

**Superconception in mammalian pregnancy can be detected and
increases reproductive output per breeding season**

Kathleen Roellig*, Frank Goeritz, Joerns Fickel, Robert Hermes, Heribert Hofer &
Thomas B. Hildebrandt

Leibniz Institute for Zoo and Wildlife Research

Alfred-Kowalke-Str. 17

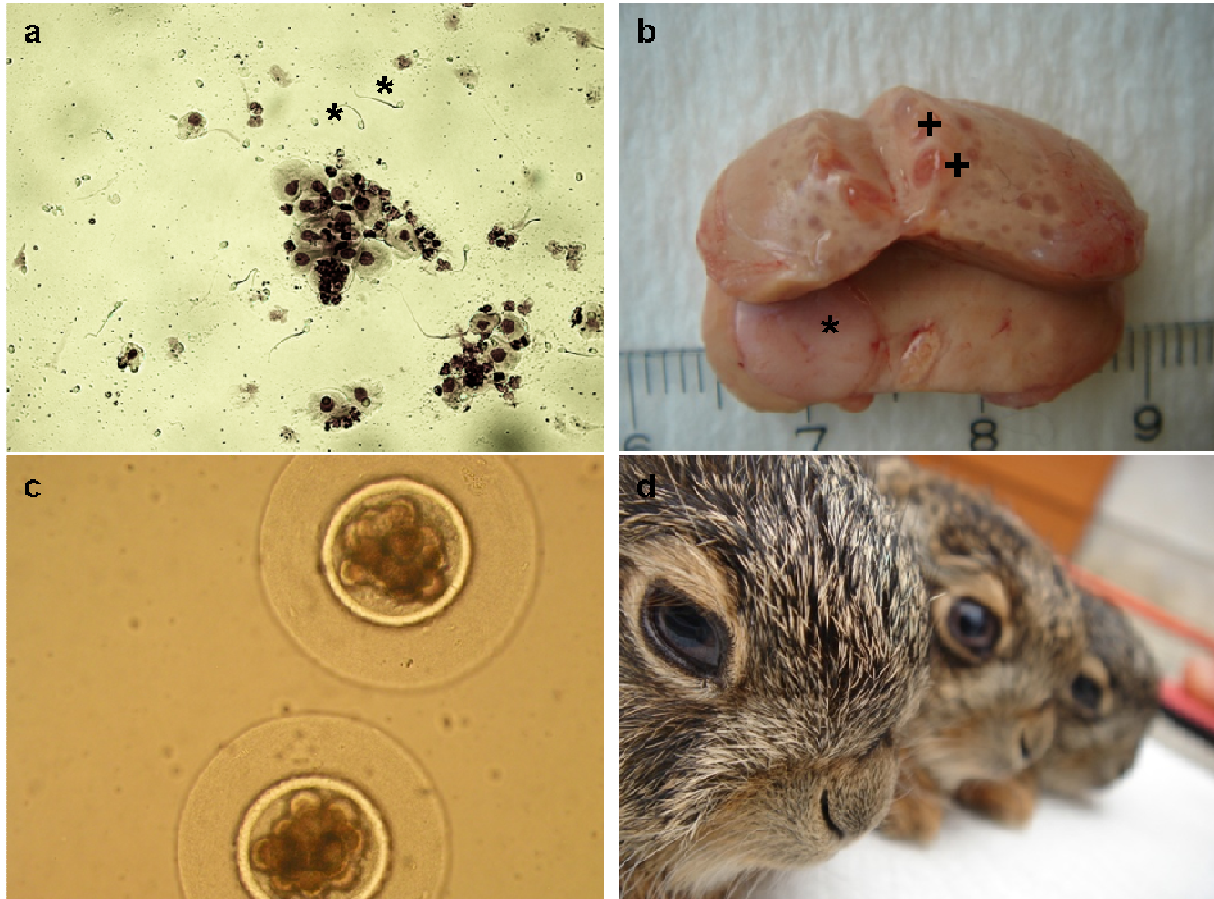
10315 Berlin, Germany

Phone: ++49-30-5168 242

Email: kathleen_roellig@gmx.de, roellig@izw-berlin.de

SUPPLEMENTARY FIGURES

Supplementary Figure S1. Illustration of oviduct flushing for the demonstration of superfetation.



