Received: 20 February 2023 | Revised: 23 June 2023 | Accepted: 3 August 2023
DOI: 10.1002/jez.2748

RESEARCH ARTICLE

JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY WILEY

Laterality in roe deer embryos implantation

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Abstract

Female reproductive success is one of the most important life-history traits to be monitored when determining population dynamics in free-ranging ungulates. Several studies have described how phenotypic characteristics of the mother, climatic conditions, population status, and habitat can impact on potential reproductive output in wild ungulates. However, little is known regarding the internal, physiological factors, that may account for differences in implantation rates. The present study investigated the differences in implantation rates and site on the basis of site and number of ovulations through the examination of about 3000 intact uteri collected from pregnant roe deer does (*Capreolus capreolus*). Although ovulation occurs with the same frequency in the left and right ovary, we revealed a higher frequency of embryos implantation in the left uterine horn in odd litter size, demonstrating that embryos can migrate between the uterine horns. In our study, a greater proportion of reproductive wastage was associated to females with three and four corpora lutea and interestingly, in relation to the site of ovulation, the percentage of corpora lutea that did not correspond to a fetus was higher in the right ovary than in the left one (73.2% vs. 26.8%). Our research described for the first time the absence of laterality in ovulation and the presence of laterality in implantation in roe deer, thus laying the foundations for in-depth studies about the functionality of this uterine side and for comparisons with populations located in other geographical areas to understand whether it is a widespread phenomenon or a local adaptation.

KEYWORDS

Capreolus capreolus, central Italy, corpus luteum, fetus, intrauterine embryo location, litter size, side of ovulation

1 | INTRODUCTION

Female reproductive success is one of the most important life-history traits to be monitored when determining population dynamics in free-ranging ungulates (Morellet et al., 2007; Vincent et al., 1995). In huntable species, female reproductive potential—before calving and post-natal mortality—can be assessed by the analyses of reproductive traits collected post-mortem and acquired data can be used to manage and monitor the populations (Chinn et al., 2022; Chirichella et al., 2019; Corlatti et al., 2018; Flajšman, Jerina, 2017; Flajšman, Pokorny, et al., 2017; Flajšman et al., 2018; Hewison & Gaillard, 2001; Malmsten & Dalin, 2014). The potential litter size is determined at the moment of ovulation by the number of ova shed, thereafter fertilization and implantation rates determine the actual reproductive output. While fertilization failures are difficult to be evaluated, the potential litter size and implantation rates can be determined by counting the number of corpora lutea (CL) and the fetuses (i.e., number, sex, developmental phase) in the ovaries and uteri collected from pregnant females. By analyzing the reproductive traits collected in culled females, several studies have described the variables that can impact on potential reproductive output in wild ungulates, mostly focusing on phenotypic characteristics of the mother, climatic conditions, population status, and habitat (Chinn et al., 2022; Chirichella et al., 2019; Corlatti et al., 2018; Flajšman, Jerina, et al., 2017; Flajšman et al., 2018; Frauendorf et al., 2016; Drimaj et al., 2020).

However, little is known regarding the internal, physiological factors, that may account for differences in implantation rates in wild ungulates. This is particularly true when dealing with species that developed unique reproductive strategies, such as the European roe deer (*Capreolus capreolus*), the only artiodactyl known to exhibit the phenomenon of delayed implantation. In this species, females have a single estrous period (Flint et al., 1994). They ovulate, and the rut takes place in mid-July to mid-August (Shams et al., 1980), while rapid embryo elongation and implantation occur 5 months later in mid-December to mid-January (van der Weijden & Ulbrich, 2020). This indicates the obligate period of embryonic diapause. The zona pellucida is shed before the diapause period and the blastocyst undergoes a low level of mitosis during diapause (Aitken, 1974; Lambert, 1999; Lengwinat & Meyer, 1996). This strategy makes the reproductive biology of this species peculiar (Bischoff, 1854; Short & Hay, 1966; van der Weijden & Ulbrich, 2020; Ziegler, 1843). A rapid embryo elongation and implantation occur 5 months later in mid-December to mid-January (van der Weijden & Ulbrich, 2020). However, Hermes et al. (2000) reported that the presence of the CL before implantation proves only the fact of the copulation and not the gestation (i.e., CL remain active during diapause also in nonpregnant females). Thus, the regression of the CL in does rutting but not fertilized takes place before implantation due to the lack of luteolisis mechanism (Faragó & Náhlik, 1997; Hoffman et al., 1978). In pregnant females, Broich et al. (1998) demonstrated that the CL remain active until parturition by using transrectal ultrasound. Therefore the CL, regardless of the number of embryos/fetuses present, remain visible throughout the gestation period in fertilized females and give birth to an average of two fawns (potential range 1–5; see Flajšman et al., 2017a) in May (Gaillard et al., 1993; Linnell & Andersen, 1998).

