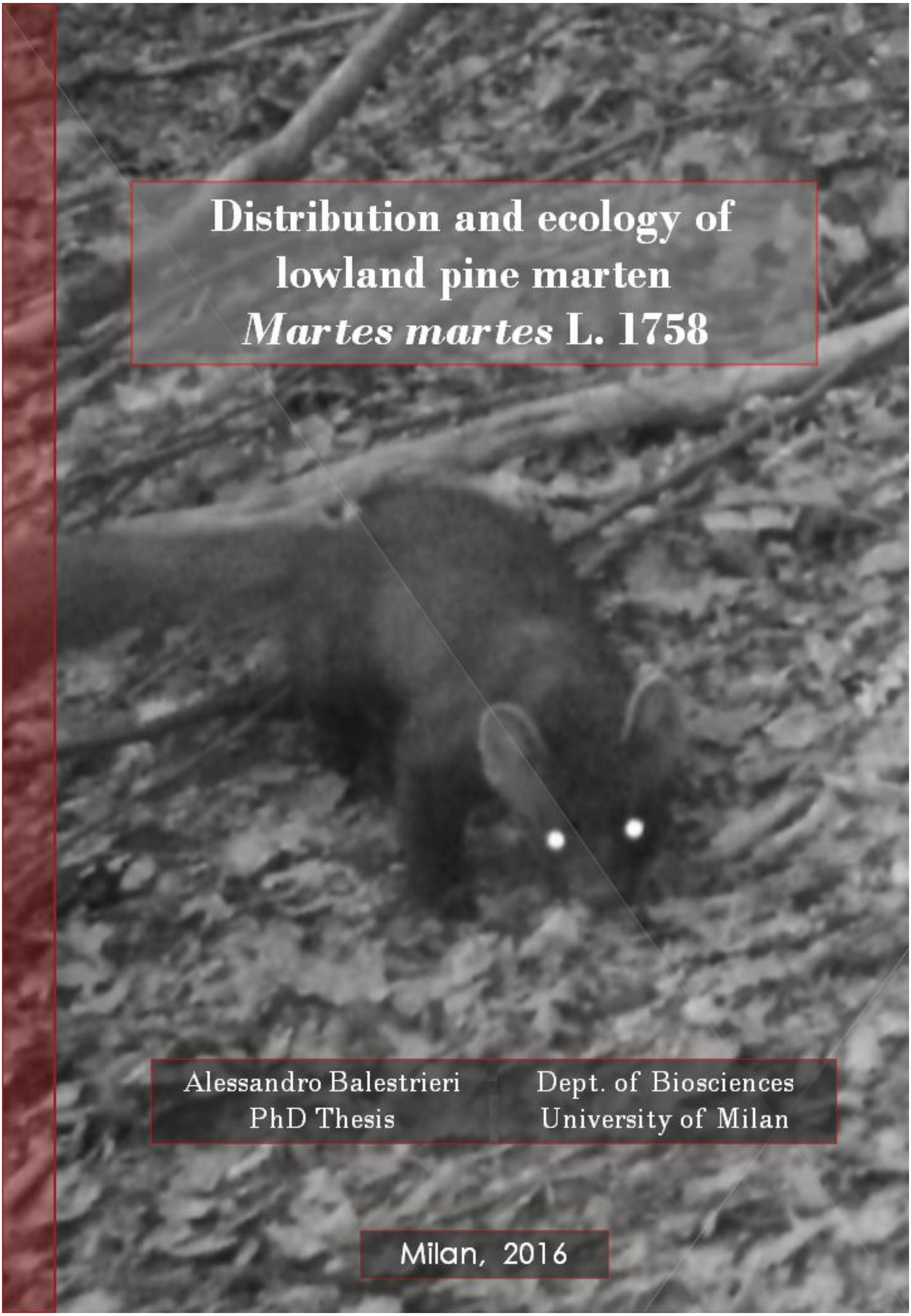
Distribution and ecology of lowland pine marten *Martes martes* L. 1758



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DISTRIBUTION AND ECOLOGY OF LOWLAND PINE MARTEN Martes martes L. 1758

Ph.D. Thesis

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Abstract

The pine marten (*Martes martes*) has been long considered a prototypical forest-specialist, but recent studies carried out in western Europe have pointed out that the species is more generalist in terms of habitat preferences than previously reported. In the western plain of the River Po residual woods mainly consist of small patches or riparian woods merged in intensively cultivated areas; nonetheless in this apparently unsuitable area the number of roadkill pine martens has increased exponentially since the beginning of the century. The expansion of the pine marten in agricultural lowlands provided an invaluable opportunity for investigating its ecological requirements and the environmental factors that shape this species' range.

To assess the actual level of pine marten penetration in lowland areas of north-western Italy, a non-invasive, faecal mDNA-based genetic method was applied at landscape scale, demonstrating that the pine marten has colonised the overall study area north of the River Po, which probably is acting as a barrier. Pine marten distribution suggests that expansion has mainly followed a north-to-south direction, following the course of major rivers as natural corridors of expansion. The unexpected high frequency of haplotypes of the central-northern European phylogroup supported the hypothesis of Alpine or trans-Alpine populations as sources of pine marten expansion.

Based on presence-absence data, Species Distribution Models were applied to identify the main factors driving the colonisation by the pine marten of the western River Po plain and predict its potential south- and eastwards expansion. The distribution of residual wood patches and availability of suitable riparian corridors played a major role in shaping the potential expansion area of the pine marten in agricultural lowlands. This was high only for the western part of the River Po plain and, secondarily, for the pine wood patches bordering the Adriatic coast.

The hypothesis that lowland woods may represent marginal habitats for the pine marten was not supported by the data collected about both its density and macronutrient balance. In the valley of the River Ticino, microsatellite analysis of faecal DNA allowed to point out one of the highest densities ever recorded in continental Europe. The high genetic relatedness of identified individuals suggested that the agricultural matrix may act as a barrier to the dispersal of juveniles. Genotyping allowed also to test for the effectiveness of a camera-trapping-based method (Random Encounter Model) for assessing pine marten density. The REM underestimated population size by ca. 40%. Dietary macronutrient ratios of pine marten differed from the target, as assessed by the meta-analysis of available diet data for Europe, to an almost negligible extent, confirming that plasticity in feeding behaviour plays a major role in pine marten expansion in agricultural areas.

Genetic surveys confirmed that pine marten expansion is coinciding with a reduction in stone marten range or abundance. When syntopic, stone marten exclusion from woodland by the pine marten is a common output, although the mechanisms underlying the higher competitive ability of the latter are still unclear. By camera-trapping, we tested the hypothesis that spatial and temporal segregation may favour the coexistence of the pine marten with the other mesocarnivores to a wider extent than that of the stone marten. Results from two study areas suggested that pine marten higher competitive ability with respect to the stone marten could be the indirect consequence of its greater plasticity in activity patterns. As different guild compositions result into different interspecific relations among coexisting species, our findings need to be further tested across different communities and study areas.

Contents

1. Introduction

> *The [pine] marten is a specialist of coniferous forests of the boreal zone and southern peninsular extensions […]. Martens prefer mesic, conifer-dominated forests with abundant physical structure near the ground and avoid areas lacking overhead cover […].*
>
> (Buskirk and Zielinski 2003)

1.1 The pine marten in fragmented habitats

The pine marten (*Martes martes*) has been long considered a strictly forest-dwelling species, generally associated with forest habitats, mainly mature coniferous and mixed forests (Proulx *et al*., 2004; Zalewski and Jędrzejewski, 2006). Deforestation and forest fragmentation affect the distribution and density of pine martens (Brainerd *et al*., 1995; Kurki *et al*., 1998), which generally avoid treeless areas (Storch *et al*., 1990; Brainerd and Rolstad, 2002; Pereboom *et al*., 2008) and are believed to need a minimum of 2 km^2 of forested habitats to survive (Zalewski and Jędrzejewski, 2006). As a consequence, the pine marten has been reported to be particularly sensitive to the effects of human activities, including habitat loss and landscape-scale habitat fragmentation (Bright, 2000; Pereboom *et al*., 2008; Mergey *et al*., 2011).

Nonetheless the pine marten can tolerate intensive deforestation - as occurred in the British Isles (Birks et al., 2005), where, anyway, the recent expansion of commercial forestry has promoted the recolonisation of large areas of northern Scotland (Balharry et al., 1996; Croose et al., 2013) and Ireland (O’Mahony *et al*., 2006, 2012) -, and on the Isle of Elba (central Italy) the species occurs also in rural areas and shrubland (Mediterranean *maquis*; De Marinis and Masseti, 1993).

Recent studies carried out in western Europe have pointed out that the species is more generalist in terms of habitat preferences than previously reported (Virgós *et al*., 2012). These studies showed that the pine marten can colonise agricultural landscapes that contain remnant forest patches and highlighted the importance of the surrounding matrix for providing food resources, den sites, and complementary habitats for enhancing connectivity among residual forest patches (Pereboom *et al*., 2008; Balestrieri *et al*., 2010; Mergey *et al*., 2011; Caryl *et al*., 2012).

In northeastern France, the pine marten can use both large wood patches and small plots and hedgerows (Perebom *et al*., 2008). Wood fragmentation does not affect neither home range size, which are smaller than expected (Mergey *et al*., 2011), nor genetic diversity (Mergey *et al*., 2012). In central Poland, habitat fragmentation has been reported to promote the coexistence of pine- and stone martens (*Martes foina*) (Pilot *et al*., 2007). In northwestern Italy, since the beginning of the century, the number of roadkill records in lowlands has increased exponentially, and faecal DNA-based investigations have confirmed the stable occurrence of the pine marten in two small wooded areas (Balestrieri *et al*., 2008, 2010). Whilst for the first two countries the landscape matrix was woodland, with small fields and urban areas scattered throughout both study areas, in the western plain of the River Po residual woods mainly consist of small patches or riparian woods merged in intensively cultivated areas.

1.2. Evidence of pine marten expansion in the Po plain

Available information on the status of Italian mustelids is incomplete; however, the pine marten has traditionally been associated with deciduous and coniferous forests ranging in elevation from 1,000 to 2,000 m a.s.l. (e.g. Spagnesi and Demarinis, 2002). Accordingly, in the last three decades of the 1900s, the stone marten was the only marten species reported for the Po-Venetian plain (Bon *et al*., 1995; Martinoli, 2001a,b; Mantovani, 2010). At the end of the 20th century, road-killed pine martens began to be reported in intensively cultivated areas of the western Po plain (Sindaco, 2006; Savoldelli and Sindaco, 2008). Twenty-four records of road-killed pine martens below the 300-m a.s.l. contour (Fig. 1), which broadly marks the upper limit of the plain (Balestrieri *et al*., 2010), revealed an exponential growth of pine marten records in the first decade of the 21st century (Fig. 2) and suggested a progressive range expansion by the pine marten throughout the interior of the plain (Balestrieri *et al*., 2010). Most records were associated with a river valley, suggesting that riparian zones may serve as natural expansion corridors for pine martens.

Successively, by a non invasive, faecal DNA-based genetic census, the stable occurrence of the pine marten was ascertained for two protected areas of the Po plain, consisting of small residual wood patches surrounded by maize and rice fields (Balestrieri *et al*., 2008, 2010). Genetic techniques were necessary to identify faecal samples to species because pine marten faeces cannot be distinguished morphometrically from those of the stone marten, and can also be confused with those of other carnivores, such as the red fox (*Vulpes vulpes*) and European polecat (*Mustela putorius*) (Davison *et al*., 2002; Birks *et al*., 2004). The identification of faecal samples to species was accomplished using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method (Ruiz-González *et al*., 2008), using two specifically designed primers that amplify fragments of DNA containing 276 bp. These primers amplify mtDNA from both *Martes* species and from 4 *Mustela* species, while red fox mtDNA will not amplify. The simultaneous use of the restriction enzymes *RsaI* and *HaeIII* differentiate pine and stone martens from each other, and from other mustelids whose mtDNA amplifies with these primers.

The colonisation of such apparently unsuitable habitats by the pine marten suggests a high degree of plasticity in both pine marten territorial (Balharry, 1993) and feeding behaviours (Jedrzejewski *et al*., 1993; Zhou *et al*., 2011). Nonetheless, agricultural areas could represent suboptimal habitats acting as dispersal sinks with high turnover of extinction-(re)colonisation events (Kawecki, 1995; Baguette, 2004). If so, then the occurrence of pine martens in the plain would depend on the immigration of non-resident martens from surrounding areas.

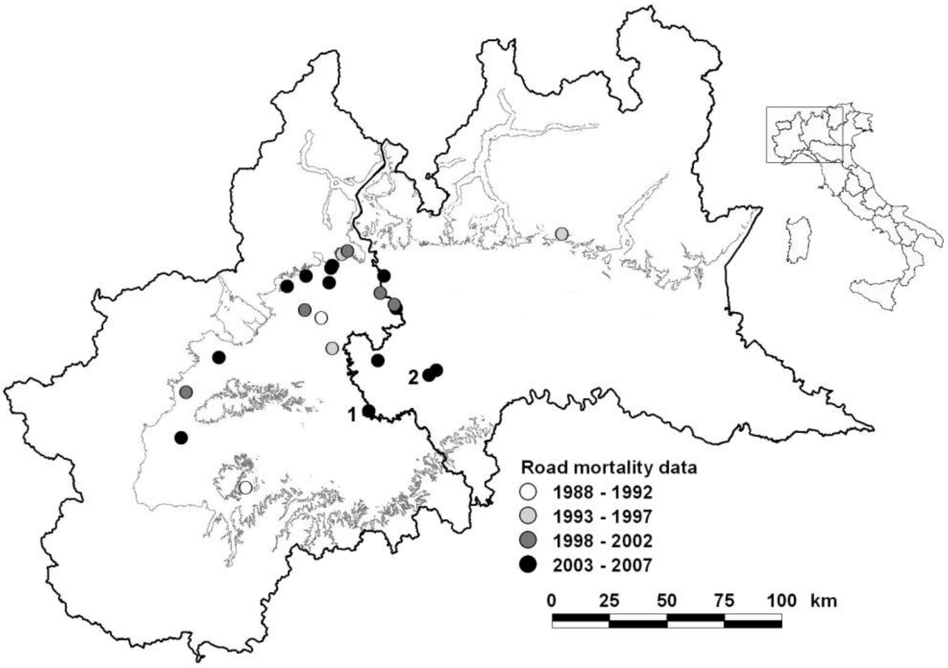

Figure 1. Road mortality of pine martens in the western River Po plain, relative to the 300m a.s.l. contour line (the two areas of genetic survey are numbered) (from Balestrieri *et al*., 2010).

1.3. Habitat change and pine marten range shift

Ranges of species can change over time, expanding or contracting according to various factors, such as landscape modification, climate change, introduction of exotics, persecution by humans, or hunting restrictions and protection measures (Lubina and Levin, 1988; Lensink, 1997; Parmesan and Yohe, 2003; Morrison *et al*., 2005). Climate variations is unanimously considered major determinants of species' range limits (Sexton *et al*., 2009) and consequent to global warming in the northern hemisphere the ranges of temperate species are shifting northwards or upwards in elevation (Parmesan and Yohe, 2003). On the opposite, in the last 20 years the reduction of human-related impact on either species or their habitats has reversed the demographic trends for several mammalian carnivores, resulting in the re-colonisation of areas where they had disappeared during the 20th century (e.g. the otter, *Lutra lutra* [Prigioni *et al*., 2007], and wolf, *Canis lupus* [Boitani, 2003], in Italy) or the occupancy of novel environments (e.g. urban red foxes, *Vulpes vulpes* [Gloor, 2002], in Switzerland).

Historical variation in the range of the pine marten in Europe has been related to changes in forest distribution. During the maximum ice expansion of the Weichselian glaciation, disjunct pine marten populations persisted within forested glacial refugia on both the Iberian and Italian Peninsulas and in the Carpathian Mountains (Sommer and Benecke, 2004), which led to the emergence of a Mediterranean mtDNA phylogroup (Ruiz-González *et al*., 2013). Both genetic and fossil data suggest that the postglacial recolonization of central and northern Europe by pine martens was from source populations that persisted in Mediterranean peninsulas and in a cryptic central-European glacial refugium (Ruiz-González *et al*., 2013), as forests advanced during glacial retreat (Sommer and Benecke, 2004).

In mountain areas of Europe, the decrease in forest cover from the expansion of low-intensity farming and livestock rearing has reversed during the last decades, following widespread agricultural abandonment (MacDonald *et al*., 2000). In the Alps, forest cover increased by about 50% from the 1960s to 2000, replacing open pastures (Falcucci *et al*., 2007), which would have had a positive effect on forest-dwelling species (MacDonald *et al*., 2000). Alpine pine marten populations, which are known to have high densities and small home ranges in mature and productive forests (indicative of high habitat carrying capacity), may have taken advantage of this increase in forest cover (Zalewski and Jędrzejewski, 2006). A similar scenario has been reported for the roe deer, *Capreolus capreolus*, a forest ungulate that has increased rapidly in density and progressively expanded into open, subalpine habitats during the 20th century (Kaluzinski, 1974; Tellería and Virgós, 1997; Jepsen and Topping, 2004).

In contrast, since the 1950s the intensification and modernisation of agricultural techniques in Europe have caused a widespread decline in landscape diversity of lowlands, due to loss of natural vegetation, fragmentation of uncultivated features, increase of field size and monocultures, and intensive use of herbicides and pesticides (Matson *et al.*, 1997; Robinson and Sutherland, 2002).

The Po-Venetian plain is the largest in Italy (*ca.* 46,000 km^2), and is one of the most densely populated areas in the country. The pedogenetic and micro-morphological characteristics of the soils of the lower plain, crossed by the River Po (652 km in length), support high levels of agricultural productivity and are intensively managed for cattle husbandry and the production of rice, maize, wheat, sugar beets, fruit, and horticultural products. Residual forests cover <5% of the Po-Venetian plain (Falcucci *et al.*, 2007). About 70% of forests are in the western and central plain (Camerano *et al.*, 2010), and either consist of small fragments (mean patch size = 4.5 ha; Lassini *et al.*, 2007) scattered within the agricultural matrix or, as in most European lowlands (Coles *et al.*, 1989; Bennett, 2003), along major rivers, such as the River Ticino, the valley of which hosts the largest and best-conserved riparian forests in northern Italy.

1.4. Interspecific relationships between pine- and stone martens

The pine marten and the closely related stone marten are quite similar in terms of body size, morphology and feeding habits (Goszczynski, 1976; Marchesi *et al.*, 1989; Lanszki, 2003; Zalewski, 2004; Lanszki *et al.*, 2007). They are medium sized mustelids with an elongated body shape. The body weight of both martens ranges between 1.0 kg and 1.6 kg, with males being, on average, 25% ca. heavier than females (Genovesi and De Marinis, 2003); however, size varies greatly with climate conditions throughout their wide distribution ranges (Reig, 1992).

Despite these similarities, the two martens occur sympatrically throughout much of Europe (Proulx *et al*., 2004). The pine marten occurs throughout most of Europe, with the exception of most of Iberia and Greece, Asia Minor, northern Iraq and Iran, the Caucasus and Western Siberia (Kranz *et al*., 2008). The stone marten is found from Spain and Portugal in the west, through central and southern Europe, the Middle East, and central Asia, extending as far east as northwestern China and India. In Europe, it is absent from the British Isles, the Scandinavian peninsula, Finland and northern European Russia (Tikhonov *et al*., 2008).

In boreal Europe, pine martens are considered to be forest specialists, being particularly associated with late-successional, structurally complex coniferous, which provide protection from predators, access to prey, and thermal insulation (Buskirk and Powell 1994; Buskirk and Ruggiero, 1994). The stone marten is synanthropic in most of its geographic range (Herr *et al*., 2009), but also inhabits more natural landscapes, particularly in the southern part of its range (Sacchi and Meriggi, 1995; Virgós *et al*., 2000).

Recent studies have shown contrasting results about the relationship between these two species at finer spatial scales: the pine marten was the only *Martes* species present in a mountainous area of northwestern Spain (Rosellini *et al*., 2008), whilst these two martens were reported to coexist in forest–field mosaics of both northern Spain and Poland (Posluszny *et al*., 2007; Ruiz-González *et al*., 2008). In contrast, in Białowieża primeval forest Wereszczuk and Zalewski (2015) recently suggested a strong ecological niche segregation between the two marten species, with the pine marten selecting deciduous woods and the stone marten preferring human settlements throughout the year.

Accordingly, by Habitat Suitability Models Vergara *et al*. (2015) showed that in sympatric areas of northern Spain the pine marten was positively associated with cooler areas with less human disturbance and high forest cover, while the stone marten preferred human associated environments, mostly villages surrounded by extensive agricultural areas.

The coexistence of these species probably depends on a combination of several factors, including the relative abundance of each predator species within the local guild, and food availability.

Geographical differences in the patterns of habitat use have suggested that in Central Europe the stone marten may avoid woods due to competition with the pine marten (Delibes, 1983). An alternative hypothesis is that stone martens would be more flexible in their behaviour and thus more prone to colonise human-impacted habitats (Marchesi, 1989).

However, current knowledge of their ecological relationships in areas of sympatry is extremely limited; further studies are needed to fully understand the factors influencing this complex relationship (Virgós *et al*., 2012).

Food availability is considered a main environmental factor influencing the population dynamics of small mustelids (King, 1989; Jędrzejewski *et al*., 1995). Early simulations showed that two or more similar-sized *Mustela* species may coexist when prey populations are high, especially if predation reduces competition and prey populations fluctuate. Anyway, stability over long periods was considered impossible (Powell and Zielinski, 1983).

In agreement with these results, in a pioneering paper Delibes (1983), by testing available explanations, identified with interspecific competition with the pine marten the cause of stone marten synanthropy in central Europe. His argumentations fit into MacArthur and Wilsons' Compression Hypothesis (1967), according to which "*as the number of competing species increases, the occupied habitats contract, but not the range of food items taken within the occupied habitats*" (Delibes, 1983), and, as reported above, have been successively supported by recent studies (Rosellini *et al*., 2008; Wereszczuk and Zalewski, 2015).

Nonetheless, it is still unclear by which mechanism the pine marten should be able to exclude the stone marten from preferred habitats.

While differential habitat use has been advocated several times for explaining coexistence, temporal niche segregation has been poorly investigated. Both species are mainly nocturnal, but the pine marten is more active during the day (Antonelli, 1996). Even small shifts in activity rhythms of stone and pine martens may play a role in facilitating their coexistence (Monterroso *et al.*, 2015). Nonetheless, evidence for temporal niche segregation between the species is considered weak (Wereszczuk and Zalewski, 2015).

About the third major dimension of ecological niches (Pianka, 1973), there is large consensus about the generalist feeding behavior of both marten species, using a wide range of resources according to their local and seasonal availability (Goszczynsky, 1976; Libois and Waechter 1991; De Marinis and Masseti, 1995; Zalewski, 2004; Zhou *et al.*, 2010).

Although the impossibility of distinguishing the faeces of the two species, and the high probability of confusing them with those of other carnivores (Davison *et al.*, 2002), make previous diet studies based on the analysis of faeces rather unreliable (Reed *et al.*, 2004), especially in areas where the two marten species were likely to be sympatric, there is plenty of data about the diet of these two marten species.

1.5 Food habits of pine- and stone martens

Small mammals are pine marten main prey throughout Europe, representing up to 81% of all prey (on average ca. 45%; Zalewski, 2004). The proportion of rodents in marten diets peaks in the temperate deciduous and mixed woodlands, declining both further north in boreal forests and southward, in Mediterranean regions (Zalewski, 2004).

.In continental Europe (Jędrzejewski *et al.*, 1993, Pulliainen and Ollinmäki, 1996; Balestrieri *et al.*, 2011), bank vole *Clethrionomys glareolus* seems to be preferred to *Microtus* voles, although the last ones are considered to be more profitable prey (Buskirk and Macdonald, 1984) and are selected by British pine martens (Gurnell *et al.*, 1994, Putman, 2000). While bank voles are habitat generalists, *Microtus* voles are generally restricted to open grassland and hedgerows (Caryl, 2008). Pine marten preference for bank voles may thus depend on the behaviour of the predator rather than the profitability of the prey, pine marten being more inclined to search for prey under the tree canopy than in open fields (Jędrzejewski *et al*., 1993).

Fruit (Marchesi and Mermod, 1989), birds (Helldin, 2000) and invertebrates (Clevenger, 1993) may sometimes represent the most frequent food item, especially in the southern European regions, while medium and large-size mammals (lagomorphs and ungulate carrion) generally constitute secondary prey at high latitudes (Zalewski, 2004). Amphibians (Reig and Jedrzejewski, 1988), fish (Lockie, 1961) and crustaceans (Ruiz-Olmo and Nadal, 1991) are eaten only occasionally.

In the River Po plain, (N Italy) mammals (especially rodents) and fruit formed the bulk of the pine marten diet, with birds and lagomorphs as important alternative sources of animal food (Balestrieri *et al*., 2011). The consumption of forest rodents, such as the bank vole, at levels greater than expected based on relative availability, confirmed pine marten preference for forested habitats (Balestrieri *et al*., 2011).

The stone marten is considered the most frugivorous European carnivore (Rosalino and Santos-Reis, 2009) and the availability of fleshy fruit seems to shape both its distribution and abundance (Mortelliti and Boitani, 2008; Virgós *et al*., 2010). In Mediterranean Europe, the diet of the stone marten includes mainly mammals and fruits (Ruiz-Olmo and Palazon, 1993 and references therein; Bertolino and Dore, 1995; Brangi, 1995; Genovesi *et al*., 1996; Barrientos and Virgós, 2006; Prigioni *et al*., 2008; Ryšavá-Nováková and Koubek, 2009), insects being also important in both Alpine (Prigioni and Sommariva, 1997) and meso-Mediterranean habitats (Amores, 1980).

Prior to pine marten expansion, in lowlands of northwestern Italy the diet of the stone marten included birds, fruit, small rodents and lagomorphs in almost similar proportions (Balestrieri *et al*., 2013). The heavy exploitation of birds might depend on the reduced availability of some key resource, namely small rodents, whose frequency of occurrence in stone marten diet was inversely related to that of birds (Balestrieri *et al*., 2013).

For both marten species fruits and berries constitute a major component of summer and autumn diets, while mammalian prey makes up a higher proportion of the diet in winter and spring (Baghli *et al*., 2002; Genovesi *et al*., 1996; Lanszki *et al*., 1999; Lodé, 1994). Large mammals, such as roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), coypu (*Myocastor coypu*) or domestic cats are presumably only consumed as carrion (Brangi, 1995; Genovesi *et al*., 1996; Baghli *et al*., 2002; Lanszki, 2003; Balestrieri *et al*., 2011).

1.6 Food niche overlap

The role played by interspecific competition in structuring communities has been recognized since the pioneering works of Volterra (1926) and Lotka (1932). The use of a limited resource (exploitation) or its non-consumptive pre-emption (interference) by one species is expected to reduce the resource availabilty to the other species (Wiens, 1989), implying resource partitioning and niche differentiation in sympatric species occupying the same trophic level (Pianka, 1969; Pimm and Rosenzweig, 1981; Ricklefs, 1990). Interspecific competition between predators sharing the same food resources should increase according to their similarity in morphology and body size (Morin, 1999; Di Bitetti *et al*., 2010).

The comparison of pine- and stone marten diets in sympatry is hindered by the difficulty of distinguishing their faeces. Consequently, to my knowledge, there are only a few studies which successfully devoted to comparative analyses.

Marchesi *et al*. (1989) used radiotelemetry to identify the faeces of radio-collared pine- and stone martens, and analysed a total of 480 faecal samples of the stone marten and 281 of the pine marten. Fruit was the main food item for both species, with the stone marten using more cultivated species than the pine marten. The latter relied on small mammals and birds significantly more than the stone marten. The use of rodent species reflected the habits of the two martens, with bank voles more preyed by the pine marten and *Microtus* spp. preferred by the stone marten, which also consumed human garbage.

