Contents

The present work studied different spermatozoa parameters and the ability of frozen rabbit spermatozoa to fertilize, in vitro, in vivo-matured oocytes, as a test to predict their in vivo fertility and prolificacy. Semen from rabbit bucks was frozen using two freezing protocols [in a freezer at \(-30^\circ\text{C}\) or in liquid nitrogen vapour (LNV)]. For the in vivo trial, females were inseminated with frozen-thawed spermatozoa. Oocytes used for in vitro testing were recovered 14 h after ovulation induction from donors and co-incubated with 2 x 10^6 frozen-thawed spermatozoa during 4 h at 37°C in Tyrode's medium under an atmosphere of 5% CO_2 in air with maximal humidity. After co-incubation period, presumptive zygotes were cultured in TCM199 supplemented with 20% foetal bovine serum (FBS), under the same conditions described above. Although no statistical differences were observed between freezing protocols in seminal parameters [motility rate: 40 and 35%, VCL: 35 and 46 μm/s, amplitude of lateral head displacement (ALH): 1.7 and 2.4 μm, for semen frozen at \(-30^\circ\text{C}\) and in LNV, respectively], significant differences were noted in the fertilizing ability in vivo and in vitro. Semen frozen at \(-30^\circ\text{C}\) showed the highest fertilizing ability in vitro (26.7% vs 6.2 and 8.7% for semen frozen at \(-30^\circ\text{C}\), in LNV and fresh semen, respectively) and the lowest fertility rate in vivo (21.7% vs 64.2% and 70.6% for semen frozen at \(-30^\circ\text{C}\), in LNV and fresh semen, respectively). Sperm frozen at \(-30^\circ\text{C}\) seemed to be more capacitated.

Introduction

After freezing, it is important to preserve a large number of spermatozoa with appropriate characteristics in order to obtain good fertility rate. For polytocos species, not only good fertility rates have to be achieved, but also good prolificacy, in order to maintain optimal productive levels. Over the years, several authors have reviewed the deleterious effects that freezing and thawing provoke in spermatozoa from different species (Berger et al. 1994, 1996; Bedford 1998; Bailey et al. 2000), leading to impaired sperm transport in the female reproductive tract and a reduction in the lifespan of the spermatozoa, which causes a reduction in the fertility and/or prolificacy of frozen semen.

Some authors have observed, in ram sperm that freezing causes similar changes in membrane spermatozoa to those produced during the capacitation process (Watson 2000), which could explain in part the worse results obtained after insemination with frozen sperm (Parks and Graham 1992; Watson 2000).

Although several diluents and protocols have been developed to freeze rabbit sperm, obtaining good results for fertility rate with some of them (between 70 and 80%; O'Shea and Wales 1969; Weitz et al. 1976; Viudes-de-Castro and Vicente 1996), only Viudes-de-Castro and Vicente (1996) found similar results for fresh and frozen semen in prolificacy (8.1 vs 8.0 total born, respectively). However, when frozen rabbit sperm were used to perform inseminations on commercial farms, results in both fertility and prolificacy were lower than those obtained with fresh semen (Theau-Clement and Roustan 1982; Mocé et al. 2003).

In order to predict fertility after insemination, different tests have been developed to determine sperm quality in vitro. Computer-assisted sperm analysis (CASA) provides a repeatable estimate of many sperm movement criteria, and this objective semen evaluation could be used to evaluate differences between bucks and try to predict the in vivo performance of semen. Several authors observed that some CASA motility parameters, or a combination of criteria (motility parameters, morphological parameters and biological test) were often highly correlated to fertility (Wood et al. 1986; Farrell et al. 1998). There are biological probes to evaluate the quality of semen in vitro, such as cervical mucus penetration, sperm chromatin decondensation, egg yolk membrane-binding assays, oocyte-binding assays or in vitro fertilization (IVF). Several authors have found significant relationships between fertility and the results obtained with some of these tests, but the reports have usually been contradictory (Graham et al. 1980). Furthermore, it seems that biological probes such as oocyte-binding assays or IVF evaluate fertility in vitro better than the other assays, as in these tests several steps that occur during the fertilization process are analysed (sperm capacitation, binding to the zona pellucida and penetration of the zona pellucida, membrane fusion, sperm head decondensation, male pronucleus formation and even embryo development). The first tests were developed with zona-free hamster oocytes for semen of different species: guinea-pig (Yanagimachi 1972), human (Yanagimachi et al. 1976), bovine (Eaglesome and Miller 1988), goat (Berger et al. 1994) and pig (Berger et al. 1996). Although a few studies in breeding species have reported results predicting semen fertility, zona-free hamster oocytes do not seem to be a good model, as they do not allow evaluation of the initial steps of the fertilization process. Since 1990, several studies have been carried out using homologous oocytes to assess the fertilizing ability of semen of breeding species. The advances in recovery and maturation of oocytes have enabled some works to use both immature and mature oocytes, obtaining good results (Ivanova and Mollova 1993; Martinez et al. 1993, 1996; Codde and Berger 1995; Graule et al. 1995).