We have previously reported that in roe deer counting CL is not an adequate method for measuring final reproductive outcome because of a high interannual variability in fetal losses (mean: 8.6%, range: 3.6%–19.8%) (Chirichella et al., 2019). Previous studies suggested that variability in reproductive output between individual roe does is the product of a two independent step process, with body mass playing a predominant role in determining potential litter size and implantation rates being subsequently limited by senescence and climatic severity (Hewison & Gaillard, 2001). In another species which also exhibits embryonic diapause, the badger (*Meles meles*), a similar pattern was suggested, with body condition affecting ovulation but not successful completion of pregnancy (Creswell et al., 1992). Thus in roe deer, ovulation and implantation rates, being separated by a time window of approximately 5 months, appear to be independent and not directly interrelated. However, other "internal" factors may cause variations in these two processes, which are likely to be related even if occurring in two different moments and seasons.

The number of ovulations has been related to differences in implantation rates in domestic ruminants, and in ewes and does it is clear that implantation rates decrease with increasing ovulation rates (for a review see Diskin & Morris, 2008). Implantation rates decrease significantly while ovulation rates increase also in roe does (Hewison & Gaillard, 2001). The site of ovulation can also influence implantation rates, with higher prevalence of embryonic loss in ewes with unilateral multiple ovulations than with single ovulation (Willingham et al., 1986), and in ewes with multiple ovulations on the right ovary than in those with multiple ovulations on the left ovary (Regassa et al., 2007).

Starting from these premises, the present study investigated the differences in implantation rates and site on the basis of site and number of ovulations through the examination of about 3000 intact uteri collected from pregnant roe does during several hunting seasons. Our hypothesis was that number and site of ovulation could impact on the implantation process and the subsequent development of the fetuses. We expected the highest probability of successful implantation in females with single or double ovulations, with implantation site being ipsilateral to the site of ovulation.

2 | METHODS

2.1 | Study area

The study took place in the Arezzo Province (3,235 km^2; 43°280N, 11°530E, Tuscany, Central Italy). In the study period (2006–2021), roe deer hunting was allowed in 22 hunting districts (average size ~9500 ha, covering 64.54% of the whole province) divided into 1910 hunting zones (average size = 109.35 ha ±1.22 SD; Figure 1). About 57% of the territory is >400 m above sea level (asl) and 7.4% is >1000 m asl. The northern part of the province is mostly mountainous, including the Apennine chain and other secondary chains, with altitudes ranging from 300 to 1654 m; 66% of the area is forested. The southern part of the area includes the lower course of the Arno River and Chiana Valley, the Chianti hills, and some low mountains, with elevations ranging from 120 to 1081 m. Approximately 50% of this area consists of cultivated fields while forests cover 32% of the area. Forests are predominantly deciduous with dominant species being beech (*Fagus sylvatica*), oaks (Turkey oak [*Quercus cerris*] and downy oak [*Q. pubescens*]), and sweet chestnut (*Castanea sativa*); the percentage of conifers accounts for only 6.5%. The climate is temperate-continental, with mean temperatures ranging from 1.4°C

