Monitoring the small-scale distribution of sympatric European pine martens (*Martes martes*) and stone martens (*Martes foina*): a multievidence approach using faecal DNA analysis and camera-traps

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Abstract. The European pine marten (Martes martes) and stone marten (Martes foina) are two closely related mustelids that live sympatrically over a large area of Europe. In the northern Iberian Peninsula, the distribution ranges of both species overlap extensively. The objectives of this study were (1) to verify whether, on a small scale, both species also live sympatrically and (2) to compare camera traps and scat DNA as methods for detecting marten species. The study was conducted in a protected area (province of Ourense, north-west Spain), which covers 6700 ha. To test the sympatry hypothesis, 90 fresh faecal samples, identified as faeces of genus Martes on the basis of their morphology, were collected from June 2004 to August 2006. The specific identification of faecal samples was conducted using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. In addition, 20 camera-traps (916 cameratrap-nights) were in operation during the study period. Of the faecal samples collected, 88.8% were attributed to the European pine marten, while the remaining 11.2% were not amplified by PCR and thus could not be assigned. The European pine marten was identified in 57.9% of the photos of carnivores and the stone marten was not detected in any. The faecal DNA analysis and camera-trap results supported previous conclusions about habitat preferences and the distribution of the two species obtained using other methods. The two non-invasive methods that were used in this study were shown to be reliable techniques that can be employed simultaneously, because each method has advantages and disadvantages that are influenced by the size of the area inventoried, sampling effort, and cost and efficiency of the method. The data gathered using these methods provided important information on the understanding of trophic and competitive interactions between the species.

Introduction

The European pine marten (*Martes martes* Linnaeus, 1758) and stone marten (*Martes foina* Erxleben, 1777), species similar in both morphology and feeding habits, are closely related mustelids that live broadly sympatrically over an extensive area of Europe. Limited knowledge of their ecological niches in sympatric areas, their extremely elusive behaviour and the high degree of difficulty in distinguishing their faeces on the basis of morphology alone, contributes to the difficulty of studying and monitoring their populations.

The European pine marten occupies most of Europe, from Mediterranean biotopes to Fennoscandia taiga, and western Siberia and Iran (Proulx *et al.* 2004). The stone marten is present throughout continental Europe, but it is absent from Great Britain and Ireland as well as most of the Mediterranean islands except Crete (Proulx *et al.* 2004). On the Iberian Peninsula, the European pine marten has a Eurosiberian distribution and is also present on the islands of Majorca and Minorca (Mitchell-Jones *et al.* 1999; Palomo and Gisbert 2002); the stone marten is present throughout the Iberian Peninsula (Fig. 1) (Palomo and Gisbert 2002).

The European pine marten is associated primarily with Eurosiberian deciduous and coniferous forests (Delibes 1983; Mitchell-Jones *et al.* 1999) and prefers mature forests and areas with permanent watercourses (Barja 2005*a*). In contrast, the stone marten can survive in a variety of habitats across its distribution range, including fragmented woodlands, villages and their periphery, and areas near farms (Waechter 1975; Delibes 1983; Sacchi and Meriggi 1995; Blanco 1998; Mitchell-Jones

et al. 1999; Virgós and Garcia 2002; Lanszki 2003). Libois and Waechter (1991) also observed that the stone marten tends to principally occupy deforested and altered areas when the European pine marten is present. In addition, its predilection for rocky habitats allows it to use buildings and other man-made structures (Delibes 1983). However, this species appears to be highly flexible as it is also found in typical forest habitats in Russia and is therefore not exclusively dependent on the presence of rocky terrain (Waechter 1975). The stone marten is also more thermophilic than the European pine marten, allowing it to occupy urban environments (Waechter 1975). The ecological requirements of both species can sometimes overlap; in these circumstances, the European pine marten stays in forested land-scapes and the stone marten seems to be displaced to more urbanised areas (Delibes 1983).

