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Embryonic resorption – a longitudinal ultrasonographic study in the model species European brown hare (*Lepus europaeus* PALLAS, 1778)

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To my mother Gisela, my father Martin, my brother Nikolas and my aunt Barbara
for their endless love and unmeasurable support

Der Tod ist kein Abschnitt des Daseins, sondern nur ein Zwischenereignis, ein Übergang aus einer Form des endlichen Wesens in eine andere.

(Wilhelm von Humboldt, Briefe an eine Freundin)

Contents

This thesis is based on the following manuscripts:

1. Schroeder, K., Drews, B., Roellig, K., Goeritz, F. and Hildebrandt, T.B. (2013) 'Embryonic resorption in context to intragestational corpus luteum regression: a longitudinal ultrasonographic study in the European brown hare (*Lepus europaeus* PALLAS 1778)', *Theriogenology*, vol. 80, pp. 778-784.
   - own contributions: ultrasonographic evaluation from day 5 to day 42 of pregnancy of 1) embryonic development, 2) embryonic resorptions and 3) CL-monitoring, 4) analysis and statistical calculations, 5) writing of the manuscript

2. Schroeder, K., Drews, B., Roellig, K., Menzies, B.R., Goeritz, F. and Hildebrandt, T.B. (2011) 'In vivo tissue sampling of embryonic resorption sites using ultrasound guided biopsy', *Theriogenology*, vol. 76, pp. 778-784.
   - own contributions: 1) implementation of the two biopsies techniques and *in vivo* extraction of resorption fluid and placental tissue, 2) ultrasonographic monitoring of embryonic resorptions and viable conceptus from day 5 to day 42, 3) histological analysis of resorption smears, 4) analysis and statistical calculations, 5) writing of the manuscript

3. Drews, B., Ringleb, J., Waurich, R., Hildebrandt, T.B., Schröder, K. and Roellig, K. (2013) 'Free blastocyst and implantation stages in the European brown hare: correlation between ultrasound and histological data', *Reproduction, Fertility and Development*, vol. 25, pp. 866-878.
   – own contributions: 1) ultrasonographic monitoring and 2) tissue collection

List of abbreviations:

AES	**agri-environment schemes**
AI	**artificial insemination**
BVDV	**bovine viral diarrhea virus**
CL	**corpus luteum, corpora lutea**
CS	**caesarean section**
CT	**computed tomography**
CVS	**chorionic villus sampling**
d	**day**
DNA	**desoxyribonucleic acid**
Ebh	**European brown hare**
EBHS	**European Brown Hare Syndrome**
eCG	**equine chorionic gonadotropin**
EED	**early embryonic death**
eFh	**Europäischer Feldhase**
em	**embryo**
EPL	**early pregnancy loss**
ET	**embryo transfer**
GK	**Gelbkörper**
h	**hour**
kg	**kilogram**
LAP	**laparatomy**
LH	**luteinizing hormone**
mg	**milligram**
Mhz	**Megahertz**
min	**minute**
ml	**millilitre**
MRI	**magnetic resonance imaging**
ng	**nanogram**
OCT	**optical coherence tomography**
OHE	**ovariohysterectomy**
pl	**placental**
PLR	**partial litter resorption**
p.m.	***post mortem***
RES	**resorption**
s.d.	**standard deviation**
SRY	**sex determining region of Y**

TLR	**total litter resorption**
UBM	**ultrasound biomicroscope**
US	**ultrasound**
µg	**microgram**
µl	**microlitre**
µm	**micrometre**
µM	**micromole**

I. Introduction

1. Embryonic resorption in mammals

1.1. Historical overview

The phenomenon of embryonic resorption in mammals has been known since the beginning of the 20th century although first records about fetal degeneration date back to ancient Greece where Aristotle [4TH C BC] already mentioned fetal retention and abortion in humans. He examined aborted fetuses in different stages of degeneration and described them as *Mola uteri* (*uterine eggs*) that were either of "fleshy" or of very hard consistence. Aristotle's book *De generatione animalium* was the first scientific work on embryology, had a profound influence on numerous naturalists, theologians and philosophers and became the basis for Arab medical research on female reproduction. Many centuries later, Jacob Rueff, a German physician, published the first version of his midwifery book [RUEFF, 1554] that became in later editions one of the early classics of obstetrical literature [RUEFF, 1600]. In his book Rueff discussed the symptoms of a healthy pregnancy and compared them to degenerated embryos and malformations occurring within the uterine lumen that were still known at this time as *Mola uteri*. A first systematic overview about the different types of *Mola uteri* was given by Mueller [1847]. Among the various regressive changes a human conceptus can undergo during embryonic and fetal development he provided the first description of a *Mola sanguinea (blood egg)* as a degenerative conceptus without embryo mainly filled with coagulated blood. The amount of blood coagulations and their location in the maternal part of the placenta (*decidua parietalis*) implied that the blood derived from maternal vessels. As the first microscopic differentiation between fetal (nucleated erythrocytes) and maternal erythrocytes (anucleated erythrocytes) had recently been presented [KÖLLIKER, 1846], Mueller showed that the blood mole mainly consisted of maternal erythrocytes. He described the mole as decreasing in size with advancing age whereby the content condensed and lost its reddish colour. It was then referred to as *Mola cruenta* (*flesh egg*). However, complete dissolution of the conceptus was not observed and in the latter part of the 19th century birth and abortion were still seen as the only outcome of pregnancy.

Embryonic resorption as a third stage of pregnancy outcome was first mentioned in 1896 by Sokoloff. In experimental studies on pregnant domestic dogs (*Canis familiaris*) he proved that the presence of the ovaries is fundamental for embryonic and fetal development. Extirpation of the ovaries caused a decrease of the abdominal girth in pregnant females. Subsequent laparotomy revealed an empty uterus or dead conceptus that showed different signs of degeneration. Since abortion or vaginal discharge was not observed the author was convinced that the conceptus underwent complete degeneration and referred to them as *atrophic eggs* [SOKOLOFF, 1896]. Strahl & Henneberg [1901] confirmed the occurrence of

embryonic resorption in several small mammalian species such as the domestic ferret (*Mustela putorius furo*), the European mole (*Talpa europaea*), the European rabbit (*Oryctolagus cuniculu*s) and the European hamster (*Cricetus cricetus*).

The findings by Sokoloff raised questions about the function of the corpus luteum (CL) as an "exocrine" gland to maintain pregnancy. Fraenkel [1903] showed that there was a direct relationship between the formation of corpora lutea (CL) and the establishment of a pregnancy in European domestic rabbits. As little was known about the embryonic development of mammals, the female European domestic rabbit became the model species with the best known reproductive physiology at this time. Cauterization of CL between the 1st and 6th day of pregnancy always prevented the implantation of the blastocyst whereas cauterization between the 8th and 20th day resulted in embryonic resorption. In none of these cases abortion was observed.

The evidence for embryonic resorption in different mammalian species became increasingly important as knowledge about pregnancy termination in women accumulated [FRAENKEL, 1902; FRAENKEL, 1907; POLANO, 1907]. In 1919, Meyer described several case studies of human fetuses at different stages of degeneration. Some of them contained huge blood coagulations and were almost completely resorbed. But since the material either derived from women that had recently aborted or from *post mortem* examinations Meyer could never bring the final proof for embryonic resorption in humans. In twin pregnancies of domestic sheep (*Ovis aries*), he discovered retarded and degenerated embryos. In the domestic guinea pig (*Cavia porcellus*), he could demonstrate the presence of erythrocytes, megakaryocytes and a few degenerative leucocytes within the degenerated material [MEYER, 1917]. These findings confirmed the results of an article published in 1915 about the development and regression of "ova" in the laboratory rat (*Rattus norvegicus*) [HUBER, 1915]. The degenerative "ova" (day 5 and day 6 after insemination) were described to lie in small masses of blood that contained leukocytes. The fact that embryonic degeneration was accompanied by the accumulation of different blood cells at the resorption site raised questions about the process of intravital aseptic autolysis [SALKOWSKI, 1880; LAQUEUR, 1912]. Schlesinger [1904] reviewed much of the literature on autolysis. He studied the autolytic activity in fetuses that died before birth and concluded that the mammalian organism provided a basic tool for aseptic tissue degeneration that should not be confused with bacterial lysis. Subsequently, several studies have been undertaken about autolytic tissue alterations of embryos under different nutritive conditions but the conclusions drawn from them were very diverse [MATHES, 1901; BERGELL & LIEPMANN, 1905; WIENER, 1905; JONES & AUSTRIAN, 1907; MENDEL & LEAVENWORTH, 1908; HARDING & YOUNG, 1918].

With the establishment of ultrasound in reproductive medicine [DONALD, MACVICAR & BROWN, 1958] early pregnancy diagnosis in domestic farm animals and pets became possible [FRICKE, 2002; STOUFFER, 2004]. Since then, research on embryonic resorptions in mammals and

humans increased substantially. Despite the constant improvement of ultrasound and other imaging techniques assisted by new developments in maternal and embryonic immunology [FRICKE, 2002; STOUFFER, 2004; VICENTE ET AL., 2012B; CHAOUAT ET AL., 2009; HE ET AL., 2011], the mechanisms involved in the process of embryonic resorption remained unexplained.

1.2. Definition

Embryonic resorption is nowadays defined as prenatal death followed by subsequent degeneration and complete resorption of the conceptus [JUBB, KENNEDY & PALMER, 2007]. It usually occurs at the zygotic/embryonic stage of pregnancy and thus can be clearly differentiated from late pregnancy failure such as mummification, maceration, putrefaction or abortion (Appendix, Table 1). If only one or some conceptus of the litter undergo embryonic resorption while others continue to develop until term [ALLEN, BRAMBELL & MILLS, 1947; ENGLAND & RUSSO, 2006], then this is referred to as *partial litter resorption* [ENGLAND, 1998], whereas resorption of the whole litter is referred to as *total litter resorption* [CONAWAY, BASKETT & TOLL, 1960]. Embryonic resorption is generally associated with a lack of clinical signs that precede the death of the conceptus. It is therefore also referred to as *spontaneous embryonic resorption* [ENGLAND, 1992; 1993; POST, 1995; SENDAG ET AL., 2010]. Often a pathologic agent is not found, implying that non-infectious factors might be responsible for the death of the embryo.

1.3. Embryonic resorption in polytocous species

1.3.1. Macroscopic description

The macroscopic appearance of embryonic resorptions has been described in several polytocous mammals listed alphabetically by family order in Table 2 of the Appendix. Polytocous females generally produce more than one offspring at each pregnancy. This is in contrast to monotocous females which give birth to one offspring at a time. In polytocous mammals the most detailed information about the macroscopic appearance of embryonic resorption is available for domestic dogs. After surgical removal of the uterus and ovaries (OHE) in pregnant domestic dog females it was noticed that resorption sites showed widespread “necrosis” without signs of inflammation. In some cases a thickening of the uterine wall attached to the resorption site was described. Old resorption sites were covered by greenish viscous zones [TROEGER, 1968; ENGLAND, 1992; MUELLER & ARBEITER, 1993; NOETHLING & VOLKMANN, 1993; ORTEGA-PACHECO, 2006; TOTTON ET AL., 2010].

England and colleagues [1992; 1993; 1998; 2006] used ultrasonography to monitor and describe the morphology of the resorption process in the domestic dog in very detailed studies. Embryos undergoing resorption showed a gradual loss of embryonic fluid over two to three days. In consequence, the conceptus increased in echodensity, became irregular in

shape and decreased in size with inward bulging and thickening of the uterine wall. Former resorption sites remained as (hypo)echoic region in the slightly enlarged uterus where free luminal fluid was often found. Complete embryonic resorption was observed after two to nine days. Since ultrasonography is now a routine diagnostic tool for pregnancy in domestic dogs these findings were confirmed in many publications [MUELLER, ARBEITER & BREITENFELLNER, 1993; YEAGER & CONCANNON, 1996; GUENZEL-APEL & HEINZE, 2001; ENGLAND, YEAGER & CONCANNON, 2003; STEIGER ET AL., 2006; FOLTIN, 2008; SENDAG ET AL., 2010]. Ultrasonographic descriptions about the resorption process in other polytocous mammals are rare. In rabbits, embryonic fluid resorption was observed after administration of aglepristone (Alizin) [ÖZALP, ZEMIZEL & ÖZOCAK-BATMAZ, 2013]. However, since all fetuses were subsequently aborted complete embryonic resorption was not documented.
A case study in the lesser hedgehog tenrec (*Echinops telfairi*) is to my knowledge the only ultrasonographic report about the resorption process in a small mammal [BERKEN, 2006].

1.3.2. Incidence

The incidence of embryonic resorption can be determined by direct counts of degenerated embryos or by estimates using a variety of indirect methods. Estimates are usually based on CL counts [HAYSSEN, VAN TIENHOVEN & VAN TIENHOVEN, 1993; STOCKLEY, 2003] or placental scar counts [DAVIS & EMLEN, 1948; BRAY ET AL., 2003; OWUSU ET AL., 2010] which are compared to the total number of viable embryos. *Post mortem* counts and counts after OHE are of limited usefulness because these parameters do not allow to distinguish total litter resorption from abortion. Therefore, resorption rates are regularly reported under the general terms *prenatal losses* or *embryonic/fetal mortality* [JOHNSTON & RAKSIL, 1987; STOCKLEY, 2003; VICENTE ET AL., 2012]. *Pre-implantation losses* are usually inferred from the difference between the number of CL and placental scars. *Post-implantation losses* are deduced from the difference between the number of placental scars and born or aborted young. According to estimates for the European rabbit [BRAMBELL, 1948] sometimes further differentiation is made into *entire litter losses* (total litter resorption and abortion) and *losses in litters that survive* (partial litter resorption). The fact that several different parameters are reported makes a simple comparison of the incidence of resorption between species difficult. Described incidence varies between 4.6 to 80% and has been so far described in 17 species (Appendix, Table 2).

1.3.2.1. Direct counts of embryonic resorptions

Direct counts of embryos undergoing resorption are performed *post mortem*, after OHE or by using ultrasound. *Post mortem* counts or counts after OHE only reflect a short period in the course of pregnancy. Hence, embryos that already underwent complete resorption and embryos that will undergo resorption in later pregnancy stages are missed by such counts. Application of ultrasound enables *in vivo* follow up examination of embryos undergoing

resorption and comparative monitoring of the remaining viable conceptus [FASSBENDER ET AL., 2001; ENGLAND & RUSSO, 2006; ROELLIG, GOERITZ & HILDEBRANDT, 2010]. The exact time of embryonic death and the subsequent morphological changes in the course of the resorption process can be documented.

1.3.2.2. The reliability of corpora lutea counts

In polytocous species almost every ovum released from the Graafian follicle is fertilized and conception rates lie close to 100% [European rabbit: ADAMS, 1960A; domestic dog: CHRISTIANSEN, 1984; domestic pig (*Sus scrofa domestica*): VAN DER LENDE, 1994]. However, a correlation between the number of CL of pregnancy and the number conceptus could so far not be shown [ANDERSON & SIMPSON, 1973; SCOFIELD, CLEGG & LAMMING, 1974]. The total number of CL generally exceeds the total number of intrauterine conceptus [ALLEN, BRAMBELL & MILLS, 1947; TSUTSUI, 1975; ALVES ET AL., 2002]. This difference is considered as an indicator of embryonic resorption and hence the incidence of resorption is often determined by comparing the total number of CL to the total number of conceptus. In most polytocous species, CL counts (*post mortem* counts, counts after OHE or by ultrasound) are a very reliable parameter to determine the incidence of embryonic resorption. They can be, however, limited under certain conditions: (i) CL can be sometimes difficult to count, e.g. in very small species that produce a high number of offspring [PARKES, 1924; PERRY, 1945]. (ii) Visualization by ultrasound can be complicated by the size and intraabdominal posititon of the ovaries [KAEHN, 1991; ENGLAND, 1992]. (iii) *Post mortem* counts can be limited in species (guinea-pigs and cats) where CL regression occurs soon after mid-gestation because progesterone production is taken over by the placenta [MCCRACKEN, CUSTER & LAMSA, 1999]. These effects are minimised by continuous ultrasonographic imaging of the number of CL and comparative monitoring of the number of conceptus which permits exact determination of the incidence of embryonic resorption in polytocous species.

1.3.2.3. The reliability of placental scars counts

Especially in small mammals, a small body size and and a low economic value limit extensive *in vivo* follow-up pregnancy examinations. Therefore, the difference between placental scars that formed *post partum* at the former implantation site and the number of viable conceptus is generally considered as a possible indicator of embryonic resorption. Moreover, fainter and smaller scars are often considered as a criterion for a former resorption site [laboratory rat: DAVIS & EMLEN, 1948; European brown hare (*Lepus europaeus*): HELL ET AL., 1997; BRAY ET AL., 2003; American mink (*Neovison vison*): ELMEROS & HAMMERHOJ, 2006; greater cane rat (*Thryonomys swinderianus*): OWUSU ET AL., 2010]. Conaway [1955] tested the reliability of this parameter by artificial induction of embryonic resorption in pregnant laboratory rats using colchicine [KERR, 1946]. Newly formed scars were of orange colour. With increasing age the scars decreased in size and darkened in colour. Only scars

of resorption sites that occurred before day 11 (of the 21 to 28 day pregnancy) were smaller and of fainter colour than those of viable embryos seen *post partum*. After day 12 of pregnancy the high variability in colour and size made a clear distinction from scars of viable embryos impossible. Despite the fact that placental scars of resorbed embryos in mid and late pregnancy show a similar macroscopic appearance to those of viable embryos they are still misleadingly referred to as an indicator of embryonic resorption [wild house mouse (*Mus musculus*): KRACKOW, 1992; domestic dog: TOTTON ET AL., 2010].

1.4. Embryonic degeneration in monotocous species

Embryonic degeneration processes have been described in several monotocous species (females producing one offspring at each pregnancy) - particularly in farm animals such as domestic horses (*Equus ferus caballus*) [VAN NIEKERK, 1965; GINTHER, 1995; PAPA ET AL., 1998; SMITH ET AL. 2003], domestic cattle (*Bos primigenius taurus*) [KASTELIC ET AL. 1988; 1991], domestic sheep [MEYER, 1917; Dutt, 1954] and domestic goats (*Capra hircus*) [HAMMOND, 1921; ENGELAND ET AL., 1997; 1998], but also in wildlife such as the moose (*Alces alces*) [SCHWARTZ & HUNDERTMARK, 1993] or the Asian elephant (*Elephas maximus*) [LUEDERS ET AL., 2010]. Reports either derive from *post mortem* studies [VAN NIEKERK, 1965; BELONJE & VAN NIEKERK, 1975; SMITH ET AL. 2003] or from long term ultrasonographic monitoring [GINTHER, 1985; GINTHER ET AL. 1985; KASTELIC, 1988; SQUIRES, MCKINNON & SHIDELER, 1988; KAEHN, 1991; KASTELIC, 1988; KAEHN, 1991; BERGFELT, WOODS & GINTHER 1992]. Different authors reported embryonic death under the term of *embryonic resorption* [CHEVALIER-CLÉMENT, 1989; MERKT, 1966; 1968; 1979; SCHERBARTH, 1980]. However, in none of these studies complete embryonic resorption was observed. Therefore, embryonic death in monotocous species is generally referred to as *early pregnancy loss (EPL)* (respectively *early embryonic death (EED)*) in horses and *fetal loss* in ruminants.
In early pregnancy stages, dying embryos usually undergo a partial degeneration process before being expelled [GINTHER, 1995; GINTHER ET AL., 1985; HAMMOND, 1921; KASTELIC ET AL., 1988; SMITH ET AL. 2003]. Fetal degeneration in late pregnancy stages is rare and generally occurs in association with infectious diseases that cause the lysis and abortion of the embryo [SMITH ET AL., 2003; JONKER, 2004; SZEREDI ET AL., 2008]. A sterile form of degeneration can be found in aborted horse fetuses that died of an overlong or twisted umbilical cord which compressed the vessels providing the maternal blood for the fetus. In consequence the fetus is disconnected from the maternal blood supply and partially degenerated before being aborted [SMITH ET AL., 2003].

1.4.1. Incidence

Since embryonic degeneration processes in domestic monotocous species are always associated with an extended interval between two estruses (prolonged interestrual period) [ERB & HOLTZ, 1958; AYALON, 1978; JUBB, KENNEDY & PALMER, 2007] they gained increasing

economic significance. Although fertilization rates after artificial insemination (AI) generally reach or exceed 90% [domestic cattle: SREENAN & DISKIN, 1986; DISKIN & MORRIS, 2008; domestic goat: ARMSTRONG ET AL., 1983; domestic horse: BALL ET AL., 1986; WOODS ET AL., 1991; domestic sheep: RESTALL ET AL., 1976; MITCHELL ET AL., 1999] the incidence of embryonic loss ranges between 30% and 40% in domestic cattle [HUMBLOT, 2000] and domestic sheep [QUINLIVAN ET AL., 1966; WILLINGHAM, SHELTON & THOMPSON, 1986; THATCHER & SANTOS, 2006; DIXON ET AL., 2007] and between 10 and 70% in domestic horses [VANDERWALL, 2008]. It also exists with more than 11% in domestic goats [ENGELAND ET AL., 1997; ENGELAND ET AL., 1998]. Average calving rates range between 50% and 55% [DISKIN & SREENAN, 1980; CHEVALIER & HUMBLOT, 1998] and are decreasing [LUCY, 2001; JONKER, 2004; DISKIN & MORRIS 2008]. Embryonic degeneration thus represents the major source of pregnancy wastage in the reproduction of domestic livestock.
In humans, the average fecundity after natural intercourse is approximately 30% [ZINAMAN ET AL., 1996]. During early pregnancy up tp 75% of the embryos are lost [BOKLAGE, 1990; NORWITZ, SCHUST & FISHER, 2001] and after implantation between 15 and 30% [KWAK-KIM ET AL., 2010; RAI & REGAN, 2006; FARQUHARSON & EXALTO, 2010]. The chance of total pregnancy loss is between 50 and 75% [BOKLAGE, 1990; NORWITZ, SCHUST & FISHER, 2001; RAI & REGAN, 2006].

1.4.2. Multiple pregnancies

Up to 25% of all pregnancies in monotocous animals start as twin pregnancies [ARTHUR, 1958; FERNANDEZ-BACA, HANSEL & NOVOA, 1970; SCANLON, GORDON & SREENAN, 1974; BOWMAN, 1986; GINTHER, 1995] but between 60 and 90% end with the unilateral loss of one twin. A total loss of both twins was observed in 10% of the pregnancies [CHEVALIER-CLÉMENT, 1989; GINTHER, 1984; 1995; NYLAND & MATTOON, 1995].
In humans, reduction of twin pregnancies to a single pregnancy is regularly reported under the phenomenon of the *vanishing twin syndrome* [LANDY & KEITH, 1998; CUNNINGHAM ET AL., 2001]. Up to 40.5% of twin pregnancies in humans suffer the loss of one twin [LANDY & KEITH, 1998]. Occasionally, systematic compression by the growing twin causes the development of a *fetus papyraceus* which remains as flattened remnant until the birth of the viable fetus [PINBORG, 2010]. Although resorption of the dead embryo by its viable twin has been mentioned as a possible explanation for its “disappearance”, only partial degeneration of the dead embryo could be confirmed [SULAK & DODSON, 1986]. Until now, the pathophysiological mechanisms of embryo reduction in monotocous animals and humans remain unexplained [CUNNINGHAM ET AL., 2001; PINBORG, 2010].

2. The European brown hare

The European brown hare (Ebh) (*Lepus europaeus*) is a small polytocous animal belonging to the mammalian order Lagomorpha which comprises the families Leporidae (hares and rabbits) and Ochotonidae (pikas) [AVERIANOV, 1999; WILSON & REEDERS, 2005]. The current

distribution of the Ebh extends from Continental Europe and the Middle East to Central and North Asia. As a species for sports hunting it has been introduced on several islands such as Great Britain and New Zealand and in several states in both North and South America and Australia [DIETRICH, 1984; FLUX UND ANGERMANN, 1990; BONINO & MONTENEGRO, 1997; WILSON & REEDERS, 2005]. In Great Britain the Ebh is considered as a native species [JNCC, 2007] because of its long-established population that can be backdated to Roman times [BATTERSBY, 2005]. There is recent evidence for an introduced population in Ireland [REID, 2011].

2.1. Population decline

The Ebh is a characteristic species of European agricultural landscapes and underwent a range expansion through the spread of pastoral agriculture in the 1950s [SCHÄFERS, 1996]. Over the last decades dramatic population declines have been recorded across Europe [NINOV, 1990; MARBOUTIN & PEROUX, 1995; MARY & TROUVILLIER, 1995; BATTERSBY, 2005; SANTILLI & GALARDI, 2006; PÉPIN & AGRIBAULT, 2007; NIEMINEN, NIEMI & JUSSILA, 2011]. As a consequence the species is now protected through *Appendix III* of the *Berne Convention on the Conservation of European Wildlife and Natural Habitats* and red-listed as *near threatened*, *vulnerable or endangered* in several European countries (Austria, Denmark, Finland, Germany, Switzerland, UK) [EUROPEAN RED LIST, 2012]. German hunting records have reported population declines since the 1960s [SCHÄFERS, 1996]. The Ebh is therefore classified as *endangered* in Germany (category 3) [ROTE LISTE DEUTSCHLAND, 2009]. Current mean population densities vary from 11.2 to 11.8 hares/km^2 [STRAUSS ET AL., 2008; WILDTIERINFORMATIONSSYSTEM DER LÄNDER DEUTSCHLANDS, 2010]. With population densities of 0 to 7 hares/km^2 it is even classified as *critically endangered* (category 2) in the German state ("Land") of Sachsen-Anhalt [WILDTIERINFORMATIONSSYSTEM DER LÄNDER DEUTSCHLANDS, 2010].

2.2. Factors associated with population declines in Europe

The main factor responsible for the steady decline of the Ebh is habitat destruction caused by intensification of cultivation [NINOV, 1990; SMITH, JENNINGS & HARRIS, 2005; ZELLWEGER-FISCHER, KÉRY & PASINELLI, 2011]. New conservation programs such as agri-environment schemes (AES) try to mitigate the negative consequences of intensive agriculture [HACKLÄNDER, 2002; SMITH ET AL., 2004; TSCHARNTKE ET AL., 2005; KUIKEN ET AL., 2011]. Since the 1990s Ebh populations seem to have stabilised in some regions of Germany [STRAUSS ET AL., 2008]. Across Europe, however, populations continue to decrease [WILDTIERINFORMATIONSSYSTEM DER LÄNDER DEUTSCHLANDS, 2010; RED LIST OF FINNISH SPECIES, 2010; GEFÄHRDETE ARTEN IN DER SCHWEIZ, 2010/2011; DEN DANSKE RODLISTE, 2011; DEUTSCHE JAGDZEITUNG, 2011].