Shortly before or immediately after parturition, oviducts were flushed to assess whether the second, smaller set of embryos detected ultrasonographically was indeed a new set of embryos in the oviduct. (a) Vaginal smear to detect spermatozoa (indicated by *) and successful insemination in a late-pregnant female. (b) Ovary with two sets of corpora lutea (CL) from first (*) and second (+) pregnancy previously detected by ultrasonography (see also Fig. 1) (c) Four-day-old embryos flushed from the oviduct. A specific characteristic of the embryo of the European brown hare is the mucous layer around the zona pellucida which increased in its thickness during the development in the oviduct. (d) Litter of newborn offspring two hours after parturition.

Supplementary Figure S2. Evidence for growth of discordant fetuses not due to superfetation.



In three out of 159 litters massive growth discordance between offspring of the same litter was detected (displayed in the three images). Owing to the breeding management and frequent ultrasound examinations, there was unambiguous evidence that these progeny belonged to the same litter and could not have developed by superfetation. (1) and (2) normal delivery with living young; (3-7) normal delivery with two dead (3, 4) and three living (5-7) offspring; (8-11) fully developed litter found in a female that died on day 41 of pregnancy. Sex and body weights of each offspring were: (1) male, 85g; (2) female, 155 g; (3) male, 135g; (4) unknown, 40g; (5) female, 95g; (6) female, 110g; (7) female, 120g; (8) female, 145g; (9) female, 140g; (10) male, 55g; (11) female, 120g.

SUPPLEMENTARY TABLES

Supplementary Table S1. Results of oviduct flushing.

ID	Day of gestation		First pregnancy		Second pregnancy (conceived by superfetation)		
			Corpora lutea (left / right)	Litter size *	Corpora lutea (left / right)	Litter size †	Cell stage of flushed embryos
64	[41]	pre-P	1/1	1	2/1	3	4
201	[43]	pre-P	3/1	4	3/1	4	16-20
4	41	post-P	2/1	3	2/2	4	6-12
33	40	post-P	1/2	3	2/1	3	12-16
47	42	post-P	0/2	2	3/1	4	32
61	42	post-P	1/1	2	2/1	3	20

Oviduct flushing was used to demonstrate that at the time of birth a new set of intact embryos was still in the oviduct next to fully developed fetuses in the uterus (see also Supplementary Figure S1). Flushing was performed before parturition (pre-P) or immediately after parturition (post-P). The table contains the identity of the female (ID), the day of gestation of the first pregnancy at which flushing was performed and details for the first and subsequent second pregnancy conceived by superfetation: the number of corresponding corpora lutea on the left and right ovary and the litter size (* at parturition, † number of embryos flushed out of the oviduct). In all cases there were as many embryos recovered from the respective oviducts as new corpora lutea were found.

Supplementary Table S2. Results of microsatellite analysis to determine paternity

Offspring	Microsatellite markers						Summary
	D7	OCE	OCL	Sat3	Sat8	Sat2	
(1) ♀ 001, ♂₁ 11, ♂₂ 077							
OS1	+	+	+	-	-	<i>n.a.</i>	+
OS2	+	+	-	-	-	+	+
OS3	+	+	-	-	-	+	+
OS4	-	+	+	-	-	+	+
(2) ♀ 037, ♂₁ 061, ♂₂ 074							
OS1	-	+	+	-	-	+	+
OS2	-	+	+	-	+	-	+
OS3	+	-	-	-	+	-	+
OS4	-	-	-	-	-	-	-
(3) ♀ 045, ♂₁ 053, ♂₂ 075							
OS1	+	-	+	-	-	<i>n.a.</i>	+
OS2	+	-	+	-	-	-	+
OS3	+	-	+	-	-	<i>n.a.</i>	+
OS4	<i>n.a.</i>	-	<i>n.a.</i>	-	-	<i>n.a.</i>	-
OS5	+	-	+	-	-	<i>n.a.</i>	+
(4) ♀ 063, ♂₁ 075, ♂₂ 053							
OS1	-	-	+	-	-	-	+
OS2	+	-	+	-	-	-	+
OS3	+	-	+	-	-	+	+
(5) ♀ 073, ♂₁ 052, ♂₂ 076							
OS1	+	-	+	-	+	<i>n.a.</i>	+
OS2	+	-	-	-	+	<i>n.a.</i>	+
OS3	+	-	+	-	-	<i>n.a.</i>	+