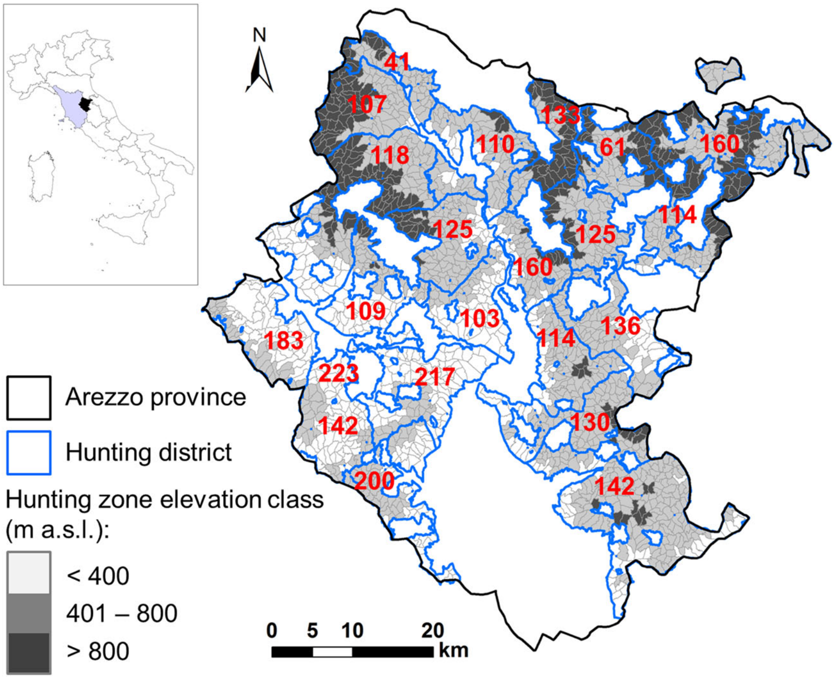

FIGURE 1 The study area located in the Arezzo Province, Tuscany, central Italy. Hunting zones ($n = 1910$) where roe deer females were legally hunted from 2006 to 2021 are shown in the digital elevation model classes of the study area (darker color corresponds to higher elevation). Sample size as the number of intact uteri were highlighted in red for each hunting district.

in the coldest months (January–February) to 24.9°C in the hottest ones (July–August).

Besides roe deer, which can be found in the 80% of the province, the study area is inhabited by fallow deer (*Dama dama*), red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and mouflon (*Ovis aries musimon*) (Apollonio & Mattioli, 2006). Wolf (*Canis lupus*), with a mean estimated number of 25 packs during the study period (Bassi et al., 2015), and red fox (*Vulpes vulpes*) were also observed in the study area.

2.2 | Data collection

We collected 2953 intact uteri of roe deer females that were legally hunted during the annual winter harvest (1 January–15 March) during the 2006–2021 period. For each individual, hunters recorded date of culling, hunting zone, and body mass expressed as eviscerated body mass to the nearest 0.5 kg.

Immediately after the culling, hunters made dissection of the animals, placing uteri into plastic bags and storing them frozen until analysis. Samples were defrosted and analyzed in the laboratory at the Casa Stabbi field station, in Chitignano (Arezzo Province, Tuscany, Central Italy). To determine the potential litter size, we counted the corpora lutea (CL) after the dissection of each ovary (L: left; R: right); moreover, we determined the number, position (L: left; R: right), sex (F: female; M: male), size, and weight of fetuses.

For a subset of collected reproductive traits ($N = 446$), we weighted the right and left ovaries ($X \pm 0.01$ g), and we measured their length, width, and thickness with a digital caliper ($X \pm 0.01$ mm).

Hunters also collected mandibles of all studied individuals for age assessment made by macroscopic inspection of teeth development and tooth wear (Ratcliffe & Mayle, 1992). Age was evaluated using a tooth wear table developed locally, and validated age by histological examination of teeth through counting annual cementum layers in a sample set of >300 individuals from a previous data collection (Capitani et al., 2005). Total length of mandibles, a proxy for skeletal size, was measured with a digital calliper to the nearest 0.01 mm.

Only adult females (i.e., ≥2-year-olds) were considered in this research. These animals were culled during the hunting activity regulated by the national authorities of Italy based on yearly hunting management plans. The study complies with all relevant national, regional, and provincial Italian laws and with the ethical standards of scholarly research.

2.3 | Data analysis

We considered only females with ≥1 corpus luteum in our analysis, representing all the pregnant females. Considering the different litter size, we calculated the percentage of CL in the left and right ovaries to evaluate the laterality of ovulation. The percentage of fetuses in the left and right uterine horns was calculated to establish the prominent embryo implantation and gestation in one side of the reproductive tract. CL and fetus positions in each female were compared in each litter size to determine the percentage of intrauterine embryo migration (i.e., when implantation site was not ipsilateral to the site of ovulation). In our data set we were able to determine only the minimum number of migrations resulting from unequal positions between the CL and the embryos in the right and left portion of the reproductive tract for each female. Based on our observations, the number of migrations could be even higher as all the embryos could pass from the left horn to the right and vice versa. Female body mass and body condition (i.e., body mass/total mandible length) were evaluated in relation to the choice of left or right implantation in odd litters.