Several investigations have been undertaken to test the possible impact of additional factors that might be responsible for the decline. Infectious diseases such as the European brown hare syndrome (EBHS), pseudotuberculosis, pasteurellosis and coccidiosis are present in many populations [DUFF ET AL., 1994; EDWARDS, FLETCHER & BERNY, 2000; BARTLING ET AL., 2004; DREWS ET AL., 2011]. Some of them (coccidiosis, pseudotuberculosis) are associated with wet weather which can increase mortality rates dramatically, especially during mild winter months [EDWARDS, FLETCHER & BERNY, 2000; SANTILLI & GALARDI, 2006]. As the main predator of the Ebh in Europe, the red fox (*Vupes vulpes*) may influence populations substantially [SCHMIDT, ASFERG & FORCHHAMMER, 2004; REYNOLDS ET AL., 2010]. The use of agro-chemicals (fertilisers and pesticides) was longtime thought to reduce survival rates [EDWARDS, FLETCHER & BERNY, 2000; SPITTLER, 2000]. None of these additional factors apparently contributed substantially on their own to the long-term population trend [EDWARDS, FLETCHERS & BERNY, 2000; SMITH, JENNINGS & HARRIS, 2005; KUDRYAVTSEVA & SMIRNOV, 2012]. All of them were, however, positively associated with agricultural intensification [PÉPIN, 1989; SCHNEIDER, 2001; SMITH, JENNINGS & HARRIS, 2005; REYNOLDS ET AL., 2010].

2.3. Reproductive research

Reproduction of the Ebh was examined in numerous field studies [RACZYNSKI, 1964, FLUX, 1965, 1967; FRYLESTAM, 1980; BROEKHUIZEN AND MAASKAMP, 1981; BONINO AND MONTENEGRO, 1997; BLOTTNER ET AL., 2001; FASSBENDER ET AL., 2001; GOERITZ ET AL., 2001; BRAY ET AL., 2003; STOTT AND WIGHT, 2004]. In the course of these investigations several reproductive disorders were detected but none of them reduced fertility in male or female hares [PÉPIN 1989; BLOTTNER ET AL., 2001; FASSBENDER ET AL., 2001; GÖRITZ ET AL., 2001; HACKLÄNDER ET AL., 2001; STOTT AND WIGHT, 2004]. The establishment of captive breeding colonies facilitated fundamental research on the reproductive biology considerably. However, the high susceptibility to stress and the solitary life of the Ebh make captive management difficult [HEDIGER, 1948]. Until now, few breeding colonies exist across Europe (Czech Republic, France, Germany, Italy, Poland). Specific hare breeding cages [Article I, II] permitted *in vivo* long term studies of the Ebh's reproductive physiology. In several investigations Caillol and colleagues developed physiological hormone profiles across the functional life span of the CL for non-pregnant, pregnant, pseudopregnant and lactating hares [CAILLOL, MARTINET & LACROIX, 1990; CAILLOL ET AL., 1990; CAILLOL, MONDAIN-MONVAL & MCNEILLY, 1990; CAILLOL ET AL., 1992]. The application of ultrasound permitted imaging of the reproductive organs in male and female hares [GÖRITZ ET AL., 2001; HACKLÄNDER ET AL., 2003; HILDEBRANDT ET AL., 2009]. By detailed longitudinal monitoring of the embryonic development the establishment of exact growth curves for prenatal age determination became possible [ROELLIG, GOERITZ & HILDEBRANDT, 2010]. With the implementation of AI [HILDEBRANDT ET AL., 2010] recently superfetation could be experimentally demonstrated for the first time [ROELLIG ET AL., 2010].

2.4. Incidence of embryonic resorption in the European brown hare

In the Ebh, direct counts of resorption sites have only been undertaken once in *post mortem* examinations by Raczynski [1964]. He reported incidence values which ranged between 6 and 80% depending on the breeding month. By comparing the total number of CL to the number of implanted embryos Broekhuizen and Maaskamp [1981] estimated the pre-implantation loss rate to be around 6%. Flux [1967] reported a 2.3% loss rate for entire litters in the pre-implantation period and a 21.9% loss rate for litters that survived. Post-implantation losses of entire litters occurred in 14.3% and were 9% in litters that survived. A total prenatal loss rate of 47.5% was reported.

3. Biopsy procedures and Amniocentesis

Percutaneous biopsy procedures represent a routine tool for medical diagnostics. Depending on the type of needle used, punch biopsies for the extraction of small tissue cylinders can be distinguished from needle biopsies (needle aspirations and vacuum-assisted core biopsies) for cell and fluid sampling. Because of their minimally invasive nature biopsies have many applications. They are commonly used for the diagnosis of organic diseases (tumors, inflammations, liquor punctures) [DUFFY, 1969; SMITH, 1985; VOSS ET AL., 2000; KANEGAYE, SOLIEMANZADEH & BRADLEY, 2001] but can also be used in therapeutical treatment, e.g. as in forceps biopsy for extraction of intestinal polyps or removal of pulmonary nodules [KARITA ET AL., 1991; EBERHARDT ET AL., 2010]. In human reproductive medicine they represent the leading technique for embryonic and fetal assessment. Prenatal collection of fetal cells enables sex determination and detection of genetic abnormalities [ALFIREVIC, MUJEZINOVIC & SUNDBERG, 2009]. Among other techniques, cells can be either gained by amniocentesis [CUNNINGHAM ET AL., 2001] or chorionic villus sampling (CVS) [WAPNER & TOY, 2011].

3.1. Amniocentesis

The amniotic fluid surrounds the embryo and therefore protects it from trauma. It maintains the temperature and also has a nutritive function for the embryo [MULVIHILL ET AL., 1985]. Several different studies in domestic sheep and humans showed that it is essential for normal embryonic development, particularly the development of the lungs and the gastrointestinal tract [ALCORN ET AL., 1977; MOESSINGER ET AL., 1983]. In humans, amniotic fluid in early pregnancy is an ultrafiltrate of the maternal plasma. During later stages of pregnancy it largely consists of extracellular fluid which diffuses through the embryonic skin, thereby reflecting the composition of the fetal plasma. After cornification of the skin the amniotic cavity mainly contains fetal urine with desquamated fetal cells. Therefore, amniotic fluid sampling by ultrasound guided amniocentesis provides a useful tool to detect genetic disorders of the embryo or to determine its sex.

3.2. Chorionic villus sampling

Chorionic villi are projections (*chorion frondosum*) of the *placenta fetalis* and emerge from the outer layer of the chorion (trophoblast). In humans, they comprise the syncytiotrophoblast, the stroma of the intravillous space and the fetal capillary wall and therefore constitute the interface between maternal and fetal blood circulation [WAPNER, 1997]. CVS involves the removal of a small piece of tissue from the *chorion frondosum*. Fetal cells are isolated from the sample and the chromosomes are then either directly examined under the microscope or the cells are incubated in nutrient media for further analysis as done with cells collected by amniocentesis [WAPNER & TOY, 2011].

3.3. Ultrasound guided assistance

The use of high frequency ultrasonography for real time imaging of the biopsy needle was first presented in 1968 [KRATOCHWIL]. Since then it has become the essential tool for needle guidance in humans. Accurate needle placement under visual control reduces the risk of micro lesions and unsuccessful insertion attempts [CRESPIGNY & ROBINSON, 1986]. Ultrasound-guided biopsies are usually practiced manually or by the application of a needle guide which is fixed to the ultrasound probe [PEDERSON, 1977; RIDLEY & HAGY, 2012]. Although comparative studies have shown that probe-guided biopsies take less time [PHAL ET AL., 2005] most of the operators still prefer manual biopsies because it permits a more flexible implementation.
Other high imaging techniques such as computed tomography (CT) [SCHMIDBAUER ET AL., 2007; YILDIRIM ET AL., 2009], optical coherence tomography (OCT) [FUJIMOTO ET AL., 1995], endoscopes [AHN & GOUMNEROVA, 2010] and magnetic resonance imaging (MRI) [PONDMAN ET AL., 2008; SINGH ET AL., 2008; WEISS, NOUR & LEWIN 2008] are also used for assisted needle guidance. Compared to ultrasound, however, their implemention is not as easily adaptable and flexible. Ultrasound biomicroscopes (UBM) (frequency: 55 to 200 Mhz, maximum resolution: 14µm) represent a favourable alternative for visualising extremely small structures such as vascular anatomy and embryonic development [MU AND ADAMSON, 2006; GELSE ET AL., 2010]. Therefore, UBM are of particular use in veterinary medicine. They allow the detailed monitoring of embryonic and fetal structures and guarantee a safe and accurate needle guidance in small mammals such as rats and mice [ZHOU ET AL., 2002; SLEVIN ET AL. 2006; FOSTER, HOSSACK AND ADAMSON, 2011; PIERFELICE & GAIANO, 2010; VANDOORNE ET AL., 2011; GRECO ET AL., 2012].

4. Objectives

The Ebh can be considered to become a model species for studies on embryonic resorption in small mammals (for species overview see: IUCN SSC SMALL MAMMAL SPECIALIST GROUP, 2011). A long breeding season of 10 months (December to September in central Europe) and a short pregnancy duration of 41.9 ± 0.7 days [ROELLIG ET AL., 2010] guarantee adequate sample sizes for studying conceptus undergoing resorption. The use of a wild animal species minimises the negative impact of inbreeding on the reproductive performance which was observed in laboratory mice [GENDRON & BAINES, 1989] and certain breeds of rabbits [VICENTE ET AL., 2012B]. Due to the fact that the Ebhs were kept in specific hare breeding cages a possible negative impact on the reproductive performance by capturing and subsequent pregnancy examinations is minimized [Article I]. Since maintenance of pregnancy in the Ebh depends on CL-derived progesterone throughout the entire pregnancy [CAILLOL & MARTINET, 1976], detailed monitoring of luteal dynamics in the context of embryonic resorption is possible. As an induced ovulator the hare depends on copulatory activity that elicits ovulatory luteinizing hormone (LH) surges via the hypothalamic region and subsequently evokes ovulation [CAILLOL ET AL., 1986]. In the case of resorption this reproductive feature permits the exact determination at which gestational age the death of the embryo occurred.

The objectives of this thesis were (1) to establish a minimally invasive ultrasound guided biopsy technique which enables for the first time the *in vivo* extraction of embryonic resorption tissue for further genetic studies; prerequisite for the detection of the resorption site and the guidance of the biopsy needle under visual control was the use of high frequency real time, B-mode ultrasound and UBM; (2) to reveal the abundance of maternal and fetal cells within the resorption fluid; (3) to determine the incidence and ultrasonographic morphology of embryonic resorptions; (4) to document early embryonic development and implantation stages and to differentiate between embryonic resorptions and viable conceptus on a very early stage of pregnancy. Suggesting a direct link between each developing conceptus and one CL the last aim of this study was (5) to detect whether the total number of CL corresponds to the total number of conceptus and consequently whether each embryonic resorption is accompanied by a corresponding CL undergoing regression.

II. Articles

Article I:
Theriogenology, 2013, vol. 80, pp. 479-486.

Embryonic resorption in context to intragestational corpus luteum regression: a longitudinal ultrasonographic study in the European brown hare (*Lepus europaeus* PALLAS, 1778)

Running title: Embryonic resorption in the European brown hare

K. Schroeder, B. Drews, K. Roellig, F. Goeritz, T.B. Hildebrandt

Abstract

Embryonic resorption is frequently observed in polytocous mammals. Often it occurs as partial litter resorption affecting only single conceptus of a whole litter. The aim of our study was to describe the incidence and morphology of embryonic resorption in the European brown hare *(Lepus europaeus)*. In 154 pregnancies viable conceptus, conceptus undergoing resorption and CL of pregnancy were ultrasonographically monitored during the entire gestation period. Resorptions were classified into (i) *pre-implantation resorptions*, (ii) *peri-implantation resorptions* and (iii) *post-implantation resorptions*. The incidence of resorption in the *pre-implantation* period was 9%, in the *peri-implantation* period 9% and in the *post-implantation* period 24%. Post-implantation resorptions were found up to late pregnancy stages when fetal development was already in progress. The highest daily incidence of resorption was on day 8 of the 42 day pregnancy. In 91% of the cases the regression of one CL was observed while an embryo was undergoing resorption at the same time. The number of resorptions did not significantly differ from the number of CL in regression during gestation, suggesting an interesting one-resorption-to-one-regression-relationship. The ultrasonographic appearance of the luteal regression during pregnancy was similar to the morphology characteristic for postpartal luteolysis.

Keywords: Corpus luteum, Lagomorpha, Pregnancy ultrasound, Regression, Resorption

1. Introduction

Embryonic resorption is a widespread phenomenon among polytocous species [1,2]. It often affects only single conceptus of the litter while others continue to develop until term [3,4,5]. This is referred to as *partial litter resorption* [6] while resorption of the whole litter is referred to as *total litter resorption* [7]. Incidences of embryonic resorptions in polytocous species are generally based on the difference between the mean number of CL and mean number of embryos, placental scars and the young [2,3,7,8]. The release of the ovum from the mature follicle results in the autonomous formation of a CL. However, so far there has been no evidence of a correlation between the number of embryos and the number of CL [9,10]. The reason for this is that studies describing the incidence or morphology of embryonic resorptions are usually performed *post mortem* or after ovariohysterectomy [9,10,11,12] which explains why it has been not possible to demonstrate intragestational changes of the luteal morphology in the context of embryos undergoing resorption. Moreover, the exact day of embryonic death is usually unknown and the time course of the resorption process cannot therefore be described. Often, detection of the dead conceptus is difficult since it is already in a late stage of resorption and almost completely decomposed. The establishment of ultrasound as diagnostic tool in reproduction medicine facilitated *in vivo* longitudinal monitoring of the macroscopic process of embryonic resorptions [4,13,14]. However, in small mammals, such as rodents and rabbits, used for experimental studies on a regular basis, descriptions of the resorption process are still based on *post mortem* studies [15,16,17].

In the European brown hare (*Lepus europaeus*), hereafter referred to as the hare, regular ultrasonographic monitoring of embryonic development showed that resorptions occur on a regular basis [5,18]. However, detailed studies of the incidence and morphology of embryonic resorptions have not been conducted so far. Based on the recently established parameters for prenatal development in the hare [19] the present study is a longitudinal ultrasonographic investigation of the incidence and morphology of embryonic resorption coupled with ovarian dynamics specifically tracing the development of CL of pregnancy.

2. Materials and Methods

2.1. Animals

Hares were housed in specific hare breeding cages (type Nitra) on the field research station of the Leibniz Institute of Zoo and Wildlife Research (IZW). Each cage had an area of 2 m^2 and comprised a front part where hares had free access to food and drinking water and a back retreat area with fresh hay. Hares were fed *ad libidum* with a customized hare pellet diet and fresh hay. All hares were kept under natural weather conditions.

The study included 44 females and 38 males. 154 pregnancies were examined during three consecutive breeding seasons (2008 – 2010). Pregnancy was achieved by natural mating (n = 54) [5,19] or by artificial insemination (n = 100) [20]. The day of insemination was defined as day one of pregnancy.

2.2. Ultrasonographic examinations

A total of 1021 ultrasound exams were performed in 154 pregnancies using a standardized examination protocol. Hares were examined either under anaesthesia (n = 259) in dorsal recumbency or without anaesthesia (n = 762) in a sitting position using a specially designed restraint box [5]. For anaesthesia, isoflurane (2.5 Vol%; Isoba; Essex Pharma GmbH, Berlin, Germany) and oxygen (1.5 – 2.0 $Lmin^{-1}$) were supplied via inhalation mask or a combination of 15 mg/kg ketamine (Ketamin 10%; WDT) and 2.3 mg/kg xylazine (Rompun 2%; Bayer HealthCare) was administered.

Ultrasound examinations were performed with high frequency real time, B–mode ultrasound units (Diasus, Dynamic Imaging, Livingstone Scotland, linear probes 8-16, 10-22 MHz; Voluson i, GE HealthCare, AU, linear probe 12 MHz; Vevo 2100, VisualSonics, Canada, linear probe 13-24 and 22-55 MHz). The exams started with the visualization of both ovaries. After the number and size of the CL were documented, a first criterion for a clinically verified pregnancy was the presence of non attached expanded blastocysts in the uterine tract. Depending on the stage of pregnancy the development of the embryos was further monitored by visualizing e.g. the embryonic vesicle, the embryonic heartbeat, the extra embryonic membranes and the placenta relying on previously established reference parameters [19]. Ultrasonographic examinations were performed daily between day 5 and day 16 of pregnancy for all individuals. From day 17 on resorption sites were measured in intervals of 1 to 3 days until uterine scar tissue was formed at the former implantation site. The mean maximum diameter of the resorption site was measured in cross section including the surrounding uterine wall. The mean diameter of embryonic resorption sites was determined until day 15 of pregnancy and compared to the mean diameter of corresponding viable conceptus.

2.3. Statistical analysis

For statistical analysis PASW version 18.0 (SPSS, Chicago, IL, USA) was used. Values are presented as the mean ± s.d. Daily incidences of peri- and post-implantation resorptions were determined by calculating the relative resorption rates of dying conceptus for day 6 to day 42 of pregnancy. The mean litter size in animals with and without resorption was compared with Paired Sample T-Test. Spearman's rank correlation was performed to correlate the number of peri- and post-implantation resorptions to the intrauterine litter size. The mean diameter ± s.d. of viable conceptus was compared to the mean diameter of conceptus undergoing resorption using Paired Sample T-Test. To test whether or not the number of resorptions is similar to the number of regressing CL a Wilcoxon signed-rank test was performed. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Classification, incidence and morphology of embryonic resorptions

In 154 pregnancies (n = 44 females) 259 offspring were born healthy at term (1 to 6 offspring per litter). The overall mean litter size was 2.44 ± 1.08 offspring. A significant difference in mean litter size was detected between animals with (including total litter resorption: 1.33 ± 1.39 offspring; excluding total litter resorption: 2.23 ± 1.09) and without resorption (2.72 ± 1.00 offspring) (Paired Sample T-Test, including total litter resorption: $p < 0.0001$, excluding total litter resorption: $p < 0.0001$; n = 20 females). Further, 497 ovulations derived from the number of CL between day 3 and day 6 of pregnancy were counted giving a mean ovulation number of 3.23 ± 1.28 ovulations per pregnancy. In total, 52% of ovulations resulted in live born offspring. The total resorption rate, calculated from the difference between the number of ovulations and viable embryos, was 42%. There was a positive significant correlation between the total number of ultrasonographically detected resorptions and implanted embryos ($r_s = 0.289$, $p < 0.001$, n = 421 implanted conceptus, n = 154 resorptions). The total abortion rate was 3% of all 497 ova (n = 14 abortions in 8 pregnancies, n = 8 females). Six females died pregnant without giving birth (3% of 497 ova). Out of the 497 ova 378 viable conceptus were ultrasonographically verified on day 5 or 6 of pregnancy. The highest incidence of resorptions was detected on day 8 of pregnancy where 4.2% of the 378 viable conceptus died (n = 16 resorptions, 16 pregnancies in 16 females) (Fig. 1).

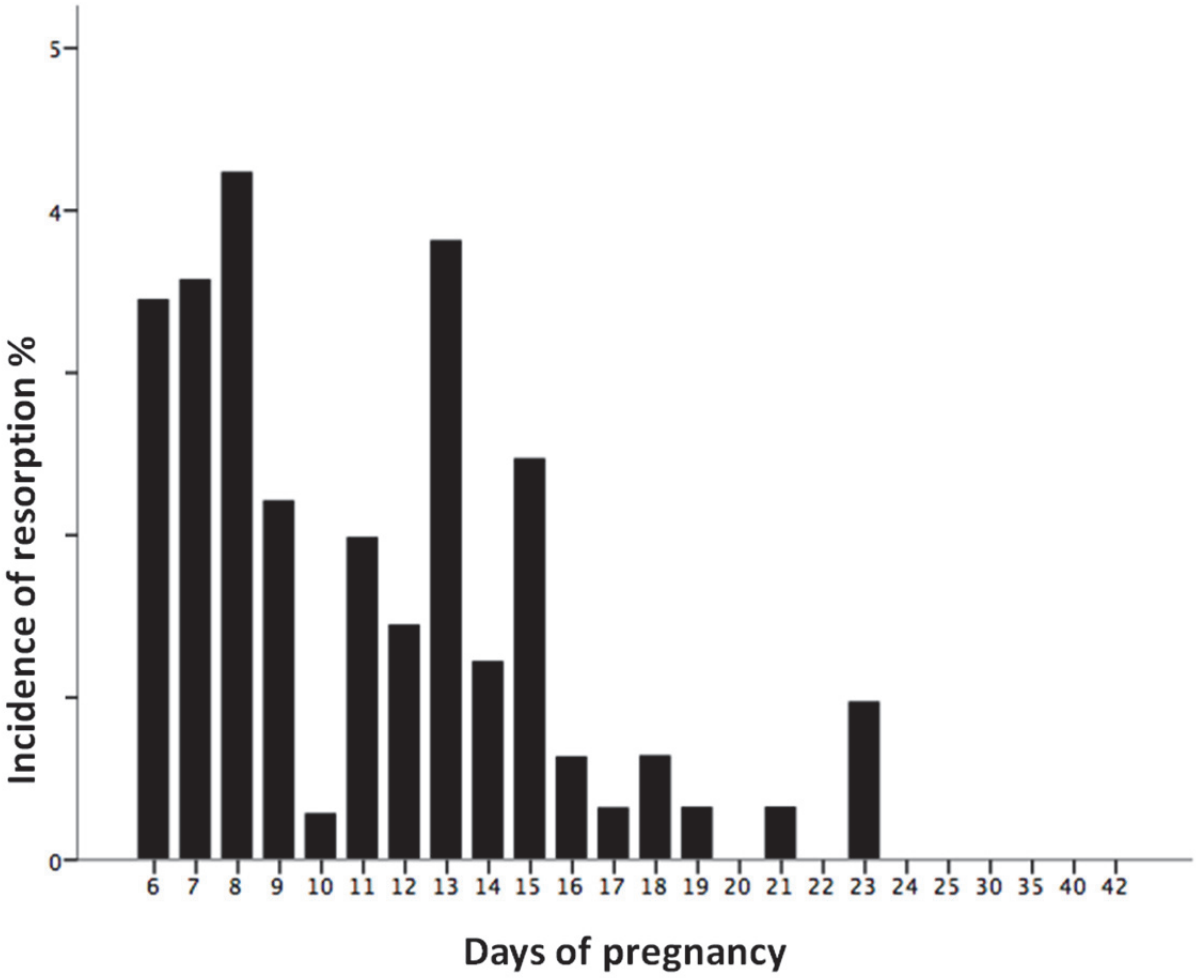

Fig. 1. Daily incidences of peri– and post-implantation resorptions in the course of pregnancy.

Since ultrasonographic examination was continuously performed every day it was possible to evaluate the exact time of death of a later embryo in resorption. The mean diameter of dead conceptus taken on the exact day of death until day 15 of pregnancy was compared to the mean diameter of corresponding viable conceptus (Fig. 2). Conceptus that were going to die or just had died were significantly smaller than their viable siblings measured the same day (Paired Sample T-Test, $p < 0.0005$, $n = 60$ dead conceptus, $n = 552$ viable conceptus) (Fig. 2, Fig. 3F).

The course of embryonic resorption was monitored over several days until morphological structures could no longer be differentiated by ultrasound. Continuous measurements revealed that the mean diameter of resorption sites increased initially before a subsequent decrease was observed (Fig. 3F&G, Fig. 3M&N). The initial increase in size of the resorption site was greater in embryonic resorptions that occurred in early pregnancy stages than in embryos undergoing resorption later in pregnancy.

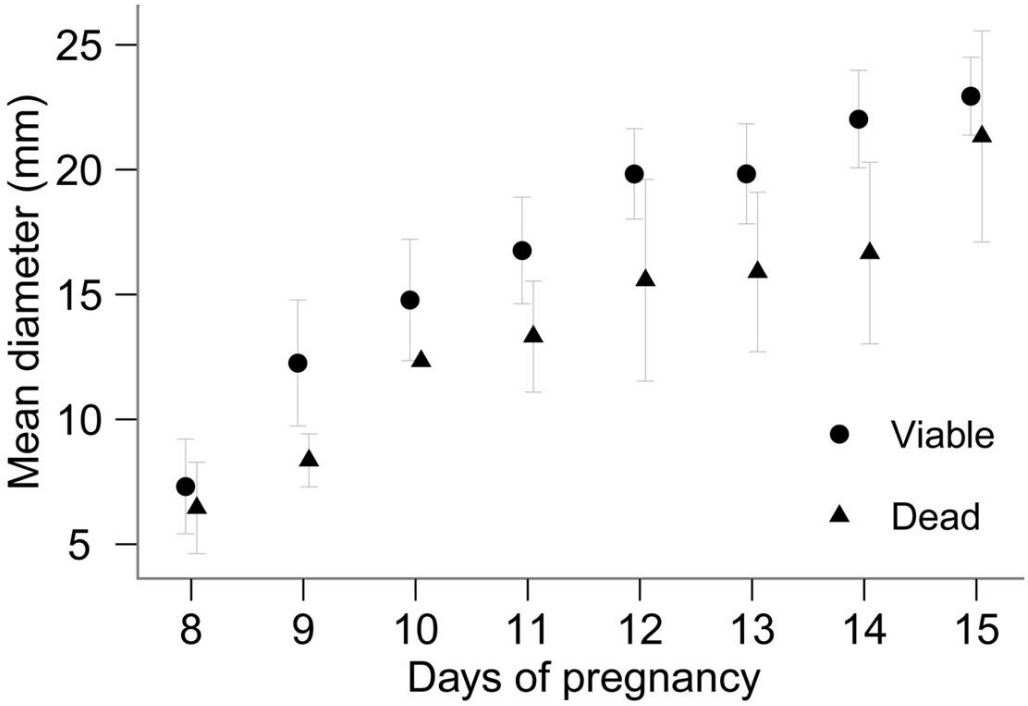

Fig. 2. Mean diameter of conceptus undergoing resorption (*dead)* compared to the mean diameter of viable conceptus (*viable)* in early pregnancy. All measurements were taken in maximum cross section.