The aim of the present work was to study the effect of cryopreservation protocol on the ability of frozen rabbit sperm to fertilize in vitro, in vivo-matured oocytes as a test to predict the in vivo fertility and prolificacy.
Material and Methods

Animals

The work was carried out at the experimental farm of the Department of Animal Science, Valencia Polytechnic University (Spain). Animals were housed in individual cages and fed (with a commercial diet) and watered ad libitum. The photoperiod employed was 16 h light/day.

The animals used in the experiment belonged to three different strains of rabbits, lines A and V selected for maternal characteristics, and line R, selected for growth rate from weaning to slaughter. Females belonged to A × V crossbreed and males belonged to R line. Selection methodologies were described by Estany et al. (1989, 1992).

Ejaculates were collected early in the morning. Bucks were accustomed to work with artificial vagina.

Semen evaluation

Semen from five to seven adult males was collected over a 7-week period. Two ejaculates per male and per week were collected using an artificial vagina and the samples were maintained a room temperature and analysed within 1 h. Only those ejaculates that presented white colour and 70% of visual motility were pooled and used in freezing. In order to minimize pool variation, each pool was split into three subsamples and processed according to the different protocols (fresh, pool was split into three subsamples and processed in freezing. In order to minimize pool variation, each pool was split into three subsamples and processed according to the different protocols (fresh, −30 and −120°C). The following measurements were taken from fresh sperm.

1 Volume: Measured in a graduated conical tube.
2 To measure motility, a fresh sample of the pool was diluted (dilution 1 : 50) in an extender composed of Tris-citric acid-glucose (pH 6.8, 300 mOsm/kg; Viudes-de-Castro et al. 1999). Motility was measured at 37°C under a microscope with phase contrast optics; two drops of 10 µl from each sample were laid on a slide and covered with a coverslip (20 × 20 mm). Images from four microscopic fields (including a minimum of 200 spermatoozoa) were taken from each pool and later analysed by CASA (VIMAS, Microptic®, Barcelona, Spain). Images were analysed by the automatic option (filter Auto-M); after that, each case was reviewed and, if there were any errors (confused trajectories, etc.), re-analysed manually until the analysis was correct. The following parameters were taken: track speed (VCL, µm/s), path velocity (VAP, µm/s), linearity [LIN = (VSL/VCL) × 100], amplitude of lateral head displacement (ALH, µm) and beat cross-frequency (BCF, Hz).
3 Concentration: One sample was fixed (dilution 1 : 50) in a solution of glutaraldehyde (2%), and concentration was measured using a Thoma-Weiss cell counting chamber (Paul Marifenfeld GmbH & Co. KG, Lauda-Koenigshofen, Germany).
4 Morphological examination: Percentage spermatoozoa with normal apical ridge (NAR), cytoplasmatic droplets and abnormal spermatoozoa were measured in a sample fixed with 2% glutaraldehyde (Pursel and Johnson 1974), at a magnification of 700× using interference contrast optics (Nomarski contrast).

Freezing protocol

All the chemicals to prepare the extenders were purchased from Sigma-Aldrich Quimica S.A. (Alcobendas, Madrid, Spain). The extender to dilute the fresh sperm (extender 1) had the following composition: 0.25 m of Tris[hydroxymethyl]aminomethane, 88 mm of citric acid anhydrous and 47 mm of D(+)-glucose (Viudes-de-Castro and Vincente, 1996).

