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ORIGINAL ARTICLE

Assessment of reproduction of brown bears in Sweden using stained placental scars

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Abstract

The Swedish brown bear *Ursus arctos* population is protected, but managed with legally defined hunting seasons. Management decisions (e.g., hunting quotas) are frequently changed and should be based on knowledge about demographic parameters, but collecting sufficient data in the field is time consuming and expensive. An efficient method to collect data on reproductive output could be counting placental scars in the uteri of female brown bears, because hunters in Sweden are required to collect samples (including reproductive organs) of harvested bears and submit them to the authorities. We assessed the reliability of placental scar counts to determine reproductive performance by counting the number of young with female radio-collared brown bears and comparing that with placental scar counts after those females had been harvested. We found that staining uteri improved the detection of placental scars. The differences between number of scars detected before and after staining the uteri, increased significantly with female age. The number of placental scars and number of observed cubs-of-the-year accompanying females corresponded well 2 and 3 years after birth; relatively small deviations between them might have occurred because of early cub mortality prior to the observations after leaving the den. Placental scar counts can provide accurate information on age of primiparity, evidence for reproductive aging (senescence), and reproductive productivity, and therefore inform decisions regarding adaptive management, sustainable hunting, and conservation.

Keywords Management · Persistence · Regeneration · Staining · Sustainable hunting · *Ursus arctos*

Introduction

Knowledge of reproductive characteristics is essential for understanding a species´ population dynamics. Reproductive success can be determined in various ways. Traditionally, reproductive performance of mammals can be determined directly in the field by observing adults accompanied by dependent offspring (Linnell et al. 1998; Schwartz and White 2008). However, direct observations can be difficult, especially in rare and elusive, or wide-ranging species or in species living in dense habitats (Thompson 2004). Direct observations commonly also result in an underestimation of the number of offspring (Zedrosser and Swenson 2005; Solberg et al. 2006) and do not provide information on past reproductive events or whether offspring from the current litter have been lost prior to observation. In addition, animals might be disturbed by observers and can change their behavior when approached by humans (Ordiz et al. 2013) or could even be a threat to human safety if females accompanied by dependent offspring feel threatened (Bombieri et al. 2019). In contrast, disturbance is reduced when using noninvasive

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monitoring (Taberlet et al. 1999). Genetic monitoring (e.g., using fecal or hair samples) can provide information on reproductive success, if parent–offspring relationships are identified (Taberlet et al. 1999). However, such analyses require intensive sampling with high recapture rates and/or a high genotyping success (Smith and Wang 2014). In doing so, bias associated with the sample detection (e.g. observer experience, Soller et al. 2020) or technical problems associated with the genetic analyses (e.g. genotyping errors, Mills et al. 2000; Smith and Wang 2014), can negatively affect the reliability of estimations of reproductive performance (Taberlet et al. 1999).

Postmortem analysis of uteri can be used to evaluate reproductive performance in female mammals, and several parameters can be assessed by counting unattached blastocysts, embryos, fetuses, scars from fetal resorptions, and/or placental scars from full-term pregnancies (Harder 2012). A placental scar is an indication of embryo implantation and placentation in the uterine wall in mammals with hemochorial and endotheliochorial placenta types (Benirschke 1983). The birth event causes a scar and dark coloration of the uterine tissue around the scar (Wydoski and Davis 1961). The method of counting placental scars to determine reproductive performance has been used widely in mammals, including soricomorphs (Nakano and Mekada 2018), rodents (Yamada et al. 1988; Santicchia et al. 2015), lagomorphs (Hackländer et al. 2001; Bray et al. 2003; Schai-Braun et al. 2017), and carnivores (Hensel et al. 1969; Vos 1994; Hauer et al. 2002; Yamanaka et al. 2011; Reynolds et al. 2017). A challenge of this method is that scars are regenerated continuously and thus become less visible over time (Harder 2012).