Six microsatellite markers were tested for each offspring from five different litters derived from superfetation. One female (♀) was temporarily mated with two different males. Mating occurred with male # 1 (♂₁) for one day to initiate the first gestation and with male # 2 (♂₂) between days 36 and 40 of gestation. Paternity of offspring (OS) of the second litter conceived by superfetation was determined: „+“ = second male was the father; „-“ = result compatible with the idea that either male could have been the father; *n.a.* = no result owing to methodological problems; “Summary” = summary of the evaluation of individual loci.

Microsatellite markers : D7=D7UTR1 ²³; Sat2, Sat3, Sat8 ²⁴; OCE=OCELAMB, OCL1 ²⁵.

Supplementary Table S3. Comparison of reproductive output of female European brown hares in pregnancies conceived with and without superfetation.

	no SF	with SF	overall mean
<u>Mean number of ovulations</u>			
Full season	3.2 ± 1.4 (N = 96)	4.0 ± 0.9 (N = 35)	3.4 ± 1.3 (N = 131)
excl. Jan/Feb	3.7 ± 1.3 (N = 66)	4.1 ± 0.8 (N = 32)	3.8 ± 1.2 (N = 98)
natural	3.0 ± 1.3 (N = 79)	4.0 ± 0.9 (N = 29)	3.3 ± 1.2 (N = 108)
AI	3.9 ± 1.5 (N = 17)	4.2 ± 0.8 (N = 6)	4.0 ± 1.3 (N = 23)
<u>Mean litter size</u>			
Full season	2.3 ± 1.1 (N = 96)	3.1 ± 1.1 (N = 35)	2.5 ± 1.2 (N = 131)
excl. Jan/Feb	2.5 ± 1.1 (N = 66)	3.2 ± 1.1 (N = 32)	2.7 ± 1.2 (N = 98)
natural	2.2 ± 1.1 (N = 79)	3.1 ± 1.1 (N = 29)	2.4 ± 1.2 (N = 108)
AI	2.6 ± 1.1 (N = 17)	3.2 ± 1.2 (N = 6)	2.7 ± 1.2 (N = 23)
<u>Prenatal loss</u>			
Full season	0.9 ± 1.1 (N = 96)	0.9 ± 1.2 (N = 35)	0.9 ± 1.1 (N = 131)
natural	0.8 ± 1.0 (N = 79)	0.9 ± 1.2 (N = 29)	0.8 ± 1.1 (N = 108)
AI	1.4 ± 1.5 (N = 17)	1.0 ± 1.3 (N = 6)	1.3 ± 1.4 (N = 23)

The number of ovulations is defined by the number of corpora lutea detected via ultrasonography at an early stage of gestation. Litter size is the number of offspring delivered in a normal parturition (including live or dead offspring). Prenatal loss shows the absolute difference between the number of ovulations and litter size at birth. Means ± standard deviations are given for the “full season” (January to September), for the season excluding January and February (“excl. Jan/Feb”) because the first litters of the year could not have been conceived by superfetation and for different modes of insemination, either with a fertile male (“natural”) or via artificial insemination (“AI”). All litters where at least one offspring was born were included. (For the statistical comparison of litter sizes and the number of corpora lutea per litter please see the results from the general linear mixed models in the main text.)