Moreover, in even litters, the sex and condition (in terms of weight and length) of fetuses were evaluated in relation to their position in the left or right uterine horn. In particular, when the litter was 2, in the presence of fetuses of the same sex, their weight and length were compared based on their position in the right or left horn of the uterus throughout the use of a paired *t*-test. In the case of fetuses of different sexes, it was evaluated whether one sex was preferably positioned in the R or L position.

For a subset of collected reproductive traits ($N = 446$), we compared the weight, the length, the width, and the thickness of the right and the left ovaries throughout the use of a paired *t*-test.

Moreover, we derived the reproductive wastage (RW) by relating ovulation rate and site (i.e., the number and R or L position of CL) to implantation rate (i.e., the number of fetuses) according to different potential litter size (i.e., the total number of CL per ♀).

We performed all statistical analyses in R version 4.0.4 (www.r-project.org; R Core Team, 2021).

3 | RESULTS

Analyzing 2953 intact uteri of roe deer adult females (i.e., ≥2 y.o.) laterality of ovulation was not observed in all litter size (0–5; see Figure 2 for further details); the total number of CL was 6176: 3058 in the left ovaries [49.51%] and 3118 in the right ovaries [50.49%]. In 98.21% of pregnant females, the number of CL was greater or equal to the number of fetuses (i.e., two fetuses were found to be generated by one CL in only 29 females out of 2953; see Table 1 for major details). Equal functionality was confirmed by the absence of differences in weight ($t = -0.08$, $df = 445$; $p = 0.93$), length ($t = -0.78$, $df = 445$; $p = 0.44$), width ($t = -1.26$, $df = 445$; $p = 0.21$), and thickness ($t = -0.14$, $df = 445$; $p = 0.88$) between right and left ovaries for a subset of collected reproductive tracts (see Supporting Information: ESM1). Although ovulation occurs with the same frequency in the left and right ovary, we observed a highest frequency of embryos implantation in the left uterine horn in odd litter size (i.e., pregnant females with 1, 3, or 5 fetuses; see Figure 3 for further details). Embryos originated from right-ovary ovulations migrated into the left uterine horn with a greater frequency than vice versa in odd litter size. Indeed, the percentage of intrauterine embryo migration is equal from right to left side (19.55%) and vice versa (18.32%) in case of twins, while in singletons only a migration from the right side to the left one was detected (18.18%). In case of triplets, a greater percentage of migration from the right side to the left one was detected (37.17% vs. 8.90%) (see Supporting Information: ESM2 for major details).

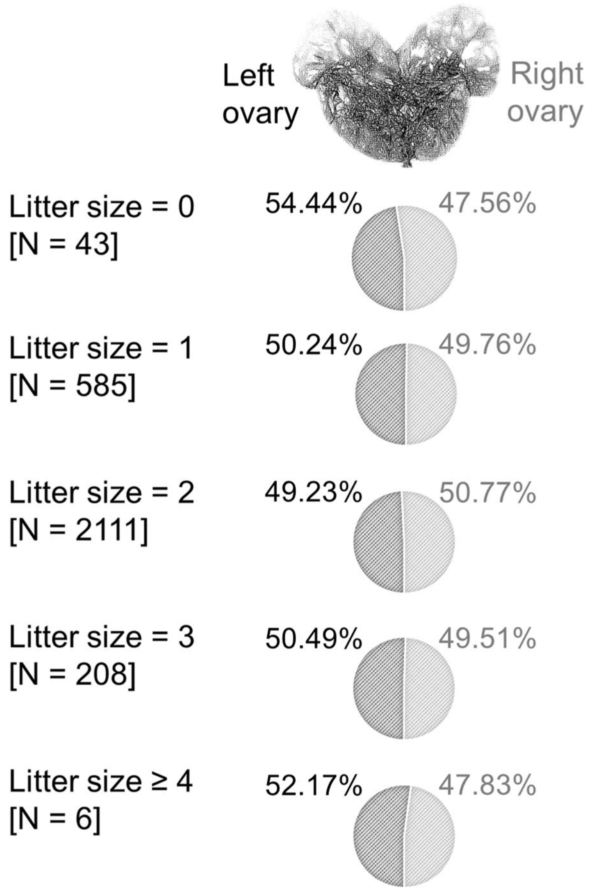

FIGURE 2 Position of corpora lutea in 2953 intact uteri from roe deer females legally hunted during the annual winter harvest (Jan 1–Mar 15) in the Arezzo Province, Tuscany, central Italy, 2006–2021. Percentages were reported according to different litter size (0–5 fetuses).

