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Research Article

The use of uterine scars to explore fecundity levels in invasive alien tree squirrels

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Abstract

In invasion ecology, reliable measures of female fecundity are necessary to infer population growth rate and develop control programs to determine the proportion of animals that should be culled to reduce population size. Here, we present a reliable staining technique of uterine scars to determine individual fecundity in terms of both seasonal and total (annual) number of young born per female. We applied this method to two alien squirrels species (grey squirrel, *Sciurus carolinensis* and Pallas's squirrel, *Callosciurus erythraeus*) introduced in Northern Italy, obtaining carcasses from control campaigns from 2011 to 2013. We also investigated environmental and phenotypic variables that might affect individual variation in fecundity and compared annual reproductive output between the two species. For grey squirrels (n=44), 25% of examined females produced a single litter and 61% two litters. Females which reproduced in both seasons tended to have larger summer than spring litters (on average 2.61 and 1.94 offspring, respectively) and mean annual fecundity was 3.4 scars/female ranging from 1 to 8 births. There was no effect of year, eye lens weight, body size or body mass on total fecundity. For Pallas's squirrel (n=31), 58% of females had a spring litter, some of these also produced a summer litter (35%) and a few even a third litter in autumn (10%). Heavier and older females (higher eye lens weight) had more uterine scars than younger animals with lower body mass. Finally, fecundity of the two IAS in Italy was similar or even higher than in the native range and/or in other countries of introduction, suggesting they are well adapted to their new environment and potentially have a high capacity to spread and recover after reduction of population size.

Introduction

Invasive alien species (IAS) are a major threat to long-term survival of local native species (Clavero and Garcia-Berthou, 2005; European Environment Agency, 2012). Among mammals, many IAS have arrived in the new environment through pet trade (e.g. many tree squirrels) or by accidental or deliberate releases from fur farms (e.g. muskrat, coypu, mink) (Kolar and Lodge, 2001; Bonesi and Palazon, 2007; Martinoli et al., 2010; Simberloff et al., 2013; Bertolino and Lurz, 2013).

Most of these species need to be controlled to avoid or reduce direct or indirect damage to native species, agriculture and forestry, buildings and infrastructure or human health (Keller et al., 2011; Simberloff et al., 2013; Genovesi et al., 2015). Control campaigns generally consist in removing a proportion of all animals present in a given population. In order for control to be effective, the number of individuals removed must be scaled to the net population growth rate to define adequate harvesting quotas (Baker, 2006). Moreover, in addition to supplying practical information to directly implement control strategies, reliable estimates of population growth rates of IAS can support further investigations aimed at disclosing impacts of such species on native species and/or human health and activities.

One of the primary parameters affecting population growth rate is the annual reproductive output of females, which is the product of the number of young weaned per litter and the number of litters produced in a year (Gurnell et al., 2004). This value is generally referred to as fecundity. In small mammals fecundity can be estimated by intensive capture-mark-recapture studies which, however, require a large investment in terms of time, manpower and funding (e.g. Wauters and Lens, 1995; Wauters et al., 2008). Therefore, in species that are harvested for hunting and/or control, alternative methods have been developed. When carcasses of pregnant females are available, the number of embryos can be directly counted (Wauters et al., 1995). Otherwise, when carcasses are obtained after parturition or for species producing more than one litter per year, counting uterine scars can be an effective method (Bengtson and Siniff, 1981; Lindström, 1981; Rolley, 1985; Bray et al., 2003). Such scars are left by the detachment of the placenta from uterine walls, hence each scar corresponds with the parturition of a single young, thus providing information on litter size at birth. Fecundity has been estimated through placental-scar counts in rodents (Nixon and McClain, 1975; Martin et al., 1976), lagomorphs (Hackländer et al., 2011) and terrestrial carnivores (Allen, 1983; Lindström, 1994; Mowat et al., 1996; Ruette and Albaret, 2011; Melero et al., 2015) and seals (Kauhala et al., 2014).

One of the issues involved in counting uterine scars resides in the difficulties to count them a long time after parturition (e.g. Martin et al., 1976; Elmeros and Hammershøj, 2006; Ruette and Albaret, 2011). Here we adapted a staining technique which allows to distinguish the number of uterine scars of different litters to two alien squirrels present in Italy (Eastern grey squirrel, *Sciurus carolinensis* and Pallas's squirrel, *Callosciurus erythraeus*). This method allows to estimate fecundity of female squirrels as a demographic parameter that may subsequently be used i) in studies on population dynamics of the alien species aimed at predicting necessary levels of control (i.e. number of animals to be

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culled) to successfully reduce population size; and ii) in spatially explicit population dynamics models to predict future range expansion and demographic trends (e.g. Lurz et al., 2001; Tattoni et al., 2006; Bertolino et al., 2008).