Accurate species identification is a key step in conservation biology and a necessary component of wildlife management and conservation studies. However, determining presence and absence of elusive or cryptic species can be logistically difficult, particularly when relying solely on field signs such as faeces, hair or tracks (Piggott and Taylor 2003). Traditionally, this kind of information is subjected to a variety of morphological analyses for definitive species identification (Kohn and Wayne 1997). Nevertheless, there are situations in which such samples, deposited by sympatrically occurring carnivores of similar body size, cannot be assigned at the species level on the basis of morphology alone (Farrell et al. 2000; Davison et al. 2002; Birks et al. 2004; Kurose et al. 2005). Misidentification of species from faeces is probably common, and has been indicated for different sympatric carnivore species (Paxinos et al. 1997; Ernest et al. 2000; Farrell et al. 2000; Davison et al. 2002; Palomares et al. 2002; Dalén et al. 2004; Sugimoto et al. 2006; Pilot et al. 2007). Traditionally, the presence of martens has been determined from data obtained from road kills or hunting information, live-trapping surveys, sighting surveys (Messenger and Birks 2000), track plates and camera-traps (Zielinski and Kucera 1995), fur-snagging devices (Messenger and Birks 2000; Lynch et al. 2006) and scat-based surveys, which have several limitations for detecting and identifying different marten species (Birks et al. 2004). Each method has advantages and disadvantages that are influenced by the size of the area inventoried, sampling effort, and cost and efficiency of the method (Birks et al. 2004). Methods of non-invasive genetic sampling of animal populations and camera-trapping are becoming more common for identification of mammalian species (Karanth 1995; Zielinski and Kucera 1995; Paxinos et al. 1997; Murakami 2002; Palomares et al. 2002; Azlan and Sharma 2003; Gómez-Moliner et al. 2004; Swann et al. 2004; Kurose et al. 2005; Trolle and Kéry 2005). Because the faeces of the European pine and stone marten cannot be reliably separated on the basis of morphology alone (Marchesi et al. 1989; Pilot et al. 2007), the application of methods that will positively distinguish the two could be an invaluable tool for conducting noninvasive surveys to determine the presence and distribution, trophic and habitat requirements, niche overlap and competitive interactions of the mustelids in sympatric areas.

Although several studies on the distribution of mammals have been conducted on the Iberian Peninsula (Blanco *et al.* 1992; Rodriguez and Delibes 1992; Virgós 2001; Palomo and Gisbert 2002), there is a remarkable absence of information on the distribution of the European pine marten and the bioecological relationship between it and the stone marten. Consequently, the goal of this work was to apply a multievidence approach incorporating two different survey techniques (faecal DNA analysis and camera-traps) to monitor the small-scale distribution of the two marten species in the northern Iberian Peninsula. This study compared the effectiveness, logistics and monetary efficacy of both techniques for this type of research.

Materials and methods

Study area

The study was performed in the Montes do Invernadeiro Natural Park, a mountainous area located in the north-west Iberian Peninsula (Universal Transverse Mercator (UTM) coordinates: 29T 064633–643 and 467462–472) (Fig. 1). The study area, which covers 6700 ha, varies in altitude from 880 to 1707 m. The climate is continental, with hot summers and cold winters





(Barja 2001). The flora is diverse, including Mediterranean plant communities and Atlantic relic forests (Castroviejo 1977; Barja 2001). The plant community is scrubland, dominated by heather (Erica australis), prickled broom (Pterospartum triden*tatum*) and sandling (*Halimium lasianthum*). The original forests remain in valleys and along watercourses and are formed principally by associations of oak (Quercus robur), birch (Betula celtiberica), holly (Ilex aquifolium) and rowanberry (Sorbus aucuparia). Large tracts of extended forestlands are formed by Scot pine (Pinus sylvestris), which has repopulated deforested areas (Barja 2001). The park is occupied by carnivores such as the Iberian wolf (Canis lupus), badger (Meles meles), European polecat (Mustela putorius), stoat (Mustela erminea), European common weasel (Mustela nivalis), genet (Genetta genetta), wildcat (Felis silvestris), red fox (Vulpes vulpes) and otter (Lutra lutra). The study area appears to have suitable resources to support both the European pine marten and the stone marten. However, the presence of both species in the study area has not previously been examined. There are no human populations in the study area and the nearest village is 4 km from the park.