TABLE 1 Number of intact uteri of roe deer females analyzed in the present study.

	No. of roe deer females			
Litter size	CL < no. of fetuses	CL = no. of fetuses	CL > no. of fetuses	Total
0	0	0	43	43
1	0	180	405	585
2	9	1889	213	2111
3	17	190	1	208
4	1	3	0	4
5	2	0	0	2
Total	29	2262	662	2953

Note: Sampled individuals were legally hunted during the annual winter harvest (Jan 1–Mar 15) in the Arezzo Province, Tuscany, central Italy, 2006–2021. Data were reported according to litter size 0–5 and relation between the number of corpora lutea (CL) and fetuses.

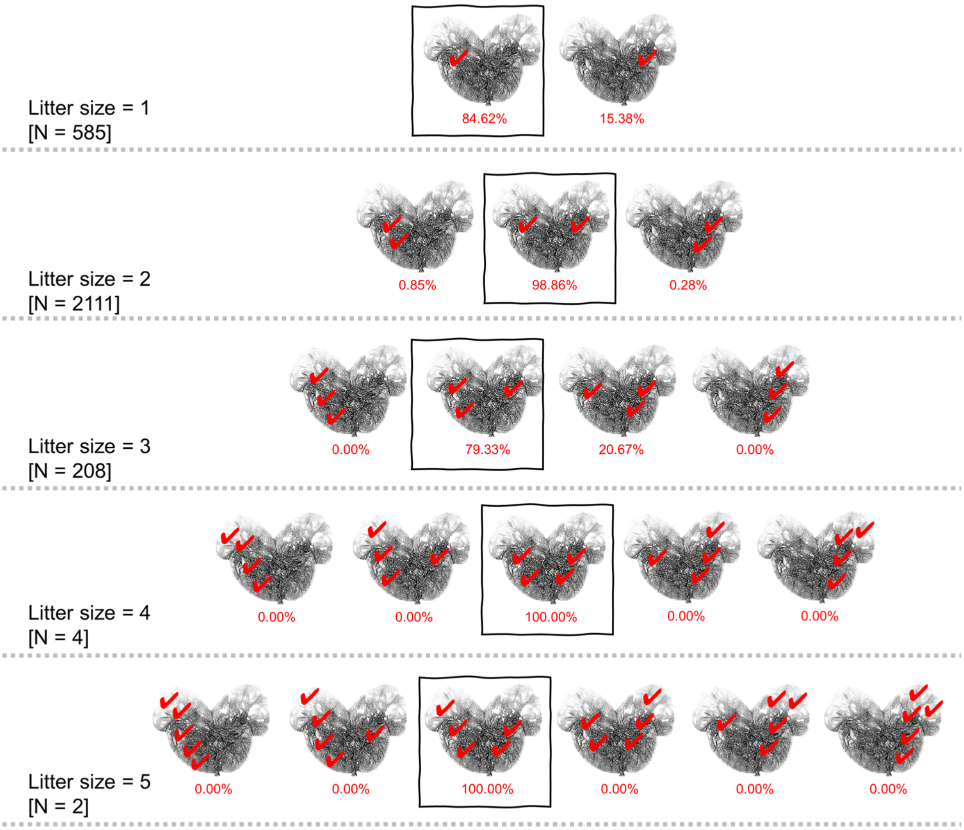

FIGURE 3 Position of fetuses in 2910 intact uteri of roe deer females that were legally hunted during the annual winter harvest (Jan 1–Mar 15) in the Arezzo Province, Tuscany, central Italy, 2006–2021. Percentages were reported according to different litter size (1–5 fetuses) and the combination with the highest frequency was highlighted in the black box.

Comparing the females with odd litters with fetuses in the left horn of the uterus with respect to the right, no differences were reported in terms of body mass ($F_{1,793} = 0.572$, $p = 0.450$) and body condition ($F_{1,777} = 0.043$, $p = 0.836$).

When the litter was two: (i) in the presence of fetuses of the same sex, no difference was found in relation to their weight and length based on their position in the right or left horn of the uterus (males fetuses: weight: $t = 1.20$, $df = 482$, $p = 0.23$; length: $t = 1.03$, $df = 482$, $p = 0.30$−females fetuses: weight: $t = 0.79$, $df = 393$, $p = 0.43$; length: $t = 1.13$, $df = 393$, $p = 0.26$), while (ii) in the presence of different sexes, no preferences were detected in the R or L position for one sex (47% males fetuses in the L-position and 53% in R-position).