Materials and methods

Trapping

Over three years (2011-2013), from September to December, grey squirrels (*Sciurus carolinensis*) and Pallas's squirrels (*Callosciurus erythraeus*) were trapped in Lombardy as part of a European Community LIFE Project (LIFE09 NAT/IT/00095 EC-SQUARE) aimed at eradicating the alien species in Italy. Traps (live-traps model 202, Tomahawk Live Trap Co., Wisconsin, USA) were set in the morning, baited with hazelnuts and checked twice to three times a day to minimize stress to captured animals during trapping. For each trapped squirrel sex and reproductive condition were recorded (Wauters and Dhondt, 1989). Animals were euthanised by CO_2 inhalation, following EC and AVMA guidelines (Close et al., 1996, 1997; Leary and American Veterinary Medical Association, 2013). Each individual was weighed to the nearest 5 g using a Pesola spring-balance and hind foot length was measured with a thin ruler ($\pm$0.5 mm). Each carcass was immediately placed in a sealed plastic bag and stored at −20 °C for further examinations.

Laboratory methods

In 2014, we analysed uterine scars of 49 female grey squirrels trapped in three different sites in Lombardy and of 40 Pallas's squirrels from the only population in Varese province, Lombardy, Northern Italy. All the analysed animals were culled at the end of the last breeding season (mid September – early December). Many authors have underlined the importance of the timing of animal sampling for uterine (placental) scars investigation with respect to parturition date(s), since scars fade over time, thus affecting their correct recognition and determination (e.g Martin et al., 1976; Lindström, 1981; Allen, 1983; Elmeros and Hammershøj, 2006). Even in our case, preliminary trials on individuals that gave birth during summer did not evidence any scars if stained after winter diapause, indicating that after winter the process of endometrium repair is completed and uterine scars can no longer be revealed by our staining techniques. Hence, collection of animals in autumn-early winter allows to reveal all the uterine scars of the previous breeding season. During *post mortem* examination, the uterus was collected, placed in clean tap water and sealed in plastic bags which were stored at −20 °C. From the same individuals, we also took both eyes and stored them in vials with a 10% formalin solution. After two weeks, eye lenses were removed and dried for 48 h at 80 °C following the procedure described in Beale (1962). Dried eye lens weight was then used to distinguish immature subadults from adults in *C. erythraeus* (body mass < 240 g and dry eye-lens weight $\leq$ 13 mg), and as a proxy for age in both species (e.g. Beale, 1962). Since, in populations at low density, grey squirrels are capable of breeding already when 7-8 months-old (Gurnell et al., 2001), we did not use eye lens weight, nor body mass, to exclude animals from our analyses. Fecundity was determined by counting the number of uterine-placental scars which are formed by the detachment of each embryo's placenta at parturition, thus allowing to estimate the total number of young born from a single female during the entire reproductive season (from February-March to July-September in these alien tree squirrels) (Koprowski, 1994; Gurnell, 1996; our unpubl. data). Uterine scars were identified after staining which reveals dark pigments of macrophages involved in processes of repairing the endometrium after detachment of the hemochorial placenta (Fig. 1, 2 and 3; Wild, 1997). After removing the connective tissue, the uterus was opened over its entire length and stained using the Turnbull reaction developed by Salewski (1964) on rats (*Rattus rattus*) and later adapted by Bray et al. (2003) on lagomorphs. Uteri were first immersed for 10 minutes in a fresh 10% solution of ammonium sulphide (H_8N_2S), rinsed thoroughly with tap water and then soaked for 10 minutes in a solution made of equal parts of 1% chlorhydric acid (HCl) and of a 20% solution of potassium hexacyanoferrate ($K_4[Fe(CN)_6], 3H_2O$). The staining process was ended by flushing the uterus with cold tap water to eliminate all traces of reagents. To prevent changes in uterine scars colour over time, they were counted within the next hour using a microscope equipped with a digital camera. Since the macrophages are rich in hemosiderin, they are coloured from blue to beige based on the age of the scar. A scar is recognized as having a paler central crater surrounded by two darker bands. In each band there are circular depressions with darker pigmentation due to concentration of blood and macrophages, indicated as antimesometrial depressions (Fig. 1, 2 and 3). We based the classification on the colours of crater, surrounding bands and antimesometrial depression. In particular, older scars, appear much paler than the more recent scars which show a darker staining. Since the animals have been collected in the same period and squirrels have synchronised seasonal parturitions, we assumed that the paler scars refer to the first parturition, while the darker ones to the second litter. In some Pallas's squirrels, three different shades of staining occurred, indicating that these females produced a total of three litters/year.

