, Material and Methods

- Describe the value of spermatozoa in fresh (SF) and frozen semen (SC) collected and evaluated (macroscopic and microscopic) in our Andrology Laboratory in Portugal (Lat 39’11´ N; Long- 8º 44´, O) from 2024 to 2020.

- Evaluate the breed and seasonal effect of semen collection periods on seminal parameters in fresh semen and after the frozen-thawing cycle.

- We have considered three periods of semen collection which is considered in latitudes ≥ 45º. 1) Breeding season from September to January (mainly fall and early winter season); 2) Non breeding season, which is short and superficial (February to April) and 3) a transition period where many ewes are mated (May to August) to sell lambs in Christmas period with high commercial values.

- Ten native sheep breeds, accounting 85 rams, with a mean age of 36 months and with 1475 good ejaculates were approved (after freezing/thawing).

Material and Methods

- From each ram 1-2 ejaculates were collected by the artificial vagina technique one time per week during several months .
- Only good quality ejaculates (SF) were approved (Volume>0.4 mL, IM (individual motility) >55%, >2000*10^6 sperm/mL and <20% abnormal sperm. And also after freezing /thawing (SC)…..
- So…. we have worked with 85 rams, 10 breeds and 1471 ejaculates.
- Fresh semen parameters evaluated: Volume, Concentration (WPA-S106), Total sperm per ejaculate, Individual Motility (MI%), Spz Viability (live sperm), and Spz Morphology (abnormal sperm).

Material and Methods

- EZN semen extender (egg yolk extender) for ram sperm cryopreservation were prepared in our Andrology Laboratory, which contains Egg Yolk (15%), 6% Glycerol, Tris, Glucose, Citric Acid and Antibiotics (Penicilin and Streptomicin). Our semen extender was adapted from Evans and Maxwell extender for semen cryopreservation.

- Ejaculates were diluted after previous semen evaluation in one step procedure (Evans and Maxwell), to to get 800* 10^6 spz /mL (total sperm) per dose (Cassou Straws®, 0.25 mL).

Material and Methods

- Sperm refrigeration and equilibration (4 Cº) lasted 3.5-4 Hours in a refrigerated chamber regulated to 4 ºC, which precedes ram sperm cryopreservation in Cassou Straws® .

- Semen straws were placed horizontally in a float freezing rack (Minitube ®) and frozen in nitrogen vapours during 20 minutes (-130 Cº).

- Semen was thawed the following day, in water at 37.5 ºC during 40 seconds. Content of individual straws were poured in 1 mL of saline physiologic solution and observations were made 4-5 later (water bath at 37.5-38ºC) in an optic microscopic.

Material and Methods

- In thawed semen, IM (%), Live Sperm (%) and sperm abnormalities were evaluated. Only good ejaculates were stored in the Portuguese National Germplasm Bank (BPGA) (>25 IM %, 30 % live Sperm, and <20 % abnormal sperm).

- Statistical analyses were performed using a multivariate mixed linear model, with the software JMP 14 for Windows (SAS Institute, Cary, NC, USA). SEM or 95 % confidence interval (95% CI) for a 0.05 level of significance were used.

Ram breeds, number of animals and ejaculates from 2004 till 2020

Breed	Number of rams	Number of ejaculates
Serra da Estrela	22	224
Churro Galego Mirandês	12	224
Saloia	11	235
Bordaleiro Entre Douro e Minho	10	253
Churro Galego Bragançano	7	176
Churro do Campo	7	51
Merino Beira Baixa	6	121
Mondegueira	5	97
Churro Algarvio	3	49
Merino Branco	2	41
Total	**85**	**1471**

Descriptive statistics of ejaculate traits (SF)

Breed	Vol (ml)($\bar{x}$)	CV (%)	Concent (*10^6/mL) ($\bar{x}$)	CV (%)	Total (*10^6) ($\bar{x}$)	CV (%)
Serra da Estrela	0.83	41	5294	44	4377	56
Churro Galego Mirandês	0.72	33	5644	34	4181	53
Saloia	0.90	34	5009	31	4571	49
Bordaleiro Entre Douro e Minho	0.56	47	5470	47	3114	60
Churro Galego Bragançano	0.88	41	4042	31	3752	57
Churro do Campo	0.53	31	3660	35	2041	59
Merino Beira Baixa	0.65	40	5422	36	3589	56
Mondegueira	0.88	80	4060	27	3516	65
Churro Algarvio	0.92	33	4491	29	4217	46
Merino Branco	0.85	31	5670	21	4928	41
Total	0.77	49	5039	38	3894	57

Fresh Semen: Volume, sperm concentration and total sperm per ejaculate in 10 local ram breeds

Breed	Volume (mL)	SPZ (*10^6/mL)	Total (*10^6)
Serra da Estrela	0.84±0.02^{a}	5283±66^{a,b}	4396±79^{a}
Churro Galego Mirandês	0.72±0.03^{a,b,c}	5916±79^{a}	4319±93^{a}
Saloia	0.88±0.03^{a}	4889±79^{a,b}	4315±93^{a}
Bordaleiro Entre Douro e Minho	0.54±0.02^{c}	5303±83^{a,b}	2881±100^{a,b}
Churro Galego Bragançano	0.81±0.03^{a,b}	3879±98^{b}	3248±115^{a,b}
Churro do Campo	0.52±0.05^{a,b}	3293±136^{b}	1764±169^{b}
Merino Beira Baixa	0.58±0.04^{b,c}	5068±125^{a,b}	2990±149^{a,b}
Mondegueira	0.83±0.04^{a,b}	3959±125^{a,b}	3291±147^{a,b}
Churro Algarvio	0.87±0.05^{a,b}	4011±159^{a,b}	3581±188^{a,b}
Merino Branco	0.85±0.06^{a,b,c}	5423±197^{a,b}	4718±234^{a,b}

Effects of breed, season and semen cryopreservation on SPZ parameters

Sperm (%)	Breed (B)	Season (S)	Thawing (T)	B*S	B*T	S*T	B*S*T
Alive	**	NS	***	NS	***	NS	NS
Individual Motility	*	NS	***	*	NS	NS	**
Abnormal Morphology	**	NS	***	**	***	*	NS

Effects of breed and breed*semen processing interactions on spermatozoa traits of fresh semen

Breed	Alive (%)	Individual motility (%)	Abnormal sperm (%)
Serra da Estrela	71±0.3^{a}	54.3±0.2	6.5±0.2^{a}
Churro Galego Mirandês	61.5±0.4^{b}	67.0±0.2	11.9±0.2^{b}
Saloia	63.0±0.4^{b}	63.04±0.2	9.5±0.2^{ab}
Bordaleiro Entre Douro e Minho	63.04±0.4^{a,b}	63.09±0.2	15.01±0.2^{b}
Churro Galego Bragançano	63.5±0.5^{a,b}	59.3±0.3	8.5±0.3^{a,b}
Churro do Campo	54.2±0.6^{b}	54.7±0.3	11.7±0.4^{a,b}
Merino Beira Baixa	74.6±0.6^{a}	67.02±0.3	13.8±0.3^{b}
Mondegueira	60.7±0.6^{a,b}	63.9±0.3	11.1±0.3^{a,b}
Churro Algarvio	64.1±0.7^{a,b}	59.7±0.4	11.7±0.4^{a,b}
Merino Branco	59.2±0.9^{a,b}	61.6±0.5	11.5±0.5^{a,b}

Effects of breed and breed*semen processing interactions on spermatozoa traits of thawed semen

Breed	Alive (%)	Individual motility (%)	Abnormal sperm (%)
Serra da Estrela	45.5±0.3	34.1±0.2	14.5±0.2[a,b]
Churro Galego Mirandês	50.08±0.4	44.9±0.2	19.2±0.2[a,b]
Saloia	45.6±0.4	40.6±0.2	14.9±0,2[a,b]
Bordaleiro Entre Douro e Minho	48.3±0.4	42.1±0.2	22.6±0.2[a]
Churro Galego Bragançano	43.0±0.5	38.6±0.3	12.2±0.3[b]
Churro do Campo	34.6±0.6	28.8±0.3	13.2±0.4[a,b]
Merino Beira Baixa	53.4±0.6	46.0±0.3	24.1±0.3[a,b]
Mondegueira	48.7±0.6	42.7±0.3	19.3±0.3[a,b]
Churro Algarvio	38.8±0.7	38.5±0.4	13.0±0.4[a,b]
Merino Branco	43.4±0.9	41.3±0.5	17.6±0.5[a,b]

Discussion and Conclusions

- Ejaculate volume and sperm concentration were respectively 0.77 mL and 5039 ±0.051*10^6/mL varying among breeds and without significant seasonal effect.
- Total sperm per ejaculate was 3895±58 *10^6/mL with differences among breeds P<0.05).
- In frozen semen there were breed diferences in sperm abnormalities without significant diferences in individual motility and live sperm.
- The freezing/thawing cycle reduced 28.5% viability (live sperm), 35.5% IM (individual motility) and increased 54.5% sperm abnormalities.
- There was a breed*thawing interaction on sperm viability, meaning significant differences among breeds.

Discussion and Conclusions

- Sperm cryopreservation is the main factor of the significant decrease in fresh semen quality ($P<0.001$).
- Seasonal effects in fresh and frozen seminal parameters is low and restricted to some breeds, except for midpiece sperm abnormalities.
- In all breeds there was an individual variation in seminal parameters in fresh and frozen semen.
- Despite breed diferences in fresh and frozen semen quality, all of the breeds were able to produce semen (with reasonable quality) during whole the year for mating, cryopreservation and assisted reproductive biotechnologies without the requirement of previous use of photoperiod and or melatonin treatments.
- There was a significant effect of breed and freezing/thawing cycle on sperm traits in these 10 Portuguese Native breeds.
- There was a limited effect of season of semen collection on sperm seminal traits.

A big thanks and recognition to Professor Jane Morrell for inviting me to participate in this outstanding digital meeting concerning Small Ruminant Semen Cryopreservation

- Many thanks to all the colleagues which participate and are engaged in this great webbinar
- If you have questions….