This book and its case studies focuses on typical local products and breeds, descriptions of the production systems and conservation techniques of endangered breeds/products in the Mediterranean area.

Traditional and extensive systems, involving local breeds, which meet the needs of the population requiring safe foods at a reasonable costs, are validated for their specific meaning to the region. It is acknowledged that natural constraints of the Mediterranean area of climate and geography, make it unfavourable to mass production at low cost. Profit related aspects are discussed considering the different economic realities of the northern part of the basin compared to the southern part.

Characteristics of typical animal production with consideration for positive and negative impacts on production systems and on the environment as well as the need to adjust to climate uncertainty and seasonal variability of feed resources, is also discussed. A focus is given to the following areas:

- animal production economy and social impact in the Mediterranean area;
- utilisation of natural resources and environmental impact of the animal production systems;
- possibilities for improving traditional systems;
- quality and traceability of typical products;
- moving from traditional to certified animal products.
Besnoitia besnoiti impact on fertility of cattle exploited in Mediterranean pastures (Alentejo)

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Summary

Besnoitia besnoiti is a bovine parasite endemic in many tropical and subtropical areas whose prevalence in the Mediterranean countries such as Portugal seems to be increasing. Most infections are mild or subclinical, characterized by the formation of numerous cutaneous and sub-cutaneous microcysts, lowering the quality of skins for the leather industry. Male sterility or impaired fertility is a common sequela in breeding bulls, and is one of the most negative aspects of the disease in animals that survive infection. Our objective was to investigate if asymptomatic Besnoitiosis leads to bovine infertility, by comparing seminal parameters pre and post-thawing, in vitro fertilization (IVF) and embryo rates between asymptomatic infected (n=3) and uninfected (n=5) bulls, exploited in an extensive production system in Alentejo-Portugal. Skin biopsies were submitted to histopathological analyses to identify B. besnoiti cysts in sires. Semen was collected by electroejaculation and sperm quality parameters before cryopreservation and after thawing were analyzed using ANOVA. The quality of semen collected from asymptomatic infected and uninfected bulls presented no differences before cryopreservation. From all the post-thawed sperm quality parameters (motility and hypoosmotic swelling test; post-swim-up motility, activity, concentration and agglutination; fertilization and embryo rates) evaluated, only post-thawed (51.0±36.3 vs. 42.3±10.6%, P≤0.05) and post-swim-up (36.3±18.8 vs 25.1±12.0 %, P≤0.009) motility were significantly different between asymptomatic infected and uninfected bulls, respectively.

Semen from asymptomatic Besnoitia besnoiti infected bulls may maintain fertilization ability. However the presence of these animals in herds represents a risk of spreading the disease leading to further economic losses.

Key words: Besnoitia besnoiti, bovine besnoitiosis, infertility, Portugal.

Introduction

Besnoitia besnoiti (Marotel 1912) is a coccidian parasite endemic in many tropical and subtropical areas where it causes both acute and chronic bovine besnoitiosis, leading to significant economic constraint to commercial cattle production (Bigalke, 1968, Kumi-Diaka et al., 1981). In Europe, it has been reported only in the Mediterranean countries, France (Besnoit & Robin, 1912), Spain
(Irigoien et al., 2000), and Portugal. In the latter country, prior to 1991, the disease was seldom recognized or reported. At present its prevalence may be increasing (Leitão, 1949, Cortes et al., 2003, 2005).

Cattle regardless of breed, sex and age are affected by this parasitosis, although clinical disease occurs rarely in calves less than 6 months of age. The occurrence is usually sporadic, and only a small proportion of infected animals develops clinical disease (Pols, 1960, Bigalke, 1968). In the acute stage of disease, parasitic tachyzoites invade blood vessels of the skin, subcutaneous tissues, fascia and testes causing widespread vasculitis and thrombosis. The result is a severe generalized reaction which is accompanied by oedema of the skin and acute orchitis. During the chronic stage, large numbers of tissue microcysts containing bradyzoites are formed. The most striking features of this stage are thickening, wrinkling and hair loss of the skin, accompanied by anorexia and severe weight loss. The case mortality rate is approximately 10% (Bigalke, 1968, Basson et al., 1970). Male sterility or impaired fertility is a common sequela in breeding bulls, and is one of the most negative aspects of the disease in animals that survive the acute and chronic stages of infection (Basson et al., 1970, Kumi-Diaka et al., 1981, Sekoni et al., 1992, Cortes et al., 2005).

The main objective of this experimental work was to compare fertility parameters between uninfected and infected bulls without clinical signs of disease and to predict some implications of their presence on farm reproduction.