The freezing extender (extender 2) was composed of extender 1, and 3.5 m of dimethyl sulphoxide (DMSO) and 0.1 m of sucrose were added as cryoprotectants (Vicente and Viudes-de-Castro 1996).

Two freezing rates were tested. Sperm was diluted (1 : 1) with extender 2 at room temperature, and packaged in 0.5 ml plastic straws (IMV®). L’Aigle cedex, France). Straws were sealed with modelling paste. The sperm was cooled at 4°C for 45 min and two different freezing rates were used to freeze it: some of the straws were put in a freeze at −30°C for 30 min, whereas the other group of straws were suspended horizontally in liquid nitrogen vapour (LNV) 5 cm above the liquid nitrogen level for 10 min (−120°C) before being plunged into the liquid nitrogen for storage (LN2, −196°C). Straws were thawed in a water bath at 50°C for 12 s.

In vitro fertilization

Oocyte donors belonging to line A were treated 48 h before ovulation induction with a single injection of 50 IU eCG (Gonaser, Hipra Laboratories S.A., Gerona, Spain). To induce ovulation, 1 µg of busereline acetate (Hoechst Marion Roussel, S.A., Madrid, Spain) was injected intramuscularly.

Oocytes used for IVF were recovered postmortem 14 h after ovulation was induced by perfusion of oviducts with Dulbecco’s phosphate-buffered saline (DPBS) medium. Donor does were slaughtered in accordance with the European slaughterhouse rules. After that, in vivo matured oocytes were pooled and washed three times in DPBS and placed in 4-well dishes containing 200 µl Tyrode medium adding albumin (6 g/l), sodium lactate (0.3%, v/v), sodium pyruvate (22 µg/l), heparin (10 µg/l), hypotaurine (11 µg/l) and adrenaline (1.8 µg/l).

Oocytes were co-incubated with frozen-thawed spermatoozoa (at a final concentration of 2 × 10⁶ spermatoozoa/ml, among 10–15 µl) during 4 h at 37°C in an atmosphere of 5% CO₂ in air with maximal humidity. After the co-incubation period, presumptive zygotes were washed twice in TC-M199 and cultured for 48 h in 500 µl of TC199 supplemented with 20% of foetal bovine serum (FBS); the incubation conditions used were the same as had been used for the IVF. Sperm motility rate at the end of the period of co-incubation with the oocytes was scored and the number of zygotes with two to four cells at the end of the culture period (36 h) was noted.
In the *in vitro* assay two control groups were used: one group of oocytes was manipulated in the same way as the group co-incubated with frozen sperm, but no spermatozoa were added to them (parthenogenetic control), and the other group was incubated with fresh rabbit sperm not previously capacitated.

Fresh and frozen semen in this experiment were not chemically capacitated before co-incubation to evaluate whether the cryopreservation method in rabbit induced the capacitation process.

**Artificial insemination**

A total of 318 inseminations were performed on A × V crossbreed multiparous lactating does. Only receptive females (red colour of vulvar lips) were inseminated. Insemination was carried out with a curved glass pipette (0.5 cm diameter). To induce ovulation, females were treated with 1 µg of busereline acetate intramuscularly at the same time as insemination was performed.

All females were inseminated with 30 million total spermatozoa, varying the inseminated volume depending on the sperm concentration.

Gestation was checked by abdominal palpation 12 days after insemination was performed, and the gestation status was noted (pregnant or non-pregnant) for each female. The fertility rate was defined as the percentage of pregnant does/inseminated does. At parturition, the following parameters were noted for each female: number of live kits born, number of stillborn kits and total number born were calculated (number of alive + number of dead).

**Statistical analysis**

Data were statistically evaluated with several statistical analysis. The following parameters were noted for each female. The fertility rate was defined as the percentage of pregnant does/inseminated does. At parturition, the following parameters were noted for each female: number of live kits born, number of stillborn kits and total number born were calculated (number of alive + number of dead).

