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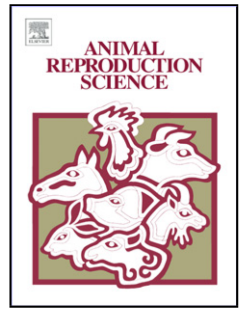

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First llama (*Lama glama*) pregnancy obtained after *in vitro* fertilization and *in vitro* culture of gametes from live animals.

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Abstract

The aim of this study was to evaluate the developmental competence and pregnancy rate of llama hatched blastocysts produced *in vitro* using gametes from live animals and two different culture conditions. Fifteen adult females were superstimulated with 1500 IU of eCG, eleven (73%) responded to the treatment and were used as oocyte donors. Follicular aspiration was conducted by flank laparotomy. Semen collections were performed under general anesthesia by electroejaculation of the male. Sixty-six COCs were recovered from 77 aspirated follicles (86% recovery) and were randomly placed in Fertil-TALP microdroplets with the sperm suspension (20 x 10^6 live spermatozoa/ml). After 24 h, they were placed in SOFaa medium supplemented with FCS and randomly assigned to one of two culture conditions. Culture condition 1 (CC1) consisted of 6 days of culture (n=28) and culture condition 2 (CC2) consisted of renewing the culture medium every 48 hours (n=35). In CC1, the blastocyst rate was 36% (10/28) and the hatched blastocyst rate was 28% (8/28) whereas in CC2, the blastocyst rate was 34% (12/35) and the hatched blastocyst rate was 20% (7/35) ($p > 0.05$). No pregnancies were obtained after embryo transfer (ET) of CC1 blastocysts (0/8) while one pregnancy was obtained (1/7) after transferring a hatched blastocyst from CC2. Forty two days after the ET, the pregnancy was lost.

This study represents the first report of a pregnancy in the llama after intrauterine transfer of embryos produced by *in vitro* fertilization using gametes from live animals.

Keywords: llama; pregnancy; IVF; IVC; SOF; embryo transfer

1. Introduction

Over the last few years, an increasing interest in the production of South American Camelids (SAC) has been developed, not only in South America but also in different countries around the world. Because these species present a long period of gestation (335 to 360 days; Johnson, 1989; Leon *et al.*, 1990) and only deliver one young per year, it is of interest to apply assisted reproductive techniques to optimize the reproductive handling of genetically superior females and to increase the genetic progress of these species. The final objective of *in vitro* embryo production is to develop high quality embryos and obtain normal pregnancies after transfer to recipient females, which finally result in the birth of healthy offsprings, a goal not yet attained in SAC.

There are few reports published on *in vitro* fertilization (IVF) in SAC. The first IVF in llamas was carried out by Del Campo *et al.* (1994). Out of the 234 zygotes cultured using epithelial oviduct cell co-culture, only 4.7% (11/234) developed to the hatched blastocyst stage and no embryo transfers were reported in this study. Gomez *et al.* (2002) reported the first production of llama-alpaca crossbreed embryos after heterologous IVF; after 6 days of culture all fertilized oocytes reached the morula stage (n=5), but none of them continued *in vitro* development. Both of these studies worked with gametes from slaughterhouse animals but it's important to apply this kind of technology in live animals. Besides, the development of a simple and viable culture system to reach embryo growth beyond the morula stage after IVF is vital for implementing an intrauterine embryo transfer (ET) program. We have recently reported the first production of *in vitro* llama embryos that developed to the hatched blastocyst stage. These were obtained after IVF using spermatozoa selected with Androcoll-E™ from raw semen, oocytes from superstimulated females and *in vitro* culture in synthetic oviduct fluid medium with amino acids (SOFaa) and with bovine serum albumin (BSA) during 6 days (Trasorras *et al.*, 2012). In dromedary, offspring were obtained from *in vitro* produced embryos achieved after adding fetal calf serum (FCS) to the embryo culture medium and reaching the hatched blastocyst stage (Khatir *et al.*, 2006).