Embryonic resorptions were classified as follows:

3.1.1. Pre-implantation resorption (day 1 to 5 of pregnancy)

After successful fertilization in the oviduct, the resulting zygote can fail to develop to blastocyst stage prior to reaching the uterine horn. This process is here defined as pre-implantation resorption. It was not possible to differentiate between unfertilized ova and zygotes that fail to develop to the expanded blastocyst stage. Based on studies that revealed fertilization rates close to 100% in different polytocous species the potential number of unfertilized ova was included in the number of pre-implantation resorptions. In the hare, current regularly used ultrasound technology allows earliest detection of expanded blastocysts on day 5 and 6 of pregnancy when they reach the uterine lumen. Therefore, the number of pre-implantation resorptions between day 1 and day 5 of pregnancy was derived from the difference between the number of ovulations (displayed as number of CL) and the number of ultrasonographically detected blastocysts on day 6 of pregnancy including unfertilized ova and zygotes. The total pre-implantation resorption rate was 9% of 497 ova (n = 46 pre-implantation resorptions in n = 29 pregnancies, n = 21 females).

3.1.2. Peri-implantation resorption (day 6 to 9 of pregnancy)

In this study, peri-implantation is defined as the period between day 6 and 9 of pregnancy. In the peri-implantation period, the blastocysts on day 6 and 7 were displayed as fluid filled

anechoic vesicles (Fig. 3A). The number of embryonic resorptions on day 7 was derived from the difference between the number of detected blastocysts on day 6 of pregnancy and the number of day 7. On day 6 and 7 the embryonic structures were still so small that the resorption process itself was not ultrasonographically detectable. From day 8 on it became possible to visualize the embryonic resorption process by ultrasound. Placental folds were seen protruding into the embryonic vesicle (Fig. 3B). In case of embryonic resorption between day 8 and 9, the embryonic fluid diminished and gained in echodensity (Fig. 3C&D). The process was terminated by the formation of echodense scar tissue (Fig. 3E). The duration of the resorption process was longer in case of partial litter resorption than in case of total litter resorption. The total peri-implantation resorption rate was 9% of 497 ova (n = 44 peri-implantation resorptions in n = 27 pregnancies, n = 15 females) with the highest incidence on day 8 of pregnancy (compare Fig. 1).

3.1.3. Post-implantation resorption (day 10 to 42 of pregnancy/until birth)

An embryo or early fetus that dies after implantation (day 10 of pregnancy) is referred to as post-implantation resorption. The typical progression of post-implantation embryonic resorption is exemplarily depicted for embryonic resorptions detected on day 10 (Fig. 3F-J) and 15 of pregnancy (Fig. 3K-O). On day 10 of pregnancy, the embryonic body became visible as a small echodense spot in the viable conceptus while in the corresponding resorption sites, that structure was absent (compare Fig. 3F). Within the extraembryonic cavity, accumulation of echodense material was observed, followed by a general increased echodensity of the extraembryonic fluid (compare Fig. 3G&H, M&N) and its subsequent diminution (compare Fig. 3I&O). In later pregnancy stages (compare Fig. 3K), cessation of the embryonic heartbeat was the first indication for embryonic death, followed by the disintegration of the embryo (compare Fig. 3L,M,N) and subsequently the extraembryonic membranes (compare Fig. 3N). The placenta was only affected at the end of the resorption process so that a clear differentiation to the former embryonic cavity was no longer possible (compare Fig. 3I&O). Similar to the peri-implantation resorption, the post-implantation resorption process was completed with formation of uterine scar tissue (compare Fig. 3J). In case the fetus died in a very late pregnancy stage between day 33 and day 36 of the 42 day pregnancy, complete disintegration of the embryo and its membranes was observed. The dissolved placental and fetal remnants were subsequently discharged from the uterus (n = 6 dead embryos in 6 pregnancies, n = 6 females). The total rate of post-implantation resorption was 24% of 497 ova (n = 117 post-implantation resorptions in 70 pregnancies, n = 32 females). The highest incidence for post-implantation resorption was found on day 13 of pregnancy (compare Fig. 1).

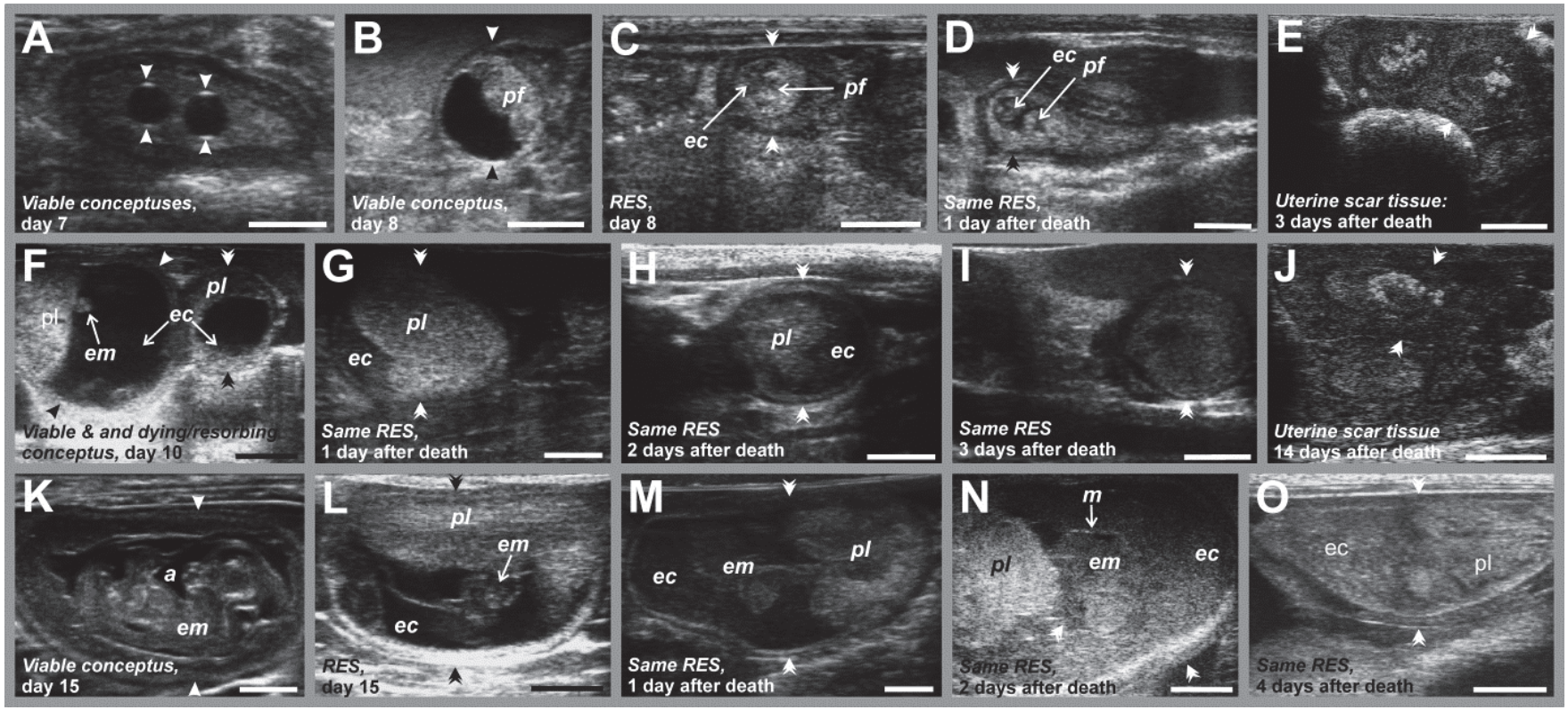

Fig. 3. Ultrasonograms of viable conceptus (arrowheads) compared to resorptions sites (RES) (pinnate arrowheads) detected on different days of pregnancy. The arrowheads point to the uterine wall surrounding the conceptus, except for those shown in Fig. 3A where they point at two free blastocysts within the uterine lumen. **(A)** Two viable conceptus on day 7 showing the two characteristic hyperechoic opposite poles. **(B)** Conceptus on day 8 with prominent placental folds (*pf*). **(C)** Resorption site on day 8. Compared to the viable conceptus (compare B) it is undersized. The embryonic cavity (*ec)* has almost disappeared. The placental folds *(pf)* are still visible. **(D)** Same resorption site 1 day after death (day 9). The placental folds (*pf)* are still distinguishable from the embryonic cavity *(ec)*. **(E)** Same resorption site 3 days after death (day 11) remaining as echodense scar tissue. **(F)** Viable conceptus with embryo (*em)* on day 10 and conceptus that subsequently underwent embryonic resorption. The diameter of the dying conceptus is smaller than the diameter of the viable conceptus. **(G)** Same resorption site 1 day after death (day 11) with increased diameter. The placenta *(pl)* and the embryonic cavity *(ec)* are visible. **(H)** Same resorption site 2 days after death (day 12). The embryonic cavity (*ec)* has decreased in size and the placenta (*pl)* lost its integrity. The diameter has now visibly decreased. **(I)** Same resorption site 3 days after death (day 13). The embryonic cavity (*ec)* and the placental folds (*pl)* have disappeared. **(J)** Same resorption site 14 days after death (day 25). Uterine scar tissue has formed. **(K)** Viable conceptus on day 15. The embryo (*em)* is surrounded by its amnion (*a)*. **(L)** Resorption site on day 15. Within the embryonic cavity (*ec)* the embryo *(em)* is disintegrated while the placenta (*pl)* is still intact. **(M)** Same resorption site 1 day after death (day 16) with progressive disintegration of the embryo (*em)*. Within the embryonic cavity *(ec)* echodense material is visible. The placenta (*pl)* remains unchanged. **(N)** Same resorption site 2 days after death (day 17). The diameter has increased. The embryo (*em)* and membranes (*m)* are disintegrated. The placenta (*pl)* shows unsharp borders. **(O)** Same resorption site 4 days after death (day 19). Within the embryonic cavity (*ec*) the embryo and its membranes have completely disappeared. The placenta (*pl)* lost its integrity. (The bar represents 0.5 cm.)

3.2. Intragestational regression of CL in context to embryonic resorption

Sonographically, viable CL of pregnancy appeared as well-defined, echoic bodies with clear demarcation to the surrounding tissue. The typical wheel-like shape was characterized by a central scaffold of echoic tissue surrounded by a less echogenic halo (Fig. 4A&B). The CL increased in echodensity and size with the proceeding pregnancy until day 35 of pregnancy. In general, when the resorption of one embryo was ultrasonographically detected, the regression of one CL of pregnancy was also detectable at the same time. In peri- and post-implantation resorptions, the first morphological evidence for embryonic death was the initial regression of one CL on the ipsilateral ovary during the ongoing pregnancy. Thus, luteal regression was observed while the embryo was still alive which was evident by the ultrasonographic detection of the embryonic heartbeat. Usually, one to two days later the death of the embryo could be confirmed by ultrasound. It was observed that embryonic resorption and CL regression happened in a one-to-one-relationship. The number of resorptions did not significantly differ from the number of regressing CL (Wilcoxon signed-rank test, V=199, p = 0.14). In 91% of the cases the regression of one CL was observed while a conceptus was undergoing resorption. The remaining 9% were explained by pre-implantation resorptions when CL regression occurred early in pregnancy due to non fertilized ova or embryos that did not survive day 5/6 of pregnancy when they were sonographically detectable (see 3.1.1.).

By ultrasound the morphology of regressing CL accompanying embryonic resorptions (Fig. 4C&D) was similar to the morphologic degradation process characteristic for postpartal luteolysis (Fig. 4E&F). Regressing CL were characterized by a decrease in echodensity and loss of the wheel-like structure (compare Fig. 4C). The CL decreased in size and lost the clear demarcation to the surrounding tissue (compare Fig. 4D). The remaining structures of a regressed CL could remain visible for several weeks (compare Fig. 4B) even throughout a following pregnancy. In case of total litter resorption during the pre-implantation period (see 3.1.1.) the remaining CL underwent regression by following a pseudopregnant cycle. 14 days after ovulation they were no longer ultrasonographically detectable. CL regression was completed faster in case of total litter resorption than in litters showing partial litter resorption where at least one live offspring was born. If only single conceptus were resorbed, regressed CL remained in form of scar tissue as long as the pregnancy was maintained and only disappeared completely after the death of the last conceptus or after birth.

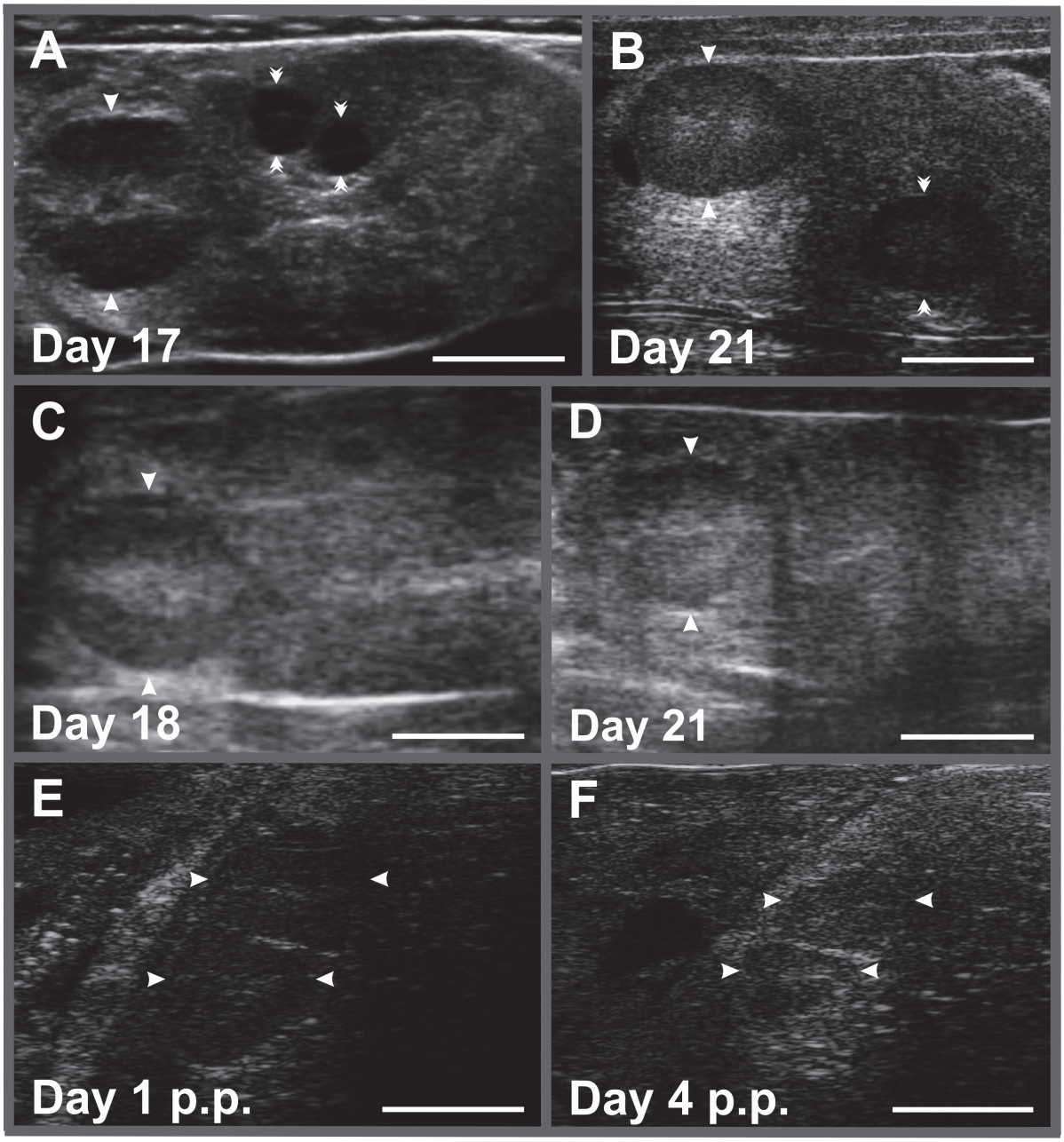

Fig. 4. Ultrasonograms of CL (arrowheads) on different pregnancy days. (A & B) CL of pregnancy. (A) Typical CL (day 17) showing the well-defined echodense body with the central scaffold. Next to the CL, two follicles are seen (pinnate arrowheads). **(B)** CL (day 21) of pregnancy showing clear demarcation to the surrounding tissue. On the same ovary, a regressed CL (pinnate arrowheads) is remaining that accompanied a former resorption process (intragestational regression). **(C & D) CL in regression in context to an embryonic resorption. (C)** Same CL (day 18) as shown in Fig. 4A, but now in regression. The CL shows demarcated borders. **(D)** Same CL as shown in Fig. 4A, now in regression (day 22). A clear differentiation between the borders of the CL and the surrounding tissue is not possible (compare Fig. 4A&B). **(E & F) Two CL on day 1 and 4 *post partum* (p.p.).** CL regression p.p. is similar to intragestational CL regression during embryonic resorption. **(E)** Two CL (day 1 p.p.). The demarcation of the borders and the loss of the wheel-like structure are seen. **(F)** Same CL (day 4 p.p.) showing a decrease in size. The luteal borders are difficult to distinguish from the surrounding tissue. (The bar represents 0.5 cm.)

4. Discussion

In this study, embryonic resorption and CL regression were monitored for the first time over the complete course of pregnancy in a small polytocous mammal, the European brown hare.

The resorption process in the hare was characterized by an initial increase in the mean diameter before a size reduction was observed. This might be due to a massive influx of maternal blood into the resorption site as shown in previous resorption studies on hares [5]. Continuous ultrasonographic imaging of the course of the resorption process revealed that the placenta (or its earlier developmental stage) was the last structure being affected by the resorption process. Although the placental barrier might already be affected by degeneration processes, the typical bilobar endometrial folds were still visible by ultrasound. It can be assumed that the constant blood supply of the maternal decidual tissue is responsible for its late degeneration [21]. The scar formation at the former resorption site can be explained by typical endometrial transforming processes that are responsible for the uterine tissue remodelling after parturition [22]. Although complete disintegration of fetal structures including bones can be observed by ultrasound until day 36 of pregnancy, after day 33, dissoluted fetal remnants were usually discharged from the uterus. Until now this phenomenon of complete embryonic disintegration in very late pregnancy stages was only described once in the rabbit [23]. Hafez [24] observed degeneration in late fetal stages but did not observe complete resorption of the bony structures. In polytocous species this late resorption might be explained by the fact that pregnancy has to be continued until the birth of the remaining viable conceptus. This is in contrast to pregnancy failure in monotocous species where abortion or discharge of the embryonic remnants terminates pregnancy and initiates the new reproduction cycle.

The highest daily incidence for embryonic resorption was determined in the peri-implantation period on day 8 of pregnancy outlining the delicate process of implantation and placentation. The daily incidences of embryonic resorptions decreased as the pregnancy progressed (compare Fig. 1). With 24% of ultrasonographically detected post-implantation resorptions the hare displays a higher resorption rate when compared to other polytocous mammals such as the dog (11%) [4]. After implantation, a relatively high proportion of conceptus died on day 13 and 15 of pregnancy during embryogenesis (until day 20 of pregnancy) and on day 23 when fetogenesis had already started [19]. To identify underlying functional mechanisms causing embryonic resorptions on certain days of pregnancy, more information on the embryonic and fetal development is required. In the dog, some authors reported that embryos showing a general retarded development during pregnancy tend to fail [4]. This idea is supported by our observations in the hare that the mean diameter of conceptus undergoing resorption measured on the exact day of death was significantly smaller compared to viable conceptus (compare Fig. 2). A negative impact of anaesthesia on

embryonic growth potentially causing subsequent resorptions could not be observed. However, in this study the majority of the females were examined without anaesthesia. The mean litter size was similar to previous breeding seasons and to mean numbers from other populations [5,18,19].

In the hare, maintenance of pregnancy is completely dependent on luteal progesterone production [25] implying that the embryo needs to send out signals to prolong the life span of the CL throughout the entire pregnancy period. This is in contrast to other species where pregnancy is not totally CL dependent and luteal regression occurs soon after midgestation when progesterone production is taken over by the placenta [26,27,28]. Until now, few embryo signals for maintaining luteal hormone production could be identified [28,29] such as interferon-τ in ruminants (bovines, sheep and goats) [30,31] and placental luteotropin in rabbits [32,33,34]. Luteotropin sustains luteal function by follicular production of estradiol, the major luteotropic hormone in the rabbit [35,36,37,38]. A recent study in the rabbit revealed embryonic resorptions in context to low serum levels of estradiol [39] indicating a signalling failure of the embryo.

Interestingly, in the hare intragestational regression of single CL was observed in context to embryonic resorption. It is possible that the general progesterone substitution in the hare is reduced by a one by one reduction of CL in case of embryonic resorption. However, this scenario is probably unlikely as such a “stepwise” reduction of the general progesterone level would dramatically reduce the survival chances of the remaining embryos. Moreover, in none of the cases was it observed that the number of embryos exceeded the number of CL. Hence, it appears that the remaining CL cannot replace the function of the regressing CL to guarantee the survival of a surplus embryo. This leads to the suggestion that the factors responsible for pregnancy maintenance might be disturbed on a local level. Our observation of ipsilateral embryonic resorption and luteal regression in the hare support that idea.

The concept that one regressing CL corresponds to one conceptus undergoing resorption implies two possible scenarios: first, the embryo fails to trigger the CL life span [39]; second, there is a primary luteal dysfunction [40] which cannot provide adequate progesterone levels for further embryonic development whereas other embryos with viable CL develop to term. For both mechanisms either a local vascular pathway between one conceptus and one CL or specific luteal receptors would be required. In several species (sheep, bovines, guinea pigs, rats and hamsters) a countercurrent transfer mechanism allows a local control between the uterine horn and the adjacent ovary [28,41]. It is not yet known how the utero-ovarian pathway is regulated in the hare but recent investigations suggest a utero-ovarian countercurrent transfer mechanism (unpublished data Schroeder & Szentiks, 2012). In contrast, there is no evidence for a countercurrent transfer in the utero-ovarian pedicle of the rabbit [41]. Research in this species mainly focuses on specific luteal receptors (estrogen receptors, LH receptors) that mediate the CL life span by the expression of certain genes

[42,43,44,45,46]. However, it is not yet possible to reveal the underlying functional mechanisms which would explain whether embryonic or luteal failure is the primary cause for resorption processes in the hare. Moreover, it is not yet known, whether this one-to-one-relationship between a fetus and the follicle from which it originated is specific or random. These ideas might be interesting aspects to be examined in future studies. Due to the high interspecies variation [31] it would be necessary in future investigations to study the vascular anatomy and the specific transfer mechanisms for luteotropic and luteolytic substances in the utero-ovarian pedicle of the hare. Ultrasonographic CL regression should be linked to functional regression by progesterone measurements and histological analysis. Further, hormonal and immunological investigations should be undertaken to identify the possible factors involved in embryo mediated CL regression during partial litter resorption in the hare.
In conclusion, this is the first study describing the occurrence of intragestational CL regression during pregnancy in detail. The very high number of regressing CL that matches the number of resorptions suggests a direct or indirect luteo-placental relationship of each conceptus to a CL, a fact that needs to be further examined in the future.

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Article II.

Theriogenology, 2011, vol. 76, pp. 778-784.

In vivo tissue sampling of embryonic resorption sites using ultrasound guided biopsy

Running title: Biopsy of embryonic resorptions sites

K. Schroeder, B. Drews, K. Roellig, B.R. Menzies, F. Goeritz, T.B. Hildebrandt

Abstract

In the polytocous European brown hare (*Lepus europaeus*) more than 23% of all successful implantations undergo embryonic resorption. The objective of the study was to establish a minimally invasive ultrasound guided biopsy technique to collect embryonic resorption tissue *in vivo*. The sampled material was genetically analysed to determine paternity and the sex of the embryo. Female hares were either mated or artificially inseminated and pregnancy was confirmed by ultrasound on day six of pregnancy. Subsequent embryonic development was ultrasonographically monitored on a regular basis to detect embryos undergoing resorption. Cell material of the resorption site was collected under ultrasonographic control via transabdominal biopsy of the placenta or aspiration of resorption fluid. To avoid breathing movements during the biopsy, the animals were intubated and a short apnoea was evoked by assisted ventilation. The presence of embryonic cells in the biopsy material was confirmed by microsatellite analysis in 11 of the fluid samples (n = 28) and six of the placental samples (n = 8). The lower success rate in the fluid samples was attributed to the abundance of maternal cells which was confirmed by the analysis of fluid sample smears. Male sex of the embryos undergoing resorption was detected by SRY analysis for ten of the fluid samples and for one of the placental samples. The two biopsy techniques did not have any negative impact on the prenatal development of the healthy siblings nor did it influence the future breeding performance of the females that were biopsied.

Keywords: Amniocentesis; Biopsy; Embryonic death; Lagomorpha; Mammals; Pregnancy

1. Introduction

Prenatal death in mammals occurs at all stages of pregnancy. In late pregnancy stages it proceeds as mummification, maceration, putrification or abortion whereas in the zygotic or embryonic stage pregnancy loss appears as embryonic resorption. Resorptions are characterized by lysis of the embryo and reabsorption of the material into the maternal organism [1].

The frequencies of clinically recognized resorptions in mammals are estimated to range between 10% and 17% [2,3]. Since hormone assays used for pregnancy diagnosis in polytocous species do not reveal information about number and health of the embryos [4] ultrasonography is the most reliable clinical method to detect embryos undergoing resorption [5].

A high incidence of prenatal loss has been described in both captive and wild populations of the European brown hare (*Lepus europaeus*) [6,7]. More than 23% of all successful implantations undergo embryonic resorption [8]. Ultrasonographic evidence for embryonic resorption can be found until late pregnancy stages when fetal development is already in progress [9]. To test the hypothesis whether the process of resorption might not be random but dependent on the degree of parental kinship or on the sex of the embryo, an *in vivo* method for sampling resorption sites was required.

In human medicine the first tools developed to diagnose sex and chromosomal patterns of living embryos were ultrasound-guided amniocentesis and chorionic villus sampling (CVS) in 1956 and 1968 [10,11]. Both techniques are now well established and serve as a routine diagnostic tool to detect fetal genetic disorders in women with high-risk pregnancies.