Material and Methods

Animals

Three different farms with cases of bovine besnoitiosis were chosen for this study. All the farms were located in Évora region (Portugal) and exploit beef cattle extensively. Eight animals, seven Limousine and one Charolais bull, aged 2-9 years, were selected. Skin samples collected from the bulls’ neck were fixed in 10% formalin solution and stained with Hematoxilin /Eosin to identify the presence of Besnoitia cysts in a light microscope (100X). The aim was to find affected males without any clinical symptoms, but with the presence of Besnoitia cysts revealed by histopathology examination. This was achieved in three bulls.

Semen collection and cryopreservation

Semen was collected by electroejaculation to asymptomatic infected (n=3) and uninfected bulls (n=5) and immediately stored in a 32°C water bath. Before cryopreservation, semen morphology was evaluated by determination of gross (1 to 5) and individual progressive (0–100%) motility, percentage of abnormal and live (supravital stain eosin–nigrosin) spermatozoa (spz). After determination of sperm concentration, dilutions were performed using TriladylÒ (ref. 13500/0250, Minitub GmbH) with egg yolk and distilled water, previously warmed at 32°C, to obtain progressively motile 20x10⁶ spz per seminal dose. Extended semen was immediately loaded into 0.25 ml straws. All the straws were vertically stored in an appropriate device and maintained at 5°C for 4 hours and then frozen by vertical freezing method (Chagas e Silva, personal communication). The rackets with straws were kept inside the RCB, Container (Air Liquide, France) in the nitrogen vapours (in a position that the device only touches the liquid phase) for 20 minutes with the lid closed, before being placed into appropriate cryogenic containers and immersed into liquid nitrogen for storage.
**Post-thawed semen evaluation**

The effect of *B. Besnoiti* on post-thawed semen quality parameters was investigated according to the following experimental design: In experiment 1 (6 replicates), semen from uninfected bulls (n=5) was tested. In experiment 2 (5 replicates), semen from two uninfected bulls, with the worst and the best fertilizing ability selected in experiment 1, and from three asymptomatic infected bulls was used. For the hypoosmotic swelling test (Host), only two straws from each infected and uninfected animals were evaluated.

*Post-thawed motility, Host and post-swim-up tests*

Individually progressive semen motility after thawing from infected or uninfected bulls was evaluated. To perform the Host test two straws from each bull were thawed. Semen aliquots were incubated with an hypoosmotic solution (100 mOsmL\(^{-1}\)) for 25 minutes and fixed with 2% glutaraldehyde in BL-1 (Ferreira *et al*., 2001).

To perform post-swim-up tests thawed semen was incubated for 1 hour in TALP medium without Ca\(^{++}\) supplemented with 48.6 mg mL\(^{-1}\) caffeine (Sigma, ref. C-0750) in an humidified atmosphere of 5% CO\(_2\) at 39°C. Spermatozoa from the upper layer were taken to evaluate their individual motility (0 – 100%), activity (1 to 5), concentration and agglutination (number of agglutinated heads in 100 observed spz).

**In vitro fertilization and embryo development**

In vitro maturation, fertilization and co-culture procedures have been described previously (Pereira *et al*., 2005). Briefly, follicles with 2-6 mm diameter were aspirated from abattoir bovine ovaries. Selected cumulus oocyte complexes were matured in TCM199 (GibCo, ref. 22340-020) with 10% superovulated oestrus cow serum (SOCS), 10 µg mL\(^{-1}\) FSH (Follotropin, Vetrepham Inc.) and antibiotics (Sigma, ref. P0781) during 22-24 hours. For fertilization, frozen-thawed semen was submitted to swim-up as above. Spermatozoa (10\(^6\) spz mL\(^{-1}\)) and 10 oocytes were placed in 40 µL droplets of TALP medium supplemented with 5.4 USP mL\(^{-1}\) heparin (Sigma, ref. H-3393), 10 mM penicillamine (Sigma, ref. P-4875), 20 mM hypotaurine (Sigma, ref. H-1384) and 0.25 mM epinephrine (Sigma, ref. E-1635). Following co-incubation for 22 hours, the presumptive zygotes were placed in 100 µL droplets of a granulosa cell monolayer cultured with TCM199, 10% SOCS and antibiotics under paraffin oil. Atmospherical conditions for IVM-IVF and embryo co-culture were 39°C and 5% CO\(_2\) in air with humidified atmosphere. Cleavage was assessed 48 hours post insemination and embryos were morphologically evaluated on day 7 and 8.

**Statistical analysis**

All data are presented as means ± standard deviations (SD). The mean values were compared by using the ANOVA and LSD and \(P<0.05\) was considered significant.
Results

Besnoitia besnoiti diagnosis

Three bulls, 2 Limousine and 1 Charolais, presented *B. besnoiti* cysts with live bradyzoites in the skin (Figure 1).

Semen evaluation

Before cryopreservation, there were no differences (*P* > 0.05) in morphological evaluation (Table 1) of semen from asymptomatic infected with *Besnoitia besnoiti* and uninfected bulls.