When an embryo culture system is developed, the amount of time that embryos will be in contact with the medium is an important factor to take into account. The culture medium should not be considered a static system; the embryos themselves alter its composition, especially when amino acids are added. Studies carried out in humans (Virant-Klun *et al.*, 2006), mice (Gardner and Lane, 1993) and sheep (Gardner *et al.*, 1994) for *in vitro* culture embryo development to the blastocyst stage, have demonstrated that although the addition of amino acids to the culture medium had a significant effect on both embryo cleavage rate and morphological development, the beneficial effects on cleavage decreased in relation to the duration of culture. It was determined that amino acids are both metabolized by embryos and spontaneously broken down at 37° C, thus producing significant levels of ammonium in the medium. The ammonium generated from the amino acids was found to not only inhibit cleavage and blastocyst development (human: Virant-Klun *et al.*, 2006; mouse: Gardner and Lane, 1993) but also to be associated with subsequent fetal retardation and neural tube defects in mice (Lane and Gardner, 1994). In sheep, increased embryo cleavage and development rates in culture, in the presence of amino acids, were obtained by placing embryos in fresh medium every 48 hours to alleviate the toxic effects of ammonium (Gardner *et al.*, 1994).

The aim of this study was to evaluate the developmental competence and pregnancy rate of llama hatched blastocysts produced *in vitro* using gametes from live animals and two different culture conditions.

2. Materials and methods

2.1. Animals

Thirty non-pregnant, non-lactating female llamas, ranging between 4 and 8 years of age and with an average body weight of 120 ± 22 kg were used in this study. Of the 30 females, 15 were used as oocyte donors and 15 as ET recipients. All females were kept separate from the males during the experiment and fed with hay and water *ad libitum*. The study was conducted at the Faculty of Veterinary Sciences of the University of Buenos Aires, Buenos Aires, Argentina, situated 34º 36' S and 58º 26' W, at sea level. This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol Nº 2010/24).

All reagents were purchased from Sigma (St. Louis, MO, USA) except where stated otherwise.

2.2. In vivo oocyte recovery

2.2.1. Management of the oocyte donor females

Ovarian dynamics were monitored by transrectal palpation and ultrasonography (Berger LC 2010 plus with a 5 MHz linear-array electronic transducer, Buenos Aires, Argentina). The absence of follicles larger than 5 mm was confirmed before beginning the superstimulation treatment and a single IM dose (1500 IU) of eCG (Novormon®, Syntex, Argentina) was administered (n=15) (Trasorras *et al.*, 2009). Positive response to eCG treatment was considered when a female presented, in each ovary, two or more follicles ≥ 7 mm (dominant follicle) at ultrasound evaluation.

2.2.2. LH surge induction

Five days after the superstimulatory treatment, females with a positive response to eCG were selected for follicle aspiration and received a single IV dose of 8 µg of buserelin (Receptal®, Intervet, Buenos Aires, Argentina) for *in vivo* oocyte maturation within the follicles. Twenty hours later, females were subjected to surgical procedures and follicular aspiration.

2.2.3. Surgical procedures and oocyte evaluation

Females selected for surgery were deprived of solid food 24 hours and water 18 hours previously. The technique was performed as previously described (Trasorras *et al.*, 2009). Briefly, general anesthesia was induced by IV administration of 0.2 mg/kg of xylazine (Rompun®, Bayer, Buenos Aires, Argentina), 1.5 mg/kg of ketamine hydrochloride (Ketamina®, Holliday, Buenos Aires, Argentina) and 0.1 mg/kg of butorphanol (Torbutrol plus®, Fort Dodge, La Plata, Argentina). Local anesthesia of the surgical area was carried out using 2% lidocaine (Equi Systems®, Buenos Aires, Argentina); general anesthesia was maintained by intravenously injecting half the induction dose of ketamine and xylazine, as needed. The superstimulated ovaries were exposed, with transrectal manual aid, through an 8 to 10 cm long surgical incision in the left flank. The ovarian bursa was moved to one side and the follicles were aspirated with a 21 G hypodermic needle attached to a 5 ml plastic syringe containing PBS as the aspiration media supplemented with 0.1% heparin (v/v) and 50 µg/ml of gentamycin.

All follicles with a diameter equal to or greater than 7 mm were aspirated. Cumulus oocyte complexes (COCs) were identified using a stereomicroscope and classified according to their maturation stage into: compact (four or more layers of granulosa cells adhered tightly to the oocyte), expanded (lax cumulus) or denuded (without granulosa cells).