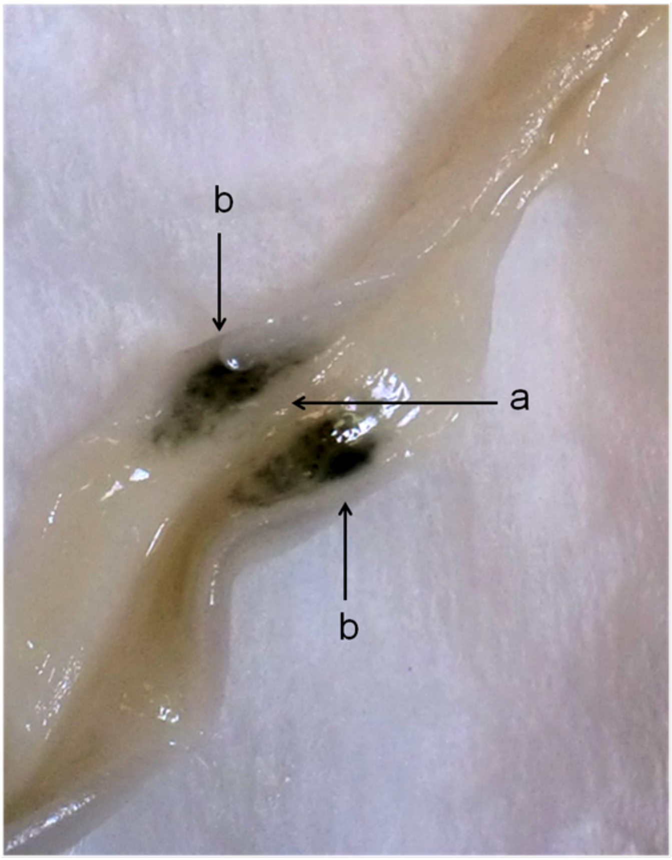

Figure 1 – Detail of a stained placental scar in a longitudinally dissected uterus of grey squirrel. A single scar is constituted by a pale, central crater (a) surrounded by two dark bands (b). Each of the bands shows a circular area with darker pigmentation due to blood and macrophages accumulation (antimesometrial depression).

Statistical analyses

For both species, we explored the effects of phenotypic and environmental factors on individual variation in number of litters/year and on total fecundity (total number of uterine scars over the entire breeding season). Since our dependent variables were counts following a Poisson distribution, we used generalized linear models (GLM) with Poisson error structure to test effects of site of collection (study area, only for grey squirrels), sampling year, body mass, foot length and eye lens weight. We also explored effects of species, period (spring vs. summer litter; spring parturition from February to April; summer parturition from June to August) and the species $\times$ period interaction on litter size (number of births per litter) thus considering only females that had at

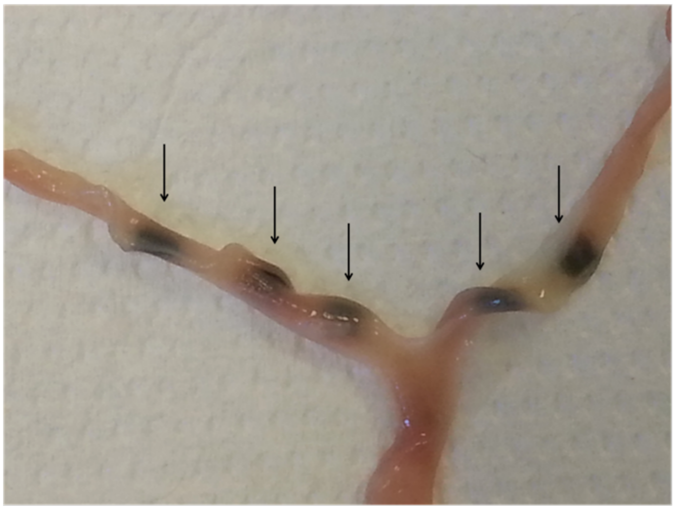

Figure 2 – Uterus of grey squirrel (*Sciurus carolinensis*) stained to highlight placental scars (indicated by arrows). Uniformity of staining indicates that all the five scars are referable to the same gestation/parturition.

least one uterine scar for a given reproductive period, using a generalized linear model with Poisson error structure. All GLM were done using the Proc Genmod procedure (SAS/STAT 9.4 software, Copyright © 2011, SAS Institute Inc., Cary, NC, USA).

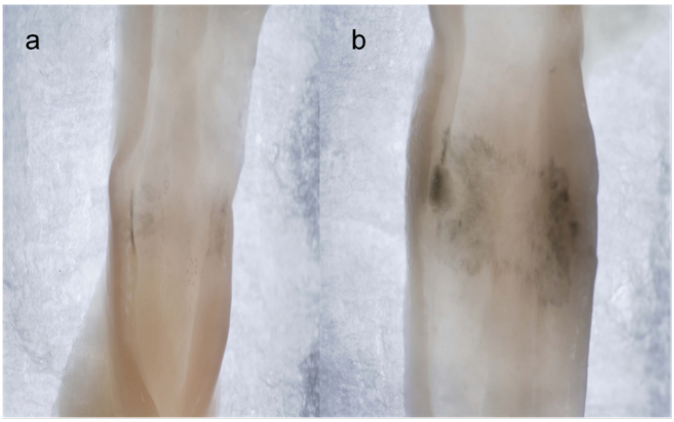

Figure 3 – Light-stained/paler scar referable to an older gestation (a) and dark-stained/darker scar referable to a more recent parturition (b).