More recently, Posluszny *et al*. (2007) identified marten faeces by DNA genotyping, collecting and analysing 287 samples for the stone marten and 155 for the pine marten. Their results mostly confirmed those of the previous study, with pine martens relying mainly on forest-dwelling rodents and birds and stone martens preferring fruit and insects.

Food niche overlap was quite high for both studies, ranging between 0.7 (Posluszny *et al*., 2007) and 0.94 (Marchesi *et al*., 1989).

In the Po plain, till now no wide enough area of sympatric occurrence of the two martens has been found. To explore the role played by intraguild competition in shaping the distribution of the two *Martes* species, Remonti *et al*. (2012) assessed trophic overlap between both the stone- (N = 91) and pine marten (N = 109) and the red fox (N = 78 and 114, respectively), which was widespread in lowlands both before and after pine marten colonization. Both marten faeces were unequivocally identified by DNA genotyping.

The food niche of stone marten and red fox overlapped almost totally ($\alpha = 0.89$) and oneway ANOSIM revealed no significant difference between the two diets, despite the latter ate insects more than the stone marten.

The index of food niche overlap between pine marten and red fox was lower, especially in the cold season (warm season: a = 0.66; cold season: a = 0.5; all year: a = 0.67). During the warm season, fruits, particularly wild cherries, were more used by pine marten, whereas red foxes relied more heavily on coleopterans. In the cold season pine marten preyed mainly on bank voles whereas red foxes relied more on wood mice (*Apodemus* sp.), rats (*Rattus* sp.) and water voles (*Arvicola terrestris*). Considering the whole study period, one-way ANOSIM revealed a small but significant difference between the two diets.

Although a high degree of food niche overlap *per se* does not implies competition for food (Schoener, 1982), these results suggest that, in the context of the meso-carnivore guild, the pine marten may have an actual competitive advantage with respect to the stone marten (Remonti *et al*., 2012).

1.7 Study aims and thesis structure

Road-kill records and preliminary genetic investigations suggested that the pine marten was expanding in the western River Po plain, apparently to the detriment of the stone marten. Anyway, road kills are unpredictable events which can help to draw a large-scale picture of species distribution, but are ineffective when trying to examine their actual range at a local scale, and genetic information was available only for two small protected areas.

Then my first aim was to ascertain the actual level of pine marten penetration in lowland areas of north-western Italy. To attain this goal, I applied at landscape scale a non-invasive, faecal mDNA-based genetic method, the effectiveness of which for identifying *Martes* species from northern Italy had already been tested before (Balestrieri *et al*., 2008, 2010). The systematic survey of field signs has been reported to yield reliable results on the distribution, abundance and habitat requirements of carnivores (Sadlier *et al*., 2004). I followed a two-step process, first assessing pine- and stone marten relative distribution and habitat requirements in a potential riparian corridor (**Chapter 2**), and secondly expanding the survey to the whole western Po plain (**Chapter 3**).

Recent studies have recognized that both the direction and rate of expansions may be influenced by external factors, such as the availability of suitable habitats or landscape heterogeneity (Darimont *et al*., 2005; Veech *et al*., 2011). In fact, when expanding in heterogeneous landscapes, individuals tend to colonise preferred habitats and avoid less-suitable ones, depending on habitat availability, landscape heterogeneity, and connectivity of suitable habitat corridors (Wilson *et al*., 2009). To identify the main factors driving the colonisation of the pine marten in the western River Po plain and predict its potential south- and eastwards expansion, I collected available occurrence data of the pine marten in the study area and relate them to a set of environmental variables by developing nine different SDMs (**Chapter 4**). To account for inter-model variability I used ensemble forecasting, which is particularly useful in modelling expanding species, which may not yet have spread to all suitable habitats, making species-environment relationships difficult to assess (Stohlgren *et al*., 2010).

Populations situated near the core of a species' geographic distribution exhibit greater abundance, less temporal variability in abundance (Vucetich and Waite, 2003) - i.e. are less prone to extinction for stochastic causes -, and often higher genetic diversity (Hoffman and Blouin, 2004; Tsumura *et al*., 2007) with respect to marginal populations.

To verify if, from an ecological point of view, lowlands represent marginal areas for the pine marten, I weighed pine marten performance with regard to both population abundance and diet composition. Pine marten density was assessed by two non invasive methods, based on camera-trapping and genetic surveys, respectively (**Chapter 5**); as the camera-trapping method had been recently developed, the estimate of pine marten density in lowland areas allowed to test for its effectiveness for mesocarnivores in fragmented habitats.

As reported in the previous paragraphs, with regard to diet, the pine marten is a generalist carnivore. Its diet has been extensively studied, showing that, throughout its wide range, this species can adapt to use several food resources depending on their local and seasonal availability. As a consequence, to investigate the frequency of use by the pine marten of prey items in the Po plain may hardly shed light on the potential of lowland habitats for sustaining a viable population of this species.

Recently, a wide literature has demonstrated that in many species foraging is guided principally by the need for regulating the amounts and balance of macronutrients – protein, lipids and carbohydrates -, to a target intake (Kohl *et al*., 2015). In agricultural landscapes dominated by crop cultivations, the decline of animal prey due to habitat loss may force predators to an unbalanced diet, with implications for either physiology or ecology (Remonti *et al*., 2011).

To test for this hypothesis, I reviewed data from the literature on the foods eaten by pine marten populations across Europe, estimated the percentage of macronutrients in each diet and then analysed both seasonal and geographic variation in the percentage of macronutrients associated with the recorded diets (**Chapter 6**).

Finally, despite being regarded as the least important of the three main niche axes, the temporal niche axis may play a major role in the case of predator species (Di Bitetti *et al*., 2009; Harrington *et al*., 2009; Wang and Fisher, 2012). Furthermore, the presence of competitors frequently influences activity patterns through interference competition, which is expected to be stronger whenever similarity in other niche dimensions and body mass are high (Donadio and Buskirk, 2006; Ritchie and Johnson, 2009). Although a recent study found weak evidence for temporal segregation between pine- and stone martens (Wereszczuk and Zalewski, 2015), intra-guild temporal adjustments may favour the coexistence of meso-carnivores (Torretta *et al*., 2015). As the pine marten has been reported to be more flexible in its activity patterns with respect to the stone marten (Monterroso *et al*., 2014), I hypothesized that differential time use may enhance the possibility for the pine marten to coexist with badgers and, particularly red foxes with respect to the stone marten. To test this hypothesis, I analysed and compared the activity patterns of these species in two study areas of the River Po plain (**Chapter 7**).

1.8 References

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2. Distribution and habitat use by pine marten *Martes martes* in a riparian corridor crossing intensively cultivated lowlands

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Distribution and habitat use by pine marten *Martes martes* in a riparian corridor crossing intensively cultivated lowlands

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Abstract

The location of pine marten records in northern Italy suggests that main rivers may play the role of natural corridors favouring this species' colonisation of cultivated lowlands. We assessed the distribution and habitat use by the pine marten on a 35 km long stretch of the River Ticino. Surveys were carried out between October 2011 and June 2012 along linear transects in a 2x2 km grid. Using the variation in marking intensity as an indicator of habitat use, habitat selection was assessed at two landscape levels – at transect-scale by the χ^2 test with Bonferroni's confidence intervals for the proportion of use, and at grid-scale by multiple linear regression. By a PCR-RFLP method, 91 faecal samples were assigned to the pine marten. Faeces were mainly located in wooded areas, while fields were avoided. At the grid-scale of analysis, marking intensity was positively related to the mean area of wooded patches and negatively to their mean perimeter-area ratio. This suggests that pine marten relative abundance may partially depend on the degree of fragmentation and structure of residual woods. The survey protocol allowed to assess the probability of detection. Occupancy models outlined that heterogeneity in detection probability may arise as a result of variation in marking intensity, *i.e.* the number of marking individuals. Our results suggest that the availability of both woodland corridors and wood patches are major factors shaping pine marten distribution in intensively cultivated plains and that non-invasive genetic surveys are a cost-effective method for future studies at a broader scale.

Key words: Non-invasive genetic sampling, detectability, faecal DNA, stone marten, Northern Italy.

Introduction

In the Mediterranean region, human population growth, agricultural intensification and the consequent loss of natural habitat have led to a general decline of biodiversity in both plain and coastal areas (Matson et al. 1997; Benton et al. 2003; Lepers et al. 2005). Plains show the lowest species richness, with the exception of some small wet areas and residual riparian woods, which support a more diverse fauna than surrounding habitats (*e.g.* Warkentin et al. 1995; Bentley and Catterall 1997; Hilty and Merenlender 2004).

A major biological effect of habitat fragmentation caused by anthropogenic modification is the decline of species that need large areas of connected natural habitats to meet their ecological requirements (Beier 1993; Mortelliti et al. 2010). Mammal distribution is particularly affected by the increased isolation and reduction in area of habitat patches (Bright 1993; Waldron et al. 2006) and habitat fragmentation is considered a major cause of the decline of forest-dwelling species (Reed 2004).

The pine marten (*Martes martes*) has been long considered a forest-specialist and its generalized decline has been imputed to the combined effects of large individual home ranges and deforestation (Buskirk 1992; Buskirk and Zielinski 2003). Nonetheless, recent studies have shown that the pine marten can colonise agricultural landscapes with highly fragmented woods (Balestrieri et al. 2010; Mergey et al. 2011; Caryl et al. 2012), suggesting that, as already observed in Mediterranean Italy (De Marinis and Masseti 1993), it is not such a strict forest-dweller as previously believed (see Virgós et al. 2012).

In Italy, the pine marten occurs sympatrically with the closely related stone marten (*Martes foina*) in mountainous areas, while in plain areas only the stone marten has been reported (Genovesi and De Marinis 2003a, b). Currently, the pine marten is colonising the western sector of the intensively cultivated Po plain (N Italy), where it probably went extinct at the end of the 1960s (Mantovani 2010), and pine marten expansion seems to coincide with the contraction of the stone marten range (Remonti et al. 2012).

The pattern of pine marten expansion seems to suggest that water systems may play the role of natural corridors favouring the dispersal of the pine marten from subalpine areas and the colonization of agricultural lowland (Fig. 1).

Riparian zones are important for maintaining landscape connectivity (Naiman et al. 1993; Taylor et al. 1993) and biological connections for wildlife (Clerici and Vogt 2013). The use by dispersing mammals of both natural and man-made linkages has been reported for several species (see Bennett 2003 for a comprehensive review). Corridors can assist the range expansion of both scarcely and highly mobile mammals, from meadow vole *Microtus pennsylvanicus* (Getz et al. 1978) to mink *Neovison vison*, which, following its introduction, has spread in Great Britain through river corridors (Harris and Woollard 1990).

As in most European lowlands (Coles et al. 1989; Bennett 2003), in northern Italy main rivers form linear habitats covered with remnant riparian vegetation, crossing clearly distinct, heavily disturbed farmland and urban areas. Lowland woods cover only 1750 km^2, of which about 70% are located in the western and central plain of the River Po (Camerano et al. 2010) as small residual fragments (mean area 4.5 ha; Lassini et al. 2007). The plain of the River Ticino includes the largest and best conserved riparian woods of northern Italy and represents the less altered river corridor of the western basin of the River Po, a critical European region from a conservation perspective (Clerici and Vogt 2013).

Records of road-killed pine marten suggested that the valley of the River Ticino may represent a suitable dispersal route for the pine marten (Balestrieri et al. 2010). Accordingly, since 2005 a stable pine marten population has dwelt in a small agricultural area, 5 km to the west of the River Ticino, about 20 km upstream its confluence in the River Po (Remonti et al. 2012). Thus, we aimed to draw the actual distribution of both pine and stone marten in the downstream, 35 km long section of the River Ticino valley and assess the pattern of pine marten habitat use in a fragmented landscape. To account for martens elusiveness and the difficulty of distinguishing its tracks from those of other medium-sizes carnivores, we applied a non-invasive genetic sampling method based on mtDNA extracted from faecal samples (Ruiz-González et al. 2008).

As the survey protocol involved repeated surveys, we also modelled the effects of both habitat variables and marking intensity on the probability of pine marten detection, in order to evaluate the cost-effectiveness of the survey method a major factor to consider in the design of occupancy surveys for carnivore mammals (Slauson et al. 2009; Long et al. 2011).

Study area

The Italian stretch of the River Ticino flows southwards from the southern edge of Lake Maggiore to the median course of the River Po, forming a 110 km long and, on average, 7 km wide valley.

The valley is partly protected by two Regional Parks: the Park of the Ticino Valley (Lombardy), covering 906.4 km^2 and the Natural Park of the Ticino Valley (Piedmont), 62.5 km^2.

The river crosses an intensively cultivated and urbanized plain, where mesophilous - *Fraxino-carpinion* - and hygrophilous - *Alno-Ulmion, Alnion glutinoso-incanae, Salicion albae* - woods are still widespread inside the weave of meanders, streams, canals and oxbow lakes which characterise the downstream stretch of the river. On the whole, water-bodies cover an area of about 48 km^2, while wet woods account for 87 km^2 (Prigioni 1995).

Pine marten monitoring focused on the lower part of the valley, from the towns of Vigevano and Abbiategrasso (Milan, Lombardy) in the north to Gropello Cairoli village (Pavia, Lombardy) in the south (Fig. 2). This *ca.* 35 km long stretch of the river has a mean annual discharge of about 300 m^{3}s^{-1} and a catchment area of more than 7000 km^2. Mean percent riparian vegetation cover, as assessed in a 100 m large belt on both river banks for seven 5 km long river stretches, is 47.8% (min-max: 12-86%; Prigioni and Balestrieri 2011).

The climate is temperate, mean annual values being 13°C for air temperature and *ca*. 700 mm for rainfall.

Methods

Collection and genetic identification of faecal samples

Surveys were carried out within a 2x2 km grid (Fig. 2), superimposed on the kilometric grid of digitalized, 1:10000 Regional Technical Maps. Grid size was chosen as to broadly correspond to pine marten mean home range in Tuscany (370 ha; Del Fante 2012).

Sampling was conducted between October 2011 and June 2012 along linear transects drawn along wood/field margins, paths and country roads to cover both open and forested habitats. Transects were surveyed 1-3 times each (mean ± SD = 2.44 ± 0.87).

A portion (ca. 1 cm) of each “marten-like” faeces (*i.e*. less than 10 mm large and then suspected to belong to the pine marten; for more *details* on faeces identification, see Remonti et al. 2012) was picked up using sticks stored in autoclaved tubes containing ethanol 96% and frozen at −20°C until processed (Ruiz-González et al. 2008).

Bi-monthly variation in marking intensity was checked by comparing the observed number of faecal samples to that expected based on the overall transect length covered during each period (N = 4).

DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer’s instructions. The specific identification of faecal samples was accomplished by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, according to Ruiz-González et al. (2008). Two primers amplify the mtDNA from *Martes martes*, *M. foina* and four *Mustela* species, of which only the European polecat *Mustela putorius* had been previously reported for our study area, whilst red fox (*Vulpes vulpes*) faeces, which can be easily mistaken for those of martens (Davison et al. 2002), render no amplicons. The simultaneous digestion of amplified mtDNA by two restriction enzymes (RsaI and HaeIII), generates different restriction patterns for each mustelid species (*i.e.* DNA fragments differing in both number and length), allowing the unambiguous identification of faecal samples (see Ruiz-González et al. 2008 for further details).

Habitat use

Based on CORINE Land-Cover maps, satellite images and ground surveys, a land-cover, digitalized, 1:10.000 map was produced by a Geographic Information System (QGis®).

Habitat selection was assessed by a use-availability design (Garshelis 2000) at two landscape levels, using faeces relative distribution as an indicator of habitat use.

Faecal counts have been widely used for assessing the abundance and habitat preferences of many mammal species (Putman 1984; Kohn and Wayne 1997; Gese 2001). Their reliability has been long debated (*e.g.* Kruuk et al. 1986; Messenger and Birks 2000), the interpretation of survey results involving several assumptions about marking activity and dropping identification (Sadlier et al. 2004). Nonetheless, faecal counts have been recently reported to be effective estimators of both relative abundance and habitat use by other carnivores (Clavero et al. 2006; Guter et al. 2008; Lanszki et al. 2008; Rosalino et al. 2008; Balestrieri et al. 2009; Kauhala and Salonen 2012).

To assess habitat use at transect-scale, faecal samples were georeferenced and assigned to a habitat type. Three main habitat types were considered: woods, poplar plantations and cultivated fields (mainly maize and rice). Samples located within a 5-m wide strip on each side of the border between two different habitat types were assigned to both habitats with a 0.5 score. The chi-squared (χ^2) test was performed to test for the goodness of fit of used habitat to available habitat types (White and Garrot 1990). Expected frequencies were calculated based on the overall transect length covered for each habitat. To determine whether a habitat was selected or avoided Bonferroni's confidence intervals for the proportion of use of each habitat were checked:

$$P_i - z_{a/2k}\cdot\sqrt{\frac{P_i(1-P_i)}{N}} < P_i < P_i + z_{a/2k}\cdot\sqrt{\frac{P_i(1-P_i)}{N}};$$

where: P_i is the proportion of use of the i^{th} habitat, N is sample size, z is the percentage point of the standard normal distribution corresponding to an upper tail probability of $\alpha/2k$, α is the level of significance and k is the number of habitat types (Neu et al. 1974; Byers et al. 1984).

To assess habitat use at grid scale, the following variables of potential importance to pine marten distribution were measured for each square:

a) W: percentage of woods,
b) U: percentage of urbanised areas,
c) C: percentage of cereal crops,
d) P: percentage of poplar plantations,
e) H: Shannon's index of habitat diversity,
f) minDr: minimum distance from the River Ticino (with minDr = 0 for all grid squares including a river stretch),
g) MWA: mean area of wooded patches, as to account for the degree of fragmentation of pine marten potentially preferred habitat,
h) MPAR: mean perimeter-area ratio of wooded patches, representing their compactness.

The influence of the measured variables on pine marten marking intensity, (MI = number of genetically identified faeces found in each grid square / total transect length per square) was tested by means of a multiple linear regression, using two different approaches.

First, we used a stepwise (forward selection) method to identify which variables were related to pine marten marking intensity (SPSS 12.0.1; SPSS, Chicago, IL, USA). Deviation from normality of the residual distribution was tested by Shapiro-Wilk's test.

Then we performed a linear multiple regression for each subset of uncorrelated (Pearson's test, $p > 0.05$) explanatory variables, entering all independent variables simultaneously. The obtained models were ranked by second order Akaike Information Criterion (AICc; Sugiura 1978), selecting those showing ΔAICc values < 2 ($\Delta AICc = AICc_i$ – min AICc, where min AICc is the AIC value of the 1st-ranked model; Burnham and Anderson 2002; Posada and Buckley 2004; Mazerolle 2006). The analysis was performed by the software SAM 4.0 - Spatial Analysis in Macroecology (Rangel et al. 2010).

Before the analyses, all variables were tested for normality. To achieve normality, U and MPAR were rank transformed, C was square-root transformed, while MWA, P and the length of linear transects were log(x+1) transformed. Being a frequency of occurrences, MI was log(x+1) transformed and modelled as a linear function of independent variables (Quinn and Keough 2002).

Detection probability

The influence of habitat variables on pine marten probability of site occupancy (Ψ) and detection probability (*p*) was analysed by the likelihood-based method for modelling occupancy data proposed by MacKenzie et al. (2002). This modelling is analogous to traditional capture-recapture methods, but uses the proportion of sites occupied by the target species as a state variable (rather than individuals). The goal is to assess Ψ knowing the species is not always detected, even when present. As the method requires multiple surveys in a demographically closed system (*i.e.* closed to changes of the occupancy state during the sampling interval; MacKenzie et al. 2003), in a sub-sample of 17 grid squares 1 transect (mean length: 3.6 ± 1.38 km) was surveyed three times between November 2011 and April 2012 (*i.e.* they were suspended before the time the cubs-of-the-year are supposed to start to scent mark; Genovesi and De Marinis 2003b), as to compile replicate observations into a sequence of 1's (detections) and 0's (non-detections). As grid size was almost equal to the size of home ranges and each home range was not occupied by more than two individuals of same-sex because of territorial behavior (Balharry 1993), we could assume detections at different sites to be independent.

As an example, the likelihood for site *i* with history 010 would be:

$$\Psi_{\mathrm{i}}(1-p_{\mathrm{i},1})p_{\mathrm{i},2}(1-p_{\mathrm{i},3}),$$

with Ψ_i: probability of site *i* occupancy; and $p_{i,n}$: detection probability for each visit. Assuming independence of sites, the product of all terms (one for each site) constructed in this manner creates the model (MacKenzie et al. 2002). Ψ may be some function of site characteristics and *p* may vary with certain variables; using a logistic model this information can be introduced to the model as, respectively, site- and survey covariates, (MacKenzie et al. 2002).

Analyses were run by the software PRESENCE (Hines 2006), using single-season models. As pine marten presence was expected to depend on wood availability and connectivity (Zalewski and Jedrzejewski 2006), 10 habitat covariates were measured: the length of the transect (L), the percent length of the transect covered by wood (Wt), the overall area of the wood patch crossed by each transect (Wa), wood patch perimeter (Wp), the minimum distance between the wood patch covered by the transect and the nearest >2 km^2 large wood patch (Dnw), the mean distance between each transect and the river (Dr), the percent cover of woods (W), urban areas (U) and crops (C) into each grid square; the mean distance between each transect and the nearest urban area (Du) was considered as an index of human disturbance. To avoid multicollinearity, Spearman's correlation (with $\alpha = 0.01$) test was used to check for any relationship between the covariates. Five covariates (L, Wa, Dnw, %Wt and W) were then selected for modelling.

Variation in marking intensity has been reported as a potential cause of heterogeneity in detection probabilities (Balestrieri et al. 2011a). As any non-invasive genetic survey for *Martes* species is a two-step process – the collection of "marten-like" faeces in the field and their later identification by genetic analyses in the laboratory -, we included as a survey covariate the abundance of "marten-like" faeces (FA = number of "marten-like" faeces per km), recording each survey as either 0 or 1 according to the results of genetic analyses.

Before the analyses, all covariates were standardised by the Z transformation (Donovan and Hines 2007). Models were first ranked according to Akaike Information Criteria values, excluding those showing delta values ≥2 (Burnham and Anderson 2002). The goodness of fit of the models was then assessed by Mackenzie and Bailey's test using 1000 simulated bootstrap detection histories (MacKenzie and Bailey 2004). To assess fit, the test use a chi-square approach, while the c-hat statistic is used for adjusting the standard errors for the model parameters (c-hat>1 indicates a lack of fit).

Naïve probabilities (*sensu* MacKenzie et al. 2003) were calculated as the number of transects or sites positive for pine marten over the total number of transects or sites surveyed.

The minimum number of surveys required to statistically establish the occurrence of the pine marten was assessed by the probability model: $Nmin = \log(\alpha) / \log(1 - p)$, where $\alpha = 0.05$ sets the confidence level (McArdle 1990; Reed 1996). To account for modelling results, mean p was calculated for three classes of faeces abundance - FA<0.5 (N=5), 0.51<FA<1 (N=6), FA>1.1 (N=6) – using top model estimates.

Results

Overall 160 "marten-like" faecal samples were collected inside 21 of the 2x2 km large sampling squares (84 km^2; Fig. 2), on a total length of 273.9 km of transects (mean transect length ± SD = 3.33 ± 1.45 km, min-max = 1.1–8.0 km), corresponding to 1.9 faeces/km^2 and 0.58 faeces/km, respectively. No time-related variation in marking intensity was recorded ($\chi^2 = 3.81$, d.f. = 3, p = 0.28).

DNA was extracted from 123 (76.9%) faecal samples, out of which 91 were assigned by our PCR-RFLP method to the pine marten and nine to the stone marten, corresponding to an overall species identification of 81.3% of analysed samples.

The pine marten occurred in all surveyed squares, averaging 4.3 ± 3.15 samples per square (min-max = 1-10; Fig. 2), while stone marten samples were found in only two squares. Grid squares overlapping the river showed 30-50% of woodland, while inside neighbouring squares the percentage of woodland decreased down to 0.9% (Tab. 3 in the Appendix).

Most pine marten faecal samples (78.8%) were collected inside or at the margins of woods, whilst 16 (17.6%) were found in poplar plantations or crops. Along surveyed transects, faeces were not distributed according to habitat availability ($\chi^2 = 15.6$, d.f. = 5, $p < 0.001$), being mainly sited in wooded areas, whilst fields were avoided (Fig. 3).

At the grid-level of analysis, the stepwise regression of the index MI on the habitat variables yielded a significant model ($R^2 = 0.28$, F = 7.49, d.f. = 20, p = 0.013), which included only the mean area of wooded patches (MWA), positively related to pine marten marking intensity ($\beta = 0.57$). Shapiro-Wilk’s test shoved a normal distribution of residuals (W = 0.93, p = 0.11). The AICc selected three models (Tab. 1). The best model included the mean area of wooded patches (MWA), positively related to pine marten marking intensity, while the second model included the mean perimeter-area ratio of wooded patches, negatively related to marking intensity ($\beta = -0.46$; Fig. 4).

On the first sampling occasion, the pine marten was genetically ascertained for 15 out of the 17 sampling squares which were surveyed three times (Tab. 4 in the Appendix). One new site was identified during the second survey and an additional one in the third survey. On average, 80.4% of squares yielded genetically identified pine marten samples from a single survey.

Two occupancy models showed to be supported by the data (ΔAIC scores <2; Tab. 2). The 2nd-ranked model was the one which best fitted the occupancy framework (MacKenzie and Bailey’s test: $\chi^2 = 0.031$, p = 0.18; c-hat = 0.26). Both models supported the inclusion of only FA as a covariate, positively related to the probability of detection. According to the best model, pine marten detection probability was similar in the first and third surveys ($p \pm SE = 0.88 \pm 0.034$ and 0.80 ± 0.012, respectively) and decreased in the second one ($p \pm SE = 0.71 \pm 0.041$). These values almost coincided with naïve probabilities (0.88, 0.82 and 0.7, respectively), as so as for the probability of site occupancy ($\Psi = 1$ for both raw data and top model).

According to the probability model, the minimum number of surveys needed to ascertain pine marten occurrence is 3.9 (CI = 3.0-5.2) where FA < 0.5 and 1.67 (1.66-1.69) where 0.51< FA< 1, while only one survey is needed where, on average, more than one “marten-like” faecal sample is found per kilometre of transect.

Discussion

Although in the last two decades of the 20th century sampling in plain areas was probably biased towards the stone marten, indirect signs of presence having been assigned to this species due to its well-known anthropophilia (Sacchi and Meriggi 1995; Lanszki 2003; Herr et al. 2009), our results draw a quite different picture, with the pine marten as the most widespread *Martes* species on the River Ticino (in terms of the number of both genotyped samples - 91 *vs*. 9 -, and positive squares - 21 *vs*. 2).

As supported by the exponential increase in road-kill reports (Balestrieri et al. 2010), the spreading of the pine marten in the valley may have occurred during the last 15 years. This rapid expansion stresses the role of riparian woods as a suitable corridor for pine marten colonisation of a landscape largely dominated by agriculture and artificial land-cover. Unfortunately, information about the status of the pine marten in Italy is insufficient to determine the causes of pine marten expansion, although, on the Alps, increased forest cover may have favoured the growth of pine marten populations (O'Mahony et al. 2012), as occurred for other forest species (*e.g*. Jepsen et al. 2004).

In our study area, the distribution of pine marten samples confirmed its preference for woodlands. Although signs were found not only in large woods but also in small, isolated wood plots and on wooded slopes and canal banks surrounded by agricultural land, the relation between marking intensity and mean wood size and perimeter-area ratio suggests that pine marten relative abundance depends on the structure and degree of fragmentation of residual woods (see also Pereboom et al. 2008).

Neither marking intensity nor occupancy rates decreased with the distance of the sampling transects from the river. As the main aim of the study was to assess pine marten distribution and habitat use along a potential river corridor, transect distance from the river never exceeded 5 km. Thus, our survey-scale may have been inadequate to detect the effect of this habitat variable.