2.3. Semen samples

To reduce variability, semen from a single *Lama glama* male (of proven fertility) was used for each IVF protocol. Semen collections were performed under general anesthesia with

0.2 mg/kg xylazine IV and 1.5 mg/kg ketamine hydrochloride IV using a P-T Electronics 304 electroejaculator (Oregon, USA) with a #4 probe and three ventral electrodes. Electrical stimulation was performed as previously described (Director *et al.*, 2007). Separation of spermatozoa from the seminal plasma was carried out according to Giuliano *et al.* (2010). Briefly, each ejaculate was diluted 4:1 in a solution of 1 mg/ml collagenase in TALP medium (Parrish *et al.*, 1986) supplemented with 15 mM Hepes (H-TALP) and 3 mg/ml BSA and incubated at 37° C for 4 min. Then it was centrifuged for 8 min at 800 g and the pellet was re-diluted in 2 ml H-TALP-BSA, layered over a 2 ml column of Androcoll-E™ and centrifuged at 600 g for 20 min in order to select the best spermatozoa. The new pellet was re-diluted in TALP medium supplemented with 6 mg/ml of BSA (Fertil-TALP) and was again centrifuged at 600 g for 10 min. After this, the pellet was re-diluted in Fertil-TALP and maintained at 38.5º C with 5% CO_2 in a humidified atmosphere until IVF. Sperm characteristics studied were: sperm motility, membrane function (Hypoosmotic Swelling test, HOS) and viability (6-Carboxifluorescein Diacetate: CFDA and Propidium Iodide: PI) (Giuliano *et al.*, 2008). In all cases 200 sperm per sample were evaluated.

2.4. In vitro fertilization

The COCs were placed in microdroplets of 40 µl of Fertil-TALP, in groups of 2-5 oocytes per droplet. The sperm suspension (10 µl at 20 x 10^6 live spermatozoa/ml) was added to each fertilization microdroplet to obtain a final concentration of 4 x 10^6 live spermatozoa/ml. Incubation was carried out for 24 hours under 5% CO_2 in air with high humidity (> 95%) at 38.5º C.

2.5. In vitro embryo culture

Following IVF, presumptive zygotes were gently pipetted to remove spermatozoa or cumulus cells still attached to them. All zygotes were then washed four times in synthetic oviductal fluid (Tervit *et al.*, 1972) supplemented with 1 µM glutamine, 2% (v/v) essential amino acids, 1% non-essential amino acids (Gardner *et al.*, 1994) and heat-treated FCS (10%) (SOFaa) before being transferred into the embryo culture microdroplets (2–6 zygotes per 20-40 µl droplet). Two culture conditions were evaluated: culture condition 1 (CC1), 6 days of culture without renewing the medium (n=28); culture condition 2 (CC2), 50% medium renovation every 48 hours (n=35). All droplets were kept under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5˚ C. Embryos were evaluated and classified according to Tibary and Anouassi (1997). The evaluation of embryos in CC1 was carried out on day 6 of culture and embryos in CC2 were evaluated on days 2, 4 and 6.

2.6. Transfer of in vitro produced embryos

2.6.1. Management of the recipient female

One day after the donor's surgery, recipients with a dominant follicle received a single IV dose of 8 µg of buserelin to induce ovulation and two days later ovulation was confirmed by transrectal ultrasonography. Transcervical ET was carried out on day 6 after buserelin administration (Trasorras *et al.*, 2010).

2.6.2. Transcervical ET technique

The maneuvers were carried out with the female either standing or in sternal recumbency. The animal was restrained in stocks, the tail was wrapped and the rectum was emptied of feces. The perineum was then scrubbed using an iodine solution, rinsed carefully with clean water and then dried. A lubricated gloved hand was inserted in the rectum to hold the cervix while an assistant separated the vulva labia and an ET pipette, covered with a sterile sheath (IMV® ET Sheath, 21'', France) and carrying the 0.25 ml straw (IMV® ET Straws, France) containing the embryo, was inserted into the vagina. Cervical threading was performed aided by transrectal manipulation and the embryo was deposited in the uterine horn ipsilateral to the corpus luteum (CL) (Trasorras *et al.*, 2010).