**Results**

Results for the sperm motility parameters and NAR are summarized in Table 1. The percentage of NAR decreased significantly (*p < 0.05*) for frozen semen (45 and 33% vs 87% for semen frozen at −30°C, LNV and fresh semen, respectively). With respect to the motility parameters, the VCL, VAP and BCF parameters were significantly higher (*p < 0.05*) for fresh semen (VCL: 78 vs 35 and 46 µm/s, VAP: 49 vs 23 and 27 µm/s, BCF: 12.3 vs 7.0 and 7.2 Hz; for fresh, frozen at −30°C and LNV, respectively). A significant decrease (*p < 0.05*) occurred in ALH in the semen samples frozen at −30°C compared with fresh semen (1.7 vs 3.4 µm) but no difference was noticed for samples frozen in LNV. On the contrary, no differences were observed in motility rate (35–55%) and LIN (55–65%) between semen frozen at −30°C, LNV or fresh semen.

Results observed after the *in vitro* fertilization assay are shown in Table 2. Significant differences between fresh and frozen semen were obtained for motility rate after the co-incubation period (37.5 vs 1.3 and 2.8% for fresh semen, semen frozen at −30°C and in LNV, respectively). No significant difference in number of two to four cell embryos was found between fresh semen and semen frozen in LNV, but the number of embryos was significantly higher (*p < 0.05*) when semen had been frozen at −30°C. Results obtained after artificial inseminations are summarized in Table 2; significant differences (*p < 0.05*) in fertility rate were found between fresh and frozen semen, and between freezing protocols (70.6 vs 21.7 vs 64.2%, for fresh semen, semen frozen at −30°C and in LNV, respectively). Semen frozen at −30°C showed 43% points less of fertility rate than semen frozen in LNV. With respect to the prolificacy, significant differences (*p < 0.05*) were observed between semen treatments, obtaining the lowest prolificacy for semen frozen at −30°C (3.4 vs 5.3 vs 8.8 total born, for semen frozen at −30°C, in LNV and fresh semen, respectively).

**Discussion**

Several causes have been suggested for the reduced fertility of frozen sperm, for example, impaired sperm transport and poor survival in the female reproductive tract (Yoshida 2000). This is due to sublethal cryodamage produced by the freeze-thaw procedure, which leads to a premature capacitation of sperm, reducing its functional lifespan (Bailey et al. 2000). This study demonstrated that the cryopreservation procedures used increased the possibility of *in vitro* fertilization without capacitation treatment, when compared with the *in vitro* performance of fresh rabbit spermatozoa.

### Table 1. Seminal parameters for fresh and frozen semen

<table>
<thead>
<tr>
<th></th>
<th>Fresh</th>
<th>Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAR (%)</td>
<td>87</td>
<td>45</td>
</tr>
<tr>
<td>Motility rate (% l.s.m.)</td>
<td>55</td>
<td>46</td>
</tr>
<tr>
<td>LIN (% l.s.m.)</td>
<td>55</td>
<td>65</td>
</tr>
<tr>
<td>VCL (µm/s) l.s.m.</td>
<td>78</td>
<td>35</td>
</tr>
<tr>
<td>VAP (µm/s) l.s.m.</td>
<td>49</td>
<td>23</td>
</tr>
<tr>
<td>ALH (µm) l.s.m.</td>
<td>3.4</td>
<td>1.7</td>
</tr>
<tr>
<td>BCF (Hz) l.s.m.</td>
<td>12.3</td>
<td>7.0</td>
</tr>
</tbody>
</table>

LNV, semen frozen in liquid nitrogen vapour; n, number of pool semen; l.s.m., least-square mean; NAR, normal apical ridge; LIN, linearity ([VSL/VCL] × 100); VCL, track speed; VAP, path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.

*a,bValues in the same column with different superscripts are statistically different (*p < 0.05*).
In conclusion, none of the parameters measured in frozen rabbit sperm between freezing protocols allowed discrimination between fertility levels. On the contrary, the in vitro fertilization test showed the differences in sperm fertilization ability between the freezing protocols studied in this work, −30°C freezing protocol seems to induce the capacitating process.

Acknowledgement

This study was supported by the CYCIT Project AGF98-0470C02-01.