Our results are consistent with those obtained by radiotelemetry in rural areas of France (Pereboom et al. 2008) and Scotland (Caryl et al. 2012), suggesting that the pine marten is more generalist in terms of habitat preferences than previously reported (Virgós et al. 2012). Such ecological generalization should increase the likelihood that individuals will find suitable resources in a new area (Mettke-Hofmann et al. 2002; Echeverría et al. 2006; Moritz et al. 2008; Pőyry et al. 2009), including man-altered habitats (Musiani et al. 2003). Accordingly, diet analyses suggested that in agricultural habitats the pine marten can cope with the reduced availability of small mammals by relying on introduced Eastern cottontail *Sylvilagus floridanus* (Balestrieri et al. 2011b).

The aims and spatial scale of occupancy surveys drive the choice of survey protocols. For large scales, the need to maximise the cost-effectiveness of the surveys may lead to lower the chance of detecting the target species (Slauson et al. 2009). By the non-invasive genetic method adopted, species identification was obtained for a rather high percentage of faecal samples with respect to other available approaches (*e.g.* 58%, Lucentini et al. 2007; 53.4%, Pilot et al. 2006), confirming the results of previous studies carried out by the same method (Rosellini et al. 2008; Ruiz-González et al. 2008, 2013; Balestrieri et al. 2010, 2011b). Although sample size was small, multiple surveys allowed us to point out that, as previously reported for the Eurasian otter *Lutra lutra* (Balestrieri et al. 2011a), heterogeneity in detection probability and hence the effort (*i.e.* the minimum number of surveys) needed to ascertain the presence of the target species may arise as a result of variation in the number of marking individuals (see also Kéry 2002 and Balestrieri et al. 2011), which should be accounted for by occupancy models (Royle and Nichols 2003).

Variation in *p* over the three surveys suggests that it may partially depend on covariates different from those tested, such as rain or cloud cover, which can affect the ability of surveyors to detect faecal samples (Olson et al. 2011). We suggest that future survey protocols have to involve multiple surveys, adjusting the overall effort per sample unit on the basis of the number of "marten-like" faeces recorded per km of transect.

In regions exposed to intense anthropogenic pressure, riparian areas are severely threatened, in spite of their crucial role for landscape connectivity (Clerici and Vogt 2013). Although further studies are needed to assess the actual pine marten distribution in the lowlands of northern Italy, on the basis of our results we can hypothesise the availability of both woodland corridors and wood patches spread in the crop matrix to be major factors shaping the distribution of this marten species in the Po-Venetian plain. The PCR-RFLP method adopted in this study, if combined with the search for faecal samples by trained surveyors (see also Ruiz-González et al. 2013), represents a cost-effective tool for future investigations on pine marten distribution at a broader scale.

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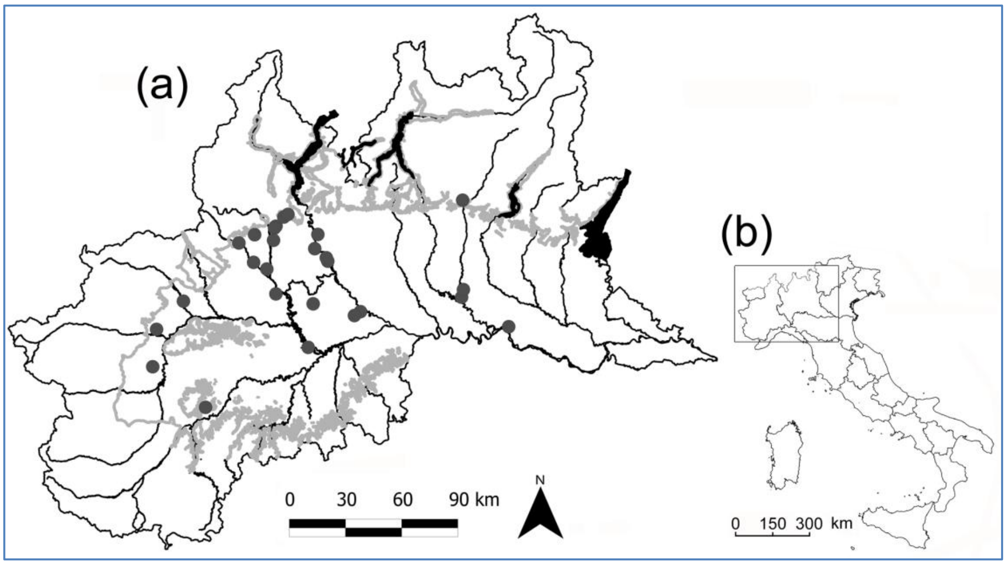

Figure 1. Distribution of pine marten road kill records (1988-2012; grey dots) in the western and central River Po plain (a) (Balestrieri et al., 2010; Mantovani, 2010) and with respect to northern Italy (b). Main rivers and lakes are shown in dark grey, while the light grey line is the 300 m a.s.l. contour line, which broadly marks the upper limit of the plain.

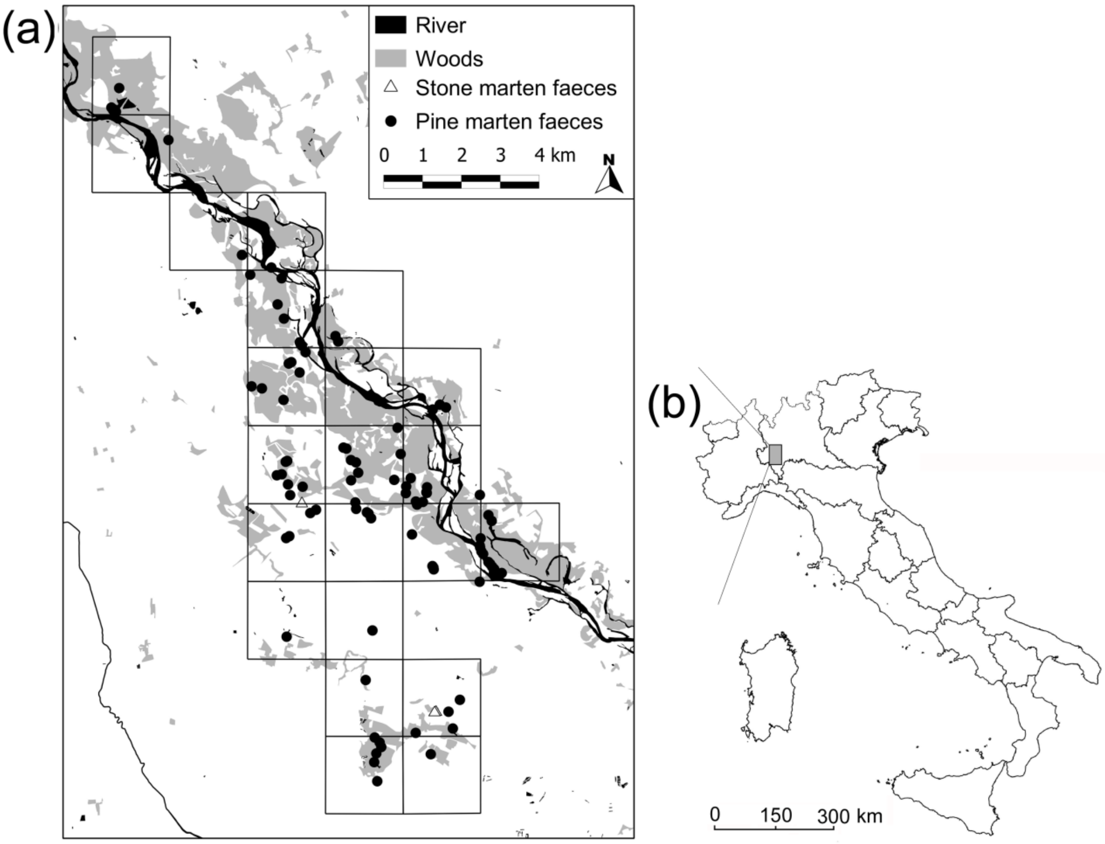

Figure 2. Genotyped faecal samples into 21, 2x2 km grid squares surveyed in the valley of the River Ticino (a), and location of the study area in Italy (b).

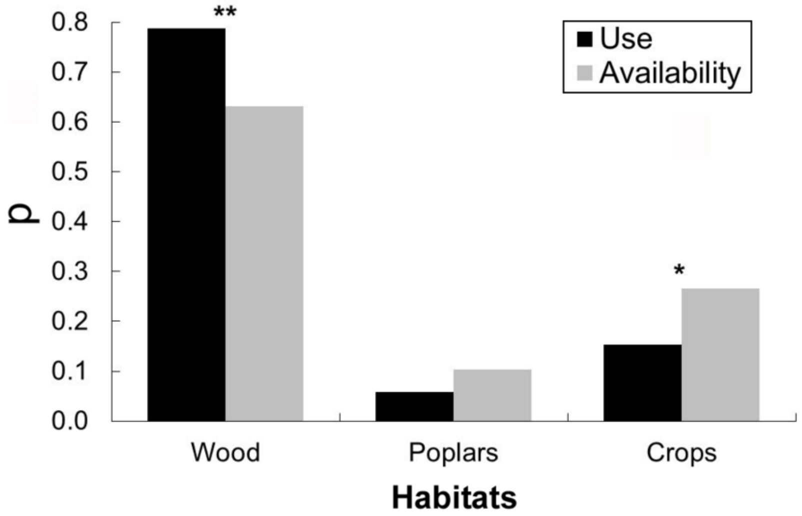

Figure 3. Habitat selection by pine marten as assessed at transect-scale by the comparison of the observed ("use") and expected ("availability") frequencies (p) of faecal samples (statistical significance according to Bonferroni's confidence intervals; * p < 0.01, ** p < 0.001).

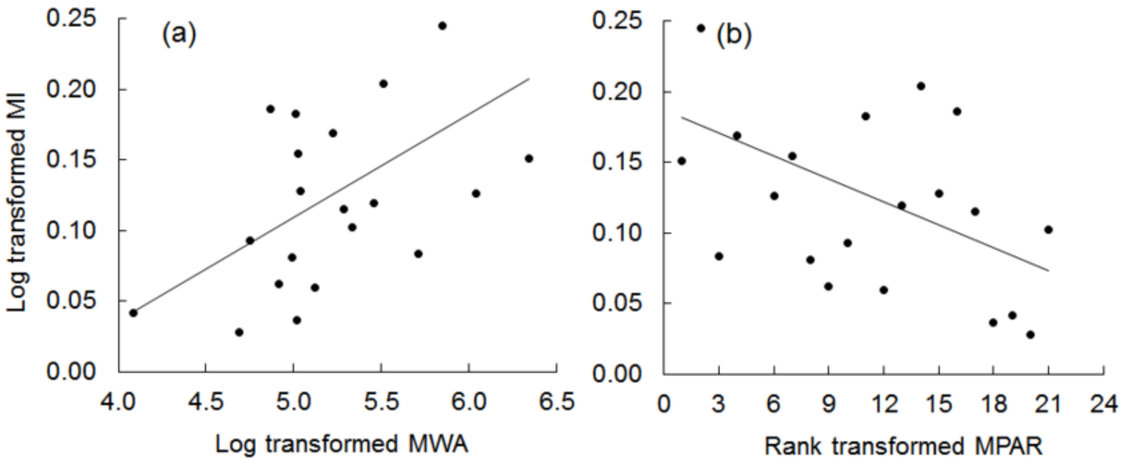

Figure 4. Relationship between pine marten marking intensity (MI) and both the mean area (MWA) (a) and mean perimeter-area ratio (MPAR) (b) of wooded patches into 21, 2x2 km wide grid squares. The two linear regressions selected by second order Akaike Information Criterion are shown (see methods).

Table 1. Models resulting from linear multiple regressions of pine marten marking intensity MI, ranked according to the Akaike Information Criterion (AICc) (MWA: mean area of wooded patches; MPAR: mean perimeter-area ratio of wooded patches; P: Percent cover of poplar plantations; ΔAICc: delta AICc, see methods).

Explanatory variables included in the model	R^2	AICc	ΔAICc	AICc weight
MWA	0.282	−52.16	0.000	0.14
MPAR	0.226	-50.57	1.593	0.07

Table 2. Top occupancy models obtained by PRESENCE (Hines 2006) and ranked according to Akaike Information Criteria (AIC) ($p_{1,2,3}$: detection probability during each survey; int: intercept; Ψ: probability of site occupancy; FA: marten-like faeces km^{-1}; ΔAICc: delta AICc, see methods).

Model	AIC	ΔAIC	AIC weight	Model likelihood	−2Log (likelihood)
Ψ(.), p(int) p(FA)	14.49	0.00	0.3353	10,000	8.49
Ψ(.), p(int) p_1(FA) p_2(FA) p_3(FA)	14.79	0.30	0.2886	0.8607	4.79

Table 3. Habitat variables of potential importance to pine marten distribution, total length of surveyed linear transects and pine marten marking intensity (MI) in 21, 2 x 2 km wide grid squares (W, U, P, C: percent cover of woods, urban areas, poplar plantations and crops, respectively, into each grid square; H: Shannon's index; minDr: minimum distance to the river; MWA: mean area of wooded patches; MPAR: mean perimeter-area ratio of wooded patches; TL: transect length; MI: marking intensity, expressed as number of faeces km^{-1}; see methods).

Grid square	W (%)	U (%)	P (%)	C (%)	H	minDr (km)	MWA (km^2)	MPAR	TL (km)	MI
1	17.7	1.7	1.0	79.5	0.29	4.61	0.70	0.009	7.92	0.76
2	8.9	6.8	2.7	81.6	1.24	3.96	0.06	0.031	4.19	0.24
3	13.5	7.9	9.1	69.3	1.57	3.05	0.13	0.032	6.77	0.15
4	11.0	0.7	6.7	81.3	1.18	1.97	0.11	0.027	9.34	0.43
5	5.8	1.5	5.5	86.5	1.22	3.22	0.05	0.094	15.27	0.07
6	0.9	0.5	9.7	88.8	0.71	1.64	0.01	0.062	10.07	0.10
7	13.8	1.7	22.0	62.1	1.72	2.88	0.11	0.036	11.64	0.34
8	3.9	0.8	12.7	82.4	1.17	0.89	0.07	0.038	7.48	0.53
9	42.2	0.3	7.4	42.7	1.96	0.00	0.51	0.021	37.93	0.21
10	52.5	1.6	0.0	36.7	1.81	0.00	0.70	0.024	8.96	1.01
11	22.1	5.0	21.4	51.4	1.88	1.16	0.10	0.031	13.37	0.52
12	54.4	0.8	18.4	26.4	1.67	0.41	1.09	0.024	29.78	0.34
13	47.5	0.1	16.8	21.5	2.53	0.00	0.33	0.034	15.02	0.60
14	53.7	0.0	15.9	28.2	2.29	0.00	0.19	0.046	23.04	0.30
15	26.2	22.2	23.0	20.7	2.87	0.00	0.17	0.021	6.30	0.48
16	57.8	0.0	17.7	8.5	2.22	0.00	0.22	0.098	26.31	0.27
17	12.3	0.7	15.6	70.3	1.44	0.19	0.10	0.030	9.73	0.21
18	20.6	1.7	0.0	68.6	1.99	0.00	0.08	0.031	6.55	0.15
19	36.5	1.2	5.9	31.7	2.73	0.00	0.10	0.048	11.45	0.09
20	30.0	18.4	5.4	29.1	2.49	0.00	0.28	0.034	3.16	0.32
21	59.3	0.0	2.7	35.1	1.23	0.00	2.21	0.007	9.61	0.42

Table 4. Detection histories and survey- and site covariates (S: survey; L: length, km; Wa: wood area, km^2; Wp: wood perimeter, km; Dr: distance to the river, km; Dnw: minimum distance between wood patches, km; Du: mean distance to urban areas, km; Wt: percent length of wood covered by each transect; W, U, C: percent cover of woods, urban areas and crops, respectively, into each grid square; see the methods for details) for each sampling site.

Site	Detection history			FA (marten faeces km^{-1})			L	Wa	Wp	Mean Dr	Dnw	Du	Wt	W	U	C
				S1	S2	S3										
1	0	0	1	0.00	0.00	0.25	4.0	0.60	1.70	4.63	2.80	1.23	10.7	6.13	1.63	91.54
2	0	1	1	0.00	0.23	0.69	4.34	2.29	20.56	0.41	0.05	2.50	74.1	28.5	17.53	37.80
3	1	0	0	0.53	0.00	0.00	3.74	6.96	62.47	0.62	0.05	3.45	82.2	54.4	0.80	44.77
4	1	0	0	0.72	0.00	0.00	2.77	0.60	1.70	5.05	3.60	0.34	37.1	6.13	1.63	91.54
5	1	1	1	0.12	0.62	0.25	8.01	6.96	62.47	0.56	0.05	0.99	41.1	38.5	0.30	54.42
6	1	0	1	0.88	0.00	0.88	2.26	0.05	1.90	3.71	0.43	2.28	39.1	13.6	1.65	84.33
7	1	0	1	1.34	0.00	0.69	3.72	0.06	17.58	3.03	2.40	1.20	13.7	0.92	0.52	98.50
8	1	1	1	0.25	1.31	0.65	3.06	2.29	20.56	0.49	0.05	3.39	92.0	48.9	0.00	37.54
9	1	1	1	0.97	0.38	1.12	4.13	6.96	62.47	2.23	0.05	1.99	53.4	54.4	0.80	44.77
10	1	1	1	0.62	1.40	0.83	4.80	2.29	20.56	0.55	0.05	2.20	63.2	52.8	0.00	44.95
11	1	1	0	0.61	2.46	0.00	1.63	6.96	62.47	2.18	0.05	2.34	53.3	3.72	0.75	95.28
12	1	1	1	1.99	0.66	0.66	3.01	0.60	2.90	5.34	4.50	1.02	58.7	17.7	1.72	80.53
13	1	1	1	0.29	0.61	2.45	3.26	1.20	8.12	1.48	0.25	1.20	98.2	52.8	0.00	44.95
14	1	1	1	1.03	0.54	2.15	2.93	0.27	3.96	2.94	0.03	0.61	32.3	23.0	5.17	71.67
15	1	1	1	1.39	2.71	1.16	2.58	6.96	62.47	0.60	0.05	2.39	79.1	40.9	0.09	46.90
16	1	1	1	0.56	3.61	2.72	3.57	2.84	10.82	0.42	0.75	1.33	68.6	52.5	1.59	36.73
17	1	1	1	2.87	1.91	1.59	3.54	0.99	15.22	0.25	0.04	2.18	95.0	12.3	0.69	85.86
Mean				0.83	0.98	0.95	3.61	2.88	25.7	2.03	0.89	1.80	58.3	29.8	2.05	64.24
SD				0.75	1.09	0.84	1.38	2.84	25.3	1.79	1.46	0.90	26.9	20.5	4.17	23.57

3. Pine marten vs. stone marten in agricultural lowlands: a landscape-scale, genetic survey

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Pine marten *vs*. stone marten in agricultural lowlands: a landscape-scale, genetic survey

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Abstract

We applied molecular methods on faecal samples to determine the overall level of occupancy for the pine marten (*Martes martes*) of the recently colonized western River Po plain (NW Italy) and assess the impact of interspecific interactions on the large-scale distribution of the similar-sized stone marten (*M. foina*).

Surveys were carried out in a 10x10 km grid, applying a hybrid sampling design. The specific identification of faecal samples was accomplished either by a PCR-RFLP method or by amplifying and sequencing a 330 bp mtDNA fragment of the control region (D-loop).

Genotyping success was 93.7% by the first method and 71.7% by DNA sequencing. Overall, we collected 47 pine marten records and 24 stone marten records. Thirty-six squares (81.8%) were found to be positive for at least one marten species, the distribution range of the two species scarcely overlapping. The pine marten was shown to be widespread in lowland areas on the left bank of the River Po, which is probably acting as a barrier to its expansion. In this area the stone marten has dramatically declined, while it is still widespread on the right bank of the river. The relative distributions of the two martens suggests that the contraction in stone marten range and/or numbers is an effect, rather than a cause, of pine marten expansion.

Six pine marten samples were from the Central-Northern European (CNE) phylogroup. The relatively high percentage of CNE martens is consistent with the hypothesis of an ongoing expansion of Alpine and trans-Alpine pine marten populations.

Key words: *Martes martes*, *Martes foina*, range variation, interspecific competition, PCR-RFLP

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Introduction

Species ranges can be highly dynamic, continuously changing in size and shifting in space as a consequence of variation in both abiotic and biotic conditions over time (Davis and Shaw 2001; Gaston 2003; Wisz et al. 2013).

Research on species' distribution ranges has recently gained a renewed interest, due to the need to face with large-scale, human-induced environmental changes (reviewed by Sexton et al. 2009). Several studies have focused on the expansion of invasive alien species (Andow et al. 1990; Hastings 1996; Von Holle and Simberloff 2005), which allow to investigate spatio-temporal dynamics in the absence of historical complexities (Svenning et al. 2014), the impact of global climate change (Parmesan 2006; Moritz et al. 2008), and the effects on ecosystems of the extinction of large carnivores (e.g. "mesopredator release"; Ritchie and Johnson 2009; Ripple et al. 2013).

Changes in species' distribution can alter basic ecological interactions, including predation, interspecific competition and disease transmission patterns (Vermeij 1991). The impact on ecosystem dynamics of the increase in population size and range of mesopredators can be severe (Prugh et al. 2009), especially for ecosystems with a simple structure (Roemer et al. 2009). Mesopredator spread has been related to the decline of several prey species (Palomares et al. 1995; Crooks and Soulé 1999; Galetti et al. 2009) and reported to facilitate the transmission of pathogens to domestic carnivores and humans (Whiteman et al. 2007). Less attention has been devoted to the intra-guild effects of expanding medium-sized carnivores (Hersteinsson and Macdonald 1992; Tannerfeldt et al. 2002).

Long considered a "forest-specialist", in recent years the pine marten *Martes martes* has been repeatedly reported to occur also in largely fragmented landscapes (reviewed by Virgos et al. 2012), suggesting a greater ecological flexibility than previously believed. In heterogeneous landscapes individuals are confined to wood patches (Vergara et al. 2015), resulting in smaller home ranges compared to forested habitats (Mergey et al. 2011). Habitat fragmentation is also a major factor producing strong genetic differentiation between pine marten populations over short geographical distances (Ruiz-González et al. 2015).

The large plain crossed by the River Po is one of the most intensively cultivated and densely populated areas in Italy. As a touchstone, residual forests cover <5% of the plain (Falcucci et al. 2007). In the last two decades, records of road-killed pine martens in the western section of this apparently unsuitable area have grown exponentially (Balestrieri et al. 2010), with evidence of its stable occurrence in two small protected wooded areas and in riparian woods on the banks of the River Ticino, a left-bank tributary of the River Po (Balestrieri et al. 2015). Although the more generalist and synanthropic stone marten *Martes foina* should be better adapted to human-impacted, fragmented landscapes than the pine marten (Sacchi and Meriggi 1995; Rondinini and Boitani 2002; Santos and Santos-Reis 2010; Vergara et al. 2015), on the River Ticino pine marten spreading seems to have been paralleled by a sharp restriction in stone marten range (Balestrieri et al. 2015).

To determine the overall level of occupancy for the pine marten and assess the impact of interspecific interactions on stone marten large-scale distribution, we applied different molecular methods on non-invasively collected faecal samples from both *Martes* spp. across the western Po plain. Faecal DNA-based genotyping has proven to be a cost-effective way to reliably verify and monitor these elusive species' presence (Ruiz-González et al. 2013a). DNA analysis was necessary because the faeces of *Martes* species, while being relatively abundant and easy to collect, thus facilitating the extensive sampling of these elusive species, cannot be distinguished from each other visually and can also be confused with those of other carnivores (Davison et al. 2002). Consequently, we applied a PCR-RFLP method for mtDNA that allows effectively identifying the two marten species (Ruiz-González et al. 2008) and reliably monitoring their distribution patterns. Additionally, and in spite of the fact that previous studies have provided important insights about pine marten phylogeographic patterns, further research is needed to get more detailed information about the distribution of main haplotypes and phylogroups in the contact zone between the Mediterranean area and continental Europe. Ruiz-González et al. (2013b) highlighted a mixed pattern of recolonization of northern Europe from both northern cryptic refugia and Mediterranean refugia (Iberia, Italy and the Balkans). As a consequence, in continental Europe this study pointed out the occurrence of both a central–northern European (CNE) phylogroup and, secondarily, a Mediterranean (MED) phylogroup, while the latter was the only phylogroup occurring in the Mediterranean basin. With one exception: the unexpected occurrence on the River Ticino of two haplotypes (i.e. Mm47 and Mm48) from the CNE lineage (Ruiz-González et al. 2013b), which potentially come from a recent colonization event from CNE populations in the Alps (Balestrieri et al. 2010, Ruiz-González et al. 2013b). To further clarify the frequency and distribution of these haplogroups in the contact zone of northern Italy, a mtDNA fragment of the control region (D-loop) was sequenced. This fragment is sufficiently informative to identify the main haplotypes and haplogroups described in previous pine marten studies (Davison et al. 2001; Pertoldi et al. 2008; Jordan et al. 2012; Ruiz-Gonzalez et al. 2013a).

Study area

The Po-Venetian alluvial plain is the largest in Italy (*ca*. 46,000 km^2). The pedogenetic and micro-morphological characteristics of the soils of the lower plain, crossed by the River Po (652 km in length), support high levels of agricultural productivity and are intensively managed for cattle husbandry and the production of rice, maize, wheat, sugar beets, fruit, and horticultural products.

Since the second half of the 19th century, widespread urbanisation and industrialisation have led to a progressive depletion of soil resources, built-up areas currently covering ca. 9% of the area (Gherardi et al. 2009). About 70% of residual forests are in the western and central plain (Camerano et al. 2010), and either consist of small fragments (mean patch size = 4.5 ha; Lassini et al. 2007) scattered within the agricultural matrix or, as in most European lowlands (Coles et al. 1989), cover the banks of major rivers.

Based on previous pine marten records (see Balestrieri et al. 2010), we focused on the western sector of the Po plain (< 300 m above sea level, Piedmont region), covering an area of ca. 7000 km^2 (Fig. 1). The Eastern limit of the study area was marked by the valley of the River Ticino, including the largest and best-conserved riparian forests of the Po-Venetian plain.

Climate was sub-continental temperate, with mean yearly temperature of 12.0°C and mean yearly rainfall of 1000 mm.

Methods

To assess the overall level of occupancy for both marten species in the western Po plain, surveys were carried out in a 10x10 km grid, superimposed on the kilometric grid of digitalized, 1:10000 Regional Technical Maps. We did not assume that occupancy and detection probabilities were constant across space (MacKenzie et al. 2003; Royle and Nichols 2003), as in Mediterranean agricultural landscapes the occurrence of both the pine marten (Pereboom et al. 2008; Balestrieri et al. 2015) and stone marten (Virgós and Garcia 2002; Mortelliti and Boitani 2008) depends on forest availability, i.e. size and degree of connectivity of residual wood patches. For each square we therefore selected 2-4 linear transects (mean transect length ±SE = 2.6 ± 0.175 km) as to cover available wood patches and their surrounding open habitats. Transects were drawn along wood/field margins, paths and country roads.

To determine how many surveys should be conducted per square, we followed the recommendations by Balestrieri et al. (2015), who assessed that the minimum number of visits needed to ascertain pine marten occurrence by faecal DNA genotyping is, on average, 3.9. We then applied a hybrid sampling design (Mackenzie and Royle 2005), with some squares surveyed only once and the others (≈ 40%) up to a maximum of four times, halting as soon as martens were detected. Repeated surveys of a same square were conducted as either multiple discrete visits (i.e. on different days) or multiple transects within a single visit (Mackenzie and Royle 2005).

Detections and non-detections of the target species were recorded, respectively, as a sequence of 1s and 0s ("detection history", *sensu* Mackenzie et al. 2002).

Sampling was conducted between January 2013 and December 2014. Hypothesising that river corridors may facilitate and drive pine marten colonisation of the interior of the plain (Balestrieri et al. 2015), surveys were mainly conducted from north to south (i.e. squares at approx. the same latitude were sampled in the same sub-period), as to follow the course of the major tributaries of the River Po (Fig. 1).