2.6.3. Pregnancy diagnosis

Pregnancy diagnosis was carried out between days 13 and 30 after ET by transrectal ultrasonographical visualization of the embryo vesicle.

2.7. Statistical analysis

The percentage of embryos at the blastocyst and hatched blastocyst stages was compared between the two culture conditions (CC1 vs. CC2) using Fisher's exact test. Statistical analyses were performed using the R 2.2.1. program and $p < 0.05$ was considered significant.

3. Results

3.1. Superstimulation treatment

After administration of 1500 IU of eCG to the oocyte donor females, multiple follicle growth required 5 days to reach dominant size. Of the treated females, 73% (11/15) responded to the superstimulatory treatment with more than two follicles and their follicles were surgically aspirated.

3.2. COCs recovery

A total of 77 dominant follicles were aspirated in five different surgical procedures from a total of 11 donor females (2-3 females per surgery) and 66 COCs were recovered (average 6 COCs/female) showing an 86% recovery rate. Only expanded COCs were used for IVF (n=66).

3.3. Semen evaluation

Semen evaluation (n=1; r=5) after Androcoll-E^{TM} treatment and before IVF, showed the following results (mean ± SD): 40 ± 14.1% progressive motility; 47 ± 9.4% sperm with swelling and 62.9 ± 6.3% live sperm.

3.4. Embryo production

After 24 h of IVF, 3 oocytes were discarded due to morphological deterioration.

In CC1, 28 presumptive zygotes were cultured for 6 days. Blastocyst rate was 36% (10/28) and the hatched blastocyst rate was 28% (8/28) (Figure 1). In CC2, 35 presumptive zygotes were also cultured for 6 days, renewing the media every 48 h. Blastocyst rate was 34% (12/35) and the hatched blastocyst rate was 20% (7/35) (Figure 2). On day 2 of culture, the cleavage rate observed at media renewal was 31% (11/35). On day 4 of culture, at media renewal, 5 hatched blastocysts were observed (5/35; 14%).

The development rate to the blastocyst and to the hatched blastocyst stages for the two culture conditions is shown in Table 1. No significant differences were found between the culture conditions when the blastocyst or hatched blastocyst stages were compared on day 6 ($p > 0.05$).

3.5. Embryo transfer and pregnancy diagnosis

For CC1, of the 8 hatched blastocysts that were transferred on day 6 of culture to synchronized female recipients (n=8), no embryo vesicle was found.

For CC2, a total of 7 hatched blastocysts were transferred to synchronized female recipients (n=7), obtaining one pregnancy (1/7) from a hatched blastocyst which was transferred to the left uterine horn with an ipsilateral CL. This embryonic vesicle was observed by transrectal ultrasonography 23 days after the ET (Figure 3) and duplicate blood samples were taken from the recipient female to corroborate high plasma levels of progesterone, resulting in 4.58 ± 0.06 ng/ml (mean ± SD). The growth of the embryonic vesicle was followed by transrectal ultrasonography (Figure 4) but by day 37 post-ET a reduction in size was observed, finally disappearing on day 42 after the ET.

4. Discussion

This study represents the first report of a pregnancy in llama after the intrauterine transfer of embryos produced by *in vitro* fertilization using oocytes from superstimulated females and fresh semen processed with Androcoll-E^{TM}.

In vitro production of embryos demands a large quantity of oocytes capable of being fertilized. Using slaughterhouse ovaries has the advantage of providing a large quantity of oocytes, but the main disadvantage is that it requires them to be *in vitro* matured. From a review of the literature, it is evident that *in vitro* maturation procedures for llama oocytes are not optimal (Del Campo *et al.*, 1992, Ratto *et al.*, 2005). In addition, because oocytes are recovered from a slaughterhouse, it is not known if follicles of a dominant size are in the growing or the regressing phase, a fact that would affect the quality of the oocyte. Therefore, obtaining gametes from live animals ensures that the oocytes are recovered from follicles in the growing phase as well as offering the possibility of producing embryos from genetically superior animals.