Collection of faecal samples

The European pine marten, the stone marten and other carnivores use forest roads and frequently defaecate on them as a way of visual-scent marking (Robinson and Delibes 1988; Barja et al. 2004; Barja 2005b; Barja et al. 2005). Initially, two scatbased surveys were conducted to estimate the distribution range of the genus Martes in the study area. The study area comprised 67 cells (UTM) of 1 km² each. In the initial survey, two 400-m transects in each cell were examined and 26 yielded Martes scats. Later, in order to collect fresh Martes faecal samples, transects 300 m long were surveyed on foot along forest roads monthly from June 2004 to August 2006 in these 26 cells alone. These surveys were conducted only in the 26 cells where signs of the presence of Martes species (scats, footprints, sightings) had previously been detected. We found fresh faeces in 17 of the 26 cells and old faeces in the remaining nine cells. Fresh faeces were characterised by their strong smell, presence of a mucus layer and lack of any sign of dehydration. Morphological characteristics, such as size and shape, were used to distinguish faeces of the genus Martes from those of medium-sized carnivores such as red fox and wildcat. The territory of the European pine marten is 1.3 km² for males and 1.0 km² for females (O. Berdión, Department of Zoology and Animal Cell Biology, Universidad País Vasco, Spain, pers. comm.). The mean territory size of the stone marten is similar to that of the European pine marten, being about 0.96 km² (López-Martín 2003). Thus, in order to increase the likelihood of obtaining faecal samples from separate individuals, the transects were placed 700 m apart. The transects were uniformly distributed throughout the study area and 218.7 km were surveyed. When fresh faeces of the genus Martes were detected, the date, time and UTM cell were recorded. A sample was collected from the fresh scat using a gloved hand. A new glove was used for each sample to avoid cross-contamination. All collected faecal samples were stored in hermetic and sterilised tubes, preserved with ethanol (96%) and maintained at -20°C until assayed (Piggott and Taylor 2003; Gómez-Moliner et al. 2004).

Genetic analysis of faecal samples

The specific identification of faecal samples was accomplished by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The faecal DNA extraction procedure was based on the protocol described in Gómez-Moliner et al. (2004). Extraction blanks were included to monitor for contamination and were processed as separate samples in the subsequent amplifications. A small fragment (276 bp) of mitochondrial DNA (mtDNA), D-loop region, was amplified with specific primers developed for Martes and Mustela by PCR. The primers were designed to amplify small fragments to maximise the probability of amplification of degraded DNA. Following DNA amplification, PCR products were digested with the restriction enzymes HaeIII and RsaI. The combined use of both enzymes produced a species-specific banding pattern allowing the scats of M. martes and M. foina to be discriminated. This method was recently developed and provides a reliable and effective molecular technique for unequivocal genetic identification of sympatric marten species through the use of species-specific differential haplotypes (Ruiz-González et al. 2008).

Camera trapping

Every three months, from June 2004 to October 2006, 10-20 automatic line-triggered cameras (models: Moultrie, Canon Prima AF 9s, Stealth Cam) were set in place and baited with chicken placed 70 cm from the ground and 2 m from the cameras. The cameras were equipped with different activation systems. Sixteen used passive and active infrared activation and four employed a plate system triggered by the pressure of the animal's weight. The cameras were placed in those cells where Martes faeces had been detected during the surveys and in optimal areas for both marten species even if signs of the animals were not detected. The optimal areas were selected on the basis of the known habitat preferences of both marten species (Delibes 1983; Sacchi and Meriggi 1995; Virgós and Garcia 2002; Barja 2005a). The cameras remained in the field for 3-12 days (916 camera-trap-nights). The cameras were placed in a total of 22 UTM cells (32.8% of cells had cameras in them). Each camera was revisited every third day.

The European pine marten and the stone marten are similar morphologically. Both species have a spot on the neck called a 'bib', which is yellow in the European pine marten and white in the stone marten (Cabrera 1914). Therefore, bib colour was used to distinguish between the two species. Spot patterns, size and symmetry of the bibs differ between individuals of the same species (Cabrera 1914). In the present study, the differences in the bib morphology and distance between cameras allowed us to identify individuals. To increase the probability of photographing the bibs, the cameras were baited with chicken placed 70 cm from the ground and 2 m from the cameras. The morphology of the bibs was analysed in the photographs obtained and when the spot pattern, size and symmetry coincided, the photographs were assigned to a single individual. Also, when the distance between cameras was greater than 1.3 km, we considered that the photographs obtained were of different individuals of the same species. This assumption was supported by the morphological differences of the bibs in the photographs.