Results

Grey squirrel

Of 49 female grey squirrels, 5 had a very small uterus, typical of immature nulliparous individuals and were not analysed. Of the remaining 44 specimens, 6 did not have any uterine scars (0 litters, 14% of females), 11 (25%) had scars of only one litter and the remaining 27 (61%) had scars from two litters. Out of the 11 females that had a single litter/year, five reproduced only in spring and six only in summer. Overall, females produced between 0 to 8 young/year, with an average of 3.4 scars/female (Tab. 1). The highest litter size at birth (maximum number of scars for a single litter) was six (Table 1). There was no difference in average number of scars between spring and summer reproduction (Mann-Whitney U-test, $p = 0.087$). However, if we only considered the 27 females that had scars for both periods, they produced on average a larger summer than spring litter (difference 0.74 ± 0.25 young/litter, Wilcoxon matched pairs signed ranks test, $p = 0.01$). Body mass of female grey squirrels varied between 450 and 660 g (mean $\pm$ SD = 533 ± 51 g) and right hind foot length between 56.0 and 67.0 mm (mean $\pm$ SD = 62.1 ± 2.5 mm). Neither body mass (Fig. 4a) nor foot length significantly affected variation in total number of uterine scars, or in number of litters produced. Also we found no effects of eye lens weight, study site or year (Tab. 2a).

Pallas's squirrel

Of 40 female Pallas's squirrels, 9 had a small uterus, typical of immature nulliparous individuals and were not used in further analyses. Of the remaining 31 specimens, 13 did not have any uterine scar (0 litters, 42% of females), 7 (22%) had scars of only one litter, 8 (26%) of two litters, and 3 females (10%) had scars from three litters. All females that produced a summer or summer and autumn litter (thus with more recent uterine scars) had also produced an early spring litter (oldest uterine scars). Hence, out of the 18 reproductive females, all of them had a spring litter, 11 reproduced in spring and summer and three in spring, summer and autumn. Overall, females produced between 0 and 9 young/year, with an average of 3.1 scars/female (Tab. 3). The highest litter size at birth (maximum number of scars for a single litter) was 6 (Tab. 3). Mean number of uterine scars of early spring litters was higher than of summer (second) litters (Mann-Whitney U-test, $p = 0.015$), but this was mainly due to more females having a spring than a summer litter resulting in more specimens with 0 scars for summer litters. In fact, considering only the 11 females with both spring and summer parturition, there was no difference in average litter size (Wilcoxon test, $p = 0.60$). Body mass of adult female Pallas's squirrels varied between 210 and 330 g (mean $\pm$ SD = 279 ± 28 g) and right hind foot length between 45.0 and 51.0 mm (mean $\pm$ SD = 49.1 ± 1.5 mm). Number of litters/year increased with eye lens weight, indicating that older female produced more litters (Table 2b). There was no effect of sampling year on number of uterine scars, but the total number of uterine scars increased with a female's foot length (estimate $\pm$ SE = 0.19 ± 0.09), body mass (Fig. 4b, estimate $\pm$ SE = 0.012 ± 0.004) and age (eye lens weight 0.064 ± 0.016; Tab. 2b). Body mass and eye lens weight were correlated (n = 31; $r = 0.39$; $p = 0.029$) suggesting an increase of body mass with age.

Grey squirrel vs Pallas's squirrel

There was a nearly significant species $\times$ period interaction ($\chi^2 = 3.75$; df = 1; $p = 0.053$) on litter size. Therefore, we compared litter size between the two species for spring and summer litters separately (for values see Tab. 1 and 3). In spring, Pallas's squirrels produced on average larger litters than grey squirrels (Kruskal-Wallis $\chi^2 = 12.0$; df = 1; p = 0.0005), but in summer there was no difference between the two species in mean litter size (Kruskal-Wallis $\chi^2 = 0.29$; df = 1; $p = 0.59$). As a consequence, total litter size was higher in Pallas's squirrel than in grey squirrel (Kruskal-Wallis $\chi^2 = 8.10$; df = 1; $p = 0.004$) with breeding females of the former producing on average 1.4 young more per year than grey squirrels.

Discussion

Reliability of uterine scar counts to estimate fecundity

The uterine staining technique based on variability in macrophage coloration described in this paper and applied to invasive tree squirrels allows to distinguish individual uterine scars and to discriminate scars produced earlier (spring litters) or later (summer – autumn litters) during the breeding season. The technique was successfully applied to both grey squirrels and Pallas's squirrels and allowed us to distinguish from two (grey squirrel) to even three (Pallas's squirrel) parturition events (litters) in a year and to determine number of births (litter size) for each litter separately as well as a female's total fecundity over the entire year (e.g. Nixon and McClain, 1975; Martin et al., 1976; Hackländer et al., 2011). Several studies have evaluated and discussed the reliability of uterine scar counts (also called placental scar counts, PSC); where possible this was done by comparing litter size and/or pregnancy rate estimates based on PSC with those based on counts of embryos (Lindström, 1981; Allen, 1983; Mowat et al., 1996; Ruette and Albaret, 2011). Using rodents, hares or carnivores held in captivity, it was shown that the time elapsed since the last (or previous) parturition(s) can strongly affect the reliability of PSC as a fecundity