Until recently, only few studies have focused on the persistence of placental scars over time. These studies have shown that the temporal process until complete regeneration of the uterine tissue varies among species, e.g., 3–7 months postpartum in the American mink (*Neovison vison*) (Elmeros and Hammershøj 2005); 12 months postpartum in the Asian house shrew (*Suncus murinus*) (Nakano and Mekada 2018); 19 months postpartum in the Arctic fox (*Alopex lagopus*) (Strand et al. 1995), and 20 months postpartum in the brown bear (*Ursus arctos*) (Moriwaki et al. 2016). Thus, time seems to have a strong effect on detection of placental scars and therefore the applicability of the scar count method. Staining of uterine tissues using, for example, Turnbull blue reaction, can improve the detection of placental scars (Salewski 1964). This staining technique improves the detection of placental scars, as shown for American mink (Fournier-Chambrillon et al. 2010) and red fox (*Vulpes vulpes*) (Ruette and Albaret 2011), but the staining of uterine tissues has only been tested in very few species.

Brown bears have interbirth intervals of 2–6 years (Hensel et al. 1969; Nawaz et al. 2008; Steyaert et al. 2012). However, litter loss is common during the mating season, for example due to sexually selected infanticide by males (Swenson et al. 2001a) or other causes (Swenson et al. 2001b; Steyaert et al. 2012). If female brown bears lose their litter during the mating season, they can mate within the same mating season and give birth to a new litter the coming year (Steyaert et al. 2014). Although hunting seems to affect the life history of brown bears (Bischof et al. 2018; Van de Walle et al. 2018) and thereby population growth (Frank et al. 2017), the determination of reproductive output of brown bears has not been performed on an annual basis (see Christiernsson 2018). In Sweden, reproductive status, age of sexual maturity (Zedrosser et al. 2009) and senescence (Schwartz et al. 2003), and litter sizes of individually-marked females have been determined in the field, based on radio-collared bears (Gonzalez et al. 2012). The observations are performed from the ground or a helicopter and therefore are either time-consuming or expensive. However, as reproductive performance is one of the parameters affecting population growth, detailed knowledge of reproductive parameters is necessary to inform adaptive management decisions and determine hunting quotas (Swenson et al. 2017), even when data from individually-marked individuals are not available.

Here we use the brown bear as model species to (1) support first indications that the staining of uteri improves the detection of placental scars and (2) test whether age of a female affects the detection of placental scars after staining. (3) We also place a special emphasis on the usefulness of the method by comparing the number of placental scars after staining with direct observations of cubs-of-the-year.

Material and methods

Study site and collection of samples

Samples of hunter-killed female brown bears were obtained from three subpopulations within 292,000 km^2 in Sweden (from 60° to 69°N) in the years 1997–2005 in the course of the Scandinavian Brown Bear Research Project (https://www.brownbearproject.com/). The regions, a southern region in south-central Sweden, a central one in north-central Sweden, and a northern one in northern Sweden (Bischof et al. 2008), corresponded to the core areas for reproduction, based on genetic data (Manel et al. 2004). They also coincided with the three female concentration areas evident from distributional information of hunter-killed individuals and were connected by male-mediated gene flow (Manel et al. 2004). The landscape is covered with coniferous forests dominated by Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*).

For further details on the study area see Zedrosser et al. (2006), Martin et al. (2010), and Ordiz et al. (2013).

In Sweden, all brown bears (including radio-marked bears) can be legally harvested by hunters from late August or early September until an annual harvest quota has been reached (Swenson et al. 2017). The only protected segment of the population is family groups, i.e., females and their dependent offspring of any age. After a bear has been killed, the carcass and information about the harvested individual (e.g., sex, body mass, harvest location) must be presented to an officially appointed inspector. Besides collecting biometric data, hunters and official inspectors are also required to collect samples (e.g., tissue, teeth, reproductive organs) (Bischof et al. 2008). These samples are submitted to the National Swedish Veterinary Institute (Statens Veterinärmedicinska Anstalt; SVA) (National Veterinary Institute 2019). Age of bears is determined by counting annual cementum layers of the first premolar tooth (Craighead et al. 1970; Matson et al. 1993). Immediately after killing, reproductive organs are soaked in water to avoid dehydration and then stored at – 20 °C at the SVA.