A portion (ca. 1 cm) of each faeces suspected to belong to *Martes* sp., was picked up using sticks, stored in autoclaved tubes containing ethanol 96% and frozen at −20°C until processed (Ruiz-González et al. 2008). All samples were georeferenced and projected onto a GIS (Arcview, ESRI).

DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. The specific identification of faecal samples was accomplished either by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method (N=35), according to Ruiz-González et al. (2008), or by amplifying and sequencing a 330 bp mtDNA fragment of the control region (D-loop) (N=46).

By the first method, two primers - Mm_L1 (5′- CCC AAA GCT GAC ATT CTA AC -3′) and Mm_H1 (5′ - ATG GGC CCG GAG CGA GAA GAG GTA CAC-3′) -, amplify the mtDNA from *M. martes*, *M. foina* and four *Mustela* species. The simultaneous digestion of amplified mtDNA by two restriction enzymes (RsaI and HaeIII), generates different restriction patterns for each mustelid species (*i.e.* DNA fragments differing in both number and length), allowing the unambiguous identification of faecal samples (see Ruiz-González et al. 2008 for further details).

By the second method, a 330 bp mtDNA fragment of the control region (D-loop) was amplified using the primers MFABDL228 (5'- AGA CTC AAG GAA GAA GCA ATA GCC -3') and CLUDH350 (5' GGG CCT GAA GTA AGA ACC AGA TGC C -3') (Randi et al. 2000). This DNA fragment was selected as it contains the main diagnostic mutations which reliably identify both the samples at species level and previously described pine marten haplotypes and/or haplogroups (Davison et al. 2001; Pertoldi et al. 2008; Rhoznov et al. 2010; Jordan et al. 2012;

Ruiz-González et al. 2013a), thus allowing for comprehensive comparisons. The standard polymerase chain reaction (PCR) amplifications were conducted in 15 µL reactions containing 3 µL of diluted template DNA, 3.2 pmol of each primer, 1.75 µM dNTP, 1.33 µM MgCl2, 1.56 mL of Gold STAR 10X buffer, and 0.6 U Taq DNA polymerase, using the following cycling conditions: an initial denaturing step at 94 °C for 5 min; 42 cycles of denaturing at 94 °C for 50 s, annealing at 58.5 °C for 45 s, and extending at 72 °C for 90 s, with a final extending step of 72 °C for 10 min. The PCR products were purified using EXO-SAP IT (USB, Cleveland, OH, USA), and sequenced using the BigDye Terminator Kit V1.1 (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM Model 3130 Genetic Analyzer (Applied Biosystems). Electropherograms were visually inspected and edited using SEQSCAPE 2.5 (Applied Biosystems), and nucleotide sequences were further aligned and edited in BIOEDIT 5.0.9 (Hall, 1999). Identical haplotypes were matched using DNASP v.5 (Librado and Rozas 2009). We then used BLAST software (Altschul et al. 1990) to determine 1) species' identity and 2) the correspondence of each discovered haplotype with already published haplotypes in GenBank and the pine marten phylogroups described by Ruiz-González et al. (2013b) (Table S1).

Additionally, we included unequivocal species records from road-killed individuals collected in 2013-14.

Results

We surveyed 44, 100 km^2 large squares, for a total of 180.9 km of transects, collecting 107 faecal samples. To maximise the cost effectiveness of genetic analyses, a subsample of 81 samples was selected (discarding apparently "old" and dubious samples) and analysed. Genotyping success was 93.7% by the PCR-RFLP method (33 out of 35 samples) and 71.7% by DNA sequencing (33 out of 46). By means of DNA sequencing, 29 samples were identified as *Martes* sp., 3 as *Vulpes vulpes*, and 1 as *Mustela putorius*. Overall, by the application of both molecular methods, 45 samples were assigned to the pine marten and 17 to the stone marten. Additionally, 2 pine- and 7 stone marten records from road-kills could be included. Thirty-six squares (81.8%) were found to be positive for at least one marten species (Fig. 1). In 88.9% of positive squares, martens were detected within the first two surveys (Tab. 1).

The distribution range of the two species scarcely overlapped, both species occurring only in four squares, two at foot hills and two in the valley of the River Ticino. Overall, the pine marten sharply prevailed north of the River Po, while the stone marten was the only *Martes* species occurring on the right bank of the river (Fig. 1). Few samples were found in the central part of the study area, which was intensively cultivated for rice.

In total, 3 different pine marten haplotypes and 6 stone marten haplotypes were identified (Table S1). The 6 different *M. foina* haplotypes (Mf_It_1-6) were identical or nearly identical (pairwise identity >97.1 %) to previously identified haplotypes by Nagai et al. (2012). All the 3 pine marten haplotypes retrieved in this study had already been found in previous studies (Davison et al. 2001; Ruiz-Gonzalez et al. 2013; see Online resource 1). Using BLAST, Hap 1 was found to be identical (pairwise identity =100%) to previously identified haplotypes of the CNE phylogroup, while Hap 2 and Hap 3 belong to the Mediterranean phylogroup (Online resource 1).

Fifteen out of the 21 pine marten samples (71,5%) assigned to the pine marten through DNA sequencing belonged to the Mediterranean phylogroup (MED), while six samples (28,5%) were from the Central-Northern European phylogroup (CNE). Pine martens from the CNE occurred throughout the northern sector of the study area (Fig. 2).

Discussion

Considering the overall high genotyping success, faecal DNA analysis proved to be an effective method for landscape-scale sampling of sympatric martens.

Based on sampling efficacy, i.e. the relatively high probability of marten detection and the 100% positivity of sites which were surveyed repeatedly, the pine marten can be considered to have colonised the overall northern section of the study area, at least wherever wood patches still occur.

The current pine marten distribution in the western Po plain suggests that expansion has mainly followed a north-to-south direction, with the River Po, which crosses the study area from the south-west to the east, acting as a natural barrier. Considering that the inner part of the plain has been reached by the pine marten only recently (Balestrieri et al. 2010), the crossing of this barrier may occur in the near future.

Spatial tracking of the environmental niche in response to changing environmental conditions has been often invoked as an explanation for range variation (Davis and Shaw 2001; Svenning et al. 2008). Variations in either climate (Sexton et al. 2009) or land-use (Brooks et al. 2002; Cousins et al. 2015) are unanimously considered major determinants of species’ range limits.

Both biogeographic and physiological studies have shown that temperature plays a major role in driving species’ ranges (Bale et al. 2002; Battisti et al. 2005) and anthropogenic climate change is being increasingly linked to changes in the distribution of both aquatic and terrestrial species (Parmesan 2006; Sexton et al. 2009). As a consequence of gradual global warming, in the northern hemisphere the ranges of temperate species are shifting northwards or upwards in elevation (Parmesan and Yohe 2003). In this context, pine marten penetration in arable lands would follow a counter-intuitive direction.

Woods are key features for the pine marten (Virgós et al. 2012); accordingly, in lowland, riparian areas its abundance has been related to the size and degree of fragmentation of wood patches (Balestrieri et al. 2015). There is no evidence that in the last decades forest cover has increased in the western Po plain, favouring pine marten colonisation. In contrast, the analysis of land cover change between 1960 and 2000 at country-scale showed that both intensive agriculture and, particularly, urbanisation have gradually increased in lowland areas of the peninsula (Falcucci et al. 2007). An opposite pattern was recorded for European mountain areas, where following widespread abandonment of low-intensity farming and livestock rearing, forest cover has increased (MacDonald et al. 2000), with a positive effect on forest-dwelling species, such as roe deer *Capreolus capreolus* (Vernesi et al. 2002; Jepsen and Topping 2004). In the Alps, forest cover has increased by about 50% from the 1960s to 2000 (Falcucci et al. 2007).

As reported for the British Isles (O'Mahony et al. 2012; Croose et al. 2013), Alpine pine marten populations may have taken advantage of this increase in forest cover, recolonising mountain districts from which pine martens had disappeared in the 20th century (Mosini 2015) and expanding in less suitable lowland areas.

The relatively high percentage of CNE martens found in the western Po plain, which currently represents an *unicum* in southern Europe (Ruiz-González et al. 2013b), is consistent with the hypothesis of an ongoing expansion of Alpine and trans-Alpine pine marten populations.

The negligible occurrence of stone marten records north of the River Po agreed with the trend reported for the valley of the River Ticino (Balestrieri et al. 2015), confirming that pine marten expansion is coinciding with a sharp reduction in stone marten range.

Although, within Mustelidae, there are several sympatric species with similar ecological requirements that have evolved mechanisms to coexist (e.g. *Mustela frenata – M. erminea*, St-Pierre et al. 2006; *M. erminea – M. nivalis*, Aunapuu and Oksanen 2003; *Lutra lutra – M. vison*, Bonesi et al. 2004), the widespread occurrence of genetically-identified stone martens on the right bank of the river, confirmed by available road-kill records (Sindaco and Carpegna 2010), suggests that the contraction in stone marten numbers and/or distribution was an effect, rather than a cause, of pine marten expansion.

Pine- and stone martens are sympatric across a large part of continental Europe (Proulx et al. 2004) and have also been reported to be syntopic (Pilot et al. 2007; Ruiz-González et al. 2008). There is still little information about the factors which may facilitate their coexistence, although some degree of spatial segregation (i.e. differential habitat use) has been reported to potentially reduce inter-specific competition (Vergara et al. 2015; Wereszczuk and Zalewski 2015).

In sympatry, stone marten preference for rocky areas and sub-urban areas has been associated to interspecific interactions with the more competitive pine marten (Delibes 1983), although it is still not clear why the latter should be able to force stone martens to use sub-optimal habitats. In fact these two martens are quite similar in size, morphology and feeding habits (Marchesi et al. 1989; Balestrieri et al. 2011, 2013). In syntopy, their trophic niches can overlap extensively (Posluszny et al. 2007). Recently, Remonti et al. (2012) compared the diets of martens and red fox *Vulpes vulpes* in northern Italy, reporting that the food niche of each marten species highly overlapped that of the fox. They concluded that the entry of the pine marten could have altered the existing ecological relationship within the carnivore guild to the detriment of the stone marten, the diet of which overlapped to a larger extent that of the red fox (Remonti et al. 2012).

As population declines are often dictated by complex combinations of environmental and biotic factors (Sinclair and Byrom 2006), particularly for same-sized Mustelidae (Powell and Zielinski 1983), further studies on niche partitioning among species belonging to the same guild are needed to predict short- and long-term outcomes of their interactions.

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Table 1. Detection histories (sensu MacKenzie et al., 2002) for the 44, 100 km^2 large squares sampled for the occurrence of pine- and stone martens in the western Po plain.

Detection history	**N of squares**	**%**
0	8	18.2
1	21	47.7
01	11	25.0
001	3	6.8
0001	1	2.3
total	*44*	

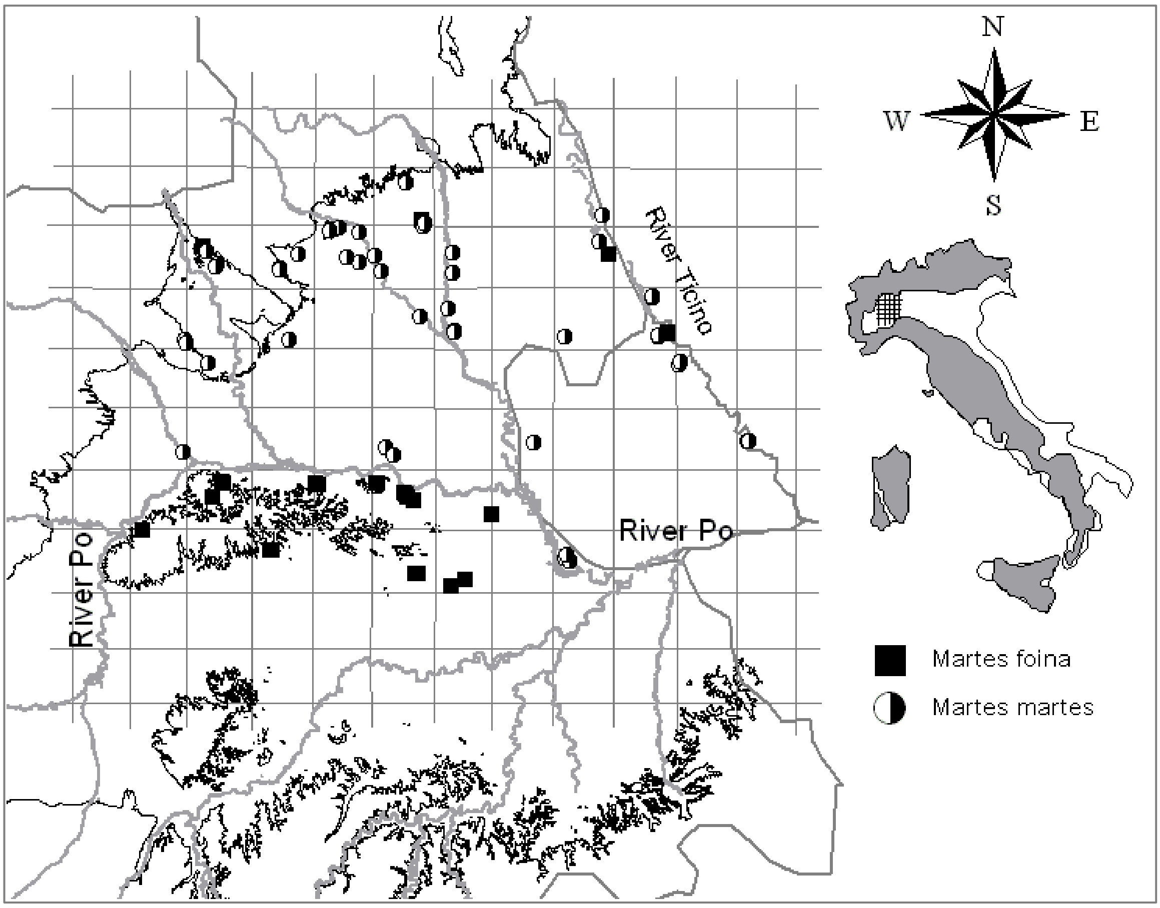

Figure 1. Distribution of both the pine- (MM) and stone marten (MF) in the western Po plain as assessed by faecal DNA genotyping; the 300 m a.s.l. contour line, which marks the limit of the River Po plain, is shown; the map of Italy shows the study area and the pine marten distribution.

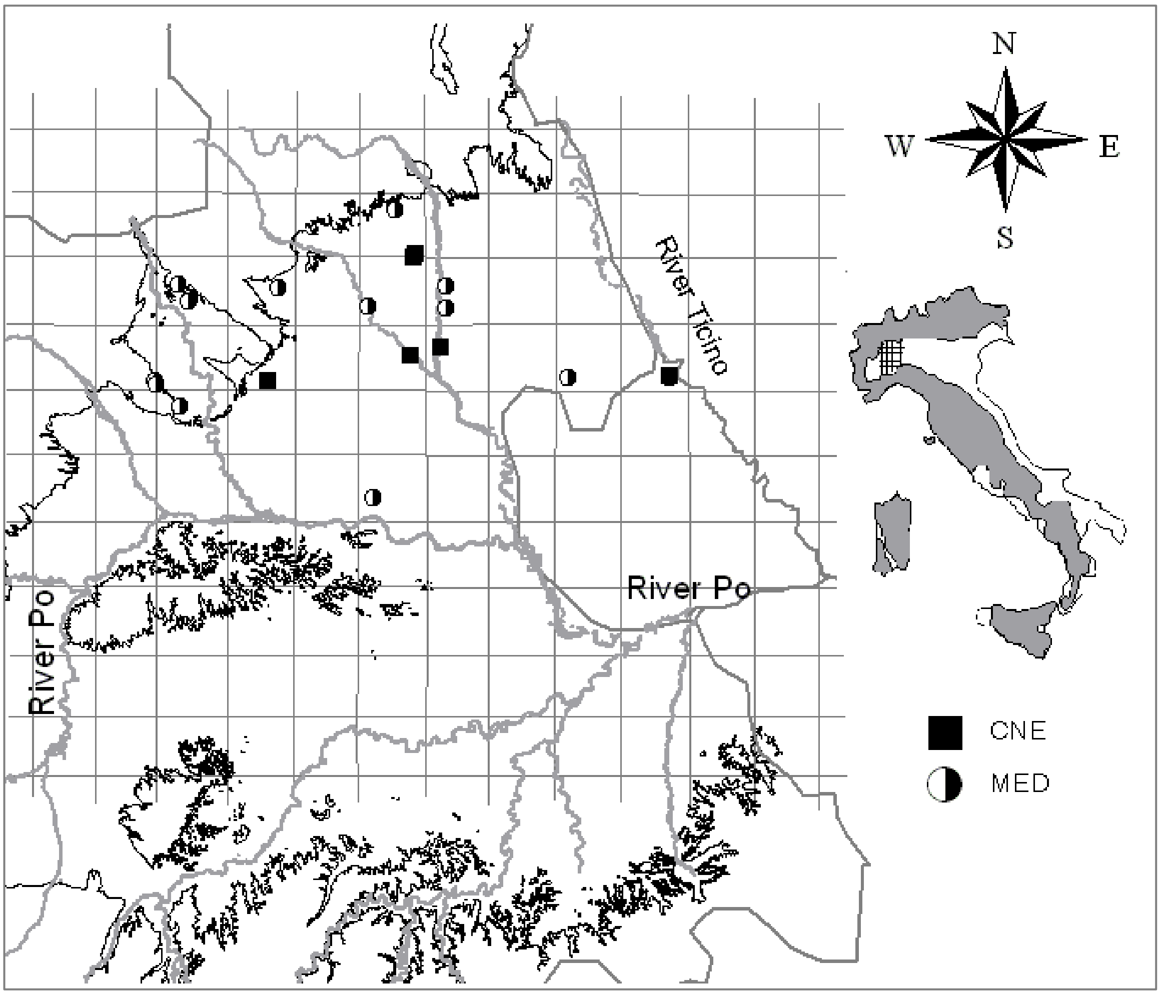

Figure 2. Distribution of pine marten samples belonging to the Mediterranean- (MED) and Central-Northern European (CNE) phylogroups; the 300 m a.s.l. contour line, which marks the limit of the River Po plain, is shown; the map of Italy shows the study area and the pine marten distribution.

4. Modelling the colonisation of human-dominated landscapes: patterns for the pine marten in intensively cultivated lowlands

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Modelling the colonisation of human-dominated landscapes: patterns for the pine marten in intensively cultivated lowlands

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Abstract

In recent years, the “forest-specialist” pine marten *Martes martes* has been reported to occur also in largely fragmented, lowland landscapes of north-western Italy. The colonization of such apparently unsuitable area provided the opportunity for investigating the environmental factors that drive pine marten expansion and predicting its potential south- and eastwards limits.

To reach these aims, we collected available occurrence data (faecal DNA samples, camera-trap records and road-kills) of the pine marten in the flood plain of the River Po (< 300 m above sea level) and relate them to 11 environmental variables by developing nine different Species Distribution Models (SDMs). To account for inter-model variability we used average ensemble predictions (EP), which are particularly useful in modelling expanding species.

A total of 184 pine marten locations were collected from January 2000 to September 2015. EP predicted a total of 482 suitable patches (4366 km^2; 8.31% of the total study area) for the pine marten, the most important variables related to the species’ occurrence being the distance from watercourses and distance from woods.

Our ensemble projection suggested that the pine marten may further expand in the western part of the River Po plain, whilst the negligible residual wood cover of large areas of the central and eastern plain makes them unsuitable marten habitat, except for some riparian corridors and the pine wood patches bordering the Adriatic coast.

The combination of recently improved modelling techniques and non-invasive survey methods, e.g. camera-trapping and fecal DNA based genotyping, represents a powerful tool to investigate spatiotemporal variation in the distribution of elusive species.

Key words: *Martes martes*, Species Distribution Models, ensemble predictions, genetic data, range expansion

Introduction

As already noted by MacArthur in the early 1970s, ranges are dynamic and their boundaries can fluctuate greatly as a consequence of both dispersal and temporal variation in environmental conditions (MacArthur 1972). Range fluctuations generally occur over historical time periods, but have recently being recorded to occur over shorter time scales due to introductions and environmental change (Kinlan and Hastings 2005; Chen et al. 2011).

Climate warming and human-driven habitat modifications are considered the main determinant of range variation, but non-climatic factors, including ecological interspecific interactions (Warren et al. 2001; Forero-Medina et al. 2011) and evolutionary dynamics (Holt 1996; Kirkpatrick and Barton 1997; Thomas et al. 2001) can also drive the magnitude, direction and pattern of range shift. While recent studies on species' distribution have mostly focused on the effects of anthropogenic climate change, demonstrating trends at Earth-scale, information on environmental determinants of range variation at the fine scale of a species' range is still scanty (Parmesan et al. 2005).

Resulting in large-scale, unintentional field manipulations, invasions can be used to test the effects of environmental factors on range limits of both alien and autochthonous species (Moyle and Light 1996; De Rivera et al. 2011; Crickenberger 2015). For invading species, the rate of range expansion has been shown to depend on both their life history and the properties of the landscape invaded (White et al. 2012).

Less attention has been devoted to range fluctuations in autochthonous species, although the (re-) colonization of previously unoccupied areas may provide an invaluable opportunity for investigating the environmental factors that shape species' ranges (*e.g.* Carone et al. 2014).

In recent years, the "forest-specialist" pine marten *Martes martes* has been reported to occur also in largely fragmented landscapes of the western part of its European range (Virgós et al. 2012). In north-western Italy, genetic surveys showed that the pine marten is currently widespread in intensively cultivated areas north of the River Po. In this area, woods mainly consist of small residual patches merged in intensively cultivated farmland, or cover the banks of main rivers (Balestrieri et al. subm.).

Whilst agro-forest systems have been reported to sustain high food availability levels for carnivores (Verdade et al. 2011), in agricultural landscapes dominated by arable land the loss and fragmentation of natural vegetation have been related to a general decline in prey biomass (Remonti et al. 2011), and the trend of land-use change in the Po plain indicates that soil depletion due to urbanisation is still increasing (Gherardi et al. 2009).

Pine marten expansion from the Alps, where pine marten populations may have benefit by the increase in forest cover occurred in the last decades (Balestrieri et al. subm.), to such apparently unsuitable area has occurred exponentially (Balestrieri et al. 2010), as is typical for species that disperse over large distances relative to home range size (Shigesada et al. 1995). Besides dispersal abilities, the rate and direction of species' expansion are influenced by a wide range of factors, including landscape connectivity, the distribution and abundance of suitable habitats and variation in climate and resource availability (Darimont et al. 2005; Veech et al. 2011; Morgia et al. 2011).

Species Distribution Models (SDMs; Guisan and Thuiller 2005) prove very useful to understand how landscape influences the spread of colonizing species, predict habitat suitability in neighbouring geographical areas which may be occupied in the near future (*i.e.* the potential geographic distribution) and, ultimately, assess species' ecological niches (Phillips et al. 2006; Warren and Seifert 2011).

In the last two decades, the development of several modelling techniques has promoted the use of SDMs in several branches of life sciences (reviewed by Thuiller et al. 2009). As different techniques can yield partially discordant results (Elith et al. 2006; Olaya-Marín et al. 2013), model evaluation is needed to assess the accuracy of predictions across modelling techniques and either select the model which "best" fits the data or derive ensemble predictions to avoid single model uncertainty (Araújo and New 2007).

Our aims were (i) to identify the main factors driving the colonisation by the pine marten of the western River Po plain and (ii) to predict its potential south- and eastwards expansion.

To reach these aims, we collected available occurrence data of the pine marten in the study area and related them to a set of environmental variables by developing nine different SDMs. To account for inter-model variability we used average ensemble predictions. Ensemble forecasting is particularly useful in modelling expanding species, which may not yet have spread to all suitable habitats, making species-environment relationships difficult to assess (Stohlgren et al. 2010).

We hypothesised that the probability of pine marten occurrence would depend on both the distribution of wood patches, as the main limiting factor, and distance from major rivers, which may play the role of natural corridors of expansion (Balestrieri et al. 2015).

Methods

Study area

The Po-Venetian alluvial plain (< 300 m above sea level) is the largest in Italy (*ca*. 46,000 km^2). The pedogenetic and micro-morphological characteristics of the soils of the lower plain, crossed by the River Po (652 km), support high levels of agricultural productivity and are intensively managed for cattle husbandry and modern-industry-based agriculture. Since the second half of the 19th century, widespread urbanisation and industrialisation have led to a progressive depletion of soil resources; built-up areas have progressively increased since the second half of the 20th century, indicating the parallel increase of human presence (Falcucci et al. 2007), and currently cover ca. 9% of the area (Gherardi et al. 2009). About 70% of residual forests (*ca.* 2,400 km^2) are in the western and central plain (Camerano et al. 2010), and either consist of small fragments (mean patch size = 4.5 ha; Lassini et al. 2007) scattered within the agricultural matrix or, as in most European lowlands (Coles et al. 1989), cover the banks of major rivers. Climate is sub-continental temperate, with mean yearly temperature of 12.0°C and mean yearly rainfall of 1000 mm.

Data collection

We collected occurrence data for the pine marten recorded between 2000 and 2015 in the Po plain, i.e. available data for north-western Italy were considered below the 300 m a.s.l. contour line, which broadly marks the upper limit of the plain (Balestrieri et al. 2010). Two main sources of data were used to assess pine marten distribution: 1) faecal DNA-based records collected between 2007 and 2015 across the whole study area (Balestrieri et al. 2015; Balestrieri et al. subm), and 2) unequivocal records from road-killed and camera-trapped individuals (Savoldelli and Sindaco 2008; Sindaco and Carpegna 2010; unpubl. data). All species locations were georeferenced in the UTM WGS84 32N coordinate system using ARCGIS 10.1 (ESRI, Redlands, California, www.esri.com/software/ arcgis).

Through Gaussian kernel density estimation based on all sampling locations we accounted for spatially biased sampling effort (Elith et al. 2010; Fourcade et al. 2014; Stolar and Nielsen 2014). Specifically, we used the resulting kernel density probability as a sampling effort map to weight bias-adjusted model estimates (Stolar and Nielsen 2014; Milanesi et al. 2015). Thus, 10,000 random points within the resulting 95% kernel density surface were generated to serve as background data.

Moreover, according to Calenge et al. (2005), we estimated the minimum convex polygon (MCP) around all species' locations to define the area available for the pine marten and to avoid any potential source of bias in the analysis (Calenge et al. 2008).

Predictor Variables

We collected data on environmental and anthropogenic features in the whole study area (Table 1). Land cover features, as well as the distance from watercourses and wood patches were derived by the Coordination of Information on the Environment (CORINE Land Cover 2006; http://www.eea.europa.eu/data-and-maps/data/clc-2006-vector-data-version-3), the European land cover database. Habitat diversity was expressed by Shannon' Index. The presence and distance from human settlements (i.e. urban areas, villages; CORINE Land Cover 2006), from roads and railways (derived from OpenStreetMap; www.openstreetmap.org) and human population density (GEOSTAT 2011 dataset - Eurostat - European Commission; ec.europa.eu/eurostat/web/gisco/ geodata/reference-data/ population-distribution-demography) were considered as a proxy for human disturbance.

All the predictor variables were re-sampled to a common resolution of 1 x 1 km cell size and the variance inflation factor (VIF) for all predictor variables was estimated in order to avoid multi-collinearity among them VIF values > 3 denoted highly correlated predictors (Zuur et al. 2010; Table 1).