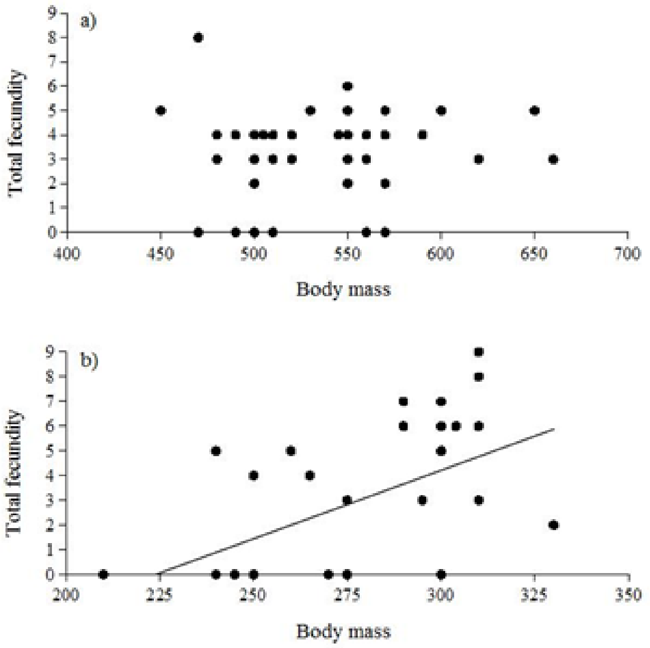

Figure 4 – Relationship between female body mass (g) and total fecundity (total number of uterine scars over the entire breeding season) in: a) grey squirrels; b) Pallas's squirrels.

measure since uterine scars tend to fade over time (Martin et al., 1976; Lindström, 1981; Bray et al., 2003; Elmeros and Hammershøj, 2006). All studies agree that, with or without staining, the darkest scars are those of the most recent parturition event and most authors underline that counting also "paler" scars will overestimate true litter size (e.g. Allen, 1983; Mowat et al., 1996). This is partly due to paler scars indicating a site where embryo resorption occurred, or being left not by the last but by previous reproduction events (e.g. hares, foxes and lynx, Allen, 1983; Mowat et al., 1996; Bray et al., 2003). Studies comparing counts of both unstained and stained uterine scars, agree that staining helps to correctly distinguish recent from older scars, both in species that produce once/year than in those potentially producing more than one litter/year (Martin et al., 1976; Bray et al., 2003; Ruette and Albaret, 2011). Overall, counting of the darkest uterine scars and counts of embryos in animals of the same population culled in the same year are strongly correlated, with –in some species- a slight overestimation of litter size at birth using PSC, probably due to embryo resorption (Allen, 1983; Mowat et al., 1996; Elmeros and Hammershøj, 2006; Ruette and Albaret, 2011). In this study, the observed variation in darkness of staining suggests that all parturitions of the year were determined and allowed us to clearly distinguish between first and second litters. Thus, since our animals were all sampled in autumns-winter, the identification of paler scars indicate that up to 9 months after parturition scars can be identified with this staining technique. However we cannot exclude the possibility of some embryo resorption, thus we could have in some cases overestimated the actual litter size, however this could be studied experimentally only by comparing number of uterine scars and number of pups shortly after parturition (e.g. voles, foxes, mink; Martin et al., 1976; Lindström, 1981; Elmeros and Hammershøj, 2006). In tree squirrels, earlier works made use of uterine scars to estimate fecundity, however details on the staining method used and on the number of litters/year are often lacking. More in detail, Tang and Alexander (1979), T'sui et al. (1982) and Tamura (1999) analysed fecundity of *C. erythraeus* in its native range without describing the staining method and with no indication of how older scars (from earlier litters) could be distinguished from more recent ones. Also, the number of scars from single and/or multiple litters was not reported and authors only analyse data of number of embryos/litter in pregnant females (see Tab. 5). Humanson (1962) on grey squirrels in their native range, refers to the staining method used as "Prussian blue reaction" without giving further details. Similarly, Shorten (1951) on grey squirrels introduced in the UK did not describe how the uterus was stained or how uterine scars were counted and apparently no attempt was made to distinguish between older and more recent scars. Moreover, this paper only presented average number of uterine scars per female for a given litter (spring or summer/autumn litter) without range of scars or a measure of variance (SD or SE, see Tab. 4). Only Nixon and McClain (1975) distinguish between older (spring litter) and more recent scars (summer litter), using darker staining as the discriminating factor.

Grey squirrel

In our sample of grey squirrels from N. Italy, collected over a period of 3 years, 61% of females produced two litters/year. This was higher than the average reported in a multi-year study in the native range (Ohio, 27%, Nixon and McClain, 1975). These authors also documented that in their study site the percentage of females producing two litters/year

Table 1 – Fecundity data of female grey squirrels trapped in Northern Italy. The mean number of uterine scars (± SD), lowest and highest number of scars (range) and mean litter size (± SD) of females which produced at least a litter (scars ≥ 1). Data for spring and summer parturition separately, and over the entire year (total).