In total, uteri of 174 female bears were provided for this study by SVA. Uteri of individuals younger than three years (n = 65) that were small in size, had narrow uterine horns, and a thin wall, in particular a thin, homogenous endometrium, indicated that these individuals had not yet reached sexual maturity. For this study, we used only the reproductive organs of female brown bears ≥ 3 years (n = 109) harvested from 1997–2005. More than 93% (n = 102) of these females were killed between the beginning of August and the end of October during the regular hunting season. The other individuals (n = 7) were killed by the authorities as nuisance bears between April and July.

Field observations of reproductive performance

We monitored radio-collared females and the young-of-the-year, yearlings, and two-year-olds that accompanied them in litters in our study sites in the southern and northern regions every year (Gonzalez et al. 2012). To evaluate the applicability of placental scar counts (PSC), we compared placental scar counts with direct observations of cubs-of-the-year from the ground or a helicopter at den emergence for female bears killed during the year of observation. We used data collected during the longitudinal long-term monitoring program from 1997–2005 (Scandinavian Brown Bear Research Project) for our subsequent analyses. In total, nine of the bears monitored, whose uteri were examined subsequently, were observed for three consecutive years before being killed by hunters.

Macro- and microscopic counting of scars before staining

Frozen samples of the female genital tract were defrosted at room temperature in fresh water. Afterwards, ovaries, oviducts, the mesometrium, and all connective tissues were removed from each uterus and only the two uterine horns connected by a single cervix were retained for further analysis (see also Ruette and Albaret 2011). Finally, both horns were opened longitudinally on the antimesometrial side, and the uterine endometrium was examined macroscopically (without any magnification device) and microscopically (using a stereo microscope with a 2–4 × magnification) to determine the presence and number of placental scars. Hereafter, uteri with at least one placental scar are referred to as positive uteri.

Staining process

To stain the uteri, we used a method based on the Turnbull blue reaction. This method has been used for staining uteri of the black rat (*Rattus rattus*) (Salewski 1964; Yamada et al. 1988), European hare (*Lepus europaeus*) (Hackländer et al. 2001, 2011; Bray et al. 2003), mountain hare (*L. timidus*) (Schai-Braun et al. 2017), American mink (Fournier-Chambrillon et al. 2010), and red fox (Ruette and Albaret 2011). The uteri were stained according to Hackländer et al. (2001): soaked in a fresh 10% solution of ammonium sulphide [$(NH_4)_2S$] for 8 min, then rinsed under tap water and immersed for another 8 min into a solution made of equal parts of 1% chlorhydric acid (HCl) and 20% of a solution of potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6] \cdot 3H_2O$). Immediately afterwards, the uteri were rinsed in water again. The stained scars were then counted microscopically (using the stereo microscope with a 2–4 × magnification).

Statistical analyses

All analyses were performed using the statistical software R version 3.6.2 (R Core Team 2022). We accepted a statistical significance of $\alpha = 0.05$. We performed Kruskal–Wallis rank sum tests to determine whether age of hunter-killed females and number of placental scars counted before or after staining differed in the three study regions. We fitted a generalized linear model (GLM) with Poisson error distribution and log-link function to test whether staining has an effect on the number of placental scars detected. We tested for overdispersion but found no indication. Residuals were plotted and visually inspected for normality. In addition, we used a paired Wilcoxon rank

sum tests to test whether number of detected placental scars differed before and after staining.