The first report of amniocentesis in veterinary medicine dates from 1975 and described fetal sex detection in cattle [12]. In a lowland gorilla it has been used to determine the stage of fetal maturity [13]. Since 1990 amniocentesis became more common in different animal species. Transabdominal or transvaginal implementation was performed in large animals [14,15,16,17]. For small polytocous species it was performed blindly or after laparatomy through the uterine wall [18,19]. The first report of an amniocentesis in a small polytocous species that was performed transabdominally under ultrasonographic control was published in 2006 [20]. As a veterinary tool for *in vivo* diagnostics CVS was applied to detect fetal sex in only two animal species,the gorilla [21] and the dog [22]. Amniocentesis and CVS are not yet performed as routine diagnostic in any animal species.

Based on these two methods used in human medicine two minimally invasive ultrasound guided biopsy techniques for resorption sites were established. They allow *in vivo* sampling of embryonic and fetal tissue in a small polytocous species like the European brown hare.

2. Materials and Methods

2.1. Animals

Hares were housed at the field research station of the Leibniz Institute for Zoo and Wildlife Research. Female hares were either mated or artificially inseminated [23]. The study included 32 individual female hares and 21 male hares aged between one and four years. According to previous studies [24], the day of insemination was defined as day one of pregnancy. In the breeding seasons 2008 and 2009, 26 females were successfully mated resulting in 64 pregnancies. Artificial insemination in 19 females resulted in 64 successful pregnancies.

2.2. Standard ultrasonographic examinations

A total of 538 ultrasound exams was performed over 128 pregnancies of which 116 were done on anaesthetized animals via isoflurane anaesthesia (2.5 Vol%; Isoba; Essex Pharma GmbH, Berlin, Germany and 1.5 – 2.0 $Lmin^{-1}$ oxygene) or via intramuscular administration of 15 mg/kg ketamine (Ketamin 10%; WDT) and 2.3 mg/kg xylazine (Rompun 2%; Bayer HealthCare). The majority of examinations was arranged without anaesthesia (n = 422), the animal being manually hold in dorsal recumbency (n = 82) or placed in a specially designed restraint box (n = 338) where other examinations (blood sampling from the ear vein, general health check) were possible (Fig. 1A & B) [Röllig, 2009]. Ultrasonographic exams were performed using a real time, B–mode ultrasound unit (Diasus; Dynamic Imaging, Livingstone Scotland) equipped with three different linear probes (5–10, 8-16, 10-22 MHz). Each pregnancy was monitored one to 17 times over its whole length of 42 days whereby the size of the ovaries, the number of corpora lutea and the development of the embryos was documented [24].

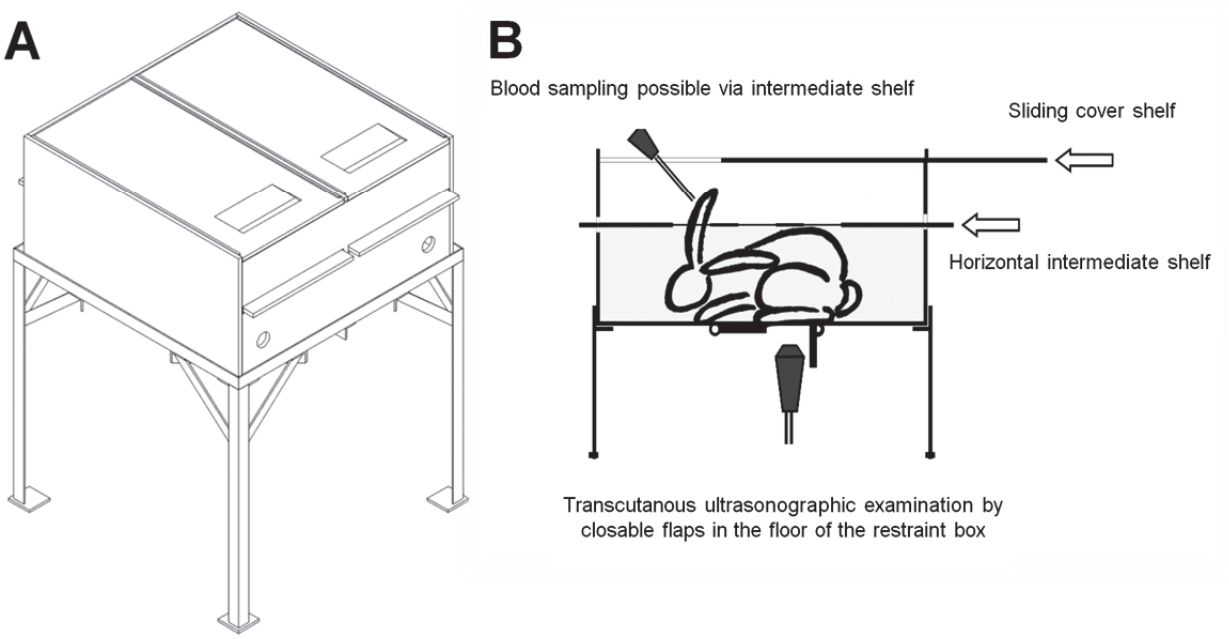

Fig. 1. (A) Restraint box (exterior view). Double examination box for two hares. Both hares are separated by an intermediate board. Transabdominal ultrasonographic examinations are performed through openings in the floor of the box. **(B) Restraint box (interior view).** After shaving and positioning of the hare, the box is closed by a cover shelf. A horizontal intermediate shelf is restraining the animal on the floor. Two flaps in the floor can be opened for transabdominal ultrasonographic examination. Openings in the intermediate shelf allow the sampling of ear blood [Röllig, 2009].

2.3. Biopsy procedure

Intubation of hares was necessary to monitor and control breathing movements during the biopsy procedure. Anaethesia was initiated by the administration of a combination of ketamine and xylazine (see 2.2.). The anaesthetised hares were placed in dorsal recumbency and endoscopically intubated (endotracheal tube, I.D. 3.5 – 4.0, O.D. 17 – 20; Rüsch; Germany) using a rigid endoscope (optic: 2.7 mm, 30°, 30 cm; Karl Storz, Germany) (Fig. 2A). Anaesthesia was maintained by a constant influx of isoflurane (see 2.2.) and oxygene (0.2 - 1 $Lmin^{-1}$).

Fig. 2. (A & B) Biopsy procedure. (A) An intubated hare with pulseoxymeter. **(B)** Aspiration of resorption fluid under ultrasonographic control. The syringe attached to the biopsy needle contains the brownish aspiration fluid. **(C) Cytology** (white bar: 20 µm). Smear of aspirated resorption material showing multiple cell necrosis of maternal red blood cells (rbc) and white blood cells (wbc) visible by a vacuolated cytoplasm (vac) with irregular shape and distorted cell walls, and cellular debris (d).

After shaving the abdominal region, ultrasonographic survey was performed to detect the position of the resorption sites and of the surrounding healthy conceptus. The area was subsequently disinfected (Braunol; B. Braun; Germany). Sterile ultrasound transmission gel (Aquasonic100, Parker Laboratories, Inc Fairfield, N.J. 07004) was applied to ensure optimal coupling.

High frequency ultrasonographic systems equipped with a linear transducer (12 MHz, Voluson i, GE HealthCare, Au; 24MHz, Vevo 2100, VisualSonics, Canada) permitted exact localisation of the area to be punctured and the ultrasonographic guidance of the biopsy needle. A short apnoea was evoked by assisted ventilation (20 breaths/min, 100% medical oxygene, 2.5 – 3.0 Vol% isoflurane) to avoid breathing movements during the biopsy.

For aspiration of embryonic resorption fluid, an echomarked biopsy needle (22 ga, 11 cm Echonox Chiba type or Chiba type, Sterylab, Italy; 20 ga, 22 ga, spine-ject, M. Schilling GmbH, Gelnhausen, Germany) with a Quincke point was used. The needle was inserted under permanent ultrasonographic control transabdominally through the uterine wall into the resorption site (Fig. 3C) and fluid was collected via aspiration (Fig. 2B). In advanced resorption sites the highly condensed material was flushed with physiological saline solution prior to aspiration. Between 0.1 and 4.0 ml fluid were aspirated. For later analysis, aspirated fluid was preserved on smears, in liquid nitrogen and in RNAprotect CellReagent (Qiagen GmbH, Hilden, Germany).

Extra embryonic tissue was taken from the placenta of the resorption site (Fig. 3D) using a 18 G, 20 G or 22 G biopsy needle with mandrin (18 to 20 ga spine-ject, M. Schilling GmbH, Gelnhausen, Germany). Tissue quantity was increased by the use of punch biopsy needles with a larger needle diameter (16 to 18 ga, TruCut, Allégiance Santé S.A. Châteaubriant, France). After the biopsy, 13.3 µg/kg antisedan (Antisedan; Pfizer) was administered and the hares were surveyed until full recovery.

All hares were ultrasonographically examined the day after the biopsy procedure. Follow-up examinations were performed in intervals of one to three days to monitor the possible impact of the anaesthesia and the biopsy on the health of the mother and the remaining fetuses. Resorption sites were observed until they could not longer be detected. The development of the remaining conceptus was documented until term and their number was compared with the number of offspring delivered.

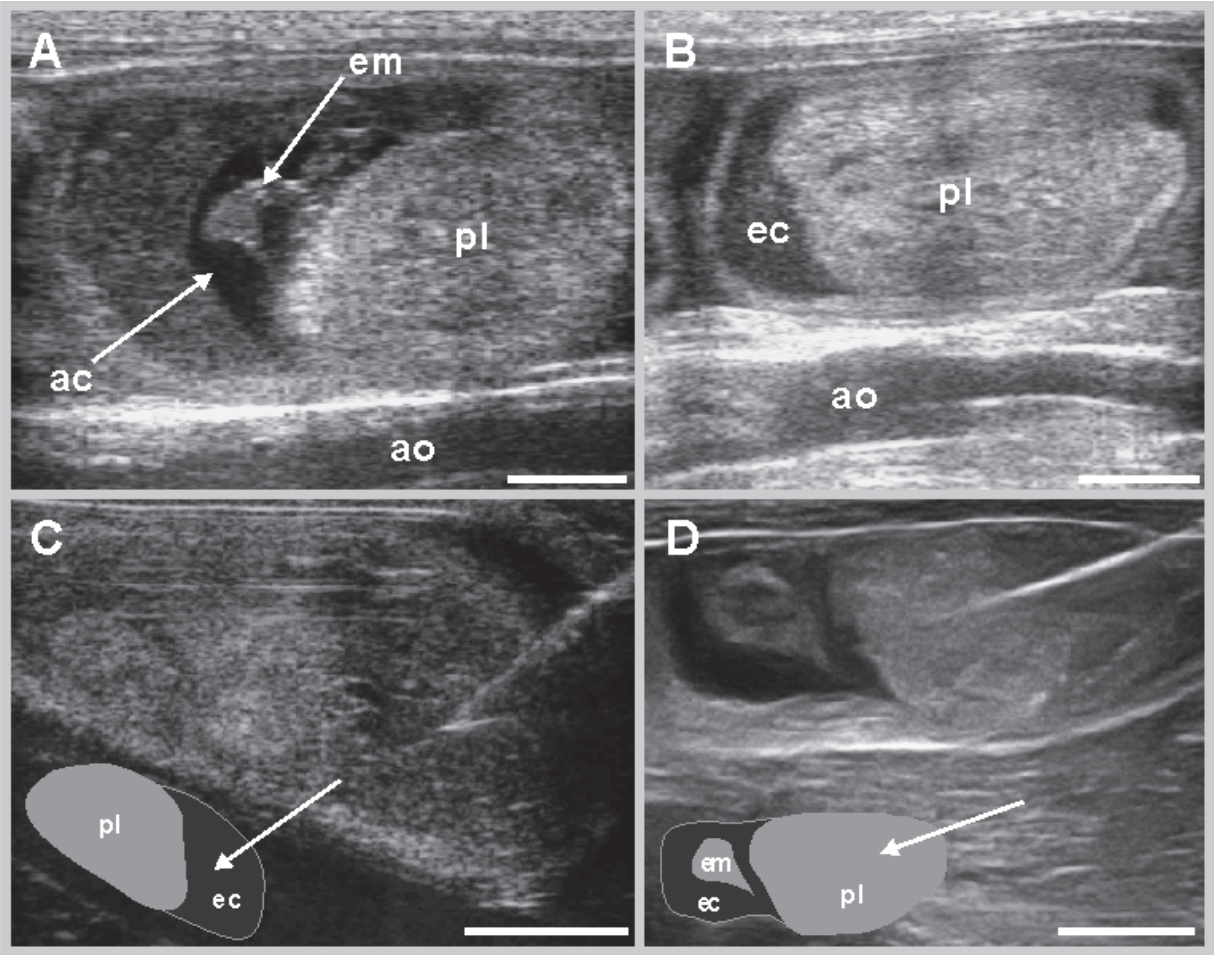

Fig. 3. (A & B) Sonograms of an embryo undergoing resorption (white bar: 0.5 cm). (A) Conceptus on day 14 of pregnancy: The embryo (em), the placenta (pl), the amniotic cavity (ac) and the aorta abdominalis (ao) are visible. **(B)** Resorption site on day 14 of pregnancy: the embryo has disappeared and the embryonic cavities (ec) are disintegrated; the placenta (pl) is still remaining; the underlying aorta (ao) is visible in longitudinal section. **(C & D) Sonograms of the biopsy procedure (white bar: 0.5 cm). (C)** Aspiration of resorption fluid from the disintegrated embryonic cavity (ec) with a spinal needle (arrow) under ultrasonographic control. The placenta (pl) appears as an echodense structure. **(D)** Biopsy of extra embryonic tissue from the placenta (pl) with a TruCut-biopsy-needle (arrow). Within the disintegrated embryonic cavity (ec) the remains of the embryo (em) can still be seen.

2.4. Detection of embryonic DNA in the resorption material via microsatellite analysis

Resorption fluid and tissue samples were immediately extracted after the biopsy procedure or frozen at -80 °C. Fluid samples were centrifuged briefly (10000 g for 30 sec) to collect cellular debris. Supernatant was removed and cells were resuspended in 250 µl 10 mM Tris HCl buffer. Total cellular DNA was extracted using a column hybridization kit (Peqlab, Germany) following the manufacturers instructions.

Embryonic DNA of the resorption sites was detected by microsatellite analysis using the previously established rabbit markers including Sol08, Sol28, Sol30 [25], Sat02, Sat12 and Sat13 [26], Lsa2 [27] and 12L4A1 [28]. The number of loci tested was limited by the quantity of DNA extracted from the small amounts of fetal tissues. Since resorption material is composed of maternal and embryonic cells, the signals of the embryonic alleles were compared to the signals of the maternal alleles. Therefore maternal microsatellites were typed before (n = 32). Detection of an embryonic rather than maternal genotype was proved by either amplification of a single embryonic microsatellite allele, or amplification of the male sex-specific SRY gene. For paternity analysis paternal microsatellites were typed (n = 21) and paternity was determined by comparison to the embryonic genotype.

SRY amplification used the hare specific primers (F: ATGTTCGGAGCACTGTGCAG; R: TCACGGCTGTAATTTATGGT) designed from genebank sequence EF437194. The primers amplify a 612 bp region of the European brown hare SRY gene using the following PCR reagents and conditions: 12.5 µl GoTaq Green Mastermix, 2X (Promega, Mannheim, Germany), 0.4 µM of each primer and 1 µl of gDNA (< 20 ng) in a 25 µl total reaction volume. PCR conditions included an initial step of 95 °C for 5 min, then 40 cycles of 95 °C for 30 sec, an annealing step for 30 sec (Table 1), 72 °C for 30 sec and a final extension step of 72 °C for 5 min. Positive bands were visualised by electrophoresis in 2% polyacrylamide gels using GelRed (Biotium Inc., Hayward, CA). Samples without template (negative controls) were made with every PCR test to exclude amplification of contaminating laboratory DNA. These were consistently negative in all tests.

Table 1

Annealing temperatures (T_A) of the different primers, their allele number and range of basepairs (bp) with references.

Locus	Alleles	Range in bp	T_A	References
Sat02	9	236-264	58 °C	Mougel et al. 1996
Sat12	9	111-145	54 °C	Mougel et al. 1996
Sat13	5	114-124	54 °C	Mougel et al. 1996
Sol08	6	107-125	58 °C	Rico et al. 1994
Sol28	9	150-202	52 °C	Rico et al. 1994
Sol30	6	180-200	58 °C	Rico et al. 1994
12L4A1	6	158-168	50 °C	Korstanje et al. 2003
Lsa2	5	245-259	50 °C	Kryger et al. 2002

2.5. Cytology

The aspirated material from resorption sites was immediately preserved on smears and stained by the Pappenheim method (May-Grünwald solution, Giemsas solution, Roth, Karsruhe, Germany; buffer tablets 7.2, Merck KGaA, Darmstadt, Germany).

2.6. Statistical analysis

For statistical analysis SPSS version 16.0 (SPSS, Chicago, IL, USA) was used. Values are presented as the mean $\pm$ s.d. Fisher's exact test was used to evaluate differences between abortion rates in pregnancies where biopsy was performed or not performed. Values of < 0.05 were considered to be statistically significant.

3. Results

3.1. Embryonic resorption and biopsy

The use of ultrasound allowed clinical detection of resorption sites on day eight of pregnancy after implantation was finished. Ultrasonographically, resorptions are characterized by the death of the embryo, the subsequent disintegration of the embryonic membranes and the reabsorption of the conceptus (Fig. 3A, 3B).

Biopsies could be taken of resorption sites that occurred on day 12 of pregnancy or later. Due to the small size of the resorption site biopsy was technically not possible in earlier pregnancy stages.

In total, 45 biopsies in 28 pregnancies (n = 16 individual females) were performed. 34 aspirations of resorption fluid were successfully performed in 25 pregnancies (n = 15 individual females). In three cases, fluid aspiration failed due to the condensed nature of the material from the resorption site. Placental samples were successfully collected from eight resorption sites in six pregnancies (n = 5 individual females).

3.2. Genetic analysis

Total DNA was extracted from 28 fluid samples and eight extra embryonic tissue samples.

In the placental samples (n = 8), embryonic DNA was detected in 6 samples by microsatellite analysis. For all of them paternity was determined. Two of the samples showed signals that overlapped with the signals of the maternal alleles. For them paternity analysis was not possible. SRY analysis was performed in five of the samples of which one was proved to be male sex.

In the fluid samples (n = 28), microsatellite analysis detected embryonic DNA in 11 samples. For 17 samples detection of embryonic DNA failed because the genotypes showed only maternal alleles. In these cases paternity analysis was not possible. Evidence for SRY gene was shown in ten cases.

If SRY analysis was negative but embryonic DNA was detected via microsatellite analysis, the sex of the resorption site was considered female.

As the fluid samples taken in 2008 and 2009 contained low quantities of embryonic DNA (see 3.3.) in 2010 only placental biopsies were performed. Paternities could be determined for all of the samples (n = 10) where embryonic DNA was detected (n = 5). Male sex was detected in six of the samples.

3.3. Cytological analysis

The aspirated material from 28 resorption sites contained brownish and slightly viscous tissue material, which was immediately preserved on smears (compare Fig. 2B). Cytological analysis of the resorption fluid revealed a high proportion of maternal erythrocytes in the samples. Few inflammatory cells were distinguished such as monocytes, lymphocytes, plasma cells and very rarely neutrophile granulocytes. Cell lysis and cell necrosis were visible by a vacuolated cytoplasm with irregular shape and distorted cell walls, karyorrhectic nuclei, and cellular debris (Fig. 2C).

3.4. Postbiopsy pregnancy progression

After the biopsy none of the females developed a uterine infection. Ultrasonographically, post partum involution of the uterus did not differ from the involution in animals that were not biopsied. All animals had successful pregnancies afterwards. A possible impact of anesthesia and biopsy was excluded because all females showed a normal pregnancy progression and the remaining embryos were born healthy at term. There was no subsequent pregnancy failure or fetal malformation detected. This is supported by a mean litter size of 2.43 + 1.09 for the breeding seasons 2008/2009 that corresponds to the mean litter size of previous breeding seasons [29].

The total abortion rate over the breeding seasons 2008 and 2009 was 11.7%. Both years separately considered showed considerable differences: In 2008, only 3.1% abortions occurred whereas in 2009 20.3% abortions have been noticed. The majority (15.6%) of abortions seen in 2009 can be attributed to an epidemic outbreak of European brown hare syndrome (EBHS) between February and April 2009 at our research station [unpublished data].

The abortion rate in pregnancies (n = 28) with biopsy procedure was 10.7% (n = 16 individual females), and in pregnancies (n = 100) without biopsy procedure 12% (n = 32 individual females) (Table 2). Abortions induced by the EBHS outbreak were included and excluded (Fisher's exact test, EBHS included: P = 0.455, n = 128; EBHS excluded: P = 0.300, n = 128). There was, however, no significant correlation between biopsy procedure and abortion rate (Fisher's exact test: P = 0.455, n = 128).

Table 2

Relationship of the number of aborted fetuses in pregnancies with and without biopsy.

Aborted fetuses	Biopsy		
	No	Yes	Total
0	93	25	118
1	3	3	6
2	3	0	3
3	1	0	1
Total	100	28	128

4. Discussion

The establishment of two minimally invasive ultrasound guided biopsy techniques in the European brown hare allows for the first time the sampling of resorption sites and thus enables the *in vivo* extraction of embryonic material. Both techniques can be prospectively used for a varity of genetic analysis such as the detection of paternity, the fetal sex or embryonic genetic disorders.

Due to the small animal size as well as the small size of the resorption site, the procedure requires considerable time, equipment and effort. Good ultrasonographic machines and knowledge ensure the detection of resorption sites and the guidance of the biopsy needle. The correct positioning of the needle point in the embryonic vesicle is complicated and needs to be performed by an experienced person. Intubation of the animals was a prerequisite for safe biopsy. Because of the very long forehead of the European brown hare, intubation can only be performed with an endoscope. An application of these techniques in larger animals showing slower breathing movements might be possible without intubation. For animals with larger body size a transvaginal or transcervical biopsy should be taken into consideration [12,14,17].

Despite the complicated procedure, implementation of this technique was completed smoothly and did not affect the health of the mother or the remaining conceptus. Regular ultrasonographic survey after the biopsy showed that females progressed normally during pregnancy to term without infection and were able to complete successive pregnancies without complication. Mean litter size corresponded to the previous breeding seasons.

In human medicine the abortion rate for CVS and for amniocentesis varies between 0.5% to 3% [30]. In the European brown hare, the influence of the biopsy procedure on the abortion

rate was tested. Although we can't exclude a negative influence of intrauterine biopsies on the remaining conceptus the results of this study show that there is no significant correlation between biopsy and abortion rate.

Cytological analysis of fluid material from resorption sites showed a very high abundance of maternal cells. This is the likely reason why microsatellite analysis of DNA isolated from aspirated fluid material did not reveal a clear fetal genotype and paternity analysis was not always possible. In our second approach, extra embryonic tissue was taken from the placenta of the resorption site to increase the amount of embryonic cells. The use of a mandrin facilitated the insertion of the needle into the placenta and allowed to get pure placental tissue. The availability of tissue was improved using punch biopsy needles with a larger diameter (16 ga). The greater amount of embryonic cells enabled the extraction of DNA for paternity analysis. A higher sensitivity of the SRY-test allowed amplification of the sex specific SRY-gene even in those cases where microsatellite analysis was not successful.

In conclusion, we have presented an *in vivo* strategy for embryonic tissue sampling in a small, polytocous mammal that displays selective resorption of its conceptus. The application of this method to other small mammalian research models will be integral for understanding whether sex or genotype contribute as a selective influence for resorption in mammalian pregnancy.

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Article III.

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Free blastocyst and implantation stages in the European brown hare: correlation between ultrasound and histological data

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Abstract

The European brown hare (*Lepus europaeus*) is the only species with superconception, whereby the maternal reproductive tract hosts two sets of conceptus at different developmental stages. The embryonic development of the hare has not yet been described. To understand the mechanism of superconception, we studied oviduct transport and implantation stages by embryo flushing and live high-resolution ultrasound. Ultrasound data of implantation stages is correlated with histology. In the oviduct, a mucin coat is deposited on the zona pellucida. The blastocysts enter the uterine horns on Day 5, 1 day later than in the rabbit, and directly expand approximately threefold. Spacing is accompanied by peristaltic movement of the endometrium. The mucin coat disappears and the conceptus attach. The yolk-sac expands in the blastocoel and syncytial knobs invade the antimesometrial endometrium. Maternal blood lacunae appear in the mesometrial endometrial folds, which are subsequently invaded by the syncytiotrophoblast. The haemochorial chorioallantoic placenta forms. The yolk-sac cavity is gradually replaced by the allantois and finally by the exocoel. The different reproductive strategies of the precocial hare and the altricial rabbit are discussed. We assume that the lagomorph-specific mucin coat and the hare-specific delay of the oviduct–uterine transition are prerequisites for superconception.

Additional keywords: Lagomorpha, mucin coat, prenatal development, superconception, syncytiotrophoblast.

Introduction

The preimplantation and implantation stages of the hare (*Lepus europaeus*) have not yet been described. There is only one publication on the placentation of the hare in which only older specimens are described (Strauss 1957). This lack of knowledge may be attributed to the fact that, in most European countries, hare numbers are declining and hunting is forbidden during the reproductive season, so that placental tissues or free blastocyst stages are difficult to obtain. Moreover, correct developmental staging of samples collected from wild animals proves difficult. In contrast, the embryology of the domesticated form of the European rabbit (*Oryctolagus cuniculus*), hereafter referred to as the rabbit, as a model species for biomedical research is well characterised (Beneden and Julin 1884; Hafez 1964; Christie 1967; Enders and Schlafke 1969; Gottschewski and Zimmermann 1973; Hoffman *et al.* 1990; Püschel *et al.* 2010).

Two major points differ between the two lagomorph species rabbit and hare: first, the hare is precocial and has a gestation length of 42 days (Hediger 1984), whereas the rabbit is altricial with a gestation length of 30 days (Novak 1991). Second, in the hare, superconception occurs as a regular reproductive mechanism. Therefore the adoption of developmental events from the rabbit to the hare may be misleading. Superconception implies the contemporaneous presence of fully developed fetuses in the uterus and embryos in the blastocyst stage within the oviduct and increases the reproductive output (Roellig *et al.* 2010a). This paradox situation results from a second mating of a pregnant female on Day 38 of the 42-day gestation period. When the fetuses from the first mating are born, the new embryos can implant in the uterus. Ultrasonographic evidence for superconception is the detection of fresh corpora lutea (CL) 3 days after the second mating next to the fully developed CL of the almost completed first pregnancy.