Litters	Uterine scars			Litter size	
	n	mean ± SD	Range (min-max)	n	mean ± SD
Spring parturition	44	1.41 ± 1.33	0 - 6	32	1.94 ± 1.19
Summer parturition	44	1.95 ± 1.49	0 - 5	33	2.61 ± 1.12
Total	44	3.36 ± 1.71	0 - 8	38	3.89 ± 1.13

Table 2 – GLM (dependent variables: Number of litters or total annual fecundity measured by uterine scars of all litters) exploring the effects of study area (only grey squirrel), sampling year and of phenotypic parameters on variation in fecundity among (a) female grey squirrels; (b) female Pallas's squirrels.

Explanatory variables	Number of litters/year	Total annual fecundity
(a) Grey squirrel		
Body mass	χ^2 = 0.00; df = 1; p = 0.95	χ^2 = 2.06; df = 1; p = 0.15
Foot length	χ^2 = 0.01; df = 1; p = 0.91	χ^2 = 0.32; df = 1; p = 0.57
Eye lens weight	χ^2 = 0.43; df = 1; p = 0.51	χ^2 = 0.32; df = 1; p = 0.64
Year	χ^2 = 0.73; df = 2; p = 0.70	χ^2 = 3.33; df = 2; p = 0.19
Study area	χ^2 = 0.45; df = 2; p = 0.80	χ^2 = 2.26; df = 2; p = 0.32
(b) Pallas's squirrel		
Body mass	χ^2 = 2.45; df = 1; p = 0.12	χ^2 = 7.51; df = 1; p = 0.006
Foot length	χ^2 = 1.42; df = 1; p = 0.23	χ^2 = 4.39; df = 1; p = 0.036
Eye lens weight	χ^2 = 6.24; df = 1; p = 0.013	χ^2 = 15.3; df = 1; p < 0.0001
Year	χ^2 = 0.01; df = 1; p = 0.92	χ^2 = 1.46; df = 1; p = 0.23

Table 3 – Fecundity data of female Pallas's squirrels trapped in Northern Italy. The mean number of uterine scars (± SD), lowest and highest number of scars (range) and mean litter size (± SD) of females which produced a litter (scars ≥ 1). Data for spring, summer and autumn parturition separately, and over the entire year (total).

Litters	Uterine scars			Litter size	
	n	mean ± SD	Range (min-max)	n	mean ± SD
Spring parturition	31	1.90 ± 1.97	0 - 6	18	3.28 ± 1.45
Summer parturition	31	0.90 ± 1.49	0 - 6	11	2.55 ± 1.44
Autumn parturition	31	0.26 ± 0.81	0 - 3	3	2.67 ± 0.58
Total	31	3.06 ± 3.00	0 - 9	18	5.28 ± 1.87

varied between 0% and 36% in relation to tree seed abundance; consequently total number of uterine scars/female varied over years and was highest in years with good seed crops (Nixon and McClain, 1975). In contrast, we did not find a significant year effect on total number of uterine scars/female in this study. Medium to long-term studies have documented that grey squirrel reproductive rate varies markedly among years in both the native range and in areas in Great Britain where the species has been introduced (e.g. Koprowski, 1994; Gurnell, 1996; Kenward et al., 1998). Reproductive rate is density dependent (with adult female density) and increases when food abundance is high. Density dependent variation in fecundity over time was also observed in native Baltic grey seals (*Halichoerus grypus*, Kauhala et al., 2014) and invasive American mink (*Neovison vison*) in Scotland (Melero et al., 2015). Seals showed a reduction in pregnancy rates over time correlated with an increase in population size (Kauhala et al., 2014). In the case of invasive mink, the probability of conceiving a litter and litter size increased as female density declined after culling, resulting in density dependent compensation in fecundity (Melero et al., 2015). This marked increase in fecundity in females reinvading the controlled area implies that control strategies must be sufficiently robust in order to overcome this compensation and suppress densities of invasive mammals (Melero et al., 2015). Both these studies had much larger samples sizes than ours and/or were carried out over a much longer time span. Hence, in our case, the lack of significant variation in total reproductive output between years might be due to smaller sample size and/or less annual variation in food availability in the Italian sites (deciduous woodland and park habitats) than in the woods and forests in Great Britain and North America. In our study, females that had reproduced in both seasons tended to have a larger summer than spring litter (2.61 and 1.94 offspring respectively, see Tab. 4). Larger summer litters were also reported for grey squirrels introduced to England (Shorten, 1951) and in the native range, particularly for adult (> 1-year old) females (Tab. 4). In the study in Ohio, adult females tended to produce larger litters than yearlings and, consequently, their total annual reproductive output was higher (Tab. 4 and Nixon and McClain, 1975). We did not divide our sample in these two age-classes, but used eye lens weight as a continuous variable and proxy for age. However, we did not find any effect of eye lens weight on number of litters or on total fecundity. Among the grey squirrels analysed in this study, variation in number of litters and in total number of uterine scars was not correlated with body size or body mass. Body mass and age are important factors in determining female reproductive success in other tree squirrels, for example Eurasian red squirrels (*Sciurus vulgaris*), through both indirect effects (on dominance status, home range quality) and direct effects (entering oestrus, successful weaning of offspring, number of litters in lifetime) (Wauters and Dhondt, 1989, 1995; Lurz et al., 2000, 2005; Wauters et al., 2001, 2008). There can be several reasons why we did not find any effect of eye lens or body mass on number of uterine scars in this study. First, our data are few and larger datasets are necessary to investigate how individual variation in phenotypic characteristics might affect variation in reproductive rate. Second, age effects might be important only between primiparous yearlings and multiparous adults, as suggested by Nixon and McClain (1975): in this case using a continuous variable (i.e. eye lens weight) might not reveal a significant pattern. Also in red foxes (*Vulpes vulpes*), the major difference in fertility was between yearlings and adults (Ruette and Albaret, 2011), while in long-lived species, for example Baltic grey seals, age-dependent pregnancy rates were evident using multi-year age-classes (Kauhala et al., 2014). Third, body mass, which is known to fluctuate seasonally in this species (Kenward and Tonkin, 1986; Koprowski, 1994), could be important for successful reproduction at the beginning of the breeding season, but not relevant when measured at the end of it (as in this study). Fourth, the lack of effects of body mass and age might indicate an adaptation of the IAS to the new environment: grey squirrel females invest in producing offspring independent of their age, size or body condition and their relative success (variation in number of young) is more determined by other factors not measured here. Finally, it is interesting to note that mean litter size estimated with number of uterine scars was slightly higher for both spring and summer litters in Ohio than in this study (Tab. 4). In contrast, in our case more females reproduced twice a year and consequently mean number of uterine scars/female over the entire year was higher. Also, the maximum number of scars found in a female was higher in N. Italy than in the population in the native range (Tab. 4). This high reproductive rate and breeding at an early age could be adaptations developed by spreading, introduced populations and/or density-dependent compensations to culling (e.g. Gurnell et al., 2001; Melero et al., 2015). We found only one paper with partly comparable data on uterine scars of other introduced populations (grey squirrels in the UK, Shorten, 1951). However, as above mentioned, this paper only presents average number of uterine scars per female for a given litter (spring or summer/autumn litter) without any range of scars or a measure of variance. Overall, mean number of scars for both spring and summer litters, as well as the maximum number of young/litter in summer/autumn (based on embryo counts) tended to be higher in the UK populations than in Italy (Tab. 4). It has been shown that grey squirrels in the UK spread more rapidly than in Italy (Bertolino et al., 2014), but both studies presented preliminary results of fecundity and it would be speculative trying to explain differences in fecundity between invasive populations in the two countries based on these data.