We fitted a generalized additive model (GAM) with integrated smoothness estimation (R package 'mgcv', Wood 2011), Poisson error distribution, and a log-link function to test whether female age and number of scars detected before staining influenced on the absolute difference in the number of scars detected before and after staining (delta PSC). High values of delta PSC indicated a large difference between number of scars counted before and after staining. Both variables were included as single terms (spline based smooth *s* for age) and as interaction term (tensor product smooth *ti* for interaction between age and number of scars counted before staining) in the model. Residuals were visually inspected for normality.

In addition, we determined the persistence of placental scars over time by comparing the number of placental scars up to three years after birth with field observations of the total number of cubs-of-the-year accompanying females. Therefore, we compared the number of cubs born based on placental scars with the number of cubs-of-the-year based on visual observations in (i) the year when a female was killed (i.e., < 1 yr after birth), (ii) the year before a female was killed plus the year when the female was killed (< 2 yr after birth, and (iii) two years before a female was killed plus in the year when the female was killed (< 3 yr after birth).

Results

More than half (51.4%, n = 56) of the reproductive tissues we examined came from female brown bears harvested in the southern bear area in Sweden; the remaining samples were collected in the central (29.3%, n = 32) and northern (19.3%, n = 21) areas. The females' ages ranged between 3 and 24 years (mean ± SD, 7.8 ± 5.1 years, median: 6 years, n = 109), and did not differ among regions (Kruskal–Wallis rank sum test, $X^2 = 0.292$, df = 2, p = 0.864).

Of the 109 samples, we detected at least one placental scar before staining in 49 uteri (45%; Fig. 1). After staining, at least one placental scar was found in 69 uteri (63%), an increase of 40.8%. The average number of placental scars (± SD) found after staining was 2.44 ± 0.831 PSC (median: 2.00 PSC, n = 69 positive uteri). We found no significant differences in the number of placental scars among the three regions, either before (Kruskal–Wallis rank sum test, $X^2 = 0.604$, df = 2, p = 0.740) or after staining (Kruskal–Wallis rank sum test, $X^2 = 0.515$, df = 2, p = 0.773, Table 1). The number of detected placental scars after staining was positively affected by the number of placental scars detected before staining (Estimate = 0.380, SE = 0.055, p < 0.001). However, the number of detected placental scars was higher after staining of the uteri than before staining (paired Wilcoxon rank sum test, V = 36, p < 0.001, n = 109, Table 1).