A growth curve of the embryo of the European brown hare based on ultrasound has recently been published (Roellig *et al.* 2010b), enabling the staging of pregnant tissues derived from females where gestational age is not known. The aim of the present study was to establish reference data for early embryo development of the hare without superconception. We describe the morphology of the embryos in the oviduct and determine their time point of arrival in the uterine horns by embryo flushing of captive hares with known date of ovulation. Furthermore, we correlate ultrasound data of captive hares with histological data of age-matched pregnant animals derived from a wild population to describe embryo–maternal interactions during the peri-implantation period and the formation of yolk-sac, amnion, allantois and the definitive chorioallantoic placenta.

Materials and methods

Animals

Data were collected from captive and free-ranging animals. The captive population of hares was kept at the field research station of the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany, under conditions described previously (Hildebrandt *et al.* 2009). All experimental procedures were in accordance with the German Law for the Protection of Animals. Pregnancy was achieved via artificial insemination (AI). The day of AI was designated as Day 1 of pregnancy. Ultrasound data for the captive population could be exactly assigned to the day of pregnancy. The collection of histological data for the captive population was limited. Therefore, histological samples are derived from a free-ranging population in Suffolk, England, where there is an overpopulation of hares and hunting is used as a means to reduce animal numbers throughout the year. During the course of population-reduction measures, we were allowed to collect the reproductive tracts of females and to search for pregnancies.

Ultrasound examination and embryo flushing of captive hares from the field research station

Prior to the ultrasound examination, hares were anaesthetized by intramuscular administration of ketamine (15 mg kg^{-1}; Ketamin 10%; WDT, Garbsen, Germany) and xylazine (2.3 mg kg^{-1}; Rompun 2%; Bayer HealthCare, Leverkusen, Germany). Anaesthesia was maintained via isoflurane (2.5 vol%; Isoba; Essex Pharma GmbH, Munich, Germany) and oxygen (1.5–1.0 L min^{-1}) administered via an inhalation mask. Transcutaneous ultrasound was performed with a high-resolution ultrasound biomicroscope (Vevo 2100; VisualSonics, Toronto, Canada) equipped with different probes (30–70, 32–56 and 13–24 MHz).

Embryos were flushed from pregnant hares without superconception on Days 3, 4, 5 and 6. Prior to embryo flushing, the number and size of the CL of each ovary was determined by transabdominal ultrasound. Flushing of the embryos was performed in dead animals. The size of the embryos was measured retrospectively from photographs using Cell P* software (Olympus Soft Imaging Solutions, Münster, Germany).

Ultrasound and histological examination of specimens from free-ranging hares

During the course of population-reduction measures conducted by an authorised hunter, we collected the reproductive tracts of 32 female hares. The reproductive tract of pregnant females was removed and ultrasonographically (Diasus; Dynamic Imaging, Livingstone, Scotland: 5–10, 8–16, 10–22 and 16–28 MHz) depicted in a water bath. In total, we could detect 16 pregnancies, of which two were early pregnancies. Pregnancies were staged according to the morphology and size of the CL and conceptus (Roellig *et al.* 2010b). The uteri were opened and two conceptus from Day 8 (hare specimen h22) and one conceptus

from Day 13 (hare specimen h4) were collected. The implantation sites were fixed in 4% paraformaldehyde for 8–14 h depending on their size, washed four times with phosphate-buffered saline at intervals of 4–6 h and preserved in 70% ethanol. After conventional paraffin embedding, serial sections were stained with haematoxylin and eosin (HE) for light microscopic examination.

Results

The location of the embryos described in the different stages of development is shown in Fig. 1.

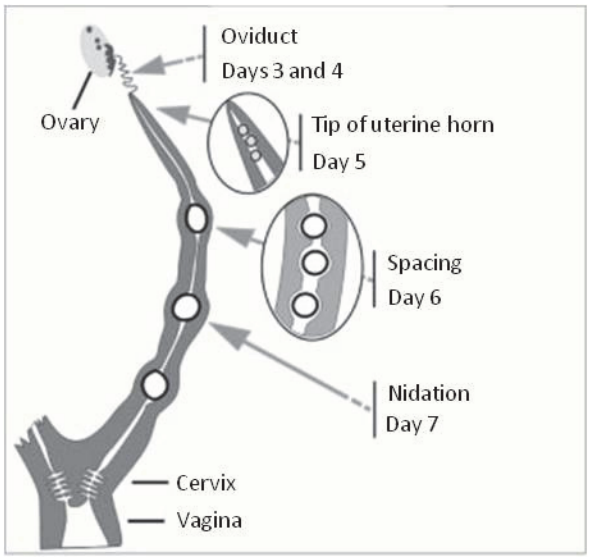

Fig. 1. Schematic drawing of a hare reproductive tract showing the localisation of blastocysts on Days 3–7. On Days 3 and 4, morulae and early blastocysts were flushed from the oviducts. On Day 5, blastocysts were found in the tip of the uterine horns. On Day 6, the free blastocysts were spaced over the length of the uterine horns. On Day 7, the blastocysts started implantation by attaching to the endometrium (nidation).

Day 3

Embryo flushing

Five embryos were collected from the oviducts from two hares. Embryos were at the 4-, 6-, 10- and 12-cell stage (*n* = 2, 1, 1 and 1, respectively). The different blastomeres could be distinguished and exclusion bodies were seen (Fig. 2*a*). Spermatozoa were found within the zona pellucida (ZP). Surrounding the ZP, an additional translucent embryonic coat, the mucin coat, became visible. At the 4-cell stage, the mucin coat was barely visible. The mucin layer increased in thickness from the 6-cell stage (2.1 µm) to the 12-cell stage (10.5 µm). The average size of embryos without the embryonic coats (i.e. ZP and mucin coat) was 90.5 $\pm$ 8.8 µm (*n* = 5).

Day 4

Embryo flushing

Twelve embryos were flushed from the oviducts of seven pregnant hares. Eleven embryos were in the late morula stage (Fig. 2*b*) and one was in the early blastocyst stage (Fig. 2*c*). The morulae were formed by a dense mass of homogeneous cells and were smaller than on Day 3 (73.7 $\pm$ 5.1 µm without embryonic coats), probably due to compaction. The diameter of the blastocyst was slightly larger (90.3 µm) than the diameter of the morulae. All embryos possessed mucin coats, which had increased by approximately 2.5-fold in thickness compared with Day 3. The thickness of the mucin coat varied considerably. Two groups could be distinguished: one group (n = 4) had a mucin coat of approximately 5 µm, whereas the thickness of the mucin coat in the other group (n = 8) was approximately 45 µm.

There were considerable differences in the thickness of the mucin coat between individual embryos (even >40 µm if considering the actual measurements, not the average of the groups with thick and thin mucin coats). For example, we flushed the uterus of one female on Day 4 and recovered four embryos. The diameter of Embryos 1, 3 and 4 was 77, 70 and 74 µm, respectively, and the mucin coat measured only 5, 7 and 7 µm, respectively. In contrast, Embryo 3 had a similar diameter (70 µm) but a thick mucin coat of 64 µm. On Day 4 we flushed the uteri of four more females. In one female, only one embryo could be recovered. This embryo had a diameter of 70 µm and a very thin mucin coat (3 µm). In the three other females, more than one embryo was recovered but no big differences within the embryo sets concerning the size of the mucin coat were observed. These animal numbers are not high enough for generalisation. However, based on the similar size of the blastocysts and the different thickness of the mucin coats within one embryo set and between different females, we conclude that the thickness of the mucin coat is not dependent on the development stage or the individual hare.

The ZP was uniform and measured 11 µm.

Day 5

Embryo flushing

In two pregnant hares, both oviducts and uterine horns were flushed separately. Whereas flushing of the oviducts revealed no embryo, four embryos were collected from the uterine horns. In one hare, two normally developed blastocysts were collected. In the other hare, the two embryos were apparently not in an appropriate stage of development: one embryo was composed of two blastomeres only and in the other embryo single cells could not be distinguished. Both abnormal embryos were surrounded by a regularly developed ZP and mucin coat. The two normal blastocysts (Fig. 2*d*) were approximately threefold greater (228

µm without the mucin coat) than the Day 4 specimens. At lower magnification, the ZP was not visible, although it was still intact (2.8 µm). A dense mass of smaller cells represented the embryoblast.

Ultrasound

Both females were scanned by ultrasound prior embryo flushing on Day 5. The endometrial layers were very prominent and fluid was detected in the uterine lumen (Fig. 2*e*). The uterine horns were characterised by homogeneous, high endometrial folds. The blastocysts could not be identified with certainty. To determine whether the mucin coat was detectable by ultrasound, one abnormal embryo with a thick mucin coat was placed in a bed of agar and scanned by high-resolution ultrasound. The mucin coat could be clearly distinguished from the amorphous cell mass in the centre (Fig. 2*f*).

Day 6

Ultrasound

The unimplanted blastocysts could be seen by ultrasound within the lumen of the cranial part of the uterine horns. The blastocysts were transported down the uterine tract by peristaltic movements of the muscular layer and the endometrium (seeVideo S1 ‘embryo movement’ available as Supplementary Material to this paper). Embryos were observed side by side or singularly. Fluid was visible between the two endometrial layers to each side of the blastocyst and between blastocysts (data not shown). The fluid most likely derives from the uterine glands (Fig. 2*g*). The blastocysts measured 1.8 $\pm$ 0.4 mm (n = 5) in diameter. Around the blastocysts, the endometrial folds had flattened. Between the blastocysts, in cross-section, the thick endometrial folds still filled the uterine lumen (Fig. 2*e*).

Embryo flushing

Two embryos flushed from the uterine horn of one female on Day 6 had diameters of 1.2 and 0.9 mm and were already visible with the naked eye. They were in the blastocyst stage and possessed a ZP (9.7 and 14.1 µm) and a mucin coat (49.7 and 67.0 µm; Fig. 2*h*). The embryoblast could not be visualised. When transferred to a Petri dish, both blastocysts sank to the bottom of the dish and assumed an oval shape. The transfer provoked a partial collapse of the blastocysts, which was reversed after a few hours when their volume augmented again. The ability of the blastocyst to contract and re-expand demonstrates the elastic nature of the mucin coat.

Day 7

Ultrasound

The blastocysts were equally spaced within the uterine horns, as depicted schematically in Fig. 1, and movement of the muscular layer and endometrium had ceased. The shape of the blastocysts was oval and measured approximately 3 mm in its greatest diameter (2.9 ± 0.1 x 2.6 ± 0.1 mm; *n* = 4; Fig. 2*i*).

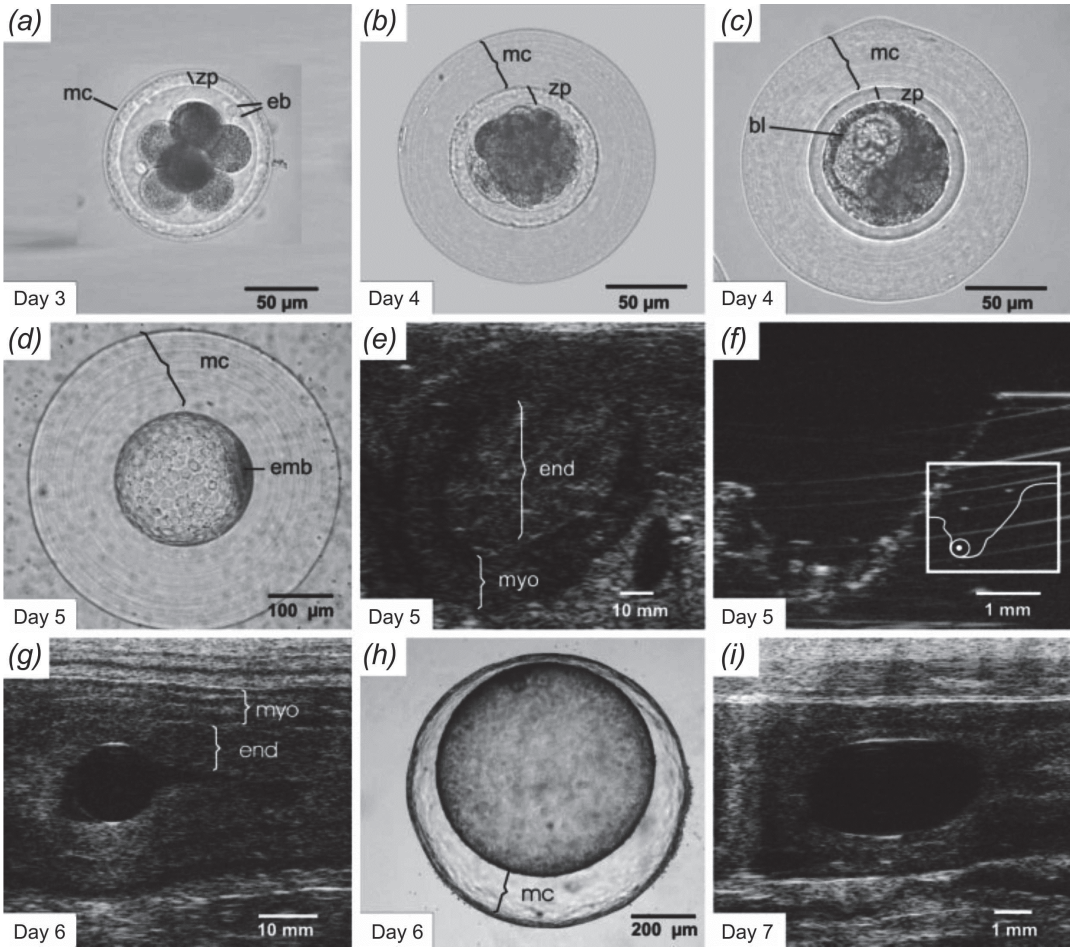

Fig. 2. Embryo flushing and ultrasonography of captive hares. ***(a)*** An 8-cellstage embryo on Day 3 with a zona pellucida (zp) and thin mucin coat (mc). eb, exclusion body. ***(b)*** Morula on Day 4 with a thick mc. ***(c)*** Early blastocyst with the beginning blastocoel (bl) formation on Day 4. ***(d)*** Expanded blastocyst flushed from the tip of the uterine horn on Day 5. The mc is prominent but the zp is not visible. Note the small cells of the embryoblast (emb). ***(e)*** Ultrasound image of the uterine horn in cross-section on Day 5. The endometrial folds and the myometrium (myo) are visible. end, endometrium. ***(f)*** Ultrasound image of a blastocyst on Day 5 placed in an agar bed. The white embryonic cells and the translucent mc are shown. The localisation of the blastocyst in the agar bed is given in the drawing. ***(g)*** Ultrasound image of a free blastocyst in the uterine lumen on Day 6 in longitudinal section. The endometrium (end) and myometrium (myo) can be distinguished. ***(h)*** Microscope image of a blastocyst on Day 6. The thickness of the mc seems unbalanced because the heavy blastocyst sank to the bottom of the Petri dish.[1] ***(i)*** Ultrasound image of an embryo on Day 7. Note the oval shape of the embryo and the second, curved echo indicating the wall of the blastocyst. The space between the endometrium and blastocyst wall is assumed to represent the mc.

[1] Erratum: The position oft the blastocyst is deconcentric because it is not yet fully reexpanded. Therefore, it can be assumed that the surrounding mc is thinner than the bracket indicates.

Day 8

Macroscopy

The position of the embryos found in a pregnant animal of the Suffolk hare population (specimen h22) was conspicuous from bulges of the uterine horns of approximately 7 mm in diameter (Fig. 3*a*). At the site of implantation, the uterine wall at the antimesometrial side appeared very thin and the spiral arteries became pronounced in the mesometrium (Fig. 3*a*, *b*). When the uterus was slit open, the thin wall of the blastocyst collapsed and fluid leaked out. The diameter of the opened implantation site returned to the diameter of the uterus between implantation sites (Fig. 3*c*). The only apparent sign of the implantation was the formation of the characteristic 'endometrial folds' at the mesometrial side, consisting of decidualised mucosa (Fig. 3*d*). The fresh CL that corresponded to the early pregnancy were of a reddish colour (Fig. 3 *a, e*).

Ultrasound

Ultrasound examinations of pregnant hares from our colony on Day 8 revealed the blood supply of the fresh CL using colour Doppler flow (Fig. 3*f*). The conceptus appeared as a fluid-filled structure with the shape of a half oval (Fig. 3*g*). Histology (see below) revealed that the fluid-filled cavity was the yolk-sac. In case of a recent previous pregnancy, old implantation spiral arteries entered the endometrial folds at the mesometrial sites were detected ultrasonographically as uterine scar tissue with a high echogenicity (data not shown). A fact that merits attention is that embryo implantation was never observed at the site of a previous pregnancy.

Histology

In overviews of histological cross-sections of the implantation site of hare specimen h22, the originally oval-shaped blastocyst (8.5 x 5.3 mm and 7.8 x 5.9 mm) as seen by ultrasound (in the live captive population and in the ultrasound examination of the uterus of specimen h22 in a water bath) had collapsed due to loss of its fluid during the dehydration process prior to embedding (Fig. 3*h*). Antimesometrially, the mucosal folds had flattened and the endometrium was thinner than at the mesometrial side, where two major 'endometrial folds' were distinguished. The two muscular layers of the uterus were also thinner on the antimesometrial than on the mesometrial side. The collapsed blastocyst was detected antimesometrially. Remnants of the mucin coat were found in the uterine lumen. The histological cross-section of an adjacent 'non-implantation' site exhibited regular endometrial folds. The endometrium and the muscular layers were of continuous thickness (Fig. 3*i*).

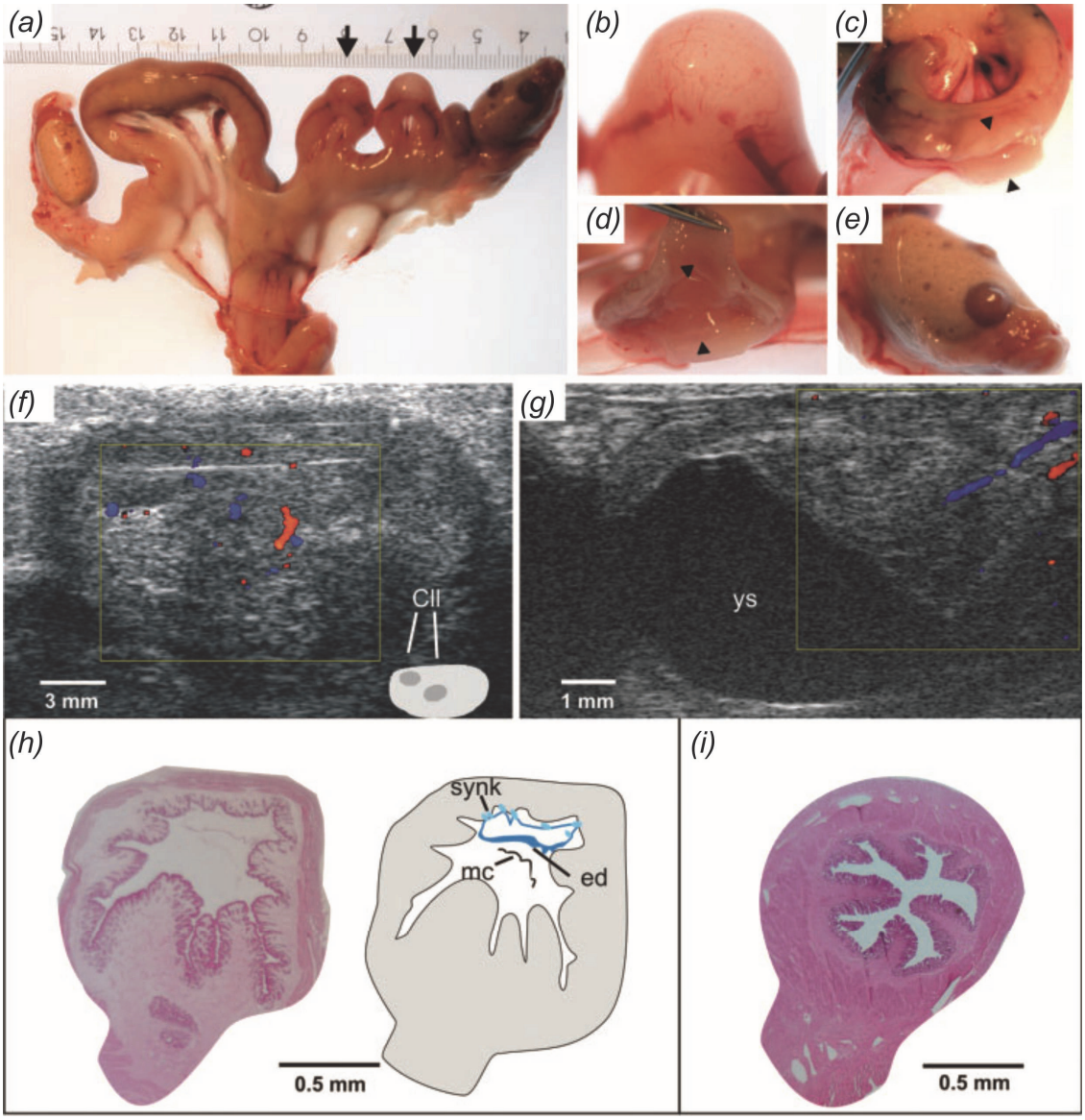

Fig. 3. Day 8 macroscopy of implantation sites found in a pregnant animal of the Suffolk hare population (specimen h22) and ultrasonography of pregnant captive hares. (*a*) Photographs of the reproductive tract. In the right uterine horn, two embryos can be seen as bulges of the uterus (arrows). The spiral arteries are pronounced. Note the corresponding fresh corpora lutea on the ovary. **(*b*)** Close up of the embryonic vesicle caudal to the ovary. Antimesometrially, the uterine wall becomes very thin. **(*c*)** The same conceptus slit open along its longitudinal axis. The diameter of the opened implantation site (arrowheads) corresponds to the diameter of the non-pregnant uterus. **(*d*)** Depiction of the enlarged endometrial folds (arrowheads) on the mesometrial side. **(*e*)** Close up of the ovary with a fresh corpus luteum (CL). **(*f*)** Ultrasound image of a hare ovary from Day 8 of the captive colony. Note the blood supply of the fresh CL depicted by Doppler colour flow (CII). **(*g*)** Ultrasound image of a blastocyst on Day 8. Blood vessels enter the thickened endometrial folds from the mesometrial side. The big anechoic cavity represents the yolk-sac (ys). **(*h*)** Histological cross-section of the implantation site shown in (*a*). Antimesometrially, the endometrial folds have flattened. Mesometrially, two endometrial folds are very pronounced. The position of the embryonic disc, and the attachment of the blastocyst via the syncytial knobs (synk), are indicated in the schematic drawing. Remnants of the mucin coat (mc) are visible between the embryo and endometrium. ed, embryonic disc. **(*i*)** Histological cross-section of the uterus shown in (*a*) at an interimplantation site. The endometrial folds are of equal size and protrude in the uterine lumen. The endometrium and surrounding muscular layer are thicker than at the site of implantation.

In serial sections, we identified the embryonic disc, the yolk-sac and syncytial knobs (Fig. 4*a*). The morphological correlates of attachment observed on Day 7 by ultrasound were the embryonic syncytial knobs. The physical attachment only occurred antimesometrially, whereas no physical attachment was present at the germ disc area at the mesometrial side, so that in histology, an artificial space between the endometrium and the embryo appeared (Fig. 4*a*). Antimesometrially, the blastocyst wall was bilaminar and consisted of thin endoderm and trophoblast. The trophoblast was differentiated into a long drawn-out cytotrophoblast and knobs of syncytiotrophoblast (Fig. 4*b*). The syncytiotrophoblast comprised multiple nuclei and exhibited foamy plasma. Where the blastocyst wall was in close proximity to the underlying endometrium, the connection of the maternal epithelial cells was loosened. Syncytial knobs were also observed at sites where remnants of the mucin coat still covered the blastocyst wall (Fig. 4*c*). At some places, the syncytial knobs attached to the epithelium and protruded between the epithelial cells. Attachment and invasion of the endometrium by syncytial knobs occurred in the close vicinity of the maternal capillaries (Fig. 4*d*).

The embryonic disc exhibited three distinct layers: ectoderm, mesoderm and endoderm. Formation of the neural plate was completed and the primitive streak was identified (Fig. 4*e*). Thick epithelial amniotic folds protruded from the caudal part of the embryonic disc (Fig. 4*f*). The intraembryonic coelom had formed. The endoderm had migrated from the germ disc along the inner side of the blastocyst (Fig. 4*c*, *e*), followed by the angiogenic extraembryonic mesoderm. Formation of the first blood islands was observed (Fig. 4*g*).

Invasion of the syncytium was not only encountered by the syncytial knobs of the blastocyst, but also by insular syncytium in the periphery of the implantation site, where the embryonic syncytium had no connection to the blastocyst (Fig. 4*h*).