Pallas's squirrel

Although our dataset was small, relationships between phenotypical characteristics of females and reproductive output revealed some interesting patterns. In the introduced population in N. Italy, females invested heavily in an early spring litter. In fact, 58% of animals examined had a spring litter, and only among the females which had already produced a spring litter, some also produced a summer litter and a few even a third litter in autumn. In contrast with grey squirrels examined in this study, individual variation in total number of uterine scars in Pallas's squirrels depended on eye lens weight (age) and body mass: older females and those of higher body mass produced more offspring in a year than younger ones and/or than females of lower body mass. The preponderance of early (spring) breeding in our study site might be related to seasonal variation in food availability or quality. In March-April, during lactation of spring litters, the invasive species was observed foraging on a wide variety of tree and shrub species, and was often seen feeding on flowers of wild cherry (*Prunus avium*), a common tree species in the mixed deciduous forest where the population occurs. Cherry flowers and, in May-June, their ripe fruits are abundant and rich in carbohydrates and may constitute an energy-rich food supply for breeding females in spring and early summer (onset of summer breeding). Furthermore, although we do not yet know if and how frequent Pallas's squirrels hoard tree seeds in our study site, in similar habitats cached tree seeds are an important food source for spring

Table 4 – Fecundity data of grey squirrels: Comparison between populations from the native range and introduced populations (this study).

		NATIVE RANGE			INTRODUCED POPULATIONS						
		Nixon and McClain (1975)			Shorten (1951)			This study			
Breading season	Age	Placental scar count		Range	Age	Placental scar count		Range	Age	Placental scar count		Range
		n	mean ± SD			n	mean			n	mean ± SD	
Spring	Yearling	23	2.43 ± 0.58	1–3	All	66	2.70	1–4*	All	32	1.94 ± 1.19	1–6
	Adult	40	2.70 ± 0.89	1-4								
Summer	Yearling	35	2.51 ± 0.77	1–4	All	36	3.04	1–7*	All	33	2.61 ± 1.12	1–5
	Adult	63	3.49 ± 0.87	2-6								
Annual	Yearling	46	2.54 ± 0.75	1–4					All	38	3.89 ± 1.13	1–8
	Adult	98	3.43 ± 0.89	1–6								

n = number of squirrels examined; mean number of scars/female with SD; Range = minimum and maximum number of uterine scars per litter or over the entire year (annual); *= embryos/female, no data on placental scars range.

Table 5 – Comparison between studies on fecundity (mean ± SD and range) in Pallas's squirrels native range (Tang and Alexander, 1979; T'sui et al., 1982) and in introduced populations (Tamura et al., 1988; Tamura, 1999; This study). Sample size between brackets, * no sample size given.