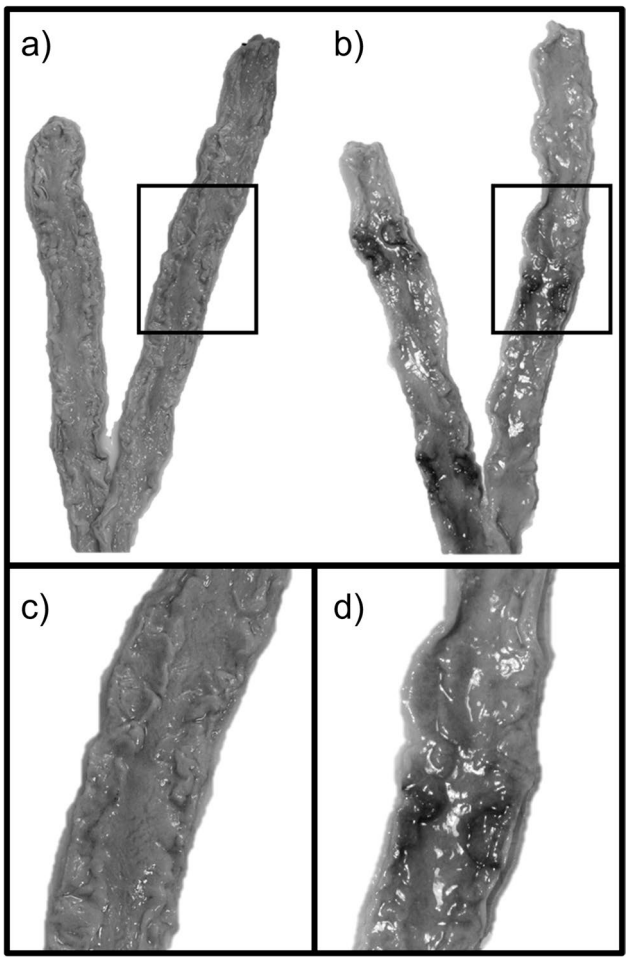

Fig. 1 Uterus of a brown bear opened longitudinally on the antimesometrial side. Placental scars were counted before (**a**, **c**) and after (**b**, **d**) staining. Detection of placental scars was improved after staining of the uterine tissue as this uterus contains three placental scars which were less visible before staining

The generalized additive model explained 54.6% of the deviance in the number of scars counted. The number of scars detected before staining had no effect on delta PSC (Estimate = − 0.286, SE = 0.189, p = 0.131). However, we found a significant effect of the interaction between female age and number of scars counted before staining on delta PSC (edf = 3.752, Ref.df = 3.950, $X^2 = 23.64$, p < 0.001), i.e., the absolute difference between number of scars detected before and after staining (delta PSC) increased with increasing age of a female (edf = 2.559, Ref.df = 3.178, $X^2 = 13.36$, p = 0.005, Fig. 2).

We further compared the number of placental scars with field observations of cubs-of-the-year accompanying nine females (Table 2). Assuming that female brown bears give birth in January each year (see Friebe et al. 2014), the

Table 1 Number of placental scars (PSC) counted before and after staining in uteri of female brown bears (≥ 3 years, n = 109) killed by hunters in three regions in Sweden during 1997–2005

Region	PSC before staining			PSC after staining			
	Min–max	Mean ± SD	Median	Min–max	Mean ± SD	Median	n
South	0–4	1.0 ± 1.2	0.5	0–4	1.6 ± 1.3	2.0	56
Central	0–3	0.8 ± 1.1	0	0–4	1.5 ± 1.3	1.5	32
North	0–4	1.1 ± 1.5	0	0–4	1.4 ± 1.5	1.0	21
Total	0–4	1.0 ± 1.2	0	0–4	1.5 ± 1.4	2.0	109

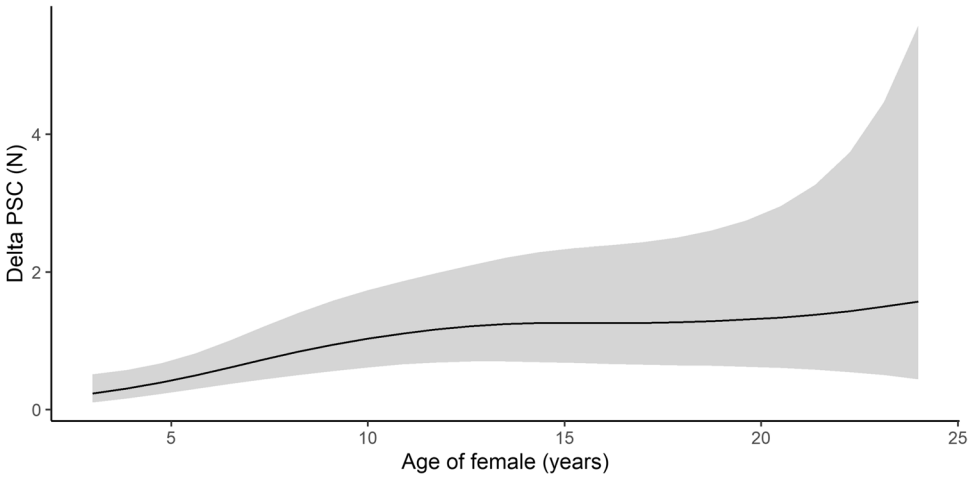

Fig. 2 Estimated smoothing curve for variation in the absolute difference between numbers of placental scars counted before and after staining the uteri (delta PSC) in relation to the age of female brown bears in Sweden, 1997–2005. The solid line represents the smoother and the gray bands represent the 95% confidence interval bands