There are only two studies on the use of a defined culture medium without co-culture with somatic cells for the *in vitro* production of llama embryos, obtaining 17% (16/94) expanded blastocysts (Conde *et al.*, 2008) and 9% (3/34) hatched blastocysts (Trasorras *et al.*, 2012). In dromedary, two systems for *in vitro* culture have been compared with regard to their ability to support the development of embryos to the blastocyst stage: semi-defined modified medium vs. co-culture with camel epithelial oviductal cells (Khatir *et al.*, 2005). They observed a slight, but not significant, superiority of the semi-defined medium over the somatic cells co-culture system in terms of blastocyst formation, hatchability and pregnancy rate. A defined medium, such as SOF, is easier to prepare than somatic cell monolayers and presents less risk of contamination. Using co-cultures with somatic cells, Berland *et al.* (2011)

reported a 21% embryo development to the blastocyst stage, which was higher than that previously reported by Del Campo *et al.* (1994) (11%). In Berland's work, all embryos that developed to blastocyst failed to hatch and totally collapsed on day 8 of culture. In contrast, Del Campo reported 4.7% zygotes reaching the hatched blastocyst stage, but no ET results were reported. In this study we obtained 28% and 20% hatched blastocysts using two types of culture conditions. The development of hatched blastocysts is very important to obtain a pregnancy after ET. In the dromedary, working with *in vivo* and *in vitro* produced embryos, it has been shown that transfer of non-hatched blastocysts into the uterus does not result in pregnancy (Tibary and Anouassi, 1997; Khatir *et al.*, 2004). We have observed that this also occurs in llamas (unpublished data).

In addition to obtaining pregnancies by ET using *in vivo* produced embryos (Trasorras *et al.*, 2010), we obtained the first pregnancy in llama by the intrauterine ET of hatched blastocysts produced *in vitro* after renewing the culture medium every 48 hours. In sheep, increased embryo cleavage and development rates in culture, in the presence of amino acids, were obtained by placing embryos in fresh medium every 48 hours to alleviate the toxic effects of ammonium (Gardner *et al.*, 1994). Nevertheless, in the present study, the hatching blastocyst rate obtained renewing culture medium every 48 hours for 6 days tended to be lower than using the same culture medium without renewal (58% vs. 80%). A rapid growth was observed 5 days post-IVF in dromedary *in vitro* embryo production using co-culture with somatic cells (Khatir *et al.*, 2004). In our study, a similar rapid growth of embryos, shown by the presence of hatched blastocysts on day 4 of culture, was observed in CC2. It would be interesting to evaluate whether this same rapid growth occurs when the culture media is not renewed. In bovines, blastocysts are usually observed on day 6-7 of *in vitro* culture (Bavister, 1995). Transcrips and proteins from the bovine oocyte govern initial embryonic development after fertilization until the fourth cell cycle. At this stage, the embryonic genome control of development becomes evident (De Sousa *et al.*, 1998). It is possible that in camelids, the embryonic genome controls the development of the embryo earlier than in the bovine species.
The embryos themselves alter the composition of the medium and the nature of such a change depends on several variables, including the mammal species and the stage of embryo development, therefore the culture conditions used cannot be considered static. Later-stage embryos, such as blastocysts, are more active than the zygote and cleavage stages and will consequently have a greater impact on medium composition (Gardner, 1998). Hence, an important characteristic of culture media is that they are temporally dynamic. If we consider

the *in vivo* embryo environment, the epithelial cells of the oviduct and uterus are constantly modifying the environment to which the embryo is exposed. The female reproductive tract is also able to remove potential embryo waste products, such as ammonium, through the maternal circulation and subsequent detoxification. In the present study, in spite of obtaining a lower hatched blastocyst rate after renewing the culture medium, it is probable that those embryos were of better quality, as one of them was able to induce maternal recognition of pregnancy and reach implantation after ET. It would be interesting to see if pregnancy rates are modified by increasing the number of *in vitro* produced embryos transferred.

5. Conclusions

Since the first *in vitro* fertilization carried out in llama in 1994 by Del Campo *et al.* using slaughterhouse gametes, this is the first report of a pregnancy obtained after the intrauterine transfer of embryos produced by *in vitro* fertilization using gametes from live animals.

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Table 1. Percentage of embryo development in SOFaa medium using two culture conditions.