According to the values of reproductive wastage (RW), we revealed a loss of implantation for 719 CL (i.e., RW = 11.64%) and a greater proportion of loss was associated to females with 3 and 4 CLs (Table 2). Interestingly, for what concerns the site of ovulation, the percentage of CL that did not correspond to a fetus is higher in the right ovary than in the left one (73.20% vs. 26.80%) (see Supporting Information: ESM3 for major details). When the number of embryos implanted in the uterus was zero ($N = 43$ females, 1.46% of our data set), we detected the presence of CL until February 15.

TABLE 2 Reproductive wastage (RW) according to different potential litter size and different combination in the position of fetuses (L: left uterine horn; R: right uterine horn) in 2953 intact uteri of roe deer females that were legally hunted during the annual winter harvest (Jan 1–Mar 15) in the Arezzo Province, Tuscany, central Italy, 2006–2021.

No. of CL per ♀	No. of ♀ (%)	No. of fetuses	No. of CL	RW (%)
1	199 (6.7)	198	199	0.50
2	2297 (77.8)	4193	4594	8.73
3	445 (15.1)	1029	1335	22.92
4	12 (0.4)	37	48	22.92
1–4	2953	5457	6176	11.64

4 | DISCUSSION

Roe deer are the only artiodactyls to display embryonic diapause that enables them to mate and to give birth in the periods of the year with favorable conditions (Andersen et al., 1998; Short & Hay, 1966; van der Weijden & Ulbrich, 2020). The high pregnancy rates in this species suggest that reproduction is highly efficient; however, certain processes are not clear even today.

To increase knowledge of reproductive biology in roe deer, in this research we investigated whether the ovulation process could influence subsequent implantation rates, considering that roe deer is the only ungulate species in which these events are timely separated by approximately 5 months. By examining approximately 3000 reproductive tracts along several years, almost no case of large numbers of fetuses congregated in one uterine horn was found. Therefore, it seems that an equal split is generally preferred, but when an odd fetus was observed, it was most often implanted in the left horn. This preference was not linked to a laterality of ovulation because it occurred with similar frequency in the left and right ovary.

Among Artiodactyla, some examples of laterality in implantation and gestation (Fernandez-Baca et al., 1970; Sumar & Adams, 2007) have been already reported, regardless of laterality of ovulation (Fernandez-Baca et al., 1970; Sumar & Leiva, 1979); this is the case of llamas (*Lama glama*) and alpacas (*Vicugna pacos*), where implantation and gestation exclusively occur in the left uterine horn (Ratto et al., 2020). Females from both species have a bicornate uterus that exhibits a clear asymmetry between uterine horns, with the left horn being larger and irrigated by a greater blood flow than its right counterpart (Del Campo et al., 1996, 2009). This asymmetry is not only observed in pluriparous and pregnant females but also in nulliparous and even in female fetuses; therefore, it is not induced by pregnancy (Del Campo et al., 2009). Therefore, since the ovaries are both functional and ovulation occurs with the same frequency in the left and right ovary (like in other species, see Fernandez-Baca et al., 1973; Sumar & Leiva, 1979), in llamas and alpaca embryos originated from right-ovary ovulations must migrate into the left uterine horn.

Unlike what occurs in these species, no asymmetries in the uterine development and function have been described in roe deer. Indeed, in our case study fetuses are located symmetrically when their number is even (i.e., 2 or 4 fetuses), but show a preference for the left uterine horn when the number of fetuses is odd (i.e., 1, 3, or 5 fetuses). The migration of preimplantated embryos is a phenomenon that has been described in farm ungulates for over half a century (e.g., Reimers et al., 1973; Scanlon, 1972). It is well documented in sheep (Doney et al., 1973), horses (Ginther, 1983), goats (Mani et al., 1992), and swine (Tummaruk et al., 2007), while no consensus about its occurrence under natural breeding circumstances in cattle was reported (Borges et al., 2017; Boyd et al., 1944; Hafez, 1964; McMillan & Peterson, 1995, 1999). In multiparous species, transuterine embryo migration is a way to regulate the spatial distribution of fetuses during pregnancy to ensure an appropriate fetal development (Nephew et al., 1992).