Table 2 Number of placental scars (PSC) counted before and after staining in the uteri and the number of cubs-of-the-year accompanying a female brown bear in the year when the female was killed (N cubs, year 1), number of cubs-of-the-year accompanying a female in the year before being killed (N cubs, year 2), and number of cubs-of-the-year accompanying a female two years before being killed (N cubs, year 3) by hunters in three regions in Sweden, 1997–2005 (n = 9)

Bear ID	Kill month	PSC before staining	PSC after staining	N cubs, year 1	N month, year 1	N cubs, year 2	N months, year 2	N cubs, year 3	N months, year 3
#1	9	0	0	0	–	2	20	0	–
#2	9	0	0	0	–	2	20	0	–
#3	8	2	2	0	–	0	–	2	31
#4	9	2	2	0	–	2	20	0	–
#5	8	2	2	2	7	0	–	0	–
#6	9	0	3	0	–	3	20	0	–
#7	10	1	3	0	–	3	21	0	–
#8	9	2	3	2	8	1	20	0	–
#9	6	3	3	3	5	0	–	2	29

Duration (N months) between giving birth and being killed was calculated by assuming that cubs-of-the-year are born in January in the respective year (see Friebe et al. 2014)

longest persistence of stained placental scars was found in a female that had been given birth 31 months before being killed. In addition, stained placental scars were visible up to 21 and 20 months before being killed in one and three females, respectively. In contrast, two females gave birth 20 month before being killed, but placental scars were not visible after staining the uterine tissue.

Discussion

Here we show that staining of uteri significantly improved the detection of placental scars and should therefore be a standard when studying reproductive status of wild bear populations. Female age had a significant effect on the

detection of placental scars after staining. Due to the persistence of scars beyond a one-year period, the number of placental scars detected after staining reflected reproduction 2–3 years before, but not specifically in the year the female brown bear was killed.

Staining of uteri increased the reliability of placental scar counts (see also Fournier-Chambrillon et al. 2010; Ruette and Albaret 2011). Although difficulties in the detection of faint scars might have occurred before staining, these scars became visible after staining. Staining of uteri should be standard, also in brown bears, when evaluating reproduction and reproductive patterns based on cross-sectioning of uteri and counting of placental scars (see also Ruette and Albaret 2011). The number of additional placental scars detected after staining uteri increased significantly between the age of 3–12 years, before reaching an inflection point that occurred at 12 to 13 years of age. At this age, approximately one to two additional placental scars were visible after staining uteri. Large confidence intervals of females between the age of 20–24 years were probably due to the low sample size in the oldest age classes in our study.

Our study provided evidence that female brown bears in Sweden already mate with males for the first time at 2 years of age, as placental scars in hunter-killed females were found in 3-year-old individuals, indicating that these females gave birth during the previous winter. Female brown bears have been documented giving birth at 3 years of age in Austria (Zedrosser et al. 2004) and Croatia (Frković et al. 2001). In contrast, the minimum age of female brown bears at first parturition in other areas ranged between 4 (Mano and Tsubota 2002) and 5 (Shimozuru et al. 2017) years in Japan, between 4 and 10 years in North America (Stringham 1989), and 7 years in Pakistan (Nawaz et al. 2008).

Although based on a small sample size, the number of placental scars counted after staining was significantly related to the total combined number of cubs-of-the-year that had accompanied a female within two to three years prior to the female's death. These cubs-of-the-year were born approximately 20 to 31 months before the female was killed, giving an indication that stained placental scars can be detected up to 31 months. Three female brown bears accompanied by cubs-of-the-year were observed after leaving the den in April/May, and were killed later in the same year. One individual was killed by authorities as a nuisance bear in June, whereas the other two individuals were killed during the regular hunting season. According to Swedish regulations, females and their accompanying offspring of any age are protected from being killed. However, these females had given birth, but may have lost their offspring during the mating season, e.g., due to sexually selected infanticide, malnutrition, or traffic accidents (Swenson et al. 2001b). It is also possible that these females were killed accidentally, as it can be difficult to observe the presence of cubs-of-the-year in a hunting situation, especially in dense vegetation.