Modelling methods

We tested spatial autocorrelation among all pine marten locations collected with Moran's *I* correlogram (Dormann et al. 2007) and then, with the resulting non-autocorrelated locations we developed nine SDMs: (1) artificial neural networks (ANN; Ripley 2007), a non-linear regression model based on hidden variables (estimated by the predictors), (2) boosted regression trees (BRT; Friedman 2001), a regression model that combines boost methods and regression trees, (3) classification tree analyses (CTA; Breiman et al. 1984), a recursive partitioning analysis which develop decision trees by applying splitting rules and partitioning data to reduce variation in the response variable, (4) flexible discriminant analyses (FDA; Hastie et al. 1994), a mixture model-based discriminant analysis, (5) generalized additive models (GAM; Hastie and Tibshirani 1990), a regression model involving smoothing parameters derived by predictor variables to estimate parametric components of linear predictors, (6) generalised linear models (GLM; McCullagh and Nelder 1989), logistic regression models that relate presence locations and pseudo-absences to the predictors, (7) maximum entropy (MAXENT; Phillips et al. 2006), a model which calculates a range of functions to identify the best approximation between the density distributions of predictors at species' occurrences and those in the rest of the study area, (8) multivariate adaptive regression splines (MARS; Friedman 1991), a non-linear regression which carries out non-linear interactions between variables, (9) random forest (RF; Breiman 2001), an ensemble classifier involving many decision trees which constitute "the forest". To avoid single model uncertainty, we calculated the ensemble prediction (EP) derived by the average predictions of the nine SDMs. We used the values of the sampling effort map as bias grid in MAXENT and as case weights in all the other methods (Elith et al. 2010; Stolar and Nielsen 2014; Milanesi et al. 2015). To estimate variable importance, we used 10,000 permutations (values close to 0 assume no influence of that variable on the model; Thuiller et al. 2009). We computed these analyses with the package BIOMOD2 (Thuiller et al. 2013) in the open-source software R (v. 3.1.2; http://www.R-project.org/). Lastly, spatial autocorrelation among the residuals of the models was verified by Moran's *I* correlogram (1 – predicted SDMs values for each location; De Marco et al. 2008).

Model validations and comparisons

To assess each model's efficiency, we compared the predicted values with the originals ones through (i) the Area Under the ROC (*Receiver Operator Characteristics*) Curve (AUC; Fawcett 2004; Ko et al. 2011), (ii) True Skill Statistics (TSS; Allouche et al. 2006) and (iii) Boyce's Index (BI; Boyce et al. 2002). AUC varies from 0 (worse than a random model with the value 0.5) to 1 (perfect model), while TSS and BI varies from −1 to 1 (positive values indicate predictions consistent with the evaluation data set, 0 indicates that the model is similar to a random model). To classify the accuracy of validations, we followed Swets (1988): 0.90-1.00 = excellent; 0.80-0.90 = good; 0.70-0.80 = fair; 0.60-0.70 = poor; 0.50-0.60 = fail. We carried out ten *k-fold cross-validations* using alternatively a random sub-sample of 50% of locations to calibrate the models and the remaining 50% to validate them (Boyce et al. 2002). *Cross-validations* were carried out in R (v. 3.1.2; http://www.R-project.org/) through the packages BIOMOD2 (Thuiller et al. 2013) and ECOSPAT (Broenniman et al. 2014). Moreover, to assess whether the nine models used provided consistent predictions in terms of variable ranking, we performed a simple linear correlation (Spearman's test), using pair-wise comparisons for all models (Williams et al. 2009).

Results

From January 2000 to September 2015, a total of 184 pine marten locations were collected in the Po plain. We obtained 116 genotyped-scat locations, 13 camera-trap videoclips, and 55 records from either road-kills or direct observations by expert zoologists. We removed 81 autocorrelated locations (within a distance of 1900 m) and thus we developed SDMs with a total of 103 pine marten locations. We did not find multicollinearity among the 11 predictor variables (Table 1) and thus we used all predictors for the further analyses. Moreover, autocorrelation among the residuals of the nine SDMs, as well as those of the EP, were not significant and thus we considered all of them as accurate.

K-fold cross-validations showed significant values for all the evaluation methods of all distribution models (Table 2), with values ranging between 0.904 and 0.998 for AUC, 0.801 and 0.989 for TSS and from 0.802 to 0.981 for BI (Table 2). Specifically, considering both AUC and TSS statistics, RF showed the highest predictive power while MARS the lowest; considering BI, EP showed the highest predictive accuracy, while ANN the lowest (Table 2).

EP predicted a total of 482 suitable patches for the pine marten, occupying a total of 4366 km^2 (8.31% of the total study area), the most important variables related to the species' occurrence being the distance from watercourses (38.1% contribution), distance from woods (37.8% contribution) and, to a smaller extent, habitat diversity (11.2% contribution) and distance from roads (10.7% contribution; Table 3). Actually, the probability of occurrence of the pine marten decreased as the distance from both water bodies and woods rose, while it increased with habitat diversity (Fig. S.1). Distance to roads showed an unimodal relationship peaking approximately at 2500 m (Fig. S.1). ANN was the only model which did not rank either distance from water bodies or distance from woods as the most important variables (Table 3). All models' ranks were significantly correlated with the consensus rank (P = 0.035 - 0.0008), except for those provided by MARS ($\rho = -0.11$, P = 0.75) and FDA ($\rho = -0.06$, P = 0.87).

Discussion

Our ensemble projection suggests that the potential for pine marten expansion is high only for the western part of the River Po plain. Currently, except for a few, recent road-kill records, on the right-side plain of the river the stone marten is the only or dominant marten species (Balestrieri et al. subm.). However, the relatively large availability of suitable areas for pine marten expansion allows to predict that in the near future the pine marten may colonize the whole western Po plain. In such a case, interspecific competition may result in stone marten decline, as happened for the left-side river plain. In contrast, the negligible residual wood cover of large areas of the central and eastern plain makes them unsuitable marten habitat, although riparian corridors may allow pine marten descent from the Alps and Apennines. The potential for pine marten colonization of the pine wood patches (*Pinus pinaster* and *P. pinea*) of the Adriatic coast is supported by the recent southward expansion of the red squirrel (*Sciurus vulgaris*), which has probably followed the major Alpine rivers (Bon et al. 2012), and penetration of the Venetian plain by the golden jackal (*Canis aureus moreoticus*), along the valley of the River Piave (Lapini et al. 2009). Nonetheless, there is no recent pine marten record for the eastern plain, the record of an individual found dead in the Po Delta in 2009 being currently considered unreliable (Mezzavilla F. com. pers.).

Our approach led us to identify the main environmental and anthropogenic factors affecting the colonization of the River Po plain by the pine marten, outlining the ecological requirements of pine martens in cultivated lowlands.

The main role played by the distance from waterbodies clearly reflects the importance of riparian corridors for marten expansion in a landscape largely dominated by crops and urban areas. Riparian zones have been reported to assist the range expansion of several mammals (Bennet 2003) and support a more diverse fauna than the surrounding landscape matrix (Hilty and Merenlender 2004).

Moreover understory vegetation of riparian forests has been associated with both prey- (Doyle 1990) and resting site availability (Baldwin and Bender 2008) to American martens (*Martes americana*). Accordingly, in a previous study at a finer scale, Balestrieri et al. (2015), demonstrated by faecal DNA analysis that pine martens are currently widespread in the valley of the River Ticino, a left-hand major tributary of the River Po, where they have displaced the stone marten.

Woods are key habitat features for the pine marten, which, accordingly, has been long considered a forest-specialist (Buskirk 1992; Zalewski and Jedrzejewski 2006). The pine marten is associated with mountainous forests and medium-extent agroforestry mosaics, while open and urban areas are generally avoided (Vergara et al. 2015; Wereszczuk and Zalewski 2015).

Also in agricultural and rural areas the abundance of pine martens has been shown to depend on the structure and degree of fragmentation of residual woods (Balestrieri et al. 2010; Vergara et al. 2015), where resting sites are almost exclusively established (Larroque et al. 2015a). Moreover in fragmented woods, martens often move along wood margins, hedgerows and corridors and tend to remain close to canopy cover (Perebom et al. 2008; Cushman et al. 2011).

Accordingly, open areas and highways have been reported to be the major obstacle to gene flow in lowlands (Larroque et al. 2015b).

In temperate regions, heterogeneous landscapes promote high biodiversity (Verdade et al. 2011), and offer larger small mammal availability to predators than continuous forests (Tattersall et al. 2002; Sidorovich et al. 2005). Wood patch borders associate cover to high prey density; therefore, below a threshold level, forest fragmentation can enhance food availability to martens and also the predator's density (Caryl et al. 2012). As an example, introduced Eastern cottontails (*Sylvilagus floridanus*) often select wood–field margins as resting sites (Althoff et al. 1997), and have been reported to be a major food resource for pine martens in the study area (Balestrieri et al. 2011).

The pattern of variation in the probability of pine marten occurrence with the distance from roads is consistent with previous results about wolves (Uboni et al. 2015), and may derive from individual differences in marten response to roads (Asceñsao et al. 2014). In turn, this response may depend on both familiarity of the landscape, traffic intensity and road passage availability.

Although model accuracy should always be tested with independent data (Araújo et al. 2005a), being pine marten expansion a recent phenomenon occurring only in the western of the River Po plain (Balestrieri et al. 2010), a statistically independent data set for validation of our ensemble prediction was not available. We are confident that our cross-validation procedure allowed a final evaluation quasi-independent of a particular realisation of random split (Thuiller et al. 2009; Falcucci et al. 2013).

Moreover, as cross-validation implies that variability in model accuracy is interpreted as a measure of the sensitivity of model results to the initial conditions rather than a measure of predictive accuracy (Araújo and Guisan 2006), model averaging may improve the accuracy of projected potential distributions (Araújo et al. 2005b).

Although our aim was not to compare the performances of individual models, based on variable ranking and statistical evaluation ANN, FDA and MARS produced forecasts very dissimilar from the ensemble prediction, while RF performed the best. In multi-model comparisons, ANN often modelled species–environment relationships less accurately than other methods (e.g. Aertsen et al. 2010; Kampichler et al. 2010), while RF gave the best results (e.g. Kampichler et al. 2010; Olaya-Marín et al. 2013). Variation in modelling success between techniques is a common output, which further emphasizes the benefits of combining several methods (Marmion et al. 2009; Stohlgren et al. 2010; Li and Wang 2012).

In the last decade, the pine marten has colonized cultivated lowlands, showing to be by far more flexible in its habitat requirements than previously believed (Virgós et al. 2012). Phenotypically plastic and/or micro-evolutionary processes have allowed martens and meso-carnivores in general to adapt to- and persist in changing environments better than large predators (Gittleman 2001; Laliberte and Ripple 2004). The same processes may also drive range expansion in previously unexploited ecosystems, as occurred for pine martens. Our study showed that recently improved distribution modelling techniques, in combination with non-invasive census methods, such as camera-trapping and feacal DNA-based genotyping, represent a powerful tool to investigate spatiotemporal variation in the distribution of elusive species.

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Figure 1. Map of the study area (i.e. the Po-Venetian alluvial plain, < 300 m above sea level) corresponding to the potential expansion range of the pine marten in northern Italy. Pine marten locations are denoted by black triangles (N = 103). Black lines indicate regional borders. The distribution range of the pine marten in Italy is shown in the upper-right corner.

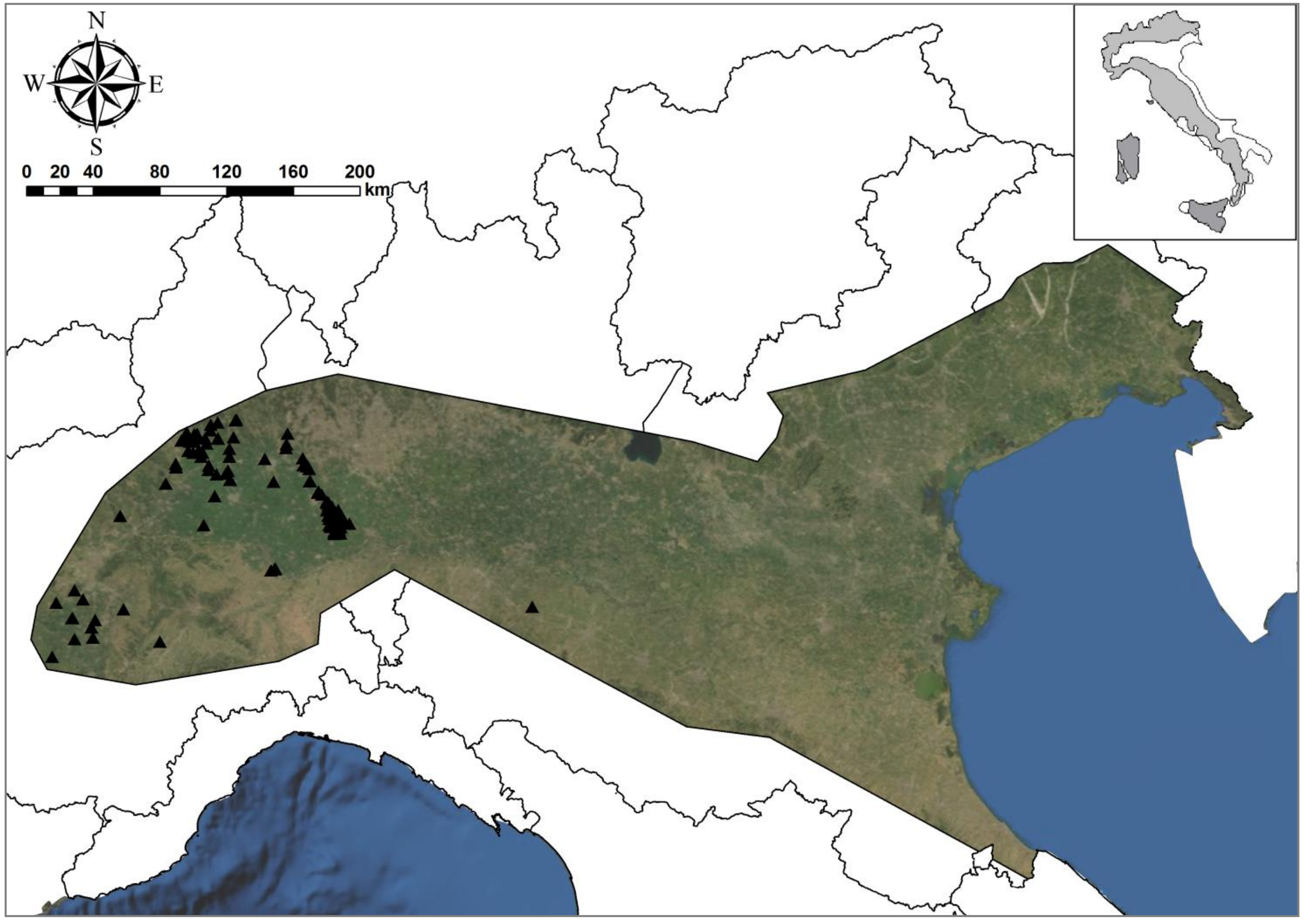


Figure 2. Habitat suitability map of the pine marten obtained by ensemble Species Distribution Models (green-red scale indicates lower-higher species occurrence probability).

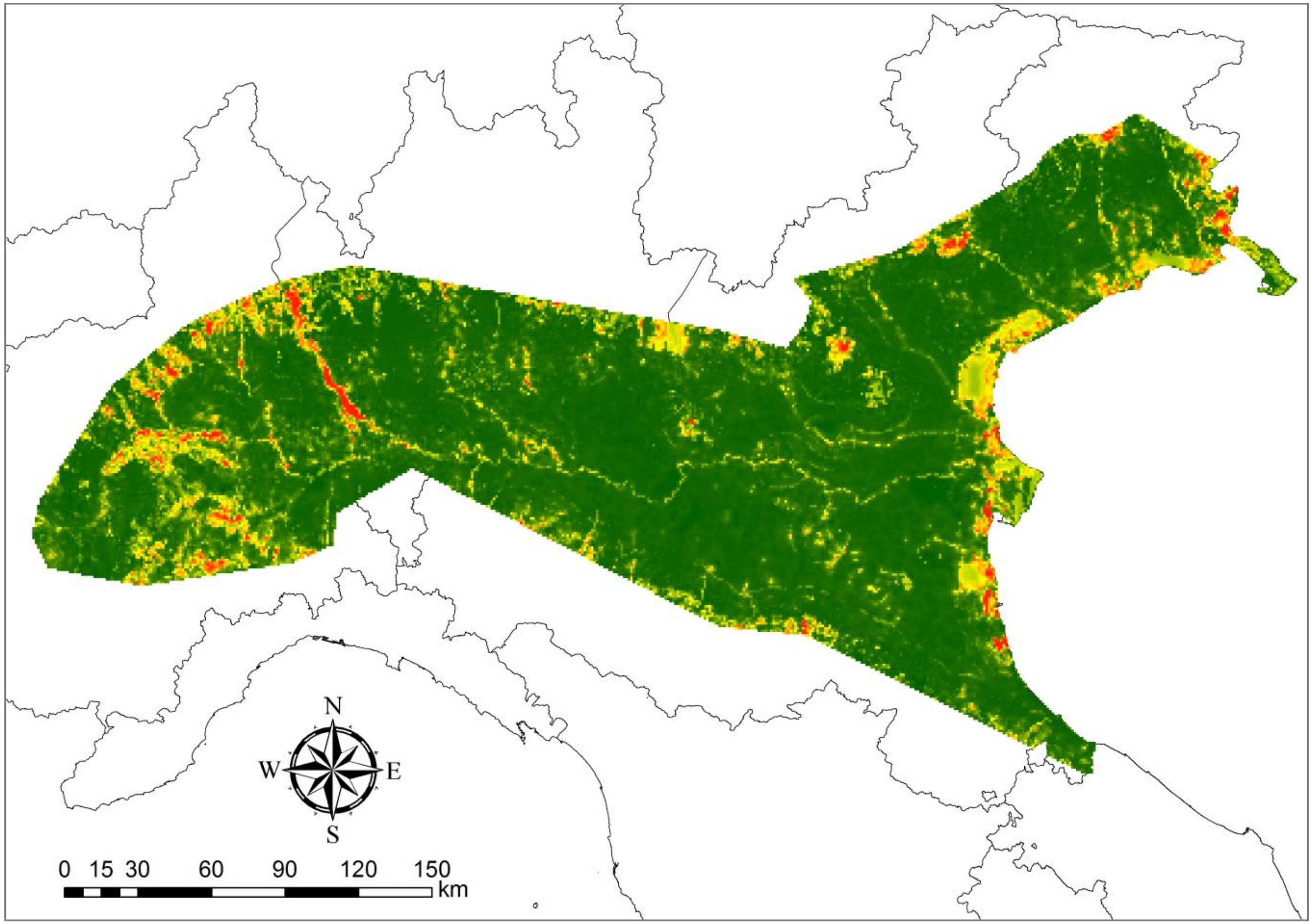


Table 1. Variables used in the development of species distribution models for the pine marten in the whole study area and in the used cells, average ± standard deviations values and variance inflation factor (VIF) values are shown.

Variables	**Unit**	**Study area**	**Used cell**	**VIF**
Cultivated fields	%	70.35 ± 36.37	34.59 ± 33.48	2.540
Grasslands	%	0.81 ± 6.18	2.21 ± 8.74	1.086
Poplar plantations	%	2.47 ± 15.47	2.39 ± 13.45	1.065
Woodlands	%	4.94 ± 16.74	40.76 ± 33.62	1.688
Habitat Diversity	$H' = -\Sigma(p_i \times \ln p_i)$	1.06 ± 0.43	1.48 ± 0.45	1.892
Distance to watercourses	m	5755.01 ± 4583.27	2189.07 ± 2528.82	1.068
Distance to woods	m	8645.01 ± 7511.79	1493.71 ± 2871.78	1.316
Human settlements	%	10.76 ± 21.72	2.17 ± 8.62	2.913
Distance to roads	m	926.11 ± 1242.26	1148.04 ± 977.08	1.284
Distance to human settlements	m	1287.11 ± 1149.11	1980.43 ± 954.02	1.622
Human population density	Number / km^2	388.81 ± 1107.11	41.31 ± 116.61	1.922

Table 2. Model evaluation of the nine species distribution methods (see the methods section for abbreviations) and their ensemble prediction (EP). Area Under the Curve (AUC) ranges between 0 and 1 (worse than a random model and best discriminating model, respectively). True Skill Statistic (TSS) and Boyce's Index (BI) ranges between −1 and 1 (higher values indicate good predictive accuracy while 0 indicates random prediction). Average values ± standard deviations are shown (*: $P < 0.001$).

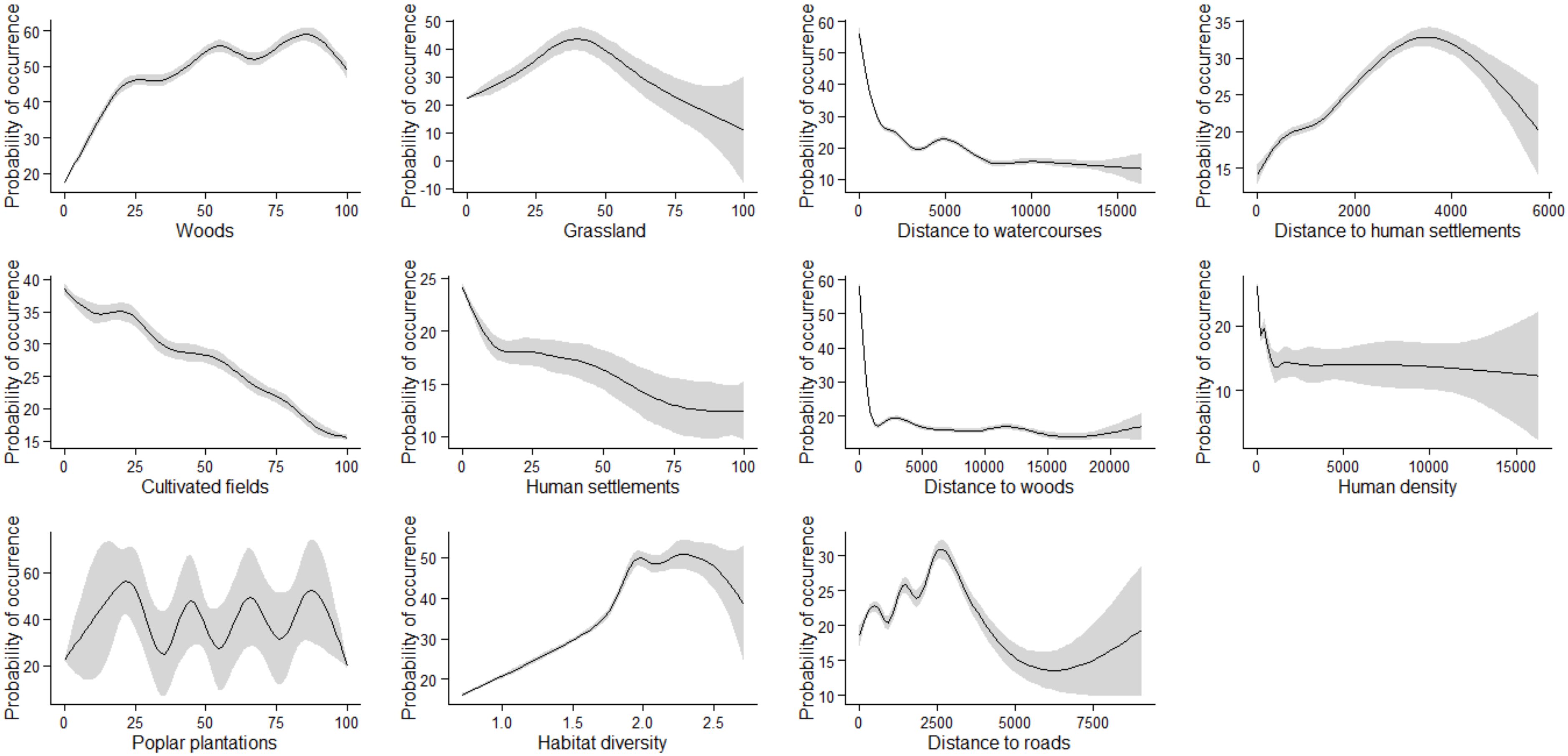
Model	**AUC**	**TSS**	**BI**
ANN	0.917 ± 0.006*	0.807 ± 0.071*	0.802 ± 0.032*
BRT	0.972 ± 0.027*	0.891 ± 0.043*	0.873 ± 0.041*
CTA	0.915 ± 0.023*	0.849 ± 0.057*	0.811 ± 0.022*
FDA	0.905 ± 0.025*	0.803 ± 0.002*	0.918 ± 0.014*
GAM	0.947 ± 0.021*	0.864 ± 0.077*	0.873 ± 0.041*
GLM	0.911 ± 0.086*	0.805 ± 0.011*	0.982 ± 0.017*
MARS	0.904 ± 0.066*	0.801 ± 0.021*	0.909 ± 0.057*
MAXENT	0.942 ± 0.056*	0.865 ± 0.082*	0.964 ± 0.035*
RF	0.998 ± 0.002*	0.989 ± 0.011*	0.804 ± 0.088*
EP	0.951 ± 0.048*	0.902 ± 0.022*	0.981 ± 0.019*

Table 3. Variable importance (%) ranking by the nine distribution methods (see the methods section for abbreviations) with respect to the ensemble prediction (EP).

Variables	**Model**									
	EP	**RF**	**BRT**	**GAM**	**CTA**	**FDA**	**MARS**	**MAXENT**	**GLM**	**ANN**
Grasslands	0.007	0.001	0.000	0.049	0.000	0.020	0.038	0.046	0.000	0.000
Poplar plantations	0.012	0.000	0.006	0.050	0.000	0.023	0.060	0.047	0.018	0.008
Human settlements	0.014	0.001	0.000	0.117	0.000	0.000	0.000	0.078	0.029	0.000
Woodlands	0.021	0.028	0.004	0.016	0.000	**0.535**	**0.940**	**0.449**	0.044	0.025
Cultivated fields	0.022	0.139	0.003	0.025	0.000	0.000	0.295	0.298	0.005	0.063
Distance to human settlements	0.030	0.052	0.028	0.127	0.000	0.050	0.062	0.105	0.047	**0.365**
Human population density	0.085	0.098	0.167	0.099	0.100	0.000	0.093	0.171	0.104	0.162
Distance to roads	0.107	0.096	0.060	0.178	0.135	0.020	0.032	0.141	0.032	0.232
Habitat Diversity (Shannon)	0.112	0.073	0.069	0.167	0.179	0.000	0.000	0.123	**0.257**	**0.282**
Distance to woods	**0.378**	**0.214**	**0.440**	**0.409**	**0.600**	0.000	0.000	0.267	0.073	0.223
Distance to watercourses	**0.381**	**0.446**	**0.384**	**0.291**	**0.590**	**0.378**	**0.552**	**0.517**	**0.194**	0.178

Supplementary materials

Figure S.1. Response curves and 95% confidence intervals (in grey) of the probability of pine marten occurrence derived by the ensemble prediction of the nine species distribution models in relation to predictor variables values.



5. Pine marten density in lowland riparian woods: a test for the *Random Encounter Model* based on genetic data

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Pine marten density in lowland riparian woods: a test for the *Random Encounter Model* based on genetic data

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Abstract

In marginal habitats, populations should reach lower densities, as a consequence of both lower overall abundance and increasing home range size. To test if recently colonized riparian woods crossing intensively cultivated lowlands of NW Italy represent marginal habitats for the forest-specialist pine marten *Martes martes*, we assessed its population density by a recently developed, camera-trap-based non-invasive method, the Random Encounter Model (REM). As the central assumption of the REM is that animals move randomly with respect to cameras, we suspected that this method may be unsuitable for species with a strong tendency to use linear elements of the territory as usual paths and select habitats, such as woodland, which are likely to be under-represented in fragmented landscapes. To test for the efficacy of the REM, we also applied a faecal DNA-based genetic census to obtain an independent estimate of the minimum number of individuals occurring in the study area.

Camera-trapping used 10 camera-traps, deployed for 10 days within a 2 km^2 large unit, for a total of 6 units and 12 km^2. Pine marten density was estimated at 0.48 (0.36-0.60) ind/km^2.