López-Gatius et al. (2017, 2020) revealed that the risk of pregnancy loss during the first trimester of gestation for cows carrying twins was from five to nine times higher for unilateral than for bilateral twins, thus highlighting the importance of embryo spacing to prevent embryo overcrowding and to preclude embryonic loss due to unnecessary nutritional and space competition. The repeatable and programmed intrauterine embryo arrangement rather than a random distribution apparently implies rigid control mechanisms, which are probably evolved from long-time selection and adaptation to various parameters in a certain species, such as uterine shape, number of embryos, walking mode, local nutritional supply, and so forth. (Chen et al., 2013). Uterine contraction mediated peristaltic movements are essential for embryo spacing along the uterine longitudinal axis (Chen et al., 2013). In the case of roe deer, uterine movements seem to favor embryo implantation in the left uterine horn when the number of fetuses is odd. Indeed, the percentage of intrauterine embryo migration in our study case is equal from right to left side and vice versa in case of twins, while in odd litter sizes a greater percentage of migration from the right side to the left one was detected. Whether these differences are associated with a higher capacity of the left horn to accommodate extra embryos still needs to be investigated.

At the moment, it has not been clarified if the selected side of intrauterine embryo location affects the reproductive performance of this species, being equal the body mass and condition of females presenting with odd litters located in the left horn of the uterus with respect to the right. Moreover, also fetus growth (i.e., their weight and length) in does with an even litter size did not show differences based on their position in the right or left horn of the uterus. In the same way, sex distribution did not differ between the left and right uterine horn in twin pregnancies, so, it seems clear that no evident case of different sex allocation occurs (Charnov, 1982).

As expected, analyzing the values of reproductive wastage (RW, we observed a greater proportion of loss associated to females with 3 and 4 CLs, highlighting the high reproductive potential (see Flajšman et al., 2013 for a review) and the ability to adjust the final litter size before substantial reproductive investment (Chirichella et al., 2019; Hewison & Gaillard, 2001). Therefore, as environmental conditions during the reproduction are unpredictable, it may be advantageous for mammalian females to first conceive, especially because ovulation is not energetically demanding, while later facing the possibility of losing offspring following unfavorable stochastic events (Creswell et al., 1992; Frauendorf et al., 2016; Hewison & Gaillard, 2001; Simard et al., 2014). It should be noticed that a small the percentage of regressing CLs (16%) was described as occurring in pregnant females in December/January (Hermes et al., 2000), thus at the moment when rapid embryo elongation and implantation take place. On the other hand, development of additional CLs was not detected (Hermes et al., 2000). Hence, we might have slightly underestimated embryo losses.

Interestingly, in relation to the site of ovulation, in the right ovary we found a percentage of CLs that did not correspond to a fetus higher than in the left one, highlighting the importance to further investigate functionality of the right ovary and robustness of ova produced in the right side. However, this finding could only partially justify the higher presence of fetuses in the left uterine horn when the litter is odd; thus, additional in-depth studies should aim to increase knowledge of the functionality of this uterine side.

5 | CONCLUSION

Clarifying the issues related to the reproduction of a widely distributed and managed species such as the roe deer (Apollonio et al., 2017) is an important requirement to be able to predict its adaptive capabilities. Our research described for the first time the absence of laterality in ovulation and the presence of laterality in implantation in roe deer, with a preference in the left uterine horn. Although we did not find any adaptive explanations and functional meanings for this preference, a minor degree of reproductive wastage in the left portion of the reproductive tract is described here. This finding lays the foundations for making comparisons with populations located in other geographical areas to understand whether it is a widespread phenomenon or a local adaptation and finally to determine if it may have an adaptive value.

ACKNOWLEDGMENTS

We are grateful to the Arezzo Province for supplying data. We are indebted to M. Meacci, E. Donaggio and L. Mattioli for logistic support in collecting data. We thank all hunters who provided samples of roe deer uteri, without their collaboration the study would not have been possible. G. Casu Finlayson revised the English and helped improve the writing. R. Nelson and an anonymous reviewer provided helpful comments on earlier drafts of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The study complies with all relevant national, regional, and provincial Italian laws, and with the ethical standards of scholarly research.

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How to cite this article: Chirichella, R., Apollonio, M., Luccarini, S., & Berlinguer, F. (2023). Laterality in roe deer embryos implantation. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 1–9. https://doi.org/10.1002/jez.2748