In our study area, most offspring separated from their mother as yearlings and most females had a 2-year cycle of reproduction during the study period from 1997–2005 (Dahle and Swenson 2003). Thus, at least in brown bears, placental scars can remain detectable with staining almost three years after implantation and 31 months after parturition. Although not directly comparable with placental scar counts in stained positive uteri (mean ± SE: 2.44 ± 0.831 PSC), the spring litter sizes recorded in the study site between 1995–2002 by research personnel averaged 2.27 ± 0.087 (SE) (Zedrosser and Swenson 2005). There are some explanations for the deviation between number of placental scars and number of observed cubs-of-the-year accompanying females. The number of placental scars could be higher than the number of cubs-of-the-year accompanying females due to cub mortality (Mano and Tsubota 2002; Yamane et al. 2009). During 1988–1998, research-obtained cub mortality was 0.35 in the southern area and 32% of the litters experienced partial loss of the cubs (Swenson et al. 2001b). Thus, coupled with direct observations of cubs, placental scar counts can provide estimates of early cub mortality. Placental scar counts, however, could also underestimate true litter size, because variation in individual hormone levels (Yamane et al. 2009) can affect the speed of regeneration of uterine tissue among females (Tsubota et al. 1989; Moriwaki et al. 2016). Therefore, in especially older females, scars might be less likely to be detected 20 months postpartum (Moriwaki et al. 2016), when placental scars are not stained.

We assume that apparently fresher and more recent scars in young females that have only reproduced once in their lifetime, led to a lower number of additional scars detected after staining uteri. In contrast, less visible and older scars in older females, which have reproduced repeatedly throughout the last 3 years, might have become visible after the staining of uteri, because placental scars vanish over time as uterine tissues regenerate (Harder 2012). Therefore, staining should be standard when determining reproductive performance of brown bears by counting placental scars in uteri.

In conclusion, our study showed that staining of uteri is a good method to enhance the visibility of placental scars in brown bears. Caution is required when estimating reproductive output, as placental scars, also in stained uteri, vanish over time and are therefore only detectable for a limited time. However, we showed that placental scars can be detected up to three years postpartum in bears.

Placental scar counts from hunter-killed bears can provide important information related to pregnancy and litter size, but the data must be interpreted with caution, as the number of cubs leaving the den might be lower than number of placental scars, due to early cub mortality. Although

data on placental scars face some limitations, it can provide sufficient information on several other aspects related to reproductive performance and can therefore inform adaptive management and conservation decisions (see also Swenson et al. 2017). Long-term data are required to study the effects of management strategies on age of primiparity, reproductive aging (senescence), or productivity in the hunted populations. Whereas sampling effort to determine the number of cubs accompanying females after leaving the den is high, reproductive organs of hunter-killed females are always collected and counting of placental scars is less time consuming and expensive. Data on reproductive performance can further be used to study whether reproductive parameters vary across regions or years and are thereby affected by food availability, climatic variables, or brown bear population density.

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Author contributions All authors contributed to the study conception and design. Sample and data collection was carried out by Andreas Zedrosser and Jon Swenson, material preparation and lab analyses were performed by Klaus Hackländer and Lisa A. Klestil. Data analysis was performed by Eva M. Schöll. The first draft of the manuscript was prepared by Eva M. Schöll and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The data analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Ethical approval Capturing and handling of bears (e.g. telemetry) was approved in accordance with relevant guidelines and regulations and were approved by the appropriate authority and ethical committee (Naturvårdsverket and Djuretiska nämden i Uppsala, Sweden). All bears were captured as part of the activities of the Scandinavian Brown Bear Research Project. The examined uteri are from bears that were legally killed during the hunting season in Sweden.

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