All the faecal samples identified by a mDNA-based PCR-RFLP method as pine marten were genotyped at 15 microsatellite loci using a multiplex protocol. We identified 15 different individuals, corresponding to a density ranging between 0.8 and 2.0 in/km^2. Using the most conservative genetic estimate, the REM underestimated population density of about 40% proving to be unreliable for estimating pine marten population size. We suggest that this may be the case for elusive species for which the assessment of average daily movements cannot be achieved without the use of invasive methods.

Key words: *Martes martes*, camera-trapping, marginal habitats, microsatellite analysis, non-invasive census.

Introduction

Population distributions, range boundaries and their variation over time are regulated by several biotic and abiotic factors, including climate, interspecific interactions, food availability and anthropogenic effects (Case et al. 2005; Holt et al. 2005; Chen et al., 2011). Although the classical biogeographical hypothesis that species' abundance declines monotonically from the centre towards the edges of their range has been recently criticized as being too simplistic (Sagarin et al., 2006), several empirical studies have demonstrated that edge populations differ from those near the core of the range in several demographic characteristics. In marginal habitats, populations reach lower densities (Vucetich and Waite, 2003), as a consequence of both lower overall abundance (Gyllenberg and Hanski 1992) and increasing home range size (Dunning et al., 1992), tend to produce less offspring (Kawecki, 2004; Thingstad et al. 2006), are more prone to extinction for stochastic causes (Antonovics et al., 2006), and often show lower genetic diversity (Hoffman and Blouin 2004; Tsumura et al. 2007) compared to core populations.

The pine marten, *Martes martes*, is distributed throughout much of Europe and northern and central Asia, where it is generally associated with mature forest habitats (Proulx *et al*., 2004; Zalewski & Jędrzejewski, 2006). Recent studies, however, have suggested that the species is more catholic in terms of habitat preferences than previously reported (Virgós *et al*., 2012), being also able to live in rural landscapes that contain remnant forest patches (Pereboom *et al*., 2008; Balestrieri *et al*., 2010; Mergey *et al*., 2011; Caryl *et al*., 2012).

Despite the supposed lower suitability of fragmented habitats, Mergey et al. (2011), in France, did not find any clear effect of the degree of habitat fragmentation on either individual space-use patterns (temporal stability and size of home ranges; Mergey et al., 2011) or genetic variability of pine marten populations (Mergey et al., 2012). However, Ruiz-González et al. (2015) found that habitat loss and fragmentation, in synergy with past overharvesting and possible interspecific competition with sympatric stone marten (*Martes foina*), are likely to be the main factors responsible for recurrent bottlenecks and low gene flow in heterogeneous landscapes.

In northern Italy, the pine marten has traditionally been associated with deciduous and coniferous forests ranging in elevation between 1,000 and 2,000 m a.s.l. (e.g. Prigioni et al., 2001). Since the end of the 20th century, pine marten records in intensively cultivated areas of the western River Po plain (NW Italy) have grown exponentially, suggesting a progressive range expansion throughout the interior of the plain (Balestrieri *et al*., 2010).

Non-invasive genetic monitoring revealed that pine marten occurrence in several residual woodland patches was stable (Remonti et al., 2012) and that riparian woods may play the role of natural corridors favouring the southward expansion of the pine marten (Balestrieri et al., 2015).

Having been colonised only recently and deeply altered by human economic activities, the agricultural habitats of the River Po plain may be expected to represent marginal habitats for the pine marten, acting as dispersal sinks with high turnover of extinction-(re)colonisation events (Kawecki, 1995; Baguette, 2004). To test for this hypothesis, we aimed to assess pine marten density on a stretch of the River Ticino for which the first records of pine marten occurrence dated back to 2005.

Like most carnivorous mammals, pine martens are elusive and difficult to monitor (Ruiz-González et al. 2008, Rosellini et al. 2008). In the last two decades, camera-trapping has become a widely used, non-invasive method to assess through mark-recapture models the density of species with individually unique coat markings (e.g. Karanth and Nichols, 1998; Maffei et al., 2005). Recently, Rowcliffe et al. (2008) have proposed a method (*Random Encounter Model;* REM) that does not depend on individual recognition and can be potentially applied to a wide variety of species. The model has been developed and tested in southern England for four non-native species, free ranging in a 226 ha large enclosure mostly consisting of open grassland (Rowcliffe et al., 2008) and then has been applied to the pine marten in central Italy, yielding density values consistent with available data for the European marten range (Manzo et al., 2011).

The central assumption of the REM is that animals move randomly with respect to cameras (Rowcliffe et al., 2013) and thus this method may be unsuitable for species with a strong tendency to use habitats which are under-represented in fragmented landscapes. Pine martens strongly avoid open habitats (Storch et al. 1990; Brainerd and Rolstad 2002) and move close to tree-cover (Perebom et al., 2008; Del Fante, 2012), suggesting that the sampling protocol required by the REM may lead to underestimation of pine marten density.

To obtain an independent estimate of pine marten density and test the efficacy of the REM for the assessment of meso-carnivore density in fragmented habitats, in the same area and period monitored by camera-trapping, we applied a non-invasive faecal-DNA based genetic census (Ruiz-González et al., 2013). Genotyping needed two steps, species identification, which was necessary to exclude the samples belonging to other sympatric meso-carnivores from further analyses (Ruiz-González et al., 2008), and microsatellite genotyping, to ascertain with >99% certainty the minimum number of individuals occurring in the study area (Ruiz-González et al., 2013).

Study area

The Italian stretch of the River Ticino flows southwards from the southern edge of Lake Maggiore to the median course of the River Po, forming a valley110 km long and, on average, 7 km wide.

The valley is partly protected by two Regional Parks: the Park of the Ticino Valley (Lombardy), covering 906.4 km^2 and the Natural Park of the Ticino Valley (Piedmont), 62.5 km^2.

The river crosses an intensively cultivated and urbanized plain, where mesophilous - *Fraxino-carpinion* - and hygrophilous - *Alno-Ulmion*, *Alnion glutinoso-incanae*, *Salicion albae* - woods are still widespread inside the weave of meanders, streams, canals and oxbow lakes which characterise the downstream stretch of the river (Fig. 1).

Pine marten monitoring focused on the lower part of the valley, between the towns of Vigevano and Abbiategrasso (Milan, Lombardy) in the north and Gropello Cairoli village (Pavia, Lombardy) in the south. Along this *ca.* 35 km long stretch of the river, mean percent riparian vegetation cover, as assessed in a 100 m large belt on both river banks, is 47.8% (min-max: 12-86%; Prigioni and Balestrieri 2011). Long-term non-invasive monitoring showed that currently stone marten occurrence in this sector of valley is negligible (Balestrieri et al., 2010, 2015).

The climate is temperate, mean annual values being 13°C for air temperature and *ca*. 700 mm for rainfall.

Materials and methods

Estimation of population size by camera-trapping

The study area was monitored by digital scouting pocket cameras (Multipir 12 and SG550 with Passive Infra-Red motion sensor), provided with 8 GB SD cards and up to 8 AA batteries. Cameras were set to record 15 s long videoclips, with a 30 s interval between two successive recordings to potentially increase the independence of videoclips. Cameras were tied to trees 30-50 cm above the ground level. In accordance with Rowcliffe et al. (2008), cameras were neither baited nor set at sites thought to have high animal traffic (trails or obligated passing places).

There are tradeoffs in the setting of camera-traps because too close trap-spacing may reduce the number of individual home ranges surveyed whereas too wide trap-spacing may result in the missing of those individuals whose home ranges are included in the “holes” between camera-traps (Kays and Slauson, 2008). Since in Italy, pine marten home range has been reported to vary between 160 and 548 ha (mean: 370 ha, N = 6; Antonelli, 1996; Del Fante, 2011), surveys were carried out using a 1x1 km grid, superimposed on the kilometric grid of digitalized, 1:10000

Regional Technical Maps; for each session, 10 camera-traps were deployed for 10 days within a 2 km^2 large unit, for a total of 6 units, 12 km^2 (Fig. 1) and, potentially, 600 camera-days.

For each unit, the distance between contiguous cameras was calculated as the inverse of the square root of the ratio between the number of trapping sites (10) and the size of the unit (2 km^2). As spatial autocorrelation is irrelevant even when cameras are placed much closer to each other (Kays et al., 2010), the resulting distance (0.45 km) was borne in mind as a general rule, subordinate to the deployment of cameras proportionally to each habitat area (woods, cultivated fields, shrubs and pastures) and the availability of suitable trapping-sites (*i.e.* those giving each camera enough clear view with respect to their potential detection radius). Camera-trap sites were georeferenced and superimposed on digital maps.

To assess pine marten pre-reproductive density and meet the model’s closure assumption, the survey was carried out in two months, between 9 March and 8 May 2015.

According to Rowcliffe et al. (2008), density (D) was calculated as:

$$D = \frac{y}{t} \times \frac{\pi}{vr(2+\theta)}$$

where y is the number of pine marten videoclips, t the total number of trap-days, *v* the average daily distance moved by pine martens, *r* the detection radius of the cameras and θ their detection arc.

As we did not have radio-tracking data for the pine marten in our study area, *v* was assumed to be 12 km/day, a distance reported for both Poland (Zalewski et al., 2004) and central Italy (Del Fante, 2012) and also used by Manzo et al. (2011) to assess pine marten density in Tuscany. The detection radius was calculated as the mean (= 7.37 m; min-max: 5-12 m) of the actual distances recorded in the field. Within 12 m from the camera, according to the manufacturer’s directions for use average θ was 0.82 radians. Overall density was also calculated as the average (± Standard Error) of the density estimates in the six units.

Faecal DNA analysis: mtDNA species identification and individual identification from microsatellite genotyping

Non-invasive scat sampling was used to collect genetic samples from *Martes* sp. in the study area. For each unit, sampling for scats was conducted twice, at the same time as camera setting and removal. Transects were drawn along wood/field margins, paths and country roads to cover both open and forested habitats (mean length per unit ± SD = 5.47 ± 1.11 km). We prioritized fresh scat samples to increase genotyping success (Ruiz-Gonzalez et al. 2013). Universal

Transversal Mercator (UTM) coordinates were recorded for all the samples collected using a GPS.

A portion (ca. 1 cm) of each faeces suspected to belong to the pine marten based on size and morphology (see Remonti et al. 2012) was picked up using sticks, stored in autoclaved tubes and stored at – 20°C until processed.

DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions.

As pine marten faeces cannot be distinguished from those of the potentially sympatric stone marten and can also be easily confused with those of other carnivores (Davison et al. 2002; Ruiz-Gonzalez et al. 2008), a molecular technique was applied for the identification of faecal samples. Species identification was accomplished by a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, providing for an effective genetic identification of sympatric marten species following the method described in Ruiz-González et al. (2008).

Identification of individual pine martens used nuclear DNA following methods in Ruiz-González *et al.* (2013). All the faecal samples identified by the PCR-RFLP method as pine marten were genotyped at 15 variable microsatellite loci (Ma1, Ma2, Ma19, Gg7, (Davis & Strobeck 1998); Mel1, (Bijlsma *et al.* 2000); Mel10,(Domingo-Roura *et al.* 2003); MLUT27, (Cabria *et al.* 2007); Mer 41, Mvis072, (Fleming *et al.* 1999); Mvi57, (O'Connell *et al.* 1996); Lut615, (Dallas & Piertney 1998); MP0059, MP0188 (Jordan *et al.* 2007) using a multiplex protocol specifically designed for degraded faecal DNA analysis (Ruiz-Gonzalez *et al.* 2013) and following a modified multitube-approach (Taberlet *et al.* 1996).

The multitube-approach of 4 independent replicates, followed by stringent criteria to construct consensus genotypes (i.e. accepting heterozygotes if the two alleles were seen at least in two replicates and homozygotes if a single allele was seen at least in three replicates) is a commonly used approach in non-invasive genetic studies leading to a low probability of retaining false homozygotes or false allele errors (e.g. Frantz *et al.* 2003; Stenglein *et al.* 2010; Brzeski *et al.* 2013). Briefly, DNA quality was initially screened by PCR-amplifying each DNA sample four times at four loci (Multiplex 1: MP0188; MP0059; Gg-7; Ma-1), since the results obtained for this four loci are indicative of the genotyping success for the full panel of 15 microsatellites (Ruiz-Gonzalez *et al.* 2013).

Only samples showing > 50% positive PCRs were further amplified four times at the remaining 11 loci. Samples with ambiguous results after four amplifications per locus or with <50% successful amplifications across loci were removed from further analysis as they were not considered reliable genotypes. Multiplex PCR products were run on an ABI (Foster City, CA) 3130XL automated sequencer (Applied Biosystems), with the internal size standard GS500 LIZ™ (Applied Biosystems). Fragment analyses were conducted using the ABI software Genemapper 4.0.

RELIOTYPE software (Miller et al. 2002) was used to assess the reliability of the genotypes obtained by 4 independent replicates. Samples that were not reliably typed at all loci after 4 replicates (at score threshold R = 0.95) were discarded from the analysis. To test the discrimination power of the 15 microsatellite set, we computed the probability of pairs of individuals bearing an identical multilocus genotype [i.e. probability of identity (PID)] by GIMLET. PID calculations were performed with both the unbiased equation for small sample size and that for siblings. The more conservative PID for full-sibs (PID-Sib) was estimated as an upper limit to the probability that pairs of individuals would share the same genotype.

Consensus genotypes from four replicates were reconstructed using GIMLET, which was also used to estimate genotyping errors: allelic dropout (ADO) and false alleles (FA) (Taberlet *et al.* 1996; Pompanon *et al.* 2005).

Estimation of population size and kinship from genetic data

Population size was assessed by capwire estimators (Miller et al, 2005), fitting data to two different models to obtain the maximum likelihood estimate (MLE) of each population size. Under the Equal Capture model all individuals were assumed to have an equal probability of being sampled, while under the Two-Innate Rates model the population was assumed to contain a mixture of easy-to-capture and difficult-to-capture individuals. The fit of the two models was compared using a Likelihood Ratio Test (LRT); the p-value was calculated by using a parametric bootstrap approach to estimate the distribution of the LRT for data simulated under the less-parameterized Equal Capture model (Pennell *et al.*, 2013). Confidence intervals (CI) for the population size were estimated using a parametric bootstrap approach (Miller et al., 2005).

As species with relatively large ranges may periodically use only some sub-areas of their whole ranges, resulting non-detection whenever the sampling method does not include them (MacKenzie, 2005), density was calculated by two different approaches: i) using all consensus genotypes and the whole wooded area on the left bank of the River Ticino (19.4 km^2; Fig. 1), assuming that pine marten move close to the tree canopy (Storch et al., 1990; Perebom et al., 2008), and ii) using only the genotypes found by the second sampling, i.e. obtained from faecal samples deposited while the cameras were active, and the area sampled by camera-trapping (12 km^2; Fig. 1).

Genetic relatedness and sibling analyses were calculated by the program ML-RELATE (Kalinowski et al., 2006) which uses a maximum likelihood method to compute pair-wise genetic relatedness (Rxy), representing the proportion of allelic composition shared between each pair of individuals. Sibship analysis was conducted using COLONY version 2.0.4 (Jones and Wang, 2010), with the typing error rate set at 0.01. This approach considers the likelihood of the entire pedigree, as opposed to relatedness on a pair-wise basis. To infer relatedness among individuals, we used all of the genotyped samples (15 individuals).

Results

Pine marten density as assessed by the REM

A total trapping effort of 494 camera trap-days allowed recording of pine martens 19 times (one video-clip per 26 trap-days) out of a total of 1722 video-clips. Other species detected were mostly red fox *Vulpes vulpes* (N = 72), European badger *Meles meles* (N = 57) and wild boar *Sus scrofa* (N = 297).

Pine marten presence was ascertained in all the sampling units, for a total of 12 sites (Fig. 1). Following Rowcliffe et al. (2008), pine marten density was estimated at 0.48 (0.36-0.60) ind/km^2.

Pine marten density and kinship as assessed by the genetic census

Sixty-one out of 63 faecal samples collected from the entire study area were classified as *M. martes*, while 2 rendered no amplicons. Thus, unequivocal species identification was possible in 96.8% of the samples.

The first quality-screening test, based on 4 replicates of four microsatellite loci, was not passed by 21 non-invasive samples (34.42%), which were then discarded. The remaining 40 samples (65.57%) were amplified at the other 11 loci. After multiple-tube genotyping, 16 samples from this subset were also discarded because they showed <50% PCR success, or because of high failure rates. Full multilocus microsatellite genotypes were obtained for the remaining 24 samples (60% of the samples that passed the screening and 39.4% of the total samples analysed), all showing reliability score >0.95 (Miller et al. 2002).

The average proportion of positive PCRs (calculated from correctly and fully genotyped samples) was 89% and varied among loci from 72% to 100%. The observed average error rates across loci were: ADO = 0.216 and FA = 0.019. The number of alleles per locus ranged from 1 to 6 (mean 3.37) (Tab. 1). Average, non-biased observed and expected heterozygosities (Ho and He) were moderated, 0.45 and 0.52 respectively (Tab. 1). PID analysis showed that the set of 15 loci would produce an identical genotype with a probability of 2.73×10^{-9}, and with a probability of 1.43×10^{-4} for a full-sib. According to these values, the 5 most informative loci could distinguish two full-siblings with a 99% probability (Mills et al., 2000), suggesting no ‘shadow effect’ (i.e. all the genotypes identified distinct individuals and matching genotypes were recaptures of the same individual).

After a regrouping procedure (i.e. pairwise comparison of the different genotypes obtained), we identified 15 pine marten individual genotypes out of the 24 samples with a complete multi-locus profile. The spatial distribution of the identified individuals is reported in Fig. 2. The average number of detections (re-samplings) per individual was 1.6, with a maximum of three detections per individual.

Using capwire estimators, population size was assessed as 22 (CI = 15-38) individuals, corresponding to a density of 1.13 (0.77-1.96) ind/km^2. Considering only the individuals sampled during camera-trapping survey, in the 6, 2 km^2 large units 9 individuals were identified, for an estimated population size of 24 (CI = 10-24) individuals and a density of 2.0 (CI = 0.84-2.0) ind/km^2.

At least three clearly distinct family groups were identified: individuals 4 and 10 were parents of individual 1; individual 5 was identified as parent of individuals 11 and 13, which were further identified as full-siblings; individuals 14 and 15 were identified as first order relatives (i.e. parent/offspring or full-sib dyad). According to COLONY all pine martens were related to each other (18 half-sib pairs), with the exception of individual 12.

Discussion

Pine marten density as assessed by the REM (0.48 ind/km^2) fell within the range of values recorded for other European countries (Switzerland: 0.1-0.8 ind/km^2, Marchesi, 1989; Poland: 0.36-0.76 ind/km^2, Zalewski and Jędrzejewksi, 2006; Central Italy: 0.34 ind/km^2, Manzo et al., 2011).

Microsatellite analysis allowed us to estimate a minimum population size that could be used as a benchmark for evaluating the efficiency of the first method, demonstrating that the number of individuals estimated by the REM (ca. 6) was 33-60% lower than expected.

Our non-invasive genetic sampling had a skewed capture rate distribution, with a few genotypes sampled up to three times and many genotypes sampled only once, suggesting that some individuals might be vagrants, with few opportunities to be camera-trapped. Moreover, when using only the samples genotyped during camera-trapping, recaptures were too few to allow a precise enough estimate of pine marten density. Therefore it is probable that true population size was within the lower range of capwire estimates. Such a conservative size would correspond to a REM's underestimation of population density of 37.7-42.8%.

The lower density provided by the REM may depend on two not mutually exclusive sources of bias: pine marten movement patterns and the estimate of *v*.

Carnivore feeding behavior can be described as a succession of feeding bouts and relatively straight and rapid relocation movements towards patches offering new feeding opportunities (Taylor et al., 1978; Getz and Saltz, 2008). Inter-patch movements follow well-defined paths often coinciding with linear features, which channel animals' movements (Stewart et al., 2002). Such a pattern would lower the probability of pine marten detection by camera-traps at sites not included in the foraging patches exploited during the trapping period, while setting cameras on paths, which often yield higher trap rates than those off paths (Towerton et al. 2011; Wang and Fisher, 2012), would violate REM's assumptions.

The selective use of the study area by the target species has been acknowledged as a major cause of bias by Rowcliffe et al. (2008). Pine marten average daily movement (*v*) was deduced from available literature. We agree with Rowcliffe et al. (2008) in considering that *v*, needing independent estimates, is a major constraint of this approach. Although available data about pine marten daily range are fairly consistent, in fragmented areas pine marten home ranges can be 2-3 times smaller than in forests (Mergey et al., 2011), suggesting that individuals may need to cover, on average, lower daily distances to satisfy their requirements.

Regrettably, for animals as elusive as the pine marten, the estimate of mobility and space use in the study area would need radiotracking data (i.e. the complementary use of an invasive and time-expensive method), an option that would nullify the pros of the REM in terms of sampling effort and ethical implications.

Faecal DNA-based genotyping is an effective non invasive method for the assessment of pine marten distribution and numbers (Ruiz-González et al., 2008, 2013; Mullins et al. 2010; O'Mahony et al., 2012; Sheehy et al. 2014), and, more generally, in the last two decades has been used to estimate population size of several elusive mammals (e.g. mountain lion *Puma* *concolor,* Ernest et al., 2000; Eurasian otter *Lutra lutra,* Prigioni et al., 2006; chimpanzee *Pan troglodytes schweinfurthii, McCarthy et al., 2015).*

Recently, faecal DNA has proven successful in correcting population size estimates of elusive animals obtained by other non-invasive methods, doubling the number of otters (*Lutra lutra*) as assessed by snow-tracking in Sweden (Arrendal et al., 2007) and that of giant pandas as estimated by transect lines (*Ailuropoda melanoleuca*) in Wanglang Nature Reserve (Western China; Zhan et al., 2006).

Finally, genetic analyses confirmed the absence of the stone marten and widespread occurrence of the pine marten recorded by previous studies (Balestrieri et al., 2015).

The record of three family groups demonstrated that the pine marten can successfully reproduce in the study area, confirming that pine marten population is not strictly depending on immigration to persist. Both high density and distribution of genotyped samples, with up to four different individuals sampled in the same 1 km^2 wide square, indicate that either cultivated areas may act as a barrier, hindering the dispersal of offsprings (Da Silva and Evans, 1994), or, as suggested by Balestrieri et al. (2015), the River Ticino is playing a major role as an expansion corridor from the Alps to the inner of the Po plain. Further sampling would be necessary to increase the number of samples per individual and reconstruct more detailed genealogical relationships among individuals.

In consistance with the findings of Mergey et al. (2011), we found no evidence that lowland habitats affected pine marten abundance, its density being among the highest ever reported in continental Europe and similar to that reported for fragmented landscapes of Ireland (0.5–3 ind/km^2, Lynch et al. 2006; Mullins et al. 2010, Sheehy et al. 2014). We believe that habitat saturation (sensu Roper 2010), i.e. constraints on dispersal imposed by the surrounding agricultural matrix, and dispersal dynamics, rather than food availability (Remonti et al., 2012), may account for such an unexpected high population density.

Reliable estimates of elusive species abundance are of utmost importance in wildlife management. The REM has proved to be unreliable for estimating pine marten population size, and we suggest that this may be the case for all elusive species for which the assessment of average daily movements cannot be achieved without the use of invasive methods. Moreover, wherever pine and stone marten are sympatric, camera-trapping does not allow their reliable identification, unless using lures, which, however, would artificially raise the encounter rate.

In turn, the main drawback of genetic analyses is their relatively high cost, which makes the effective census of large areas unlikely to be carried out. For landscape-scale sampling, an effective alternative may be the complementary use of the two methods, applying the more expensive genetic census in small, well delimited sampling areas to provide for an average correction factor.

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Table 1. Properties of the 15 microsatellite multiplexed loci used in this study and summary of the genetic variability assessed per locus. For each locus, the table includes: number of alleles (NA), observed (HO) and expected (HE) heterozygosities, rates of positive PCR (PCR+), allelic dropout (ADO) and false allele (FA)

MULTIPLEX	Locus	N_A	H_E	H_O	PCR+	ADO	FA
MULT_1	Gg-7	5	0.74	0.8	0.96	0.128	0.394
	Ma-1	3	0.55	0.6	0.9	0.37	0.133
	MP005	4	0.67	0.6	1	0.152	0.051
	MP018	2	0.36	0.47	0.96	0.028	0
MULT_2	Lut-453	1	0	0	0.99	1	0
	Mel-1	3	0.57	0.53	0.88	0.303	0.154
	Mel-10	3	0.54	0.6	0.89	0.35	0.095
MULT_3	Lut-435	4	0.62	0.2	0.74	0.083	0.017
	Ma-19	3	0.67	0.67	0.85	0.111	0.25
	Mvi-57	6	0.7	0.6	0.99	0.152	0.205
	Mvi072	4	0.67	0.33	0.72	0.5	0.167
MULT_4	Lut-615	3	0.54	0.4	0.86	0.083	0.2
	Ma-2	3	0.45	0.47	0.86	0.267	0.143
	Mer41	4	0.67	0.47	0.88	0.333	0.167
	Mlut-27	1	0	0	0.9	0	0
Mean		**3.27**	**0.52**	**0.45**	**0.89**	**0.257**	**0.131**

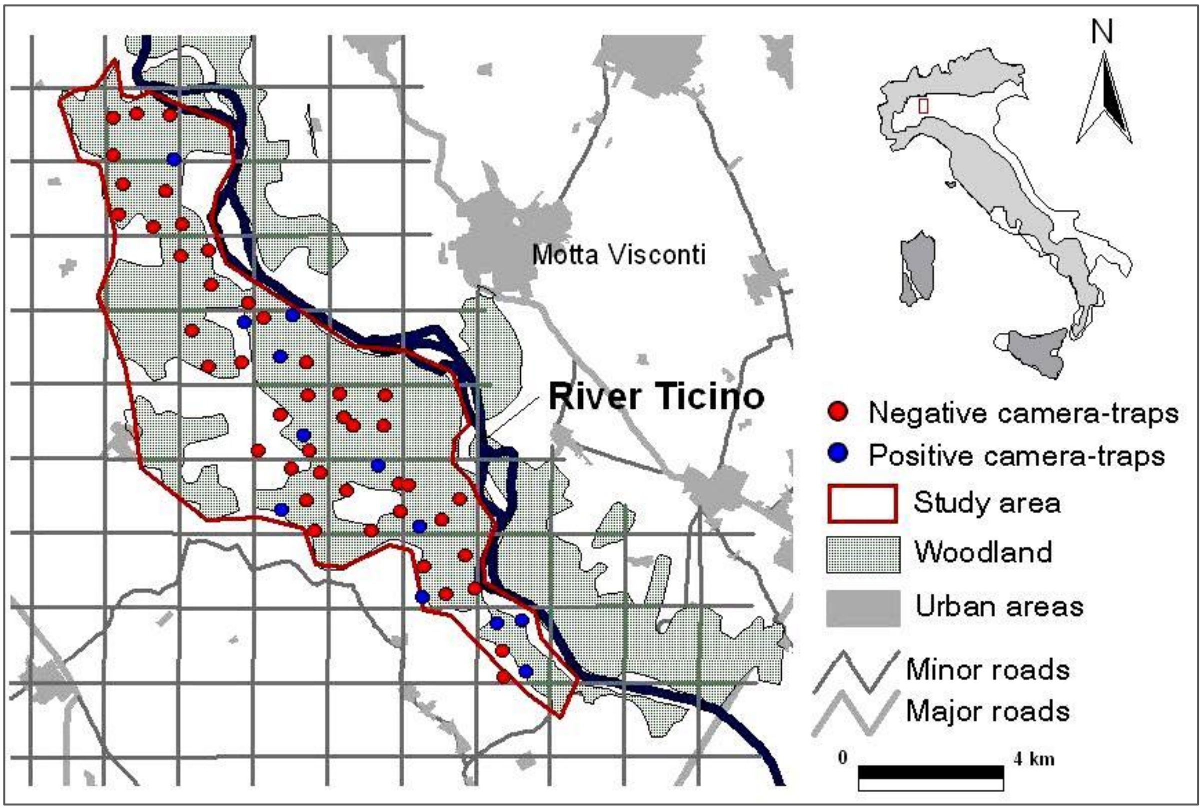

Figure 1. Positive and negative camera-traps for pine marten occurrence in the study area, which is marked in red. The 1x1 km grid sampled by both camera-trapping and scat surveys and the location of the sampling area with respect to the Italian pine marten range are also shown.