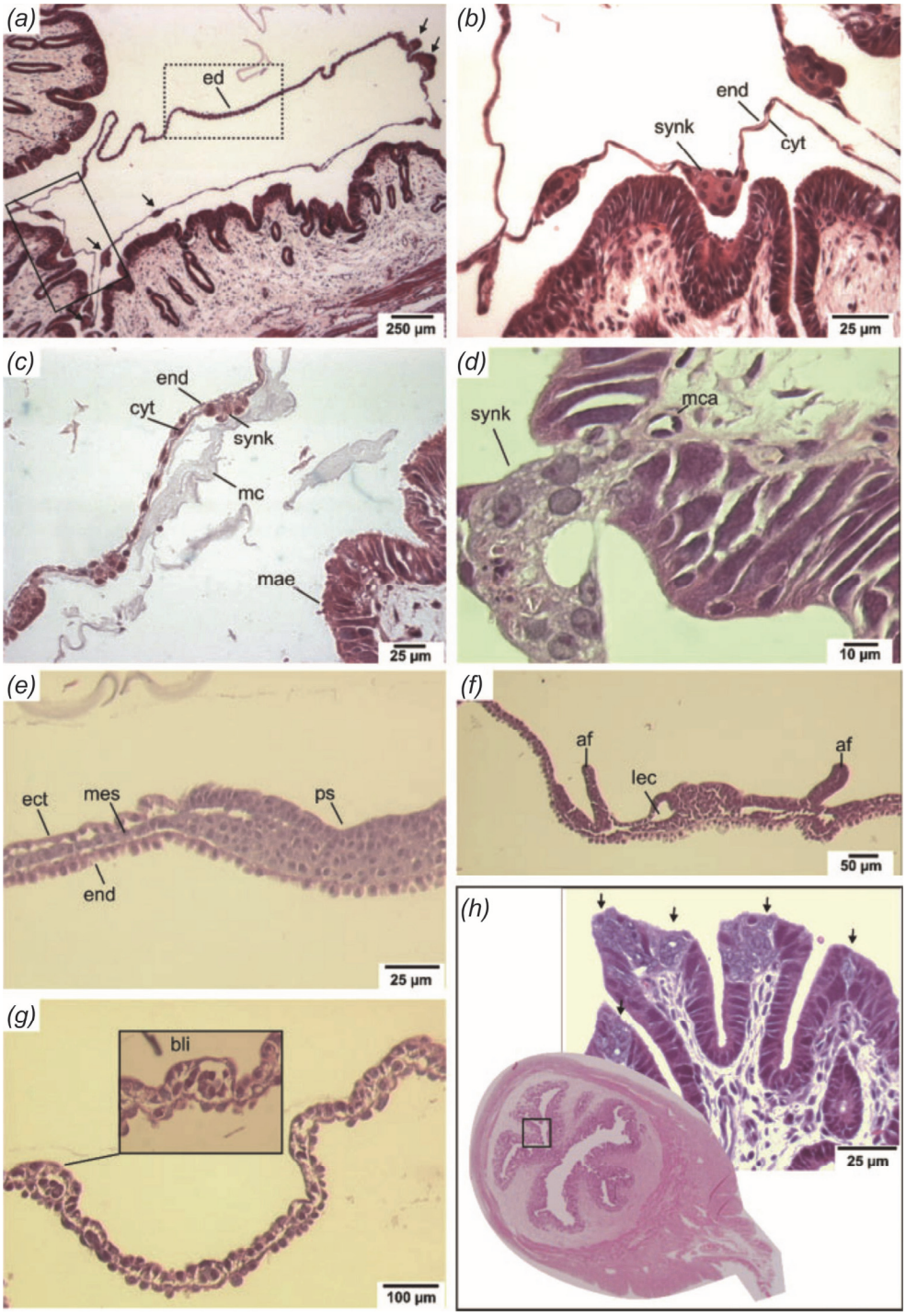

Fig. 4. Day 8 histology of the implantation site shown in Fig. 3*a*. (***a***) The expanded blastocyst as seen by ultrasound has collapsed during fixation. The embryonic disc (ed) is located mesometrially. Antimesometrially, the cytotrophoblast has fused to syncytial knobs (arrows). (***b***) Detail of the rectangle depicted in (*a*). Antimesometrially, the blastocyst is bilaminar. The trophoblast is differentiated into the cytotrophoblast (cyt) and syncytial knobs (synk). end, endoderm. (***c***) The blastocyst wall consisting of cyt and synk with underlying endometrium. At the site shown, remnants of the mucin coat (mc) can be seen between the blastocyst and the maternal epithelium (mae). (***d***) Invasion of the endometrium by a syncytial knob. Note the foamy cytoplasm of the syncytium. Invasion takes place in proximity to the maternal capillaries (mca). (***e***) An embryo with a primitive streak (ps) and distinct germ layers in a more caudal section than in the area in (*a*) delineated by the dashed rectangle. ect, ectoderm; mes, mesoderm. (***f***) Formation of the amnion and extraembryonic coelom at the caudal end of the embryo. af, amniotic fold; Iec, intraembryonic coelom. (***g***) Development of blood islands (bli). (***h***) Invasion of the endometrium by the embryonic syncytium (arrows) at an interimplantation site. The location of the invasion is indicated by the rectangle in the cross-section of the uterine horn.

Day 9

Ultrasound

The two endometrial folds exhibited numerous anechoic cavities (Fig. 5*a*), which corresponded to maternal blood lacunae. The endometrial folds had a thickness of 4.8 mm. The embryo itself was identified as a 0.6 mm long structure lying between the endometrial folds. The main cavity was the extended yolk-sac cavity.

Day 10

Ultrasound

The size of the embryo was 2.2 mm and the external heart with its heartbeat could be visualised by ultrasound. The brain vesicles appeared as an anechoic structure in the head. The allantoic sac protruded from the caudal end of the embryo and the yolk-sac membrane was loosely overlying the abembryonic pole (Fig. 5*b*). The endometrial folds had a thickness of 5.8 mm and were now referred to as placental folds. The maternal blood lacunae had increased further in size. Single lacunae measured up to 3.3 mm in their greatest diameter.

Day 11

Ultrasound

The head with the prosencephalic vesicle and the rump of the embryo became more distinct. The allantoic sac was visible between the two placental folds as a small, anechoic cavity. The placenta measured 8.5 mm in thickness. From the lateral region of the placenta, the exocoel became apparent as a slightly echodense cavity compared with the anechoic yolk-sac (Fig. 5*c*). The embryo was connected via its umbilicus to the placenta.

Day 12

Ultrasound

The embryo was slightly curved and measured 5.5 mm. Its position was at right angles to the placental folds, with its head pointing away from the placenta (Fig. 5*d*). In the head, the prosencephalon, mesencephalon and rhombencephalon were differentiated. The heart protruded from the ventral part and the pericardium could be seen as a very fine delicate membrane enveloping the heart. The thickness of the placental folds was 10.9 mm. The amniotic fluid appeared anechoic, whereas the two exocoelomic cavities, the yolk-sac and the allantois showed different grades of echogenicity. Compared with Day 11, the exocoelom and allantois had increased considerably in size. The yolk-sac still reached the endometrium at the

abembryonic pole. The amniotic cavity was wide around the head of the embryo, but narrowed towards the tail of the embryo.

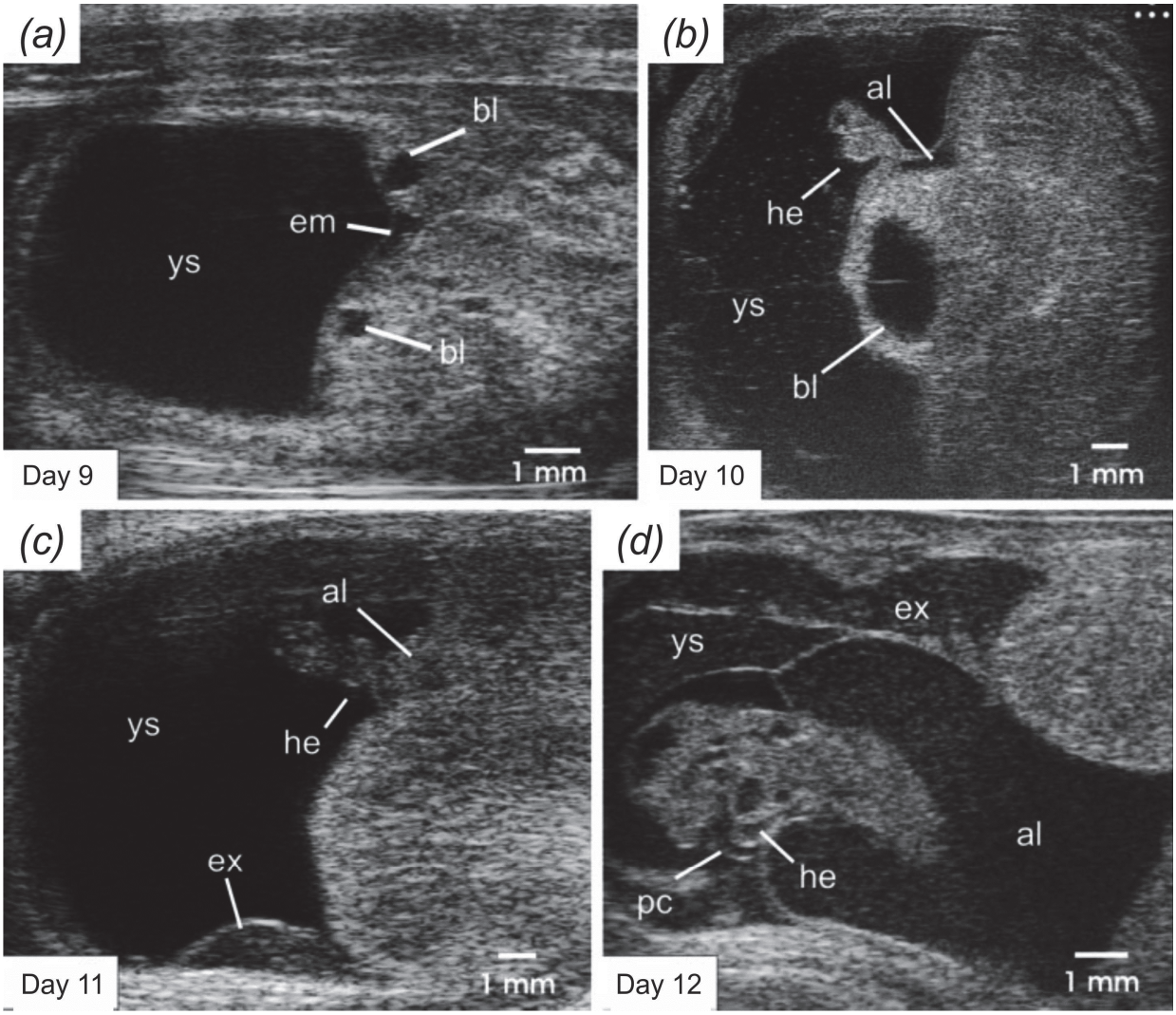

Fig. 5. Days 9–12 ultrasonography of pregnant captive hares. ***(a)*** Conceptus on Day 9. The anechoic cavities in the endometrial folds represent maternal blood lacunae. The embryo can be seen as a small hyperechoic dot between the endometrial folds. bl, blood lacuna; em, embryo; ys, yolk-sac. ***(b)*** Conceptus on Day 10. Embryonic heart (he) and brain vesicles are clearly visible. The allantois (al) is protruding from the caudal part of the embryo. The blood lacunae have increased further in size. ***(c)*** Conceptus on Day 11. The allantois at the caudal part of the embryo is located between the placental folds. At the lateral parts of the yolk-sac, the echodense exocoel (ex) can be seen. ***(d)*** Conceptus on Day 12 displaying the extraembryonic cavities. The heart is protruding from the ventral body wall. pc, pericardium.

Day 13

Macroscopy

Macroscopically, the bulge of the conceptus (hare specimen h4 of the Suffolk hare population) with the uterine wall measured approximately 1.5 cm (Fig. 6*a*). The embryo, with a length of 6.5 mm, was still small in comparison with the placenta (Fig. 6*b*). The head of the embryo was bent and its tail was curled around its protruding heart (Fig. 6*c*). The pericardium covered the heart as a translucent casing. In the embryo presented, the atrium was extended by the arrested blood column. The brain vesicles were prominent and eye cups and pharyngeal arches could be identified.

Ultrasonography

Ultrasound data of a living embryo on Day 13 showed the relationship between the big placental folds and the small embryo (Fig. 6*d*). Within the placental folds, elongated fluid accumulations, indicating maternal blood effusions, were documented. The afferent maternal spiral arteries supplying the placenta were obvious. From the ventral side of the embryo, the yolk-sac opened towards the uterine lumen and the sinus terminalis was evident (Fig. 6*e*). The curled form of the embryo and the protruding heart observed macroscopically were also evident in the living embryo seen by ultrasound (Fig. 6*f*).

Histology

The overview of the serial histological sections reflected the size relationship of the embryo and placenta as observed by macroscopy and ultrasonography. The blood lacunae were evenly distributed in the decidualised part of the placental folds. The invading trophoblast was apparent from its blue staining. It did not reach the blood lacunae (Fig. 6*g*). With higher magnification, anatomical details of the embryo and its extraembryonic membranes could be differentiated (Fig. 6*h*). The amnion was composed of a delicate, flat, monocellular layer with underlying mesenchyme surrounding the embryo. The allantoic membrane stretched over the surface of the placental folds. The inner yolk-sac membrane was composed of tall columnar epithelial cells with irregular processes and a mesenchymal layer carrying blood vessels (Fig. 6*i*) that were filled with nucleated erythrocytes. Details of the embryonic anatomy, such as the mesonephros with its typical glomerulus, the ventral aorta and the intraembryonic coelom, were visible in the histological sections (Fig. 6*j*). The heart still protruded from the ventral wall and the two ventricles could be distinguished (Fig. 6*k*). The trophoblast had formed into columns that invaded the maternal endometrium to a depth that made up approximately one-fifth of the thickness of the placental folds. In the hare placenta, we observed gradual erosion of the maternal endothelium. In Fig. 6*l*, the maternal endothelium is still visible in the left half of the picture but has disappeared in the right half. In the former, the brush border of

trophoblast syncytium is in direct contact with the maternal endothelium. The placental barrier finally consisted of syncytiotrophoblast directly in contact with maternal blood, followed by a layer of cytotrophoblast (Fig. 6*l*).

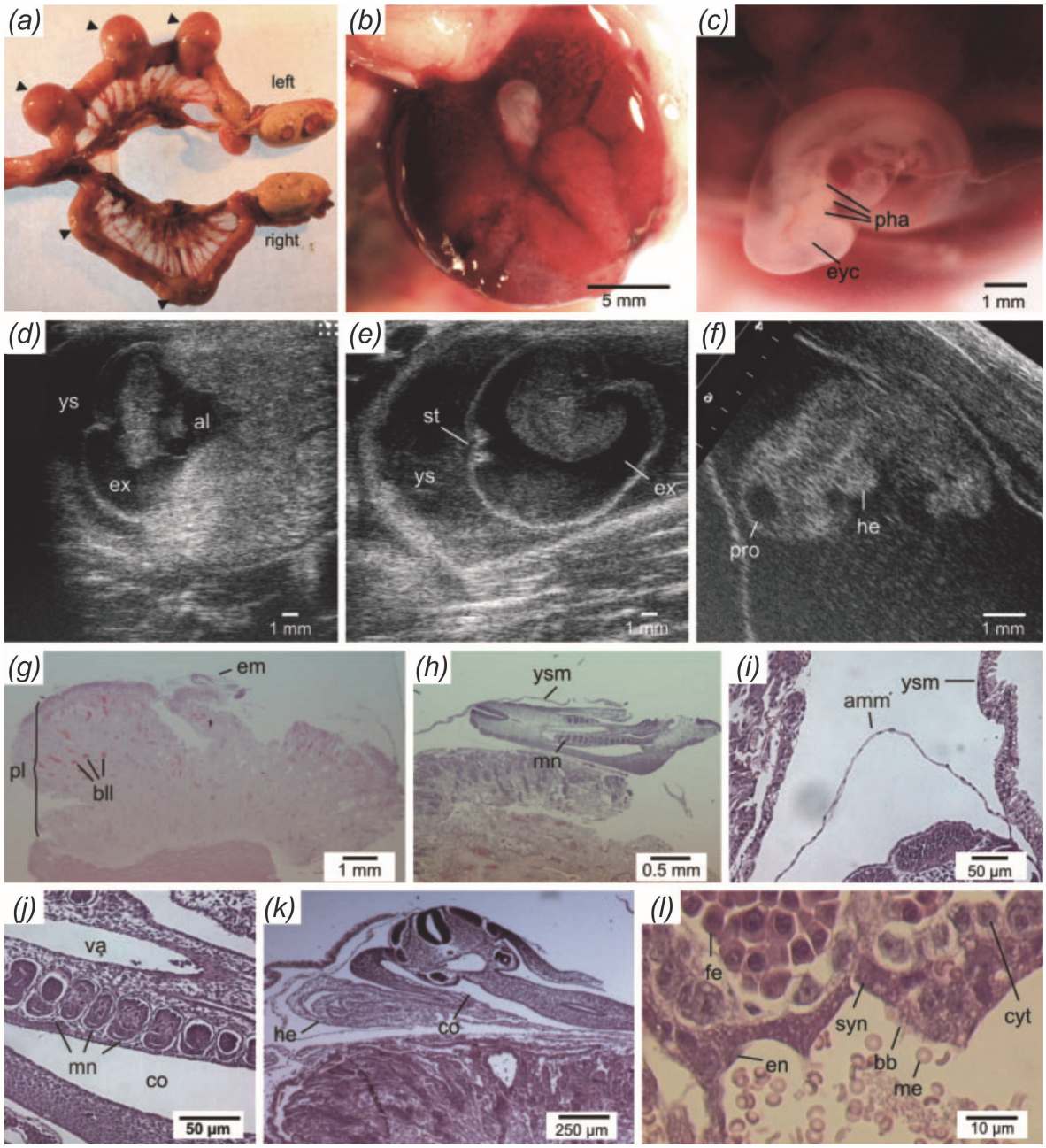

Fig. 6. Day 13 macroscopy and histology of a conceptus (h4) and age-matched ultrasonography of pregnant captive hares. ***(a)*** Pregnant reproductive tract. Note the three fresh corpora lutea (CL) on the left side corresponding to the three embryos (arrowheads). On the right side, the CL are of a greyish colour and small in size. The two irregular bulges (arrowheads) of the right uterine horn are strongly suggestive of embryonic resorptions. ***(b)*** Uterus dissected to show the very small embryo compared with the placental folds. ***(c)*** Close up of the same embryo. Note the protruding heart and the blood congestion in the atrium. The pharyngeal arches (pha) and eye cups (eyc) can be seen. ***(d)*** Whole conceptus with placenta. al, allantois; ex, exocoel; ys, yolk-sac. ***(e)*** Vessels of the sinus terminalis. The embryo is surrounded by its fine amnion (not shown) and the exocoel. ***(f)*** The prosencephalon (pro) and heart (he) are obvious. ***(g)*** Overview showing the big placenta (pl) with its numerous blood lacunae (bll) in relationship to the small embryo (em). ***(h)*** Close up of the embryo. The yolk-sac membrane (ysm) is more pronounced than the fine amniotic membrane that directly envelopes the embryo. The dorsal neural tube, the mesonephros (mn) and the intraembryonic coelom can be seen. ***(i)*** Close up of embryonic membranes. The amnion consists of epithelial cells with underlying mesenchyme. The yolk-sac epithelium exhibits irregular shaped protrusions. amm, amniotic membrane. ***(j)*** Close up of the embryonic mesonephros with the glomerula. The two cavities represent the ventral aorta (va) and intramebryonic coelom (co). ***(k)*** Embryo with external heart, ventral aorta and intraembryonic coelom. ***(l)*** The placental barrier between the nucleated fetal erythrocytes and the maternal erythrocytes consists of cytotrophoblast (cyt) overlying the syncytiotrophoblast (syn). On the left, a maternal endothelial cell isvisible and, on the right, the trophoblast brush border (bb) is in contact with maternal blood. en, maternal endothelium; fe, fetal erythrocyte; me, maternal erythrocyte.

Discussion

This is the first description of the early embryonic development of the European brown hare. We correlated ultrasound data from living pregnant hares without superconception with histological data. It is the timing of pregnancy events without superconception that makes superconception possible in the hare. The results are summarised in Fig. 7 on a time scale. The hare morula develops in the oviduct between Days 2 and 3. The blastocyst cavity appears on Day 4. A threefold expansion occurs with arrival in the uterine horns on Day 5.

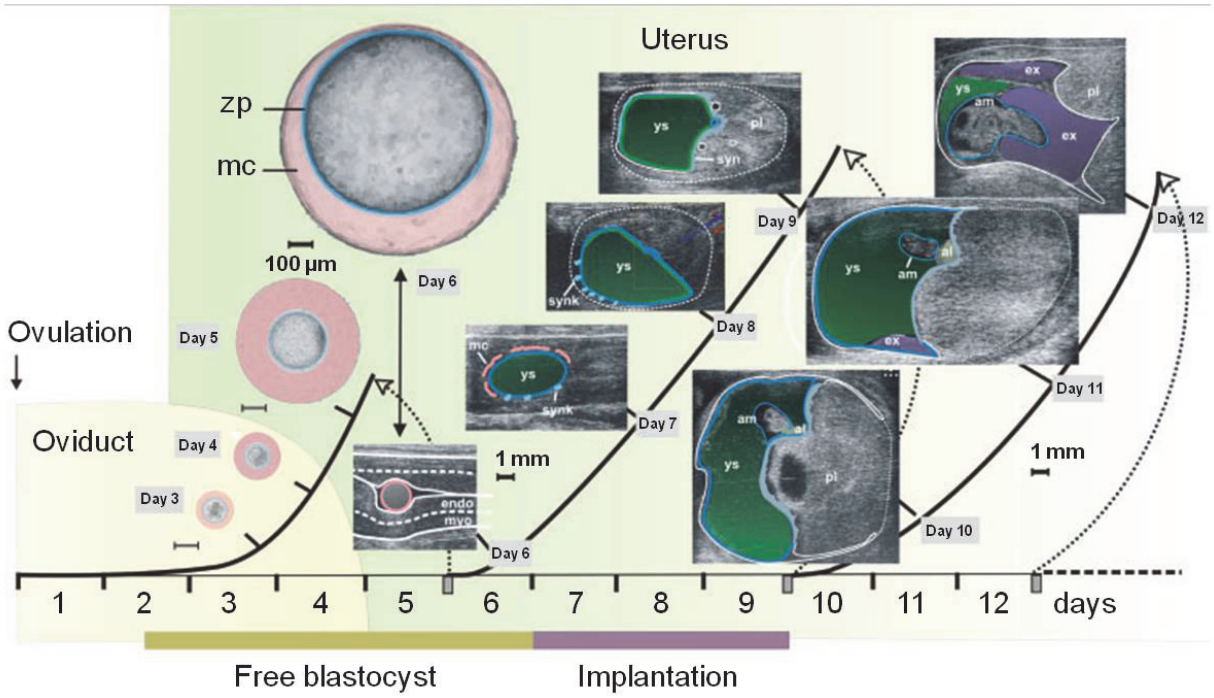

Fig. 7. Schematic overview of embryonic development in the European brown hare. The development in the oviduct involves the formation of the blastocyst and the deposition of concentric mucin layers around the zona pellucida by oviducal cells. The free blastocysts reach the uterine horn on Day 5, where they expand further. Uterine peristalsis moves the blastocysts towards the cervix. Adhesion and breakdown of the mucin coat take place on Day 7, when the blastocysts are evenly spaced within the uterine horns. Implantation is initialised antimesometrially by attachment and invasion of syncytial knobs on Day 8. The germ disc is visible and amnion formation begins. The definite chorioallantoic placenta develops mesometrially by invasion of the syncytiotrophoblast into the endometrial folds on Day 9. The allantoic sac can be seen between the two major placental folds. Head, rump and external heart become evident on Day 10. Between Days 11 and 13, the exocoel begins to displace the yolk-sac from the lateral sides of the conceptus and the embryo has grown all major organs. am, amnion (blue); al, allantois (pale green); ex, exocoel (violet); mc, mucin coat (pink); pl, placental folds; zp, zona pellucida (blue); synk, syncytial knobs (light blue); syn, syncytiotrophoblast (grey); ys, yolk-sac (dark green). The dotted white line shows the border between the endometrium and myometrium, whereas the continuous white line shows the border between the endometrium and uterine lumen/conceptus.

We observed that, during oviduct transport, a prominent mucin coat is placed on the ZP of the hare morulae, which increases in thickness. Although the ZP is common to all mammals and is derived from the oocyte or follicle cells (Boyd and Hamilton 1961), a mucin layer has only been described in the rabbit (Böving 1957; Kane 1975) and marsupial species (Selwood 2000). The mucin coat consists of polysaccharides and is produced by the epithelium of the oviduct. Even artificial objects are covered in mucin while travelling down the oviduct (El-Gayar and Holtz 2009). From the evolutionary standpoint, the mucin coat corresponds to the albumin and egg shell layers in egg-laying vertebrates (Menkhorst and Selwood 2008). The function of the mucin coat in mammals is still under discussion. Experiments in rabbits have shown that the thickness of the mucin layer seems to be an important factor for successful implantation (Murakami and Imai 1996). The mucin coat could also act as anti-adhesive substance to enable uterine spacing prior to implantation (Hohn *et al.* 2003). It is not clear by which mechanism the mucin coat is finally dissolved, but specific proteases in the uterine secretions combined with embryonic factors seem to be responsible (Kirchner and Mootz 1969; Kirchner and Seitz 1972; Denker and Hafez 1975; Fischer *et al.* 1991).

The early embryo development documented in the hare corresponds to the development in the rabbit, where the morula stage is also reached on Day 3 and blastocyst formation begins on Day 4 (Alliston and Pardee 1973). The main difference to the rabbit is the time point of arrival in the uterine horns. In the rabbit, the morula stage enters the uterus between Days 3 and 4 (Daniel 1964; Alliston and Pardee 1973; Warner *et al.* 2003), whereas in the hare the embryo arrives in the tip of the uterine horns on Day 5 at the blastocyst stage (Fig. 7). On Day 6, the hare blastocyst increases approximately 10-fold in size to an average diameter of 1.8 mm and becomes visible by ultrasound (Fig. 7). The preimplantation blastocyst of the rabbit has a diameter of 3 mm (Daniel 1964; Alliston and Pardee 1973; Warner *et al.* 2003). This is almost double the size of the preimplantation blastocyst of the hare. The greater size of the rabbit blastocyst may be attributed to the one ‘extra’ day it spends in the uterine lumen, which enables further fluid uptake and expansion. In both species, implantation occurs on Day 7.

In the hare, we identified peristaltic movements of the myo- and endometrium as the mechanism responsible for the transport of embryos down the uterine lumen on Day 6. In the rat (Kaulenas *et al.* 1991), the pig (Rexroad and Guthrie 1983) and the mouse (McLaren and Michie 1959), only myometrial activity is claimed to be responsible for blastocyst transport. Hormone-dependent endometrial motion has also been described in ultrasonographic studies in humans (Fukuda and Fukuda 1994; Ijland *et al.* 1996).

The implantation stages of the hare were clearly visible with high-resolution ultrasound, which depicts the extended, oval-shaped and equally spaced blastocysts. The cellular details of implantation are below the resolution of ultrasound and are only visible in the histological series of the Day 8 implantation site. In the dorsal and mesometrial moiety of the hare blastocyst, the trilaminar germ disc develops. The embryonic disc is stretched out like an

umbrella above the balloon-like yolk-sac. This corresponds to the development of the germ disc in birds and therefore may be the original form of germ disc development in placental mammals.