	NATIVE RANGE			INTRODUCED POPULATIONS		
	Tang and Alexander (1979)	T'sui et al. (1982)		Tamura et al. (1988) Tamura (1999)	This study	
Litters/year	1-2 (40)	1-2 (15)		1-3*	1-3 (31)	
Embryos/female	1.68 (40)	Spring	1.83(6)	2.4 ± 0.7 (SD)		
		Summer	1.75 (8)	range 1-4 (44)		
Scars/ breeding female					Spring	3.28 ± 1.45 (1-6) (18)
					Summer	2.55 ± 1.44 (1-6) (11)
					Autumn	2.67 ± 0.58 (1-3) (3)

breeding in native red squirrels (Wauters and Casale, 1996; Zong et al., 2010, 2014). We found few data in the literature reporting measures of fecundity in this species, either in its native or introduction range (Tab. 5). Data from the native range, based on counts of embryos in animals shot throughout the year, reveal two litters per year (concentrated in spring and summer) with only 1-2 embryos/litter (Tang and Alexander, 1979; T'sui et al., 1982). These authors suggest that small litter size was related to high population density in the areas where animals were collected (commercial conifer forests, orchards and woods). In any case, all data from populations introduced to other countries show higher fecundity than in the native range (Tab. 5), suggesting that this species might be extremely adaptive to new environments and habitats, as supported by its high invasiveness (Bertolino and Lurz, 2013). High fecundity in colonizing populations in Italy and Japan could also be due to low squirrel density. However, in our case, large number of animals removed for control and high estimated population density (ca. 7 individuals/ha, Mazzamuto unpubl. data) seem to contradict this. Comparing fecundity between Japan and Italy, although based on different methods, showed that in both countries females could produce up to 3 litters per year, with average and maximum litter size slightly higher in Italy than in Japan (Tab. 5) (Tamura et al., 1988; Tamura, 1999). In contrast, data from a population introduced at Antibes (France), suggest lower fecundity in this country (n = 84, mean ± SE = 1.8 ± 0.1 embryos/female, range = 1-3, Chapuis et al. unpubl. data) maybe due to poor habitat quality at the collection site.

Conclusions

Our first data of uterine scar counts in female grey squirrels culled in N. Italy suggest than fecundity over an entire year was slightly larger than in populations in the native range. Hence, this IAS seems well adapted to the new environment in Italy, where habitats most commonly occupied are mixed lowland woods with presence of oaks (*Quercus* sp.) and urban, suburban or private parks where densities tend to be high (Wauters et al., 1997; Venturini et al., 2005; Bertolino et al., 2008; Martinoli et al., 2010). Furthermore, it has been reported that the grey squirrels in Italy have lost many of the macroparasite species they normally host in their native range, setting the premises for positive effects of parasite release on host fitness (Romeo et al., 2014), which could also include a higher fecundity in animals with low helminth abundance in the Italian populations. A similar pattern was found in the Pallas's squirrel where uterine scar counts definitively indicate a high fecundity of individual females when compared with the (scarce) data available for the native range. Also for this species, the extremely poor parasite fauna and absence of gastrointestinal helminths in most animals, suggest that parasite release might be involved in the high fecundity of the IAS in this area (Mazzamuto et al., 2016) and in other countries where it has been introduced (Dozières et al., 2010; Gozzi et al., 2014). Although there are no directly comparable fecundity data for native red squirrels, which can have large litters in some circumstances (Mari et al., 2008), estimates of reproductive rate from percentage females breeding and litter size at weaning in lowland mixed woodlands (e.g. Wauters and Lens, 1995; Wauters et al., 2001) suggest that the two IAS have similar or even higher fecundity than the native species. Thus, not only grey but also Pallas's squirrels might become a risk for the long-term persistence of populations of the native red squirrel. Preliminary data on trapping success and removal of the IAS linked with occurrence/density of red squirrels (Mazzamuto unpubl. data) suggest that this is actually the case. Our data also have implications for Spatially Explicit Population Dynamics Models (SEPMs) used to predict future range expansion of invasive squirrels. In fact, in these models, parameter input of fecundity values have a high impact on model results of future population size and distribution (Lurz et al., 2001; Guichón and Doncaster, 2008). Based on fecundity values found in our study, the range of litter sizes and % females breeding used in SEPMs predicting future expansion of grey squirrels in Italy are realistic (litter size range: 2–4 in Lurz et al., 2001; 1.5–3.5 in Tattoni et al., 2006; 2–3.5 in Bertolino et al., 2008). However, a SEPM developed to predict range expansion of Pallas's squirrel in Argentina used a mean litter size of 2 young/female per year (Guichón and Doncaster, 2008), which, based on our data, seems an underestimation of true fecundity. In conclusion, the results presented in this paper indicate that uterine scar counts are a good method to estimate reproductive output in the two introduced squirrel species and that both IAS have a high reproductive output in their new range in Italy. Therefore, and since both species negatively affect native red squirrels (Gurnell et al., 2004; Bertolino et al., 2014; Mazzamuto, unpubll. data), management plans must aim to eradicate both species in Italy, or, when in the case of the grey squirrel this is considered impossible, control populations to avoid further expansion.

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