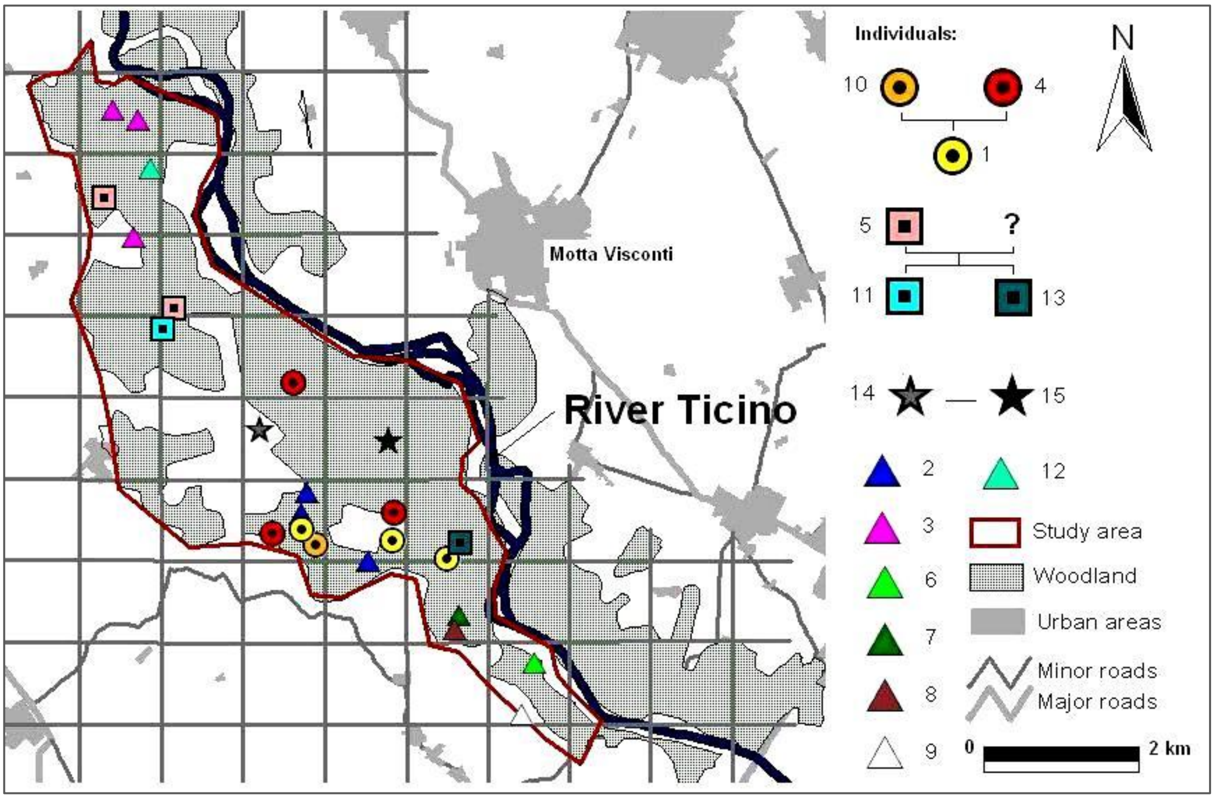

Figure 2. Spatial distribution of the genotyped individuals in the study area; individuals belonging to the same family groups are identified with same-shaped marks.

6. Functional implications of omnivory for dietary nutrient balance

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Functional implications of omnivory for dietary nutrient balance

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Abstract

Captive experiments have shown that many species regulate their macronutrient (i.e. protein, lipid, and carbohydrate) intake by selecting complementary food types, but the relationships between foraging strategies in the wild and nutrient regulation remain poorly understood. Using the pine marten as a model species, we collated available data from the literature to investigate effects of inter-annual, seasonal and geographic variation in diet on dietary macronutrient balance. Our analysis showed that despite high variation in foods comprising the diet, typical of a generalist predator, the macronutrient energy ratios of pine martens were limited to a range of 50-55% of protein, 38-42% of lipids and 5-10% of carbohydrates. This broad annual stabilisation of macronutrient ratios was achieved by using alternative animal foods to compensate for the high fluctuation of particular prey items, and sourcing non-protein energy (carbohydrates and fats) from plant-derived foods, particularly fruits. Macronutrient balance varied seasonally, with higher carbohydrate intake in summer-autumn, due to opportunistic fruit consumption, and higher protein intake in winter-spring. The pine marten's nutritional strategy fell between those of true carnivores (e.g. the wolf) and more omnivorous feeders (e.g. the European badger). In terms of energy contributed by protein, however, pine martens are equivalent to obligate carnivores such as the wolf and domesticated cat, and different to some omnivorous carnivores such as the domesticated dog and grizzly bears.

Key words: diet, macronutrient regulation, diet balancing, generalist carnivore, feeding strategy

Introduction

Predators can be aligned along a gradient in resource use, from specialists that use only one or a few food items, to generalists, which rely on a wide range of resources. The relative advantages of these alternative feeding strategies are still debated. According to optimal foraging models, specialists are favoured both during periods of environmental stability and when their preferred prey is abundant, implying that dietary generalism is favoured when the environment is not stable or prey availability unpredictable (Britt et al. 2006; Dennis et al. 2011; Terraube et al. 2011). Specialists are expected to achieve better performance when exploiting their preferred prey (MacArthur and Pianka 1966), although recent studies have shown that the relation between niche breadth and performance level is influenced by several environmental variables and may be more complex than previously believed (Whitfield et al. 2009; Barkae et al. 2012; Peers et al. 2012).

Optimal foraging models focus on the net rate of energy intake, in relation to prey availability and the time costs associated with foraging, capturing and handling different prey types (Křivan 1996). However, a wide literature has demonstrated that in many species foraging is guided principally by specific macronutrients in the diet. Animals typically regulate the amounts and balance of macronutrients to a target intake, either by selecting nutritionally balanced foods or by combining complementary foods in appropriate proportions (Raubenheimer and Simpson 1997, 2003a, 2003b; Felton et al. 2009; Mayntz et al. 2009). This has been demonstrated in laboratory studies on both vertebrates and invertebrates, including herbivores (e.g. locusts *Schistocerca gregaria*, Raubenheimer and Simpson 1993, 2004), omnivores (e.g. cockroaches *Blatella germanica*, Raubenheimer and Jones 2006; brown bears *Ursus arctos*, Erlenbach et al. 2014) and carnivores (e.g. predatory beetles *Anchomenus dorsalis*, Jensen et al. 2012; domestic cats *Felis catus*, Hewson-Hughes et al. 2011; dogs *Canis lupus familiaris*, Hewson-Hughes et al. 2012).

An interesting question, however, is how nutrient regulation interacts with broader, ecological factors in the wild, including spatial and temporal variation in resource availability and intra- and inter-specific competition. A few experimental studies have attempted to address such issues, for example by manipulating the relative abundance (Behmer et al. 2001) or spacing (Behmer et al. 2003) of different foods offered to locusts *Locusta migratoria* in experimental arenas, or offering to free-ranging Mormon crickets *Anabrus simplex* artificial foods varying in macronutrient composition to assess their nutritional priorities during migration (Simpson et al. 2006). Such studies benefit from the control offered by experimental manipulation, but are typically short-term and address only a limited subset of factors that might interact with nutrient regulation in the nutritional ecology of the target species. Other studies have therefore recorded the feeding behaviour of animals in an unmanipulated context in the wild, including spider monkeys (*Ateles chamek*; Felton et al. 2009), mountain gorillas (*Gorilla beringei*; Rothman et al. 2008), baboons (*Papio hamadryas ursinus*; Johnson et al. 2013) and giant pandas (*Ailuropoda melanoleuca*; Nie et al. 2015). These studies have, however, been restricted to herbivorous our near-herbivorous species, principally primates, which can be habituated to the presence of human observers.

By contrast, very little is known about nutrient regulation of free-ranging omnivores and carnivores, which tend to be elusive and feed infrequently, and are thus difficult to observe in the wild. For these animals, field-based nutritional studies can at present be performed only using indirect methods, including faecal analysis (Putman 1984) and gut contents analysis (Tait et al. 2014), which provide estimates of the frequency of occurrence or, with greater uncertainty, relative bulk of each food item in the diet (Zabala and Zuberogoitia 2003), but cannot provide reliable data on the absolute amounts of food ingested (Balestrieri et al. submitted). On the contrary, the review of proportional data can provide interesting insight into nutrient regulation in the wild and its interaction with such factors as habitat, climate, and population dynamics of both predators and prey There is, nonetheless, a large amount that can be learned about nutrient regulation in the wild and its interaction with such factors as habitat, climate, and population dynamics of both predators and prey, using analysis of proportional data (Raubenheimer 2011, Raubenheimer et al. 2015). To date such studies have been performed for feral domesticated cats (Plantinga et al. 2011), wolves (*Canis lupus*; Bosch et al. 2014) and predatory seabirds (Tait et al. 2014), but only one comparable study has been performed on an omnivore, the European badger (*Meles meles*; Remonti et al. 2011). This is an important omission, given the considerable evolutionary and ecological importance of omnivory (Coll and Guershon 2002, Eubanks 2005, Chubaty et al. 2014), and its significance for foraging theory (Singer and Bernays 2003).

Our aim in this study was to investigate seasonal and habitat-related variation in the diet of an opportunistic carnivore, the pine marten *Martes martes*, and its effect on short term (i.e. seasonal) and long-term (i.e. inter-annual) macronutrient balance. We hypothesised that a broad-based diet could be associated with several, mutually non-exclusive functions (Raubenheimer and Jones 2006):

1. *Nutrient balancing*, in which a wide diet provides a variety of complementary food options for diet balancing. This would predict that the nutrient composition of the diets of geographically and ecologically distinct populations is similar (Raubenheimer et al. 2015).

2. *Nutrient generalism*, in which a species has the flexibility to tolerate a wide range of nutrient intakes thus enabling it to occupy diverse habitats. This predicts between-population variation in the nutritional composition of the diet.

3. *Periodic complementarity*, in which a species compensates for periodic shortages of one nutrient resource (or takes advantage of the disproportionate availability of one resource) by targeting it according to availability, for example increasing the intake of non-protein energy when fruits are available. This predicts seasonal variation in diet, but a similar annual average across populations.

4. *Risk tolerance*, in which a species targets a particular balance of nutrients but has the ability to survive periodic food shortages by eating otherwise imbalanced foods. This predicts species-typical dietary nutrient intake, with irregular outliers.

We chose the pine marten as a model species because it is a generalist polyphagous predator that consumes a wide range of animals, including small mammals, birds, insects (i.e. a generalist carnivore), as well as plant-derived foods (omnivory), and can switch to alternative prey depending on the local and temporal availability of foods (Jędrzejewski et al. 1993; Zalewski 2005). As a consequence of this feeding flexibility, pine martens have an ecologically and geographically wide distribution: they occur throughout much of Europe and northern and central Asia and, although often associated with forest habitats (Proulx et al. 2004; Zalewski and Jędrzejewski, 2006), can also colonise fragmented landscapes (Pereboom et al. 2008; Balestrieri et al. 2010; Mergey et al. 2011; Caryl et al. 2012). To test our hypotheses of the functional associations between a broad-based diet and macronutrient gain, we reviewed data from the literature on the foods eaten by pine marten populations across Europe, estimated the percentage of macronutrients (i.e., protein, lipids, and carbohydrates) in each diet and then analysed both seasonal and geographic variation in the percentage of macronutrients associated with the recorded diets.

Methods

Nutritional composition of diets

We selected the available literature on the diet of pine martens, using the following criteria: 1) the results were expressed either as percent biomass or volume (hereafter: %VOL), both of which estimate the relative bulk of each food item as ingested; 2) the study covered at least one entire year, in order to avoid biases due to temporarily unbalanced diets; 3) not only dominant foods, but all food items were reported; 4) sample sizes exceeded 60 faecal samples (Zhou et al. 2011). Sixteen studies, based on 10238 faecal analyses, met these criteria (Tab. 1).

After pooling data from the same study sites to avoid pseudo-replication, we estimated the percentage of macronutrients in each diet by multiplying the per cent biomass or volume of the various food categories by the proportion (g/100g) of macronutrients (protein, lipids and carbohydrates) in each food (Remonti et al. 2011). For this purpose, the nutritional composition of thirteen food categories was obtained from available literature:

1) Flesh fruit: mean nutritional composition of seven species (*Rubus* sp., *Pirus* sp., *Malus* sp., *Prunus* sp., *Vitis* sp. and *Sambucus* sp.; Fidanza and Versilioni 1989, Johnson et al. 1985)
2) Acorns (*Quercus* sp.; Nieto et al. 2002)
3) Hazelnuts (*Corylus avellana*; Fidanza and Versilioni 1989)
4) Chestnut (*Castanea sativa*; Borges 2008)
5) Mushrooms (De Román et al. 2006)
6) Earthworms: mean nutritional composition of three species (*Lumbricus terrestris*, *Eisenia foetida* and *Dendrobaena veneta*; Stafford and Tacon 1988)
7) Insects: nutritional composition of Orthoptera (Zwart 1980)
8) Insect larvae (Zwart 1980)
9) Frogs: mean nutritional composition of two species (*Rana clamitans* and *Rana esculenta*; Schairer et al. 1998, Tokur et al. 2008)
10) Reptiles: nutritional composition of lizards (Cosgrove et al. 2002)
11) Birds: mean nutritional composition of two species (*Turdus merula* and *Columba livia*; Fidanza and Versilioni 1989)
12) Mammals: mean nutritional composition of mice and rats (Douglas et al. 1994)
13) Bird eggs (Fidanza and Versilioni 1989).

The macronutrient energy ratios (MER) in each diet were calculated using the following coefficients: protein = 14.64 kJ/g; fat = 35.56 kJ/g; non-structural carbohydrates = 14.64 kJ/g (Hewson-Hughes et al. 2011).

Right-angled mixture triangles (Raubenheimer 2011) were used to examine in three dimensions the annual and seasonal dietary macronutrient ratios of different pine marten populations. Additionally, for each location, latitude, elevation and habitat characteristics were taken from information provided in the studies. The correlation between MER and latitude and latitude x altitude, and the correlation between the %VOL of mammals in the diet of pine martens and the %VOL of alternative sources of protein were tested using Pearson's correlation test. Levene's test for homogeneity of variance, based on medians, was used to compare the variance of protein energy ratios in the diet to the variance in the %VOL of the main foods that contributed protein to the diet.

Seasonal variation in macronutrient balance

Seasonal diet data were available for a sub-sample of eight localities (numbers 2, 4, 10, 12, 13, 14, 15, 16; Tab. 1), and were used to calculate the macronutrient energy ratios for each locality and for the four seasons. For localities 2, 4, 10, 15 and 16 (Tab. 1), seasons were defined as follows: spring: April-June, summer: July-September, autumn: October-December, winter: January-March; studies 12, 13 and 14 reported seasonal data without specifying the months. Seasonal macronutrient energy ratios were compared by Nonparametric Multivariate Analysis of Variance (NPMANOVA) with 10000 permutations and post-hoc pairwise comparisons using Bonferroni’s correction to correct for multiple comparisons (Anderson 2001; Hammer et al. 2001).

Results

Mammals, fruit and birds were the main foods of pine martens (Tab. 2). Although mammals contributed the greatest volume to the diet in 15/16 (93.8%) localities, their %VOL varied from 26.7% in Killarney National Park, SE Ireland to 84% in Bialowieza National Park, Eastern Poland (Tab. 1 and 2). Fruit was the main food type in Killarney National Park, and in terms of volume comprised the second major food type in 60% of the reviewed localities, with a %VOL ranging from 4.4 to 51.4%. Birds were the second major source of protein in 31.3% of localities, while other vertebrates (i.e. reptiles and amphibians) were in general consumed in small quantities. Except for the Morvern peninsula, W Scotland, where insects were the second most important animal food after mammals (%VOL = 27.3%), and a few more localities (i.e. number 1 and 6, Tab. 2), invertebrates were a minor source of protein.

Despite the high variation in the composition of the diets of pine martens, annual macronutrient energy ratios clustered within a range of 50-55% protein, 38-42% lipids and 5-10% carbohydrates. Two populations showed apparently unbalanced diets that diverged from the main cluster (Fig 1). In the Morvern peninsula (number 4; Tab. 1), pine martens were reported to eat a proportionately high volume of nuts (%VOL = 20.3 in autumn), leading to a disproportionately high percentage of lipids in the diet. In Killarney National Park (number 11; Tab. 1), an abundant consumption of fruit led to a high percentage of carbohydrates in the diet. Finally, the diet of pine martens in the western River Po plain (number 15, Tab.1) differentiated marginally from the main cluster of diets due to a low percentage of lipids and a relatively high percentage of carbohydrates. By excluding these three sites, a 53.5% protein- to 46.5% non protein- (40.3% lipids, 6.2% carbohydrates) mean energy intake target was obtained (Fig. 1).

Annual macronutrient energy ratios did not vary either with latitude (protein: r = -0.12 p = 0.66; lipids: r = 0.41 p = 0.11; carbohydrates: r = -0.29 p = 0.28) or latitude x altitude (protein: r = -0.05 p = 0.85; lipids: r = 0.19 p = 0.94; carbohydrates: r = -0.06 p = 0.83; N = 16 for all comparisons). The %VOL of mammals in the diet of pine martens was inversely correlated to the %VOL of alternative sources of protein, i.e. birds + insects (N = 16 r = -0.58 p = 0.018). As a consequence of this alternative use of animal foods, the variation of protein energy in the diet was lower than the variation in the use by pine martens of the main food items (Levene's test, p = 0.0003; Fig 2).

Seasonal macronutrient energy ratios for each locality were more dispersed than annual ratios, particularly for summer and autumn diets, while spring and winter diets clustered more tightly around low percentages of carbohydrates (Fig. 3). Overall, seasonal macronutrient energy ratios were significantly different (N = 96, 4, 3 F = 3.953, p = 0.0045). Post-hoc comparisons revealed that macronutrient ratios in summer and autumn were significantly different from those in spring and winter (spring-summer: p = 0.0102; spring-autumn: p =0.0258; winter-summer: p = 0.0036; winter- autumn: p = 0.0078). In contrast, macronutrient energy ratios between spring and winter and between summer and autumn were not significantly different (p > 0.05 for both comparisons). In detail, winter/spring diets were shifted towards a lower percentage of carbohydrates and a higher percentage of protein with respect to the annual mean ratio, while summer/autumn diets were shifted to the opposite direction, with a lower percentage of protein and higher percentage of carbohydrates (Fig. 4.). Overall, the seasonal macronutrient energy ratios aligned on a fixed protein:lipid ratio of 1.3:1, especially for summer and autumn (Fig. 3).

Discussion

The pine marten has been reported to be an opportunistic feeder with a flexible diet that can include a wide range of foods in relation to their availability (Zalewski 2005; Zhou et al. 2011). However, our results provide evidence that this species uses its dietary versatility as a means to achieve long-term stabilisation of macronutrient energy ratios across populations living over a wide latitudinal range. Specifically, the use of alternative sources of protein (i.e. mammals, birds, insects) compensated for the high fluctuation in the use of any one prey type, thus leading to a broad annual stabilisation of the proportional contribution of protein energy to the diet. The tight similarity observed in the nutrient composition of diets from most pine marten populations supports the first of our hypotheses listed in the Introduction: broad diets based on both animal and plant foods allow pine martens to stabilise their dietary macronutrient balance in the face of geographic and temporal variation in food availability.

Two localities hosted populations with dietary macronutrient ratios that did not follow this general pattern. The presence of such outlier populations is consistent with our risk tolerance hypothesis, namely that pine martens target a particular balance of nutrients but have the ability to survive periods of food shortages by eating otherwise imbalanced foods. The risk tolerance hypothesis is related to the concept of the "fallback food" as used by primatologists (Malenky and Wrangham 1994, Marshall et al. 2009, Irwin et al. 2014),

For the Morvern peninsula, Putman (2000) reported a "remarkably species-poor" prey base, with the absence of any mammal (i.e. squirrels, rats, rabbits, hares) that could represent potential prey for pine martens. Moreover, in this area pine martens were reported to suffer dramatic, periodic population declines, probably in relation to the "relative scarcity of high-quality foods" (Putman 2000). Only 14% of Killarney National Park was covered by semi-natural woodland, dispersed in a mosaic of lakes, upland blanket bog, heathland, rivers and streams (Lynch & McCann, 2007). It is possible that in sub-optimal habitats with scarce availability of prey, pine martens may be unable to achieve fully balanced diets, as observed for the European badgers in habitats dominated by cereal crops (Remonti et al. 2011). Other than these two areas, in the recently colonised western River Po plain (Tab. 1, number 15) the macronutrient ratios of pine marten diets differed only slightly from the target. In this area, the negative impact of intensive agriculture has been reported to affect the small mammal community in terms of both species richness and population abundance (Balestrieri et al. 2015), although pine martens partially compensated by relying on introduced Eastern cottontail *Sylvilagus floridanus* (Balestrieri et al. 2011).

The between-season variation in macronutrient intakes within populations that had similar proportional annual macronutrient ratios is consistent with our third hypothesis, that of seasonal complementarity. In winter and spring the diet was dominated by animal-source foods with a high proportional protein content. Consequently, in summer and autumn carbohydrate-rich fruits were disproportionately exploited to redress the accrued deficit of non-protein energy. The great variation observed in summer and autumn in dietary carbohydrate content could be related to differences in fruit availability across the wide latitudinal range considered. The seasonal impact of fruit-derived carbohydrates to the diet is clearly illustrated in the extent to which seasonal diets aligned along a protein:lipid ratio of 1.3:1 (Fig. 3). This shows that the principle dimension of variance in macronutrient balance across seasons was for carbohydrate to displace a fixed ratio of protein:lipid (i.e. variation along a carbohydrate:protein+lipid trajectory). Alternatively, carbohydrates may play the role of short-term substitutes for fat as sources of non-protein energy, as has previously been observed in experiments on carnivorous fish (Ruohonen et al. 2007), dogs (Hewson-Hughes et al. 2012) and grizzly bears (Ehrlenback et al. 2014). This would, however, predict that the principle source of variance would be along a protein isoline (i.e. negative diagonal representing fixed dietary protein content) which was clearly not the case in our study, as shown by Fig. 3. The consequence of seasonal carbohydrate intake for the annual diet can be seen in Fig. 1. This plot shows that, despite the considerably tighter clustering of diets around a protein density of approximately 55% (shown by the solid diagonal grey line), there was nonetheless some residual spread along the carbohydrate axis (i.e., the C:P+L trajectory).

Several carnivores exhibit a wide seasonal variation in their diet composition, mainly according to fruit availability (Rosalino and Santos-Reis 2009). For example, both red foxes *Vulpes vulpes* and stone marten *Martes foina* are able to shift from a diet mainly based on fruit (i.e. 35-50% in volume) in the warm seasons, to a diet based on animal food in the cold season (i.e. more than 90% in volume; Padial et al. 2002; Lanszki et al. 2007; Posłuszny et al. 2007; Remonti et al. 2012). Such opportunism, which allows these predators to exploit temporary, highly profitable resources such as fruits, is likely adaptive, although the exact benefits are expected to differ with species and circumstances. For example, Tremblay et al. (1998) reported a negative effect of summer fruit-rich diet on the survival and/or fecundity of coyotes *Canis latrans*, while Larivière et al. (2001) did not observe any negative impact of temporarily high consumption of fruit on the reproductive biology of red foxes.

Considering European free-living mesocarnivores, the percent protein energy in the annual diet of pine martens was very similar to that reported for wolves (54%; Bosch et al. 2014), feral cats (52%; Plantinga et al. 2011) and European badgers (≈ 50%; Balestrieri et al. submitted), confirming the importance of protein regulation (Simpson et al. 2003). Percent lipid energy was similar in pine martens, wolves (45%; Bosch et al. 2014) and feral cats (46%; Plantinga et al. 2011), while in badgers it decreases to about 20% (Balestrieri et al. submitted). Pine marten percent carbohydrate energy was intermediate between that of wolf and feral cats (1-2%; Plantinga et al. 2011; Bosch et al. 2014) and that of the European badger (≈20%; Balestrieri et al. submitted). We suggest that these species could be aligned along a carnivore-omnivore continuum, based on a gradient of increasing tolerance towards carbohydrates. Obligate carnivores, such as wolves and wildcats, which feed almost exclusively on animal prey (Jędrzejewski et al. 2000; Lozano et al. 2006; Meriggi et al. 2011), ingest negligible amounts of carbohydrates. Opportunistic predators such as pine martens, in contrast, can seasonally consume high quantities of fruits and achieve slightly higher proportional carbohydrate intakes. Finally, European badgers, which exhibit a more omnivorous strategy, had a notably wider trophic diversity and a higher tolerance towards carbohydrates (Remonti et al. 2011).

Secondary analyses of literature data on the one hand have limitations due to the inevitable heterogeneity in both the methods and aims of studies, but on the other hand provide the opportunity to integrate data across geographic and temporal scales that would not be feasible in any one study. A limitation of our study and others that have used compilations of published dietary reports (Remonti et al. 2011, Plantinga et al. 2011, Bosch et al. 2014) is that they are potentially subject to greater error and sampling imbalance than purposed-designed observational studies of dietary intake (e.g. Johnson et al. 2013). Moreover, dietary studies based on faecal analysis enable estimates of the relative bulk of different foods in the diet, but not the absolute amounts of food consumed (Remonti et al. 2011). Such issues are, however, more likely to create artificial variation in the observed diets of a species, than to account for the tight clustering of diets that we have observed in our study. Furthermore, using similar methods Plantinga et al. (2011) demonstrated that feral domestic cats ingest macronutrient ratios similar to those recorded for captive cats in tightly controlled experimental conditions (Hewson-Hughes et al. 2013). A further limitation of our analysis could be that the nutritional composition of plant items can vary between species and with latitude (Johnson et al. 1985). This is, however, unlikely to impact on our analysis, as our main conclusions rest on the alternate consumption of fruit vs. animals foods which differ considerably more in their macronutrient composition than do different fruits.

In conclusion, our study has demonstrated that synthetic analyses of dietary data from the literature can provide interesting insight into the nutritional ecology of wild, free-ranging predators, in relation to locally- and seasonally-shaped food availability. By elucidating the macronutrient correlates of diet breadth in an omnivorous carnivore, our analysis has suggested functional reasons why some carnivores diversify their diet beyond animal-derived foods, providing a nutrient-focussed basis for understanding the diversity of foraging strategies. Equivalent studies across a broad range of species are needed for increase a general understanding of functional implications of polyphagous feeding.

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Table 1. Selected studies on pine martens diet.

N	Latitude	Altitude	Locality	N	References
1	59°40'	150	Sweden	760	Helldin 2000
2	57°48'	200	Morangie forest, north-east Scotland, UK	2449	Caryl et al. 2012
3	57°33'	150	West Ross-shire, England	337	Lockie 1961
4	56°33'	200	Morvern peninsula, W Scotland	174	Putman 2000
5	55°34'	90	Anyksciai district, Lithuania	335	Baltrūnaitė 2002
6	55°09'	180	Moletai district, E Lithuania	156	Baltrūnaitė 2001
7	55°00'	140	Polotsk district, Vitebsk region, N Belarus	1430	Sidorovich et al. 2005, 2010
8	55°00'	200	Gorodok district, Vitebsk region, NE Belarus	674	Sidorovich et al. 2005
9	53°00'	200	Minsk and Smolevichi d., Minsk region, C Belarus	742	Sidorovich et al. 2010
10	52°45'	160	Bialowieza National Park, Eastern Poland	1735	Jędrzejewski et al. 1993
11	52°01'	70	Killarney National Park, SE Ireland	387	Lynch and McCann 2007
12	51°48'	200	Rogow, C Poland	155	Posłuszny et al. 2007
13	46°24'	150	Boronka Nature Conservation Area, SW Hungary	332	Lanszki et al. 2007
14	46°01'	440	Luino, N Italy	148	Biancardi and Rinetti 2001
15	45°10'	100	Western Po plain, NW Italy	195	Balestrieri et al., 2011
16	42°06'	1293	Macizo Central Ourensano, Galicia, NW Spain	229	Rossellini et al. 2007

Table 2. Percent estimated volume (%VOL) of the main food types in pine marten diets (numbers are the same of Table 1; S.D. = Standard Deviation).