Results

The faecal DNA analysis allowed us to detect the European pine marten in 17 UTM cells and the camera-trapping in 9 cells (in 6 cells the species was detected by both methods). Therefore, the presence of the European pine marten was confirmed in 20 cells (29.9% of cells) (Fig. 2). However, neither method detected the stone marten. Of 90 analysed faecal samples from throughout the study area, after applying the PCR-RFLP method, we successfully identified 80 as being from the European pine marten and none from the stone marten. Consequently, 88.8% of the collected samples could be assigned to a species; in the remaining 11.2% the DNA extracted was not amplified by the primers used. The mean number of fresh faecal samples analysed per cell was 5.3; the mean number of scats detected was 1.97 per month per kilometre surveyed. In 65.4% of the study cells (17 of 26), fresh scats were collected. The faecal DNA analysis confirmed the presence of the European pine marten in 100% of the cells in which fresh faeces were collected.

The camera-traps captured 126 photographs of four different terrestrial carnivores (Fig. 3). The European pine marten was the most photographed species (57.9%), followed by red fox (33.3%), wildcat (5.6%) and Iberian wolf (3.2%). This sampling method confirmed the presence of the European pine marten in

40.9% of camera-equipped cells, red fox in 36.4% of the cells, wildcat in 27.3% and Iberian wolf in only 18.2%. The intercamera distance (>1.3 km) allowed for the identification of nine different individuals of European pine marten. Also, seven different individuals were identified according to the morphology of the bibs. The cells where the presence of the European pine marten was confirmed (using faecal DNA analysis and cameratrapping) included a high percentage of tree cover (coniferous and deciduous forests), permanent watercourses and a high percentage of rocky substrate and outcroppings. In contrast, the cells in which pine martens were not detected were dominated by scrubland.

Discussion

In this study, both research methods indicated that, while the European pine marten was present in the study area, the stone marten was not. The PCR-RFLP technique allowed us to achieve a high rate of species identification (88.8%). These genetic analyses open the possibility for studying population genetics of the species, recognising that nuclear marker genotyping from scats is more difficult than mtDNA amplification. The remaining 11.2% of samples did not amplify. The possibility that some scat samples had been incorrectly identified in the





field as belonging to the marten would mean an even higher amplification success. Misidentifying fox scat as marten scat is a particularly common error (Davison *et al.* 2002). However, the primers developed for *Martes* and *Mustela* by PCR did not amplify in fox scats.

Camera-traps detected martens in only 40.9% of the cells (9 of 22), whereas faecal DNA detected them in 100% of the cells in which fresh faeces were collected (17 of 17). However, both methods have advantages and disadvantages that are influenced by the size of the area inventoried, sampling effort, detectability and cost and efficiency of the method. Although a large number of faecal samples can be collected, the faecal DNA is highly degraded (Taberlet et al. 1999) and the faeces collected can be from other carnivore species for which the primers used do not amplify, implying important economic costs. Also, the collection of fresh faeces requires a high field effort. However, this method is advantageous for both large- and small-scale investigations. An advantage of camera-trapping is the possibility of obtaining a broad range of significant information regarding the carnivore's community. The absence of specific attractants does not allow the species of interest to be selectively photographed. Also, the greater effort required for instrument placement, physical monitoring of the cameras and revisits to the study area are a disadvantage for large-scale research efforts. In the current study, camera-traps detected the European pine marten in cells in which the faecal DNA analysis did not detect its presence. Also, the use of camera-traps allowed us to visually detect the European pine marten (and identify individuals) and other carnivores (red fox, wildcat and Iberian wolf), which are difficult to study due to their elusive and nocturnal habits. Camera-trapping allows the collection of valuable information



about multiple species, including herbivores and carnivores, within any given community (Karanth 1995; Peterson and Thomas 1998; Azlan and Sharma 2003; Swann *et al.* 2004; Trolle and Kéry 2005). Nevertheless, the simultaneous use of both methods in a small-scale marten survey can enhance the accuracy of data and result in a more ecologically relevant study.