In the hare, as in the rabbit, the trilaminar embryonic disc integrates into the trophoblast. In the hare, we observed that the amniotic folds elevate actively, as indicated by the unusually tall columnar amniotic epithelium. Ultrasound depicts the true firm distension of yolk-sac, whereas the yolk-sac is collapsed in histological sections. The ventral antimesometrially located moiety of the hare blastocyst is lined by the endodermal yolk-sac and, after dissolution of the mucin coat and ZP, becomes attached to the uterine epithelium via syncytial knobs. Syncytial knobs have only been described before in the rabbit (Böving 1962; Enders and Schlafke 1971). In our sections, syncytial knobs did not form at the embryonic pole. The induction of syncytial knobs seems to be independent from direct contact of the blastocyst with the endometrium because syncytial knobs were also observed where remnants of the mucin coat were located between the blastocyst and the maternal epithelium. In our serial sections, all the syncytial knobs had direct contact with a subepithelial capillary after invasion of the uterine epithelium, as documented by Böving (1962) in the rabbit. We observed syncytial knobs in the endometrium between implantation sites in the hare, which have not been described in the rabbit. These syncytial knobs were not connected to the trophoblast of the blastocyst. We assume that these knobs were originally coherent with the embryo, but were disconnected from the blastocyst during the early phase of implantation, when the embryo was still moving around by the peristaltic contractions of the endometrium. The fate of these cells remains unclear.

Expression of endogenous retroviral DNA is responsible for syncytial trophoblast formation (Blaise *et al.* 2003; Dupressoir *et al.* 2005). Because the endogenous retroviral DNA also includes an immunosuppressive component, the syncytial knobs between implantation sites observed by us may not only play a role in attachment and spacing, but may also modulate the maternal immune system. The symplasma in the endometrial epithelium (Böving 1962) and the embryo–maternal symplasma described in the rabbit (Larsen 1961; Enders and Schlafke 1969) require confirmation by immunohistochemical or *in situ* hybridisation methods.

After implantation on Day 10 in the hare, the whole embryo with the amniotic cavity protrudes into the yolk-sac (Fig. 7). The yolk-sac cavity then forms a sickle-shaped cavity between the embryo and uterine epithelium. The outer layer of trophoblast and yolk-sac regresses so that the inner layer of the sickle-shaped yolk-sac becomes the definitive outer lining of the conceptus (late inversion in the rabbit; Amoroso 1961; Krespi and Davies 1963; Hamilton *et al.* 1972). The marginal sinus forms in the outer margin of the yolk-sac mesoderm and is clearly visible by ultrasound. On ultrasound, the allantois during early pregnancy appears as a fluid-filled sac. The fluid most likely derives from the functional mesonephros, as depicted by histology (cf. Fig. 6 *j*). The allantoic membrane covers the symmetrical cushions of the

endometrium at the embryonic pole and forms the allantochorionic placenta. The exocoelom (chorionic cavity) is the space between the trophoblast mesoderm and yolk-sac mesoderm. On the ultrasound scans, the exocoelomic cavities appear at the dorsal rim of the sickle-shaped yolk-sac on Day 11 (Fig. 7) and, with further development, also become covered by the extension of the yolk-sac, which invests the whole conceptus and, with its visceral layer, forms the embryonic border towards the uterine lumen.

The main cavity retained until birth is species specific. In the elephant, it is the allantoic cavity. In the human, the chorionic (extracoelomic) cavity is replaced by the amniotic cavity. In the hare, the exocoel is the main cavity.

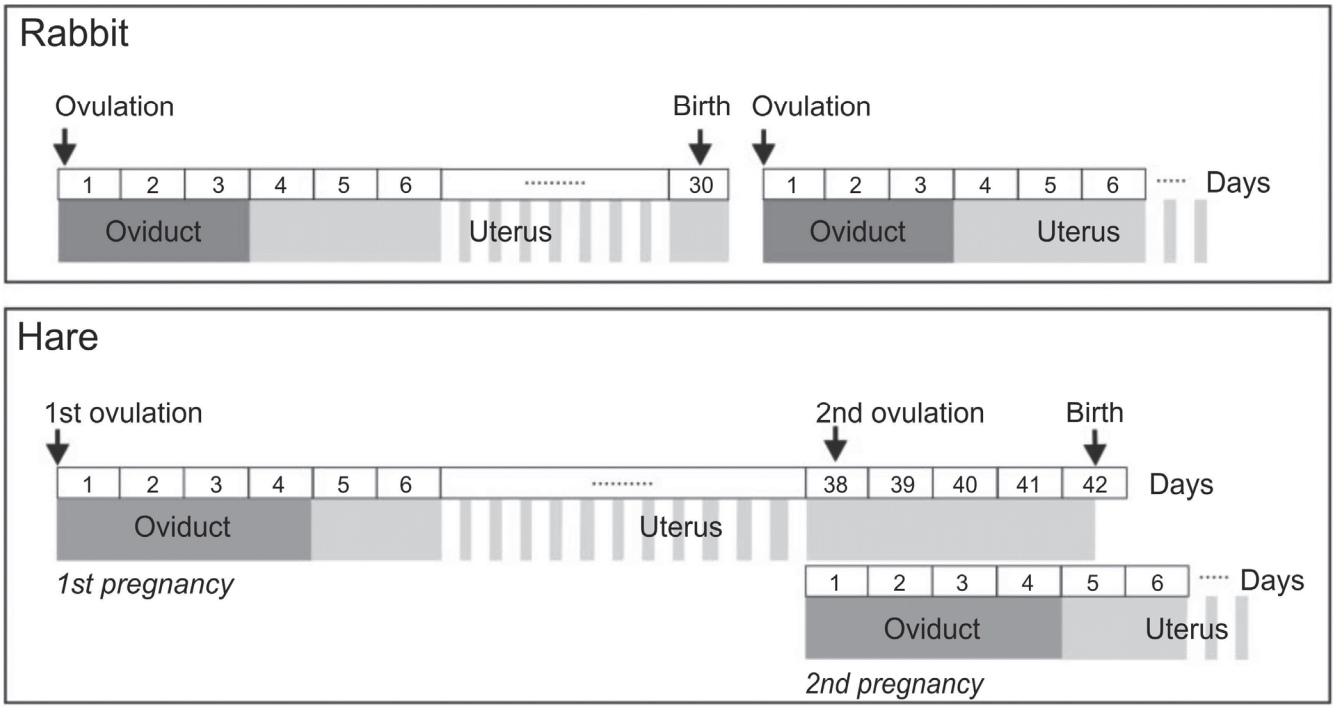

Fig. 8. Comparison of the developmental time scale in the hare and the rabbit. The reproductive strategy of the hare is characterised by superconception and a prolonged gestational time. Our results show that the oviducal phase of the hare is also elongated compared with that in the rabbit.

Figure 8 describes differences in reproductive strategies between the rabbit and hare. The complicated reproduction mechanism of the hare is characterised by superconception and prolongation of pregnancy. Here we observed in addition a prolonged oviduct phase of at least 1 day. We assume that the selective pressure is the development of the precocial mode from the original altricial mode. The CL-dependant endocrine environment of the endometrium is the necessary support of the first pregnancy, which usually involves both uterine horns. Therefore, in case of superconception, the second set of embryos must be placed on hold in the oviduct. Only after delivery of the first litter can priming of the endometrium for the next implantation by the new set of CL occur. If the hare blastocyst reached the uterus 3–4 days after ovulation, as it does in the rabbit, in cases of superconception it would be flushed out with the birth of the first pregnancy. The mucin coat is possibly also a prerequisite for the evolution of superconception, because it may protect the early blastocyst in the environment of a *post partum* uterus. Other aspects concerning superconception, such as the question as to how spermatozoa can bypass the first pregnancy and whether other hare species (e.g. the Japanese hare *Lepus brachyurus*) also exhibit superconception, need further investigation.

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III. General Discussion

This thesis is the first longitudinal *in vivo* study of embryos undergoing resorption in a small polytocous species, the European brown hare (Ebh) (Article I). *In vivo* studies generally focus on long-term ultrasound investigations in the domestic dog [ENGLAND, 1992; ENGLAND, 1993; MUELLER & ARBEITER, 1993; MUELLER, ARBEITER & BREITENFELLNER, 1993; ENGLAND, 1998; ENGLAND & RUSSO, 2006; SENDAG ET AL., 2010]. Small mammals (domestic ferrets, domestic guinea pigs, European hamsters, European moles, European rabbits and house mice) were the first model species to investigate in embryonic resorptions [STRAHL & HENNEBERG, 1901; FRAENKEL, 1903; HENNEBERG, 1903; MEYER, 1917; FURTUYN, 1919; 1929] but since then studies have been exclusively based on *post mortem* findings or case studies performed after OHE [STOCKLEY, 2003; ZHAO ET AL., 2010; OWUSU ET AL., 2010]. Therefore, the exact time of embryonic death is unknown and the incidences of resorption are generally estimated by comparing the total number of CL to the total number of embryos, placental scars and the young [ALLEN, BRAMBELL & MILLS, 1947; CONAWAY, BASKETT & TOLL, 1960; FLUX, 1967; STOCKLEY, 2003].

Through the use of high frequency ultrasound, detailed description of the macroscopic process of embryonic resorption and exact determination of the resorption rate in the small polytocous Ebh became possible. Ultrasonographic monitoring is facilitated by the large size of the Ebh's reproductive organs (uterus horn, non-pregnant: length: 8-9 cm, width: 4-6 mm [ZÖRNER, 1981], ovary in the breeding season: length: 20-33 mm, width: 12-17.5 mm [STIEVE, 1952; BLOCH & STRAUSS, 1958]) which allow accurate documentation of the embryonic structures and the number and appearance of the CL. First ultrasonographic detection of a resorption site was possible on day 8 of pregnancy (Article I) after attachment of the blastocyst (Article III). Subsequently, resorption sites were characterised by an initial increase of the mean diameter (Article I) that might be attributed to the influx and accumulation of maternal blood within the resorption site [STEIGER ET AL., 2006]. This is confirmed by the cytological analysis of resorption fluid gained by biopsy that revealed a very high abundance of maternal erythrocytes (Article II). In the course of pregnancy, resorption sites decreased in size and gained in echodensity until scar tissue was formed at the former implantation site. The placenta was the last structure to be affected by the resorption process (Article I). This corresponds to ultrasonographic findings in the domestic dog where placental structures also persisted until the end of the resorption process [ENGLAND & RUSSO, 2006]. Resorption processes were observed up to day 36 of the 42 day pregnancy but dissolved fetal remnants were then discharged from the uterus. This very late dissolution might be explained by the fact that in polytocous species pregnancy has to be maintained until the birth of the remaining conceptus. Previously, this phenomenon has only been described once by Köbner [1910] in *post mortem* examinations in the European domestic rabbit. In contrast, in monotocous species it has been suggested that degenerated conceptus are usually

expelled early in pregnancy since this allows a faster return to the estrus cycle for a new pregnancy.

Measurements from the exact day of death of conceptus which were subsequently resorbed revealed that they were initially smaller than their viable siblings. In domestic dogs, embryos showing a general retarded development tended to be resorbed [ENGLAND & RUSSO, 2006]. Retarded embryonic development and subsequent resorption might result from implantation sites that lack sufficient vascularisation, e.g. old uterine scar tissue or space limitation by other embryos. In aborted domestic horse fetuses that died of an overlong or twisted umbilical cord the compression of umbilical vessels initiated fetal degeneration [SMITH ET AL., 2003].

The macroscopic process of embryonic resorption was studied in the context of intragestational luteal regression (Article I). To my knowledge, this has not been done before in polytocous species. Few studies tried to correlate the number of CL to the number of conceptus [ANDERSON & SIMPSON, 1973; SCOFIELD, CLEGG & LAMMING, 1974; FISCHER, 2009] but failed. Since these studies relied on post mortem investigations in domestic pigs or were performed after OHE in domestic dogs they could not provide a long-term observation of luteal dynamics in the context of the resorption process. In the pig production industry, the number of successfully fertilized ova gained increasing importance for embryo transfer (ET). However, the experimental conditions complicate long-term observations of the luteal dynamics. Moreover, advanced hormonal treatment (superovulation) of donor and recipient animals can make it difficult to draw any conclusions concerning embryonic survival [FISCHER, 2009]. Interestingly, in a very recent study on nonsurgical ET in the pig a significant correlation between the number of CL and the number of embryos was found in a very early pregnancy stage. But there was no correlation between the number of CL and degenerated blastocysts/oocytes [ANGEL ET AL., 2014]. For further studies ultrasonographic long-term observation of the ovaries should be provided to monitor the luteal dynamics of the female pig.

Moreover, observations in polytocous species are complicated by the fact that in some species CL regression occurs soon after midgestation when the placenta takes over progesterone synthesis (luteo-placental shift) [CSAPO, 1969; CSAPO, PURI & TARRO, 1981; MCCRACKEN, CUSTER & LAMSA, 1999]. In contrast, the Ebh placenta does not produce progesterone and the embryo is completely dependent on luteal progesterone production [CAILLOL & MARTINET, 1976]. This allowed the study of the morphology of embryonic resorptions in the context of luteal dynamics over the entire pregnancy period. Interestingly, regression of one CL was observed while an embryo was undergoing resorption. This one-to-one-relationship between one embryo undergoing resorption and one regressing CL strongly suggests that a withdrawal of the total systemic progesterone level is not the only key for for pregnancy failure but that there might be important local interactions between one conceptus

and one CL. As in other small mammalian species [DELCAMPO & GINTHER, 1972; MCCRACKEN, CUSTER & LAMSA, 1999] future studies should investigate the vascular anatomy between the embryo and the ovary (Article I). Because there is substantial variation in the embryo-luteal-interactions between species research should specifically focus on the factors mediating embryonic survival and life span of the CL in the Ebh.

The use of ultrasound/UBM in combination with histological data permitted the detailed documentation of early embryonic development between day 3 and day 13 of pregnancy (Article III). In the Ebh the blastocyst entered the uterine lumen on day 5 of pregnancy (day 1 is defined as the day of conception). This is one to two days later than in the European domestic rabbit where the timepoint of arrival in the uterine horn is between day 3 and 4 of pregnancy (day 1 is defined as the day of conception) [DANIEL, 1964; ALLISTON & PARDEE, 1973; WARNER, CONLON & KANE, 2003]. Since the Ebh is capable to host two sets of pregnancies (superconception) [ROELLIG ET AL., 2010] the prolonged oviduct phase might have the advantage to prevent embryos reaching the uterine horn earlier than day 5 being flushed out during the ongoing process of birth. Interestingly, a thick mucin layer covers the zona pellucida (ZP) of the Ebh morulae/blastocyst (Article III). Previously, this has only been observed in the European domestic rabbit [BÖVING, 1957; KANE, 1975] and in some marsupial species [SELWOOD, 2000]. The function of the mucin coat still remains unclear. It appears to play an important role during the implantation process [MURAKAMI & IMAI, 1996] and might also provide a protection from the environment of a *post partum* uterus in case of superconception. The implantation process in the Ebh was determined for the period between day 7 to 9 of pregnancy (Article III). This corresponds to findings in the European domestic rabbit where the first blastocyst attachment also occurs on day 7 of pregnancy [HOFFMAN, BREINAN & BLAEUER, 1999]. In the Ebh, the highest daily incidence of embryonic resorption was detected on day 8 of pregnancy (Article I), implying that a high percentage of embryos is lost during the delicate process of implantation.

In the present study, a total of 42% of embryos underwent embryonic resorption of which 24% were ultrasonographically detected in the post-implantation period (Article I). In other polytocous mammals reported resorption rates were lower, e.g. in the domestic dog (5-13%) [ENGLAND, YEAGER & CONCANNON, 2003; ENGLAND & RUSSO, 2006], in the wild brown rat (9.07%) [PERRY, 1945], in the wild house mouse (7-19%) [KRACKOW, 1992], in the American beaver (*Castor fiber*) (22.27%) [OSBORN, 1953] or in the cotton rat (*Sigmodon hispidus*) (5.5-11.8%) [BERGSTROM & ROSE, 2004] (Appendix, Table 2). In other Lagomorpha, a similar incidence was estimated (white-tailed jackrabbit (*Lepus townsendii*): 39.6% [KLINE, 1963], European rabbit: >43% [BRAMBELL, 1948]). With some weather conditions the incidence of embryonic resorption could even exceed 80% [BRAMBELL & MILLS, 1948; RACZYNSKI, 1964; ROGOWITZ, 1992], suggesting that resorptions might be part of the reproductive biology of lagomorphs.

To elucidate whether the embryonic sex or genotype might contribute to higher resorption rates in the Ebh, two ultrasound guided biopsy techniques were established. They allow for the first time *in vivo* sampling of embryonic material in a small polytocous species (Article II). Prenatal diagnostic procedures such as amniocentesis and CVS represent routine diagnostic tools in human reproductive medicine for the analysis of fetal chromosomal patterns [ALFIREVIC, MUJEZINOVIC & SUNDBERG, 2009]. In polytocous animals, application of these techniques is complicated by the presence of adjacent conceptus. A particular challenge is the typical bicornuate uterus, which is surrounded by the intestines and easily escapes under the pressure of the needle. By using different needle types and high resolution ultrasonographic machines, a flexible adaptation to the size of the animal, the thickness of the uterus and the consistency of the resorption site is feasible. Therefore, both techniques can be performed in other polytocous mammals, on both resorption sites and on viable conceptus. Extended professional experience and equipment help to ensure a successful biopsy. The total amount of extracted embryonic material might be restricted by the small size of the animal. Usually, a minimum of 5 mg of chorionic villus tissue [WAPNER & TOY, 2011] and between 20 and 30 ml of amniotic fluid [CUNNINGHAM ET AL., 2001] are required for further genetic analysis in humans. In contrast, from the resorption sites approximately 1 mg of chorionic villus tissue and between 0.1 to 4.0 ml of amniotic fluid was gained. Moreover, the quantity of viable fetal cells that could be isolated from the specimen was limited by the lytic material of the resorption site. Preserved smears of aspirated resorption material showed that it mainly consisted of maternal erythrocytes and a few white blood cells that underwent multiple cell necrosis. Therefore, embryonic DNA was not successfully detected in any of the samples. In human medicine, the isolated embryonic cells are grown in cell cultures which enable subsequent microscopical analysis of the chromosomes [VAN DYKE, 2010; WAPNER & TOY, 2011]. Future research in veterinary prenatal diagnostics should focus on developing species-specific cell culture media that allow fetal cell multiplication for further cytogenetic analysis.

IV. Conclusion: Embryonic resorption as a physiological phenomenon in mammals

Terms such as “embryonic loss” or “reproductive wastage” often suggest embryonic resorptions to be of pathological origin. Several infectious diseases have been identified in association with resorption processes. Although severe maternal infection with any type of infectious agent can implicate embryonic resorption by systemic toxemia [SCOFIELD, CLEGG & LAMMING, 1974; JOHNSTON & RAKSIL, 1987; POST, 1995] infectious diseases account only for a small proportion of embryonic resorption [SCOFIELD, CLEGG & LAMMING, 1974]. The high extent of embryonic resorptions in some polytocous mammals such as the lagomorphs suggests that

selective resorption of single conceptus might be of advantage for maternal and/or offspring survival.

The possible impact of genes on embryonic survival has been subject of extensive research [BEREPUBO & LONG, 1983; BOLET, 1986; JOHNSTON & RAKSIL, 1987; BLASCO ET AL., 1993; BASRUR & KING, 2005; GEISERT & SCHMITT, 2002; ROSS ET AL., 2009; VICENTE ET AL., 2012A]. In humans, up to 75% of embryos are lost during early pregnancy [BOKLAGE, 1990; NORWITZ, SCHUST & FISHER, 2001; CHRISTIANSEN, 2006; KWAK-KIM ET AL., 2010] and the majority (60%) of these *miscarriages* occur because embryos are genetically abnormal [HASSOLD, 1980; CLARK, 2008]. However, in polytocous species chromosomal anomalies occur with relatively low incidences of 5 to 10% [MCFEELY, 1967; FECHHEIMER & BEATTY, 1974; KING, 1990]. In laboratory mice (*Mus musculus*), Bradford [1979] showed that embryonic survival depends on certain genes that control ovulation rate and litter size. Moreover, embryonic survival can be affected by the expression of lethal genes [PAIGEN, 2003; LOBO, 2008]. In domestic cats (*Felis catus*) and dogs, chromosomal anomalies cause abortion [BEREPUBO & LONG, 1983; JOHNSTON & RAKSIL, 1987]. However, the incidence of embryonic resorption caused by chromosomal anomalies is unknown.

A hereditary predisposition for embryonic resorption occurs in certain lines of laboratory mice [HOLT, VANGEN & FARSTAD, 2004] and European domestic rabbits [PEIRÓ ET AL., 2007; VICENTE ET AL., 2012B]. Also, strains of domestic pigs, laboratory mice and different breeds of cattle and and rabbits showed an increased resorption rate for offspring originating of the same sire [RAMPACEK, ROBISON & ULBERG, 1975; BOLET, 1986; GENDRON & BAINES, 1989; HUMBLOT, 2000; BAMBER ET AL., 2009; VICENTE ET AL., 2012B]. There is evidence for a heritability of the number of ova relased [BRADFORD, 1979; BOLET, 1986; BLASCO ET AL., 1993; DIXON ET AL., 2007; PEIRÓ ET AL., 2007]. In the pig, superovulated animals treated with eCG for ET showed higher loss rates than non-treated animals [LONGENECKER & DAY, 1968; GUTHRIE, HENRICKS & HANDLIN, 1974; MARTINAT-BOTTÉ ET AL., 2010]. Exceptionally high ovulation rates have been found in certain polyovulatory species such as the plains vizcacha (*Lagostomus maximus*) [JENSEN ET AL., 2008], the tailless tenrec (*Tenrec ecaudatus*) [NICOLL & RACEY, 1985] and the eastern rock elephant shrew (*Elephantulus myurus*) [VAN DER HORST & GILLMAN, 1941]. But although several hundred ova are released per reproductive cycle only few of them are fertilised. Moreover, after implantation the majority of fertilised ova is again reduced by pre- or postimplantation resorption [VAN DER HORST & GILLMAN, 1941; WEIR, 1971; NICOLL & RACEY, 1985]. Several studies tried to increase litter size by selecting for high ovulation rates in some breeds of European domestic rabbits, domestic pigs and laboratory mice but they always resulted in increased postimplantation resorption rates [BRADFORD, 1979; BOLET, 1986; ARGENTE, ET AL., 1997; GEISERT & SCHMITT, 2002; ARGENTE ET AL., 2003]. This suggests a trade-off between ovulation rate and number of implanted embryos [domestic dog: ALLEN, 1982; ENGLAND, 1992; 1998; greater cane rat: ADU & YEBOAH, 2000; OWUSU ET AL., 2010; domestic pig: BOLET, 1986; Botta's pocket gopher

(*Thomomys bottae navus*) & Gambel's white-footed mouse (*Peromyscus maniculatus gambelii*): LOEB & SCHWAB, 1987; European rabbit: BRAMBELL & MILLS, 1947; BRAMBELL, 1948; ADAMS, 1960B; ARGENTE ET AL. 1997].

Uterine space reduction by overcrowding is often suggested as another main reason for embryos undergoing resorption [ARGENTE ET AL., 2003; FOXCROFT ET AL., 2006]. Uterine crowding does not seem to be a limiting factor during the first stages of gestation but becomes problematic during later gestation stages [BRAMBELL & MILLS, 1948; ANDERSON, 1957; BOLET, 1986; PEIRÓ ET AL., 2007]. It appears to be a physiological phenomenon in large litters [ALLEN, 1982; ARGENETE ET AL., 1997; ENGLAND & RUSSO, 2006] or in litters containing embryos of large size [LOEB & SCHWAB, 1987]. This could be explained by an inter-embryo inhibition effect as shown in twin pregnancies of domestic horse mares [NEWCOMBE & ENGLAND, 2002]. However, in polytocous species remaining conceptus do not make use of the space vacated by embryos undergoing resorption [LAMBERSON & ECKARDT, 1996; FREKING ET AL., 2007], so that the uterine space surrounding each embryo might be fixed early during implantation. The spacing of blastocysts along the uterine horn is the result of ciliary action, muscular contraction and unoccupied sites of uterine receptivity. Embryonic resorptions might result from implantation sites that lack sufficient vascularisation, e.g. old uterine scar tissue or space limitation by other embryos. Interestingly, in the Meishan pig a higher prolificacy has been successfully obtained by selecting for an increased placental vascular supply. This allowed an increased embryonic survival with the result that Meishan sows farrow three to five more piglets per litter than European and US breeds [WILSON ET AL., 1998].

Complex interactions have been described between maternal and environmental conditions. The influence of climatic factors on reproductive performance was reported by Bergstrom & Rose [2004] in cotton rats (*Sigmodon hispidus*) from Virginia and Georgia. Although the mean number of ovulations did not differ significantly in both populations, Georgia cotton rats had smaller litters during the winter months. The authors concluded that cotton rats are capable to reduce intrauterine litter size by embryonic resorption in response to harsh weather conditions. A seasonal peak of embryonic resorption during the summer months was documented in stray domestic dogs in Yucatan (Mexico), indicating a possible negative influence of heat on reproductive performance [ORTEGA-PACHECO ET AL., 2007]. The effect of heat on mammalian embryonic development has been also shown by Hansen [2009], Wolfenson and Blum [1988] and Ulberg and Burfening [1967]. Geographically, litter size variation was reported in many different mammals such as rabbits [ADAMS, 1960A; BRAMBELL & MILLS, 1948], hares [CONAWAY, BASKETT & TOLL, 1960; NEWSON, 1964; ROGOWITZ, 1992], several African murids [DIETERLEN, 1967; NEAL, 1968] and across a wide variety of canids [MOEHLMANN & HOFER, 1997]. Observation of the reproductive biology in the big brown bat (*Eptesicus fuscus*) revealed that embryonic resorption represents an important physiological tool for intrauterine litter size reduction in this species. Here, females usually give birth to two offspring in the

eastern parts of the United States whereas in the western parts only one offspring is raised [COCKRUM, 1955]. Moreover, post mortem examinations revealed a total intrauterine litter size that generally exceeds the number of born offspring [KUNZ, 1974]. Sex ratio in favour of female offspring has been proposed as a possible explanation since the birth of female pups early in the breeding season enables these to reproduce in the same year [BARCLAY, 2012]. Sex-selective fetal resorption was also discussed for wild house mice [KRACKOW, 1992], golden hamsters (*Mesocricetus auratus*) [PRATT & LISK, 1989], coypus (*Myocastor coypus*) [COCCHI & RIGA, 2008] and the greater cane rat [OWUSU ET AL., 2010]. The effect of social interaction on the incidence of embryonic resorption was tested on a laboratory population of golden hamsters. Socially subordinate hamster females suffered from increased resorption rates in the presence of strange males [HUCK ET AL., 1988]. Social stress as a potential factor causing embryonic resorption has also been shown in wild [ROWE, TAYLOR & CHUDLEY, 1964] and laboratory house mice [BLOIS ET AL., 2004] and prairie deer mice (*Peromyscus maniculatus bairdii*), with higher incidences of embryonic resorption under crowded conditions [HELMREICH, 1960].