References


Courtens JL, Bolet G, Theau-Clément M, 1994: Effect of acrosome defects and sperm chromatin decondensation on work, we demonstrated the possibility to freezing rabbit semen at −30°C, with fertility and prolificacy rates similar to fresh semen in a selected maternal line (Viudes-de-Castro and Vicente 1996). Furthermore, when this freezing protocol was used in other selected rabbit lines both, fertility and prolificacy were lower than frozen semen in LNV (Mocé et al. 2003) and fresh semen (Vicente et al. 2000). Fertility and prolificacy rates from the growth selected line used in this study were particularly affected by the freezing protocol (16% vs 47% and 4.5 vs 7.3 total rabbit born; Mocé et al. 2003), in spite of motility and acrosome integrity rates were favourable to −30°C freezing protocol. The differences in the fertilizing ability of fresh and frozen sperm between males or lines could be due to seminal plasma composition (Killian et al. 1993) or differential structural changes induced to cryopreservation process (sperm membranes or chromatin decondensation, Courtens et al. 1994).

In general, the cryopreservation procedure affected the state of the acrosome and different motility parameters, reducing curvilinear and path velocity, ALH and BCF. However, motile sperm trajectory was not affected (LINV). In addition to the lower parameters observed for frozen sperm immediately after thawing when compared with those observed for fresh semen, we observed a high reduction in motility rate of frozen semen after the co-incubation period (4 h), and only some frozen sperm in the cumulus–oocyte complex conserved movement. This reduced longevity and motility of frozen sperm has been related with the generation of reactive oxygen species (ROS) during cryopreservation process in ram and in bull (Fraser et al. 1995; Upreti et al. 1998).

Although acrosome reaction is a prerequisite to penetration of the zona pellucida by sperm in mammalian species, spermatozoa with intact acrosomes adhering to the zona pellucida have been seen to indent it deeply on occasion in human and in rabbit (Bedford 1998; Mocé and Vicente 2002). Mocé and Vicente (2002) demonstrated, at least, that immature or in vitro matured rabbit oocytes allowed the adherence of frozen semen which had the highest value of morphologically intact acrosomes (95%), but zona pellucida did not seem to have been penetrated, as no heads of spermatozoa were observed in the ooplasm. Working with boar sperm and ovulated or immature pig oocytes, Martinez et al. (1993) observed that immature oocytes presented decondensed sperm in the ooplasm (so immature pig oocytes allow spermatozoa penetration), but there was no male pronucleus formation (as the oocyte ooplasm was immature).

Results show that freezing in LNV improved both fertility rate and total born when compared with the freezing at −30°C (Table 2), but the results with frozen spermatozoa did not reach the mean values obtained with fresh semen. It is well-known that spermatozoa are subjected to different stresses while being frozen, and that freezing has to be slow enough to prevent intracellular ice formation (which is lethal) but fast enough to permit extracellular water freezing to minimize the harmful effects of prolonged exposure to high salt concentrations. We observed in this assay that motility and NAR were not statistically different between the two freezing protocols (Table 1), although the sperm frozen at −30°C seemed to be more capacitated, obtaining an in vitro fertilization rate higher than that observed for control groups and sperm frozen in LNV.

Table 2. In vitro and in vivo fertilizing ability of fresh and frozen semen

<table>
<thead>
<tr>
<th></th>
<th>Number of oocytes</th>
<th>Motility rate after incubation (l.s.m. ± SE)</th>
<th>Number of two to four cell embryos (%)</th>
<th>Number of female</th>
<th>In vivo fertility rate (on day 2)</th>
<th>Total number born (l.s.m. ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parthenogenetic control</td>
<td>82</td>
<td>8 (9.8)b</td>
<td></td>
<td></td>
<td>70.6a</td>
<td>8.8 ± 0.26a</td>
</tr>
<tr>
<td>Fresh semen</td>
<td>89</td>
<td>37.5 ± 2.1a</td>
<td></td>
<td>72</td>
<td>70.6a</td>
<td>8.8 ± 0.26a</td>
</tr>
<tr>
<td>Frozen semen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−30 LNV</td>
<td>131</td>
<td>1.3 ± 2.1b</td>
<td>35 (26.7)a</td>
<td>88</td>
<td>21.7c</td>
<td>3.4 ± 1.99c</td>
</tr>
<tr>
<td>LNV</td>
<td>145</td>
<td>2.8 ± 2.1b</td>
<td>9 (6.2)b</td>
<td>158</td>
<td>64.2b</td>
<td>5.3 ± 0.34b</td>
</tr>
</tbody>
</table>

Number of oocytes, number of in vivo matured oocytes; number of female, number of females inseminated.