	EMBRYO DEVELOPMENT (%)	
Culture condition	**Blastocysts/Zygotes**	**Hatched blastocysts/Blastocysts**
CC1	36% [a] (10/28)	80% [b] (8/10)
CC2	34% [a] (12/35)	58% [b] (7/12)

CC1: the same microdroplet of medium for 6 days.

CC2: renew the microdroplet of medium every 48 hours for 6 days.

Values with different letters within columns are different ($p < 0.05$).

Figure 1. Hatched llama blastocyst produced on the sixth day of culture in CC1. PZ: pellucid zone.

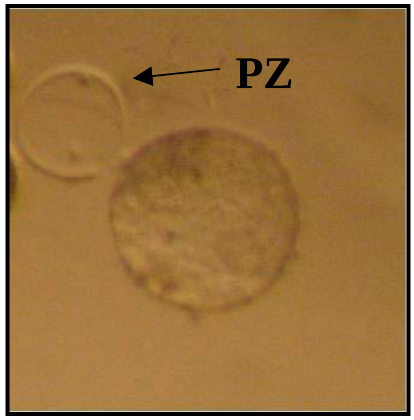

Figure 2. Hatched llama blastocyst produced on the sixth day of culture in CC2. PZ: pellucid zone.

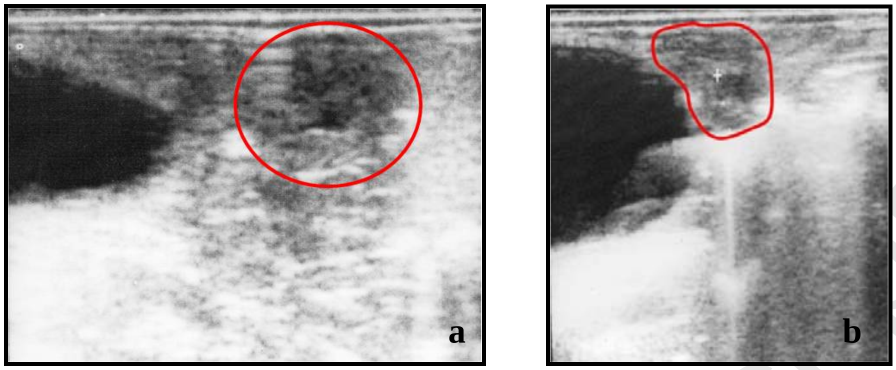

Figure 3. Ultrasound images of the recipient female's reproduction tract. (a) Embryonic vesicle 23 days after ET (red circle indicates the uterus). (b) Corpus luteum in left ovary; +: 1.23 cm diameter. Red line delimits the ovary with de CL.

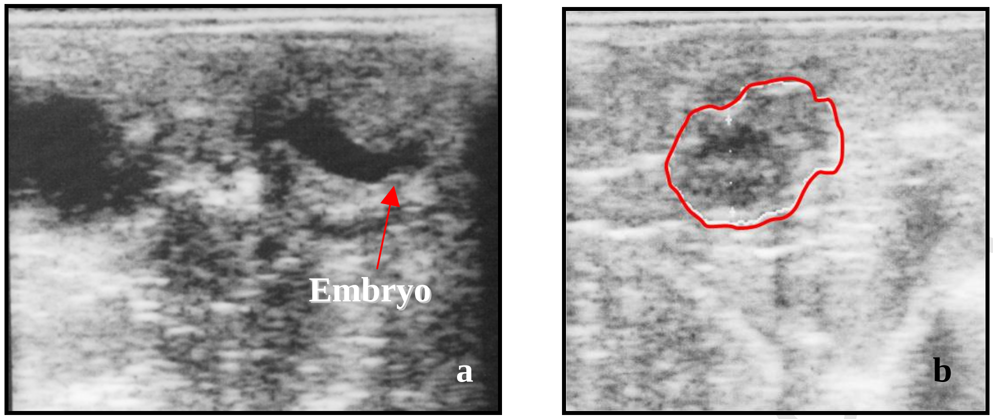

Figure 4. Ultrasound images of the recipient female's reproductive tract. (a) Embryonic vesicle 30 days after ET. Observe the increase in embryonic vesicle size between this figure and figure 3. (b) Corpus luteum in left ovary; +: 1.37 cm diameter, this structure also increased in size. Red line delimits the ovary with the CL.