N	Mammals	Birds	Other vertebrates	Fruit	Insects
1	58.5	14.6	2.8	14.0	9.8
2	43.1	25.0	0.0	30.4	1.4
3	72.3	10.8	0.0	11.3	4.3
4	29.1	13.5	0.7	16.5	27.3
5	68.9	13.0	1.5	15.1	1.5
6	50.1	9.6	0.3	28.6	8.7
7	61.5	11.3	2.2	19.9	3.4
8	66.8	13.5	1.9	13.0	4.0
9	68.3	17.6	1.0	4.8	4.8
10	84.0	7.7	2.1	4.4	0.0
11	26.7	12.7	5.4	51.4	2.0
12	61.9	24.3	0.0	13.4	0.4
13	57.1	17.9	2.2	21.6	1.2
14	53.8	14.2	0.9	21.9	3.7
15	54.2	13.0	0.5	26.5	2.4
16	50.0	20.9	0.2	28.1	0.8
Mean	56.64	14.97	1.35	20.06	4.73
S.D.	14.99	4.97	1.41	11.54	6.62

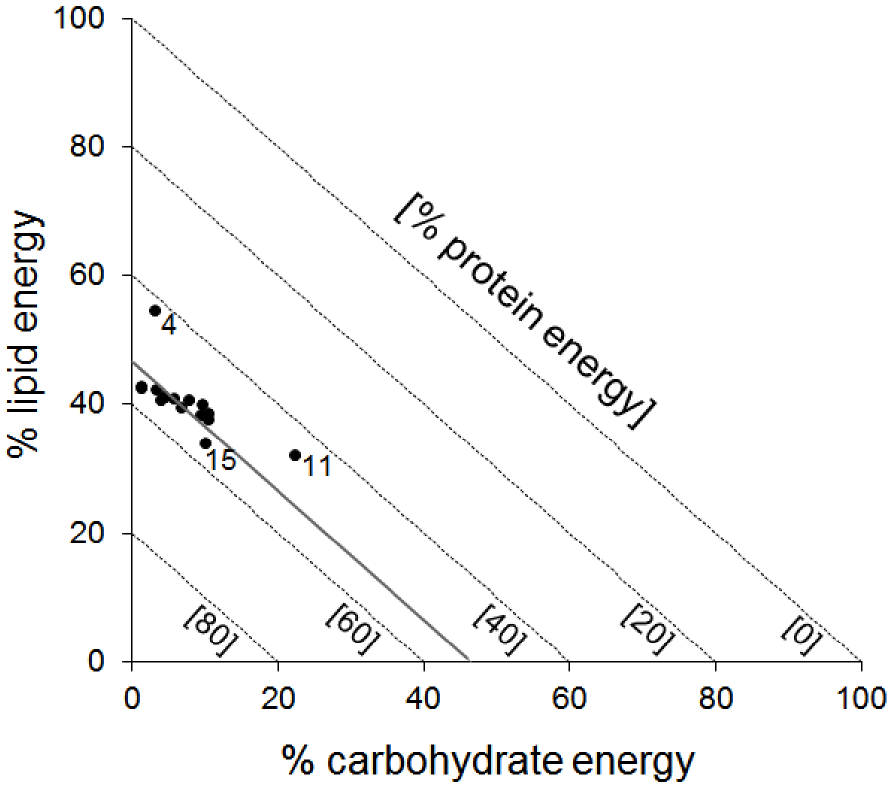

Figure 1. Right-angled mixture triangle plot (Raubenheimer, 2011) showing the estimated macronutrient (lipid, carbohydrate, protein) intake by pine martens from sixteen European localities, expressed as percentage of metabolizable energy. Protein percentage, which is represented by diagonal protein isolines, varies inversely as distance from the origin. The % dietary protein on which most populations converged (approximately 55%) is shown by the solid grey line. The numbers of the three sites correspond to those in Table 1 (see text).

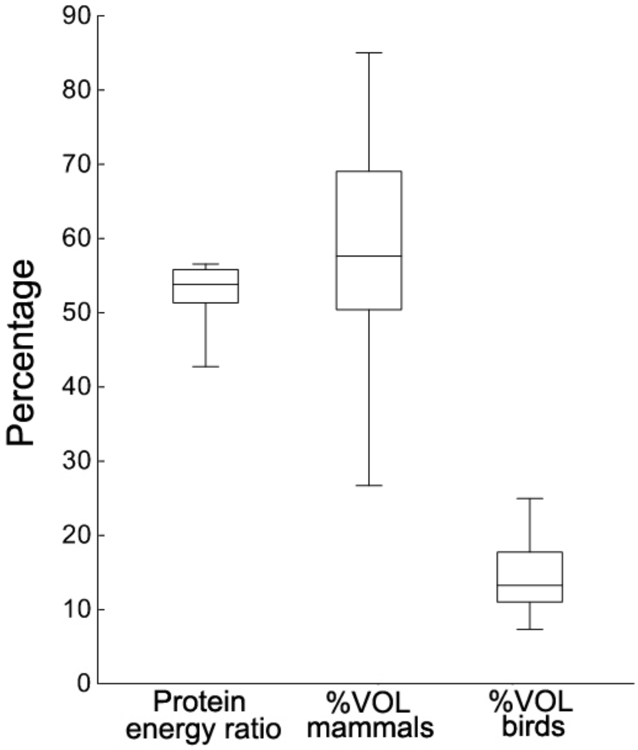

Figure 2. Variation in the protein energy ratio in the diet of pine martens, compared with the variation in the percent estimated volume (%VOL) of the main sources of protein. The 25-75 percent quartiles are drawn in the boxes, the median is shown by a horizontal line inside the box and the minimum and maximum values are shown by short horizontal lines.

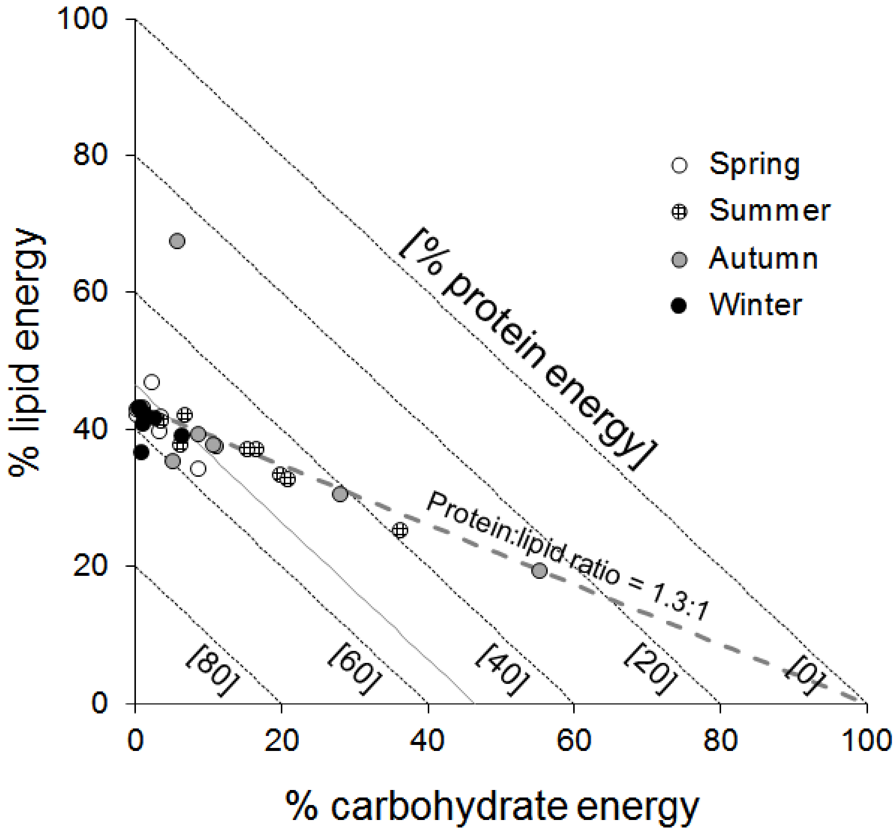

Figure 3. Right-angled mixture triangle showing the estimated seasonal macronutrient (lipid, carbohydrate, protein) intake by pine martens from sixteen European localities, given as percentage of metabolizable energy. Protein percentage varies inversely as distance from the origin increases and is represented by diagonal isolines. The % protein target is shown by a grey line; the dashed grey line represents the 1.3:1 protein:lipid ratio (see text).

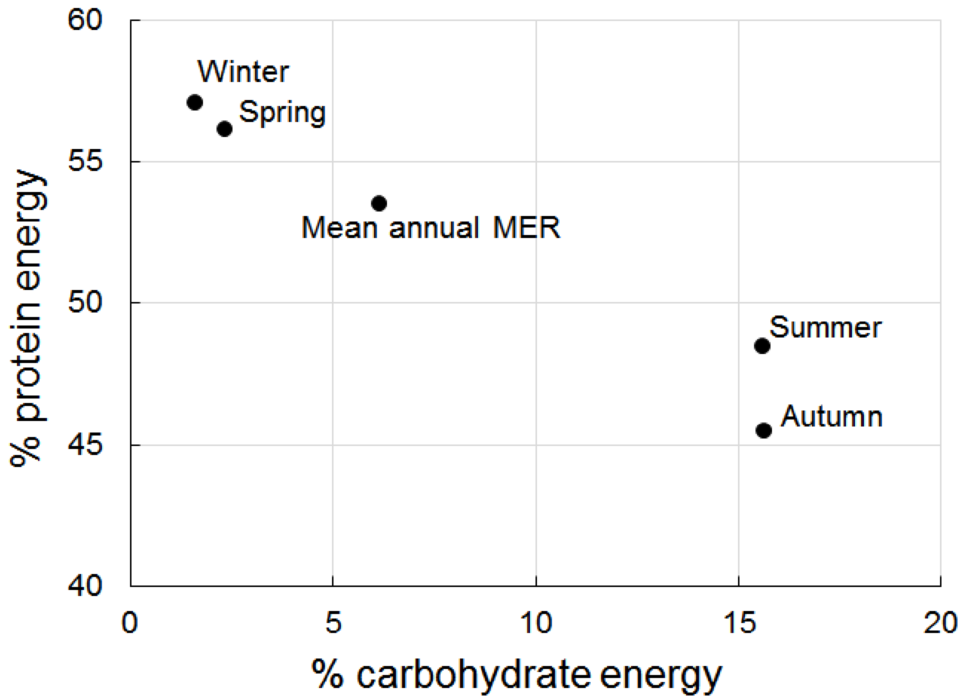

Figure 4. Seasonal and annual carbohydrate *vs*. protein energy ratio in the diet of pine martens from eight European localities.

7. Activity patterns of martens (*Martes* spp.) and larger mesocarnivores in cultivated lowlands of NW Italy.

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Manuscript

Activity patterns of martens (*Martes* spp.) and larger mesocarnivores in cultivated lowlands of NW Italy.

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Abstract

Interference competition is considered the main factor driving temporal partitioning in carnivore assemblages. Recently, the pine marten (*Martes martes*) has expanded in agricultural habitats of the western part of the River Po plain, where its arrival has coincided with the contraction of stone marten's (*M. foina*) range With the aim of testing the hypothesis that pine marten success may depend on its ability to avoid intra-guild competitive interactions more effectively than stone martens, we investigated and compared the spatial and temporal behaviours of mesocarnivores in two study areas of the River Po plain. Based on camera-trapping data, time-use was assessed through the probability density function using Kernel Density, while space use by pairs of species was compared by Sørensen's similarity index.

While stone martens, red foxes (*Vulpes vulpes*) and European badgers (*Meles meles*) were strictly nocturnal, the pine marten showed a cathemeral pattern. Both marten species tended to avoid the other mesocarnivores, with the lowest index recorded for the pair pine-marten – red fox.

Combining space and time pattern of use, the smallest species, the pine marten, was the most efficient in avoiding encounters with the two larger mesocarnivores, particularly with the red fox. We suggest that the replacement of the stone marten by the pine marten in residual woodland could be the consequence of the latter higher ability to avoid interference competition within the local guild of mammalian predators.

Key words: interference competition, cathemerality, camera-trapping, Kernel density.

Introduction

Diel activity pattern along are regulated by each species' endogenous clock (Kronfeld-Schor and Dayan, 2003) and by external abiotic and biotic factors, which, in the case of predator species, include prey accessibility and interspecific competition (Halle, 2000; Arias-Del Razo et al., 2011).

In carnivore assemblages, spatial and temporal shifts in the use of key resources may arise in response to interference intra-guild competition (Palomares and Caro, 1999; Donadio and Buskirk, 2006). Interference may take the extreme form of interspecific killing, when a larger, and thus dominant, species kills a smaller one (Polis and Holt, 1992; Linnell and Strand, 2000; Palomares and Caro 1999). Aggressive behaviour provides clear benefits to the larger competitor, which, restricting subordinates to suboptimal habitats (Creel and Creel, 2002; Macdonald et al., 2004; Mitchell and Banks, 2005), gains the exclusive access to food resources (Robertson et al., 1976; Whitehouse, 1997). On the opposite site, spatial and/or temporal segregation may enable the coexistence of subordinates with top predators (Palomares et al., 1996; Torretta et al., 2015).

In lowlands of Northern Italy, large carnivores are absent; the most widespread mesocarnivores are, from the largest to the smallest, the European badger *Meles meles*, the red fox (*Vulpes vulpes*), and the stone marten (*Martes foina*) (Spagnesi and De Marinis, 2002). Recently, the pine marten (*M. martes*) has expanded in agricultural habitats of the western part of the River Po plain, where its arrival has coincided with the contraction of stone marten's range (Balestrieri et al., subm. a).

The two martens are the most similar sympatric carnivores in Europe in terms of morphology, foraging behaviour and activity pattern (Larroque et al., 2015). Where they co-occur, the stone marten is usually slightly heavier than the pine marten (Marchesi, 1989). Nonetheless, when syntopic, both species exhibit different habitat selection, with the stone marten often associated with rural and suburban areas while the pine marten occupies woodland. This differential habitat use has been related to the supposed higher competitive ability of pine martens, although it is still not clear why this species should be able to displace the stone marten from forested areas.

We hypothesized that pine marten dominance on the stone marten may depend on the former higher capability to avoid direct and/or exploitative interactions with the two larger mesocarnivores.

Available information about direct interactions between pairs of carnivores has been reviewed by Palomares and Caro (1999). Interspecific killing occurs between foxes and badgers, each one being able to kill only non-adult individuals of the other species, but rates of killing are probably low if we consider that they often share the same burrows (Mori et al., 2014). Foxes can kill adult American martens (*M. americana*) and pine martens, even sharply limiting their population density (Thompson, 1994; Lindström et al., 1995), as suggested by pine marten recovery following an epidemic of scabies among red foxes (Lindström et al., 1995; Smedshaug et al., 1999). Accordingly. both martens are believed to select shelters above ground to avoid foxes (Pulliainen, 1981; Hermann, 1994; Webster, 2001). On the contrary, no attacks are known between badgers and martens (Palomares and Caro, 1999). In the western River Po plain, dietary overlap between red foxes and both marten species is quite high, particularly for the pair red fox-stone marten (Remonti et al., 2012). In areas with low earthworm availability, such as both Alpine and cultivated areas, food overlap can be high also between badgers and martens (Prigioni et al., 2008).

In the last few decades, most studies on the mechanisms of species coexistence have been directed at the differential use of habitat and food resources (Dayan and Simberloff, 1994; Barrientos and Virgos, 2006; Prigioni et al., 2008), while time as a niche axis has seldom been investigated. Nonetheless, in many cases, niche displacement at the temporal scale seems to play a major role (Valeix et al., 2007; Di Bitetti et al., 2009).

With the aim of testing the hypothesis that pine martens can avoid intra-guild competitive interactions more effectively than stone martens, we investigated and compared the spatial and temporal behaviours of mesocarnivores in two study areas of the River Po plain by camera-trapping. Time-use was assessed through the probability density function using Kernel Density (Ridout and Linkie 2009). The major advantages of using kernel functions to fit activity data are related to their continuous and circular structure, which allows to avoid problems associated with both timescale categorization and definition of the origin. Additionally, being nonparametric functions, they can fit either bimodal or multimodal patterns, which are both widespread among carnivorous mammals.

Study areas

The first study area coincided with the right bank of the stretch of River Ticino flowing between the towns of Vigevano and Abbiategrasso (Milan, Lombardy) in the north and Gropello Cairoli village (Pavia, Lombardy) in the south. Along this *ca.* 35 km long stretch of the river, mean percent riparian vegetation cover, as assessed in a 100 m large belt on both river banks, was 47.8% (min-max: 12-86%; Prigioni and Balestrieri, 2011). Mesophilous - *Fraxino-carpinion* - and hygrophilous - *Alno-Ulmion*, *Alnion glutinoso-incanae*, *Salicion albae* – riparian woods were surrounded by an intensively cultivated and urbanized lowland.

The second study area consisted of an isolated, 14.5 km^2 wide, hill (up to 147 m a.s.l.) in the middle River Po plain (San Colombano al Lambro, Lombardy region, ca. 45°11’N, 9°29’E), bordered to the north-east by the River Lambro and to the south by the River Po. Woodland (24% ca.) consisted of oaks (*Quercus robur*, *Q. petraea*), alder (*Alnus glutinosa*), chestnut (*Castanea sativa*) and black locust (*Robinia pesudoacacia*), with shrubs of *Cornus sanguinea*, *Corlylus avellana* and *Crateaegus monogyna*. Permanent cover also included grasslands, fallow fields and shrublands (14%). Cropland (55%), mostly vineyards (39%), was mainly located in the south-eastern part of the hill. The surrounding matrix consisted of an intensively cultivated agricultural area, being mostly maize and rice fields and small urban areas.

For both areas, the climate was sub-continental temperate, with an average yearly temperature of 11.4-13°C and an average yearly precipitation of ca. 700 mm.

Methods

Both study areas were monitored by digital scouting pocket cameras (Multipir 12 and SG550 with Passive Infra-Red motion sensor), provided with 8 GB SD cards and up to 8 AA batteries. Cameras were set to record 15 s long videoclips, with a 30 s interval between two successive recordings to potentially increase the independence of videoclips. Cameras were tied to trees 30-50 cm above the ground level. No attractant was used.

Surveys were carried out within a 1x1 km grid, superimposed on the kilometric grid of digitalized, 1:10000 Regional Technical Maps; for each session, 10 camera-traps were deployed for 10 days within a 2 km^2 large unit, for a total of 6 units, 12 km^2 and, potentially, 600 camera-days (Fig. 1, 2). For each unit, the distance between contiguous cameras (0.45 km) was calculated as the inverse of the square root of the ratio between the number of trapping sites (10) and the size of the unit (2 km^2). With this distance borne in mind as a general rule, cameras were deployed proportionally to each main habitat (woods, cultivated fields, shrubs and pastures) area, choosing trap-sites as to give each camera enough clear view with respect to their potential detection radius. Camera-trap sites were georeferenced and superimposed on digital maps.

The co-occurrence between carnivores was evaluated using presence-absence data from camera-sites by Sørensen’s similarity index (SI; Sørensen, 1948):

$$Ss_{i,j} = \frac{2a_{ij}}{2a_{ij} + b_{ij} + c_{ij}}$$

where a_{ij} represents the number of sample stations with the presence of both species i and j, and b_{ij} and c_{ij} are the number of sample stations with the presence of only one species.

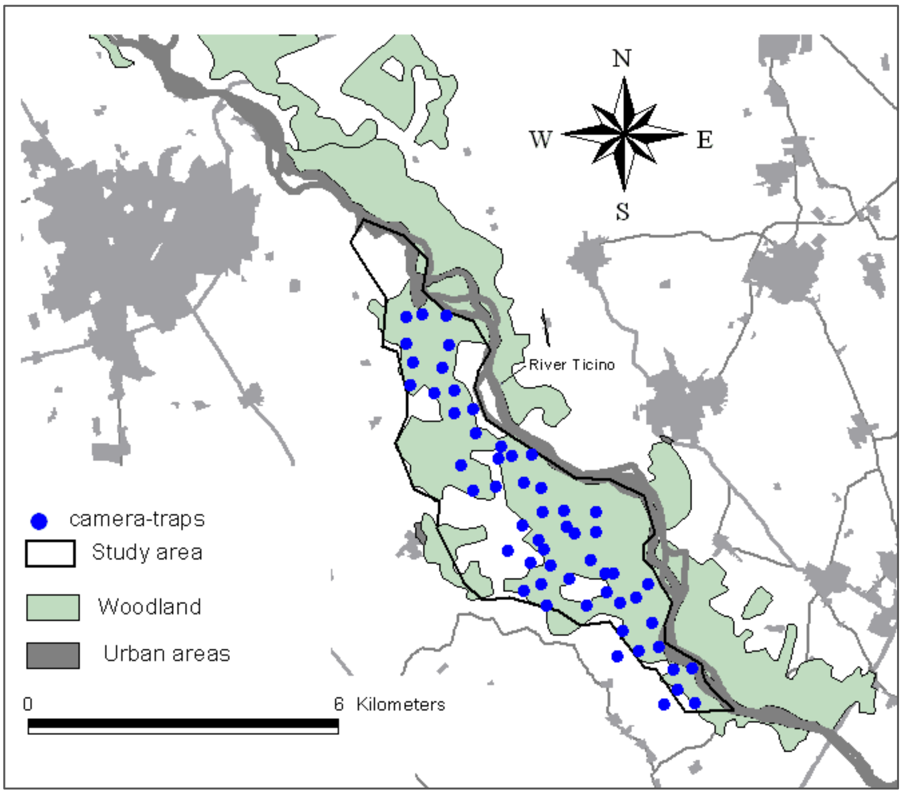

Figure 1. Study area and camera-trap locations on the River Ticino.

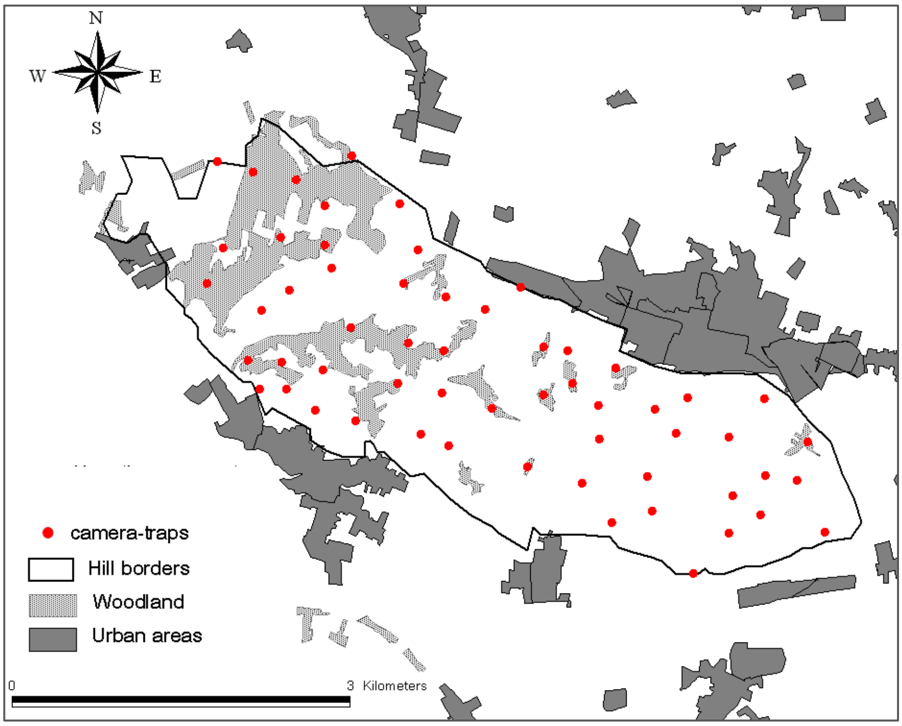

Figure 2. Study area and camera-trap locations in the hilly area of San Colombano al Lambro.

This index ranges from 0 (maximum segregation) to 1 (maximum co-occurrence, i.e. all considered species are present in all sample units).

Activity patterns were estimated non-parametrically through the probability density function using Kernel Density Estimate (Ridout and Linkie, 2009). Distribution uniformity was tested by Watson's test (U^2).

Pairwise carnivore activity patterns were compared by estimating the coefficient of overlap (Δ_1), ranging between 0 (no overlap) and 1 (complete overlap) (Ridout and Linkie, 2009; Linkie and Ridout, 2011). Following the approach of Monterroso *et al.* (2014), activity patterns with Δ_1 values ≤50th percentile of the sample were considered as "low overlap values", while activity patterns with Δ_1 >75th were defined as "high overlap values". Intermediate Δ_1 values were considered as "moderate overlap values". To test for the reliability of the index and obtain 95% confidence intervals a smoothed bootstrap was performed generating 1000 bootstrap samples (Meredith and Ridout, 2014).

To evaluate statistically the activity overlap value pairwise activity patterns of different carnivores were compared through Watson's two-sample test (two-sample U^2) (Monterroso *et al.*, 2014). The analyses were performed using "circular" and "overlap" packages for R software (Lund *et al.*, 2013; Meredith and Ridout, 2014).

Results

On the River Ticino (TI), during 503 trap-days we collected 143 independent videos of mesocarnivores (61 records of the red fox, 61 of the badger and 21 of the pine marten). The red fox occurred at 24 (40.7%) sample stations, the badger at 20 (33.9%) and the pine marten at 12 (20.3%). In the hilly area (SCL), trapping effort was 585 trap-days, and the number of mesocarnivore records was 181 (62 records of the red fox, 98 of the badger and 21 of the pine marten). The red fox occurred at 33 (55.9%) sample stations, the badger at 30 (50.8%) and the pine marten at 14 (23.7%). In both study areas, Sørensen's index was the highest for the pair fox-badger (Tab. 1). In TI, the pine marten tended to avoid the red fox, whilst in SCL the lowest index was recorded for the pair stone marten-badger.

Table 1. Number of videos (a-b: both species) and Sørensen similarity index (SI) for all species pairs in the two study areas (TI: River Ticino; SCL: San Colombano al Lambro).

Area	**Species pairs**	**a-b**	**a**	**b**	**SI**
TI	Fox-Badger	11	13	9	0.50
	Fox-Pine marten	6	18	6	0.33
	Pine-marten-Badger	7	13	5	0.44
SCL	Fox-Badger	20	16	10	0.61
	Fox-Stone marten	11	25	3	0.44
	Stone-marten-Badger	8	22	6	0.36

The diel activity patterns of all species were non-uniform ($0.23 < U^2 < 2.44$, $P < 0.025$ for all tests). The red fox showed a bi-modal pattern in SCL, with peaks at sunrise and sunset, while in TI the pattern tended to be uni-modal, with a main peak between 21:00 and 22:00 h. The percentage of records during daylight was the same (16.2-17.4%) in both areas. The badger was always active in both areas from sunset to sunrise. In TI showed a bi-modal pattern, with peaks between 21:00 and 22:00 h and 02:00 and 03:00 h, while in SCL was active from 19:00 to 02:00 h. The pine marten showed a peak of activity between 09:00 and 10:00 h, 51% of records having been collected during daylight. In contrast, the stone marten was mainly nocturnal (86% of records).

The threshold value between the low-moderate activity overlap was 0.71 (50th percentile), whereas between the moderate-high activity overlap was 0.75 (75th percentile). On the whole, activity overlap was high between stone marten and badgers, and between red foxes and pine martens, while