LNV, semen frozen in liquid nitrogen vapour; l.s.m., least-square mean.

Values in the same column with different superscripts are statistically different (p < 0.05).

In vitro Test for Fertilizing Ability Evaluation

Codde JM, Berger T, 1995: In vivo fertility of rams in selected maternal line (Viudes-de-Castro and Vicente 1996). Furthermore, when this freezing protocol was used in other selected rabbit lines both, fertility and prolificacy were lower than frozen semen in LNV (Mocé et al. 2003) and fresh semen (Vicente et al. 2000). Fertility and prolificacy rates from the growth selected line used in this study were particularly affected by the freezing protocol (16% vs 47% and 4.5 vs 7.3 total rabbit born; Mocé et al. 2003), in spite of motility and acrosome integrity rates were favourable to −30°C freezing protocol. The differences in the fertilizing ability of fresh and frozen sperm between males or lines could be due to seminal plasma composition (Killian et al. 1993) or differential structural changes induced to cryopreservation process (sperm membranes or chromatin decondensation, Courtens et al. 1994).

In general, the cryopreservation procedure affected the state of the acrosome and different motility parameters, reducing curvilinear and path velocity, ALH and BCF. However, motile sperm trajectory was not affected (LINV). In addition to the lower parameters observed for frozen sperm immediately after thawing when compared with those observed for fresh semen, we observed a high reduction in motility rate of frozen semen after the co-incubation period (4 h), and only some frozen sperm in the cumulus–oocyte complex conserved movement. This reduced longevity and motility of frozen sperm has been related with the generation of reactive oxygen species (ROS) during cryopreservation process in ram and in bull (Fraser et al. 1995; Upreti et al. 1998).

Although acrosome reaction is a prerequisite to penetration of the zona pellucida by sperm in mammalian species, spermatozoa with intact acrosomes adhering to the zona pellucida have been seen to indent it deeply on occasion in human and in rabbit (Bedford 1998; Mocé and Vicente 2002). Mocé and Vicente (2002) demonstrated, at least, that immature or in vitro matured rabbit oocytes allowed the adherence of fresh semen which had the highest value of morphologically intact acrosomes (95%), but zona pellucida did not seem to have been penetrated, as no heads of spermatozoa were observed in the ooplasm. Working with boar sperm and ovulated or immature pig oocytes, Martinez et al. (1993) observed that immature oocytes presented decondensed sperm in the ooplasm (so immature pig oocytes allow spermatozoa penetration), but there was no male pronucleus formation (as the oocyte ooplasm was immature).

Results show that freezing in LNV improved both fertility rate and total born when compared with the freezing at −30°C (Table 2), but the results with frozen spermatozoa did not reach the mean values obtained with fresh semen. It is well-known that spermatozoa are subjected to different stresses while being frozen, and that freezing has to be slow enough to prevent intracellular ice formation (which is lethal) but fast enough to permit extracellular water freezing to minimize the harmful effects of prolonged exposure to high salt concentrations. We observed in this assay that motility and NAR were not statistically different between the two freezing protocols (Table 1), although the sperm frozen at −30°C seemed to be more capacitated, obtaining an in vitro fertilization rate higher than that observed for control groups and sperm frozen in LNV.

In conclusion, none of the parameters measured in frozen rabbit sperm between freezing protocols allowed discrimination between fertility levels. On the contrary, the in vitro fertilization test showed the differences in sperm fertilization ability between the freezing protocols studied in this work, −30°C freezing protocol seems to induce the capacitating process.

Acknowledgement

This study was supported by the CYCIT Project AGF98-0470C02-01.

References


Martín et al., 1996: Oocyte penetration by fresh or stored diluted boar spermatozoa before and after in vitro capacitation treatments. Biol Reprod 55, 134–140.
Mocé E, Vicente JS, 2002: Effect of cooling and freezing, the two first steps of a freezing protocol, on the fertilizing ability of the rabbit sperm. Reprod Nutr Dev 42, 189–196.


Submitted: 11.10.2004

Author’s address (for correspondence): MP Viudes-de-Castro, Instituto Valenciano de Investigaciones Agrarias: Centro de Investigación y Tecnología Animal, Ctra Náquera-Moncada, Km 4.5, 46113 Moncada, Valencia, Spain. E-mail: mpviudes@ivia.es