Both young and increasing age is reported to have a high influence on embryonic loss [ENGELAND 1997; 1998; HUMBLOT, 2000; ARMSTONG, 2001; ORTEGA-PACHECO ET AL., 2007]. Additionally, energy imbalances such as lactation or nutritional deficiencies [FORTUN-LAMOTHE, PRUNIER & LEBAS, 1993; GAERESKOG, ERIKSSON & WENTZEL, 2006; ASHWORTH, TOMA & HUNTER, 2009; PICKELL ET AL., 2011; GREWAL, SING & KAUR, 2011] can cause embryonic resorption, as well as metabolic diseases (hyper- and hypothyreoidism, diabetes mellitus, hyper- and hypoadrenocorticism) [POST, 1995], hypoluteoidism or other hormonal imbalances leading to chronic endometrial hyperplasia [FELDMAN & NELSON, 2004; ENGLAND, 1998]. Since embryonic survival directly depends on the intrauterine environment and placental nutrition [FOXCROFT, 1997; FOXCROFT ET AL., 2006; WILSHER, LEFRANC & ALLEN, 2012] aged or sick animals might have an insufficient nutritive supply or uterine environment.

The uterine fluid provides the nutritive supply of the embryo prior to implantation. It mainly consists of proteins secreted by the endometrium [LIN ET AL., 2005]. The pattern of proteins is modified throughout gestation. An altered composition of the uterine fluid could prevent normal embryonic development and subsequently result in resorption. Uteroglobin which constitutes the major component of the uterine fluid during the critical period of implantation is suggested to prevent maternal inflammatory and/or immune response from damaging the embryo by binding to the embryonic surface and masking cell surface antigens [KIRCHNER 1976; BEIER, 2000; MUKHERJEE, ZHANG & CHILTON, 2007]. An insufficient expression of cell surface antigens might result in the failure to protect the embryo from maternal immune response and hence induce embryonic resorption [LIN ET AL., 2005]. Another protective function is provided by T (T_{Reg}) cells ($CD4^+$ and $CD25^+$) and regulatory natural killer cells (NK cells) which maintain feto-maternal immunotolerance and therefore guarantee embryonic survival

[SOMERSET ET AL., 2004; SAITO ET AL., 2007; LEBER ET AL., 2011]. In pregnant laboratory mice, activation of $CD4^+$ and $CD25^+$ depends on levels of progesterone and 17β-estradiol [MAO ET AL., 2010]. Low levels of progesterone and estradiol might lead to the failure of activating $CD4^+$ and $CD25^+$ cells and consequently induce embryonic resorption. This is supported by a recent study in the European domestic rabbit where embryonic resorption was related to low serum levels of estradiol [VICENTE ET AL., 2012B]. The angiogenic function during pregnancy is controlled by NK cells. They are activated by different cytokines (interferone inducers) such as IL-12, IL-15 and IL-28 [LEBER ET AL., 2011; LÉDÉE ET AL. 2011]. However, NK cells were deleterious for embryo development in case of a dysregulated cytokine milieu [BOYSON ET AL., 2006; LIN ET AL., 2009]. Laboratory mouse models could show an upregulation of T cells (TH1/TH2) and NK cells during embryonic resorption processes [CLARK, MCDERMOTT & SZEWCZUK, 1980; DUCLOS, POMERANTZ & BAINES, 1994; BLOIS ET AL., 2004; JIANG ET AL., 2009]. Also, the activated products of macrophages (TNF-α and nitric oxide) were present in association with embryonic resorption in laboratory mice. Treatment with immunoglobulins, interleukins and dendritic cells had an anti-resorption effect [TAKEDA, ET AL., 2007; JIANG ET AL., 2009; ZHAO ET AL., 2010]. Therefore, non-specific maternal inflammatory response plays a major role in embryonic resorption processes [CHAOUAT ET AL., 1997; CHAOUAT ET AL., 2009; WANG ET AL. 2010; BAINES ET AL., 2011]. Future research should focus on hormonal-immunological investigations concerning the feto-maternal allograft.

V. Summary

Embryonic resorption is a common phenomenon in polytocous mammals. The process of resorption is not associated with any obvious clinical symptoms. Therefore, studies are usually based on *post mortem* examinations or counts after ovariohysterectomy (OHE). The present study is the first longitudinal *in vivo* investigation describing the incidence and morphology of embryos undergoing resorption in a small polytocous species, the European brown hare (Ebh) (*Lepus europaeus*). High frequency ultrasonographic examinations enabled early detection of resorption sites from day 8 of pregnancy onwards. Resorptions were monitored in context of embryonic and fetal development and classified into (i) *pre-implantation resorptions*, (ii) *peri-implatation resorptions* and (iii) *post-implantation resorptions*. In total, 42% of embryos underwent resorption. Parallel monitoring of the ovaries revealed in 91% of the cases the regression of a CL while an embryo underwent resorption. The number of resorptions did not significantly differ from the number of CL in regression suggesting a one-resorption-to-one-regression-relationship. Two minimally invasive ultrasound guided biopsy techniques were established for the genetic analysis of resorption sites. Resorption material was gained *in vivo* by aspiration of resorption fluid or by biopsy of extraembryonic tissue from the placenta (CVS). Further analysis was performed by microsatellite analysis (paternity testing) and SRY testing. Paternity was determined in 11 of the fluid aspirates (n = 28) and six of the placental biopsies (n = 8). The lower success rate in the fluid samples is attributed to the high abundance of maternal cells which was confirmed by analysis of fluid sample smears. By SRY testing male sex of the resorbing embryo was identified in ten of the fluid samples (n =28) and one of the placental samples (n = 8). A negative influence of the biopsy techniques on the viability of the remaining embryos or the reproductive performance of the mother was not observed.

V. Zusammenfassung

Die embryonale Resorption – eine ultrasonographische Längsschnittstudie an der Modellart Europäischer Feldhase (*Lepus europaeus* PALLAS, 1778)

Die embryonale Resorption ist ein Phänomen, das bei allen polytoken Säugetiere auftritt. Da der Resorptionsprozess nicht mit deutlich erkennbaren klinischen Symptomen assoziiert ist, beruhen Studien in der Regel auf *post mortem* Zählungen oder Zählungen nach Ovariohysterektomie (OHE). Die vorliegende Studie beschäftigt sich mit der Resorptionsrate und Morphologie embryonaler Resorptionen beim Europäischen Feldhasen (EFh) (*Lepus europaeus*). Zum ersten Mal wurden bei einem kleinen polytoken Säugetier *in vivo* Langzeituntersuchungen von embryonalen Resorptionen durchgeführt. Hochauflösende Ultraschalluntersuchungen ermöglichten die Detektion embryonaler Resorptionen ab Tag 8 der Trächtigkeit. Dabei wurden die Resorptionen im Zusammenhang mit der embryonalen und fetalen Entwicklung ultrasonographisch untersucht und in (i) *prä-implantative*, (ii) *peri-implantative* und (iii) *post-implantative* Resorptionsstadien eingeteilt. Insgesamt wurden 42% aller Embryonen resorbiert. Parallele Verlaufsuntersuchungen der Ovarien zeigten, dass in 91% der Fälle der Resorptionsprozess von einem regressierenden GK begleitet wurde. Dabei unterschied sich die Anzahl an Resorptionen nicht signifikant von der Anzahl regressierender GK, was auf einen Eins-zu-eins-Zusammenhang zwischen Resorption und GK-Regression hinweisen könnte. Die Entwicklung von zwei ultraschall-gestützten Biopsietechniken ermöglichte weitergehende genetische Untersuchungen des in Resorption befindlichen Embryos. Resorptionsmaterial wurde *in vivo* gewonnen durch Aspiration von Resorptionsflüssigkeit oder durch Stanzbiopsie von extraembryonalem Plazentagewebe. Anschließend wurde das Material mittels Mikrosatelliten-Analyse (Vaterschaftsnachweis) und SRY-Gen-Bestimmung analysiert. Bei 11 der Aspirationsbiopsien (n = 28) und sechs der Plazentabiopsien (n = 8) konnte die Vaterschaft bestimmt werden. Die geringere Erfolgsrate bei den Aspirationsbiopsien lässt sich auf eine hohe Abundanz mütterlicher Zellen in der Aspirationsflüssigkeit zurückführen, was durch Analyse des Aspirationsbioptats in Ausstrichen bestätigt werden konnte. Die Identifizierung des männlichen Geschlechts durch SRY-Gen-Bestimmung gelang bei 10 der Aspirationsbiopsien (n = 28) und einer der Plazentabiopsien (n = 8). Ein negativer Einfluss der Biopsietechniken auf die weitere Entwicklung der verbleibenden Embryonen oder die zukünftige Fortpflanzungsleistung der Mutter konnte nicht beobachtet werden.

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VII. Appendix

Table 1. Clinical classification of prenatal death.

(There can be some overlap between the states of prenatal death making exact classification difficult.)

State	Stage of embryonic / fetal development	Embryonic / fetal changes	Causes	Complications
Resorption	- usually occurring in the embryonic stage (but can affect fetuses (see III.)	- degeneration and complete resorption of the conceptus - no expulsion of embryonic / fetal remnants	- several infectious agents (bacterial and viral diseases) - under research: non-infectious agents (hormonal-immunological factors, nutritional imbalances, metabolic diseases etc.) (see IV.)	- in monotocous species: extended interval between two estruses (prolonged interestrual period)
Mummification	- usually occurring in the fetal stage	- fluid resorption and subsequent dehydration of the conceptus - eventual mineralization	- failure to abort (fetal retention) - cervical closure prevents local infection of the uterus and conceptus - non-infectious factors (torsion of the umbilical cord) - infectious diseases that cause minimal inflammation (Campylobacter fetus, bovine viral diarrhea virus (BVDV))	- mummified conceptus is often retained within the uterus and has to be removed by fetotomy or caesarean section (CS) - mineralization of the conceptus with phosphorus and calcium salts (lithopedion) - no return to estrus

State	Stage of embryonic / fetal development	Embryonic / fetal changes	Causes	Complications
Maceration	- usually occurring during late embryonic and fetal development	- degeneration of the conceptus showing the signs of bacterial and autolytic decomposition - no resorption - odour development	- failure to abort (fetal retention) - cervical dilation - local infection of the the uterus and conceptus - systemic infection	- conceptus is often retained within the uterus and has to be removed by fetotomy or CS - infection affects maternal health (fever, anorexia, declining milk production, death) - no return to estrus
Putrefaction	- usually occurring during late embryonic and fetal development	- degeneration of the conceptus with emphysematous changes (swelling of the conceptus) - no resorption - odour of putrefaction - gas crepitation of the conceptus	- failure to abort (fetal retention) - infection of the uterus and conceptus with tissue-dissolving and gas-producing anaerobic bacterias	- swollen conceptus has to be removed by fetotomy or CS - rupture of the uterus and its adnexa - infection affects maternal health (fever, systemic toxemia, anorexia, declining milk production, death); - no return to estrus
Abortion	- usually occurring in the fetal stage - under research: abortion during early embryonic development in monotocous species and humans (see 1.4.)	- expulsion of the dead conceptus with and without different signs of degeneration	- several infectious agents (bacterial and viral diseases) - several non-infectious agents (nutritional imbalances, metabolic diseases etc.) (see IV.)	- infection affects maternal health (fever, anorexia, declining milk production, death)

Table 2. Publications citing embryonic resorption, the incidence of embryonic resorption and the mean litter size in different polytocous mammalian species.

(RES = resorption; TLR = total litter resorption; PLR = partial litter resorption; CL = corpora lutea; em = embryo; US = ultrasound; OHE = ovariohysterectomy; LAP = laparatomy; p.m. = post mortem)

Order **Family** Species	**Scientific name**	**Incidence**	**TLR**	**PLR**	**Females showing RES**	**Mean litter size**	**RES inferred from**	**Examined**	**References**
Afrosoricida									
Tenrecidae									
Lesser hedgehog tenrec	*Echinops telfairi*	n/a	n/a	n/a	n/a	n/a	n/a	US	BERKEN, 2006
Tailless tenrec	*Tenrec ecaudatus*	n/a	n/a	n/a	74%	n/a	em	*p.m.*	NICOLL & RACEY, 1985
Artiodactyla									
Suidae									
Domestic pig	*Sus scrofa domestica*	30%	n/a	n/a	n/a	n/a	CL	*p.m.*	BOLET, 1986
Domestic pig	*Sus scrofa domestica*	30-40%	n/a	n/a	n/a	n/a	CL	*p.m.*	FLINT, SAUNDERS & ZIECIK, 1982
Domestic pig	*Sus scrofa domestica*	25.6%	n/a	n/a	n/a	n/a	em	*p.m.*	DYCK, 1991
Domestic pig	*Sus scrofa domestica*	27% 11.9%	n/a	n/a	n/a	n/a	CL em	*p.m.*	HAMMOND, 1914
Domestic pig	*Sus scrofa domestica*	32.6% 12.4%	n/a	n/a	n/a	n/a 12.1 ± 3.85	CL em	*p.m.*	HAMMOND, 1921

Order Family Species	Scientific name	Incidence	TLR	PLR	Females showing RES	Mean litter size	RES inferred from	Examined	References
Domestic pig	*Sus scrofa domestica*	38.8%	n/a	n/a	n/a	9.1 ± 0.54	CL[1]	*p.m.*	LAMBERT ET AL., 1991
Domestic pig (Landrace & Yorkshire)	*Sus scrofa domestica*	47%	n/a	n/a	n/a	12.8	em	*p.m.*	NISSEN ET AL., 1997
Domestic pig (Large White)	*Sus scrofa domestica*	34.8%	n/a	n/a	n/a	n/a	CL	*p.m.*	PERRY & ROWLANDS, 1962
Domestic pig	*Sus scrofa domestica*	46%	n/a	n/a	n/a	n/a	CL	*p.m.*	SQUIERS, DICKERSON & MAYER, 1952
Carnivora									
Canidae									
Domestic dog (Beagle)	*Canis familiaris*	11%	n/a	n/a	n/a	5.6	CL	OHE	ANDERSON & SIMPSON, 1973
Domestic dog	*Canis familiaris*	4.8%	5%	n/a	n/a	n/a	em	US	ENGLAND, 1992
Domestic dog (Beagle)	*Canis familiaris*	10.6%	n/a	n/a	n/a	n/a	em	US	ENGLAND & RUSSO, 2006
Domestic dog	*Canis familiaris*	5-13%	n/a	n/a	n/a	n/a	em	US	ENGLAND, YEAGER & CONCANNON, 2003
Domestic dog	*Canis familiaris*	n/a	n/a	n/a	10%	n/a	n/a	US	MUELLER & ARBEITER, 1993
Domestic dog	*Canis familiaris*	n/a	n/a	n/a	12%	n/a	n/a	US	MUELLER, ARBEITER & BREITENFELLNER, 1993
Domestic dog (Stray dogs)	*Canis familiaris*	17.3%	n/a	n/a	n/a	n/a	em	*p.m.*	ORTEGA-PACHECO ET AL., 2006
Domestic dog (Stray dogs)	*Canis familiaris*	25.9%	15.2%	17.3%	42.9%	n/a	CL	*p.m.*	ORTEGA-PACHECO ET AL., 2006

Order Family Species	Scientific name	Incidence	TLR	PLR	Females showing RES	Mean litter size	RES inferred from	Examined	References
Domestic dog (Stray dogs)	*Canis familiaris*	n/a	n/a	n/a	45.6%	n/a	n/a	OHE	ORTEGA-PACHECO ET AL., 2007
Domestic dog (Kangal)	*Canis familiaris*	12.8%	n/a	n/a	n/a	6.2 ± 3.8	em	US	SENDAG ET AL., 2010
Domestic dog	*Canis familiaris*	n/a	n/a	n/a	n/a	n/a	n/a	US	TAVERNE, OKKENS & VAN OORD, 1985
Domestic dog (Stray dogs)	*Canis familiaris*	n/a	7%	32.6%	n/a	4.6	em / pl. scars	OHE	TOTTON ET AL., 2010
Felidae									
Domestic cat	*Felis catus*	n/a	n/a	n/a	n/a	n/a	n/a	n/a	FELDMAN & NELSON, 2004
Domestic cat	*Felis catus*	n/a	n/a	n/a	n/a	n/a	n/a	*p.m.*	KUNTZ, 1920
Domestic cat	*Felis catus*	30%[4]	n/a	n/a	n/a	n/a	n/a	OHE	SWANSON, ROTH & WILDT, 1994
Domestic cat	*Felis catus*	16%[4]	n/a	n/a	n/a	n/a	CL	OHE	TSUTSUI ET AL., 1989
Mustelidae									
Domestic ferret	*Mustela putorius furo*	n/a	n/a	n/a	n/a	n/a	n/a	*p.m.*	ROBINSON, 1921
Domestic ferret	*Mustela putorius furo*	n/a	n/a	n/a	n/a	n/a	n/a	*p.m.*	STRAHL & HENNEBERG, 1901
Chiroptera									
Vespertilionidae									
Hoary Bat	*Lasiurus cinereus*	n/a	n/a	n/a	n/a	n/a	n/a	*p.m.*	BOUCHARD, ZIGOURIS & FENTON, 2001
Vespertilionid Bat	*Eptesicus fuscus*	n/a	n/a	n/a.	n/a	2	em	*p.m.*	KUNZ, 1974

Order Family Species	Scientific name	Incidence	TLR	PLR	Females showing RES	Mean litter size	RES inferred from	Examined	References
Lagomorpha									
Leporidae									
Black-tailed jackrabbit	*Lepus californicus*	8.6%	n/a	n/a	n/a	n/a	CL	*p.m.*	LECHLEITNER, 1959
European brown hare	*Lepus europaeus*	6-80%	n/a	n/a	n/a	n/a	em	*p.m.*	RACZYNSKI, 1964
European domestic rabbit	*Oryctolagus cuniculus*	4.8%[4] 20.2%[5]	8.1%[4] 3.3%[5]	9.7%[4] 18.3%[5]	53.3%[2,4]	n/a	CL / em / pl. scars	*p.m.*	ADAMS, 1960b
European wild rabbit	*Oryctolagus cuniculus*	>43% 9.5%[4] 19.7%[5]	13%[4]	10.2%[4]	n/a	n/a	n/a	*p.m.*	BRAMBELL, 1948
European wild rabbit	*Oryctolagus cuniculus*	9.5%[4]	n/a	n/a	n/a	n/a	n/a	*p.m.*	BRAMBELL & MILLS, 1947
European rabbit	*Oryctolagus cuniculus*	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a	n/a n/a n/a	em em em	*p.m.* *p.m.* *p.m.*	FRAENKEL, 1903 HAMMOND, 1914 STRAHL & HENNEBERG, 1901
Iberian hare	*Lepus granatensis*	21%[4]	n/a	n/a	n/a	1.6 ± 0.1	CL	*p.m.*	ALVES ET AL., 2002
Mountain hare	*Lepus timidus scoticus*	n/a	6.5%[4] 24.2%[5]	14.6% 5.9%	n/a	1.9	CL / pl.scars	*p.m.*	FLUX, 1970
Mountain hare	*Lepus timidus scoticus*	7.2-21.4%	n/a	n/a	n/a	n/a	CL	*p.m.*	IASON, 1990

Order Family Species	Scientific name	Incidence	TLR	PLR	Females showing RES	Mean litter size	RES inferred from	Examined	References
Snowshoe hare	*Lepus americanus*	5-12%[4]	20%[5]	10%[5]	n/a	n/a	CL / pl. scars	*p.m.*	NEWSON, 1964
White-tailed jackrabbit	*Lepus townsendii*	4.6%	n/a	n/a	19%	n/a	CL	*p.m.*	JAMES & SEABLOOM, 1969
White-tailed jackrabbit	*Lepus townsendii*	39.6%[4]	n/a	n/a	n/a	3.6 ± 0.6	CL	*p.m.*	KLINE, 1963
White-tailed jackrabbit	*Lepus townsendii*	5-86%	n/a	n/a	n/a		CL / pl. scars	*p.m.*	ROGOWITZ, 1992
Rodentia									
Bathyergidae									
Naked Mole Rat	*Heterocephalus glaber*	n/a	n/a	n/a	n/a	n/a	em	US	ROELLIG ET AL., 2011
Castoridae									
Eurasian beaver	*Castor fiber*	n/a	n/a	n/a	n/a	n/a	n/a	n/a	HAMMOND, 1943
North American beaver	*Castor canadensis*	22.27%	n/a	n/a	n/a	n/a	CL	*p.m.*	OSBORN, 1953
Caviidae									
Domestic guinea pig	*Cavia porcellus*	n/a	n/a	n/a	n/a	n/a	n/a	*p.m.*	MEYER, 1917
Cricetidae									
Cotton rat (Virginia)	*Sigmodon hispidus*	5.5%	n/a	n/a	n/a	5.9 ± 1.41	CL	*p.m.*	BERGSTROM & ROSE, 2004

Order **Family** Species	**Scientific name**	**Incidence**	**TLR**	**PLR**	**Females showing RES**	**Mean litter size**	**RES inferred from**	**Examined**	**References**
Cotton rat (Georgia)	*Sigmodon hispidus*	11.8%	n/a	n/a	n/a	5.2 ± 1.79	CL	*p.m.*	BERGSTROM & ROSE, 2004
Prairie deer mouse	*Peromyscus maniculatus bairdii*	n/a	n/a	n/a	n/a	n/a	em	*p.m.*	HELMREICH, 1960
Gambel's white-footed mouse	*Peromyscus maniculatus gambelii*	n/a	n/a	n/a	21.8%	4.6 ± 0.04	em	*p.m.*	LOEB & SCHWAB, 1987
Chinese hamster	*Cricetulus griseus*	n/a	n/a	n/a	n/a	n/a	em	*p.m.*	FORTUYN, 1929
Golden hamster	*Mesocricetus auratus*	12-35%	n/a	n/a	n/a	n/a	em	*p.m.*	HUCK, 1988
European hamster	*Cricetus cricetus*	n/a	n/a	n/a	n/a	n/a	em	*p.m.*	STRAHL & HENNEBERG, 1901
Geomyidae									
Botta's pocket gopher	*Thomomys bottae navus*	n/a	n/a	n/a	8.4%	5.1 ± 0.19	em	*p.m.*	LOEB & SCHWAB, 1987

Order Family Species	Scientific name	Incidence	TLR	PLR	Females showing RES	Mean litter size	RES inferred from	Examined	References
Muridae									
Laboratory house mouse (DBA/J & CBA/J)	*Mus musculus*	25 – 30%	n/a	n/a	n/a	n/a	em	*p.m.*	CLARK, MCDERMOTT & SZEWCZUK, 1980
Wild house mouse	*Mus musculus*	17-19%	n/a	n/a	n/a	5.2 ± 0.28[3]	pl. scars	*p.m.*	KRACKOW, 1992
Laboratory brown rat	*Rattus norvegicus*	n/a	n/a	n/a	n/a	n/a	n/a	LAP	COREY, 1933
Laboratory brown rat (Albino)	*Rattus norvegicus*	n/a	n/a	n/a	n/a	n/a	n/a	*p.m.*	HUBER, 1896
Wild brown rat	*Rattus norvegicus*	9.07	n/a	n/a	n/a	n/a	em	*p.m.*	PERRY, 1945
Myocastoridae									
Coypu	*Myocastor coypus*	8.74%[4]	n/a	n/a	n/a	5.1 ± 1.91[6] 5.5 ± 1.73[6]	em	*p.m.*	COCCHI & RIGA, 2008
Spalacidae									
East African Mole Rat	*Tachyoryctes splendens*	n/a	n/a	n/a	n/a	1.2 – 1.65	em	*p.m.*	JARVIS, 1969
Thryonomyidae									
Greater Cane Rat	*Thryonomys swinderianus*	42.7%	n/a	n/a	n/a	3.4 ± 0.33	em	*p.m.*	ADU & YEBOAH, 2000
Greater Cane Rat	*Thryonomys swinderianus*	n/a	n/a	n/a	5.8%	n/a	em / pl. scars	*p.m.*	ASIBEY, 1974

Order Family Species	Scientific name	Incidence	TLR	PLR	Females showing RES	Mean litter size	RES inferred from	Examined	References
Greater Cane Rat	*Thryonomys swinderianus*	41.6%	n/a	n/a	n/a	3.6 ± 0.06	em / pl. scars	*p.m.*	OWUSU ET AL., 2010
Soricomorpha **Talpidae**									
European mole	*Talpa europaea*	n/a	n/a	n/a	n/a	n/a	em	*p.m.*	STRAHL & HENNEBERG, 1901

[1] corrected for fertilization rate

[2] total litter loss excluded

[3] other means: 5.05 ± 0.27, 5.33 ± 0.35, 5.36 ± 0.25

[4] pre-implantation

[5] post-implantation

[6] calculations only for the period of March to December

Publication list

1. Schroeder K, Drews B, Roellig K, Goeritz F, Hildebrandt TB. Ultraschallgestützte Biopsietechniken beim europäischen Feldhasen (Lepus europaeus). In: Ergebnisse der Fachtagung Feldhase - Der aktuelle Stand der Hasenforschung 19. - 20. März 2010 in Kassel, Lang J, Godt J & Rosenthal G (eds), Lutra Verlags- und Vertriebsgesellschaft, Tauer: 2010.

2. Schroeder, K., Drews, B., Roellig, K., Menzies, B.R., Goeritz, F. and Hildebrandt, T.B. (2011) 'In vivo tissue sampling of embryonic resorption sites using ultrasound guided biopsy', *Theriogenology*, vol. 76, pp. 778-784.

3. Drews, B., Ringleb, J., Waurich, R., Hildebrandt, T.B., Schröder, K. and Roellig, K. (2013) 'Free blastocyst and implantation stages in the European brown hare: correlation between ultrasound and histological data', *Reproduction, Fertility and Development*, vol. 25, pp. 866-878.

4. Schroeder, K., Drews, B., Roellig, K., Goeritz, F. and Hildebrandt, T.B. (2013) 'Embryonic resorption in context to intragestational corpus luteum regression: a longitudinal ultrasonographic study in the European brown hare (*Lepus europaeus* PALLAS 1778)', *Theriogenology*, vol. 80, pp. 778-784.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 01.11.2013

Katharina Schröder