Small mammals are relatively abundant in the study area (64.4 animals ha⁻¹) (I. Barja, S. Rosellini and A. Piñeiro, unpubl. data) and are the primary prey of the European pine marten all year round (Rosellini et al. 2007). In some areas of the northern Iberian Peninsula small mammals have also been found to constitute the largest proportion of the stone marten diet (Delibes 1978). Nevertheless, despite the apparent presence of adequate food resources for the survival of both species within the study area, only the European pine marten is present. The ecological displacement that occurs when both species overlap in the same area is likely related to their relative densities and to the availability of trophic resources, even though prey items appear to be abundant. Competition for these resources can play a fundamental role in the interactions between individuals and species (Schoener 1983; Begon et al. 1986; Keddy 1989). Interspecific and intraspecific competition can cause a decrease in individual fitness (Begon et al. 1986). Since reduced fitness has significant negative consequences from an evolutionary standpoint, some kind of differentiation in the utilisation of trophic resources and habitat must occur in order for both species to coexist (Schoener 1974). Such differentiation can allow both species and individuals to avoid depressed fitness, that is, a decrease in fecundity and/or survival (Schoener 1974; Barrientos and Virgós 2006). In sympatric carnivores, there is ample evidence that ecological competition determines size and stability of populations (Palomares et al. 1996; Barrientos and Virgós 2006). Similar carnivores that coexist in the same area, therefore, normally differ in their trophic strategies (Schoener 1974; Clevenger 1994; Barrientos and Virgós 2006). In mustelids, the sympatry among species has been explained by differences in body size, which reduces interspecific competition (Erlinge 1986). While the high degree of trophic overlap between the two martens studied here has been reported by some authors (Marchesi et al. 1989; Clevenger 1994), the mechanisms of coexistence have been proposed only by Clevenger (1994).

The results of this work suggest a functional ecological mechanism for competition between the two studied species. On the Iberian Peninsula, the distribution ranges of the European pine marten and the stone marten overlap within the study area, providing a situation for ecological competition, and species segregation, on both a large and small scale. The exclusive detection of the European pine marten within the study area is likely associated with its preference for uniform and well conserved forests (Barja 2005*a*), resulting in the displacement of the stone marten to disturbed, deforested and urban areas (Delibes 1983; Libois and Waechter 1991).

On a smaller scale, habitat fragmentation can allow for sympatric distribution of very similar species (Pilot *et al.* 2007). The observed spatial segregation of the two mustelids is probably due to both physical size, the European pine marten being the larger of the two (Delibes 1983; Barea and Ballesteros 1999; Barja 2005*a*), and the more general and flexible habits of the

stone marten (Waechter 1975; Delibes 1983; Sacchi and Meriggi 1995; Mitchell-Jones *et al.* 1999; Virgós and Garcia 2002; Lanszki 2003). Interspecific competition is, therefore, avoided and the stone marten, due to its more generalist habits, shows a higher level of adaptability to human-altered environments, which may have implications for long-term viability of the species (Clevenger 1994).

The genetic analysis of four faecal samples collected in the periphery of the closest village to the study area, and the location of several dead mustelids, indicated that the European pine marten was also present there. This information and the other results obtained during this study seem to indicate that the scarce human-caused environmental alterations might influence in the absence of stone marten. In the study area and surrounding areas the density of humans is very low and the forest habitats are well conserved, with a progressive abandonment of agricultural activities, including crops and animal husbandry. We suggest, therefore, that further studies are needed to analyse the sympatry hypothesis on a small scale, considering different degrees of urbanisation and environmental disturbance. The possible competition and habitat segregation between the two marten species in the forests of the northern Iberian Peninsula is a basis for future studies regarding the animal community of forest habitats. These investigations provide fundamental information relative to habitat management directed principally at the conservation of the European pine marten since it apparently depends a great deal, if not exclusively, on unimpacted forest resources.

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