![Figure 1. Hematoxilin-eosin stain of skin presenting Besnoitia cysts.](image)

After cryopreservation, there were no significant differences between the 2 uninfected bulls running simultaneously in both experiments. Consequently, post-thawing results from experiment 1 and 2 were treated together (Table 2). From all the sperm quality parameters evaluated only post-thawed (*P* = 0.05) and post-swim-up (*P* = 0.009) motility were significantly different between asymptomatic infected and uninfected bulls, respectively. Cleavage and day 7-8 embryo production rates obtained with semen from uninfected and asymptomatic infected bulls presented no differences (*P* > 0.05).

Discussion

*Besnoitia besnoiti* infections cause economic losses to cattle owners in endemic areas due to mortality, loss of condition, low market value and temporary or permanent sterility (Bigalke, 1968, Kumi-Diaka *et al.*, 1981). Prevalence of bovine besnoitiosis in Portugal, especially the asymptomatic form, is not yet well determined. In the present study live bradyzoites within *Besnoitia* cyst were identified although all the bulls tested were asymptomatic. Previous studies (Cortes *et al.*, 2001, 2005) in farms located in Évora region diagnosed 42% of *B. besnoiti* infected Limousine bulls without clinical signs of the disease.

Asymptomatic *B. besnoiti* infected bulls presence in herds implies a potential transmission of besnoitiosis although as demonstrated sire fertility is not affected. Infertility or sterility seems to be a late consequence of *B. besnoiti* infection (Sekoni *et al.*, 1992). Semen quality pre and post cryopreservation was not diminished in infected asymptomatic bulls. Neither single parameters of semen quality (motility, sperm number and morphology) having moderate value as predictors of absolute fertility levels (Parkinson, 2004), nor supravital stain and Host tests which can be considered important variables for normal sperm function (Tartaglione & Ritta, 2004), were affected. IVF and
Table 1. Evaluation (mean±SD) of semen collected by electroejaculation to asymptomatic infected with Besnoitia besnoiti (INF, n=3) and uninfected bulls (control, n=5) before cryopreservation.

<table>
<thead>
<tr>
<th>Bulls</th>
<th>Volume (mL)</th>
<th>Gross motility</th>
<th>Individual motility (%)</th>
<th>Concentration (10⁵ spz mL⁻¹)</th>
<th>Dead spz (%)</th>
<th>Morphological abnormalities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.2±2.4</td>
<td>3.0±0.7</td>
<td>82.0±11.0</td>
<td>10.6±3.7</td>
<td>23.6±13.7</td>
<td>23.4±3.2</td>
</tr>
<tr>
<td>INF</td>
<td>6.7±2.8</td>
<td>3.3±0.6</td>
<td>76.7±11.7</td>
<td>8.4±3.6</td>
<td>16.0±1.7</td>
<td>25.0±1.0</td>
</tr>
</tbody>
</table>

Table 2. Evaluation (mean±SD) of post-thawed semen collected to asymptomatic infected with Besnoitia besnoiti (INF) and uninfected (control) bulls.

<table>
<thead>
<tr>
<th>Bulls</th>
<th>Post-thawed</th>
<th>Post-swim-up tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Motility (%)</td>
<td>Host (%)</td>
</tr>
<tr>
<td>control</td>
<td>45</td>
<td>43.3±10.6ᵃ</td>
</tr>
<tr>
<td>INF</td>
<td>15</td>
<td>51.0±18.6ᵇ</td>
</tr>
</tbody>
</table>

Within columns, means with different letters are significantly different (P<0.05, ANOVA).
embryo production rates also presented no differences between asymptomatic infected bulls and control. IVF and embryo production rates are useful indicators of bull fertility. Relevant reports correlated those results with the non-return rates using cryopreserved semen (Marquant-LeGuienne et al., 1990) and the conception rates in natural service (Brahmkshtri et al., 1999).

These results suggest that in sub-clinically infected bulls there are no implications on the fertility. However, in advanced stages of infection infertility is a well characterised consequence and, therefore, in endemic farms, the simple elimination of infertile bulls is not recommended since when this manifestation is established, the disease is already in an advanced stage and dissemination to other animals may already have occurred. Under these circumstances early detection of infection and adequate control measures have to be implemented in order to attenuate the economical impact of besnoitiosis. In addition, we strongly disagree with the transport of infected animals, even if subclinically, to other areas or herds without any contact with this parasite. In fact, due to the mechanical transmission of B. besnoiti by blood-sucking insects or iatrogenically from infected to susceptible cattle (Bigalke, 1968), and the high number of tabanids and Stomoxys calcitrans during summer season, moving infected animals to Besnoitia free herds will contribute to the spread of the disease.

Acknowledgements

We wish to thank Andrew Hemphill for fruitful discussions and for revising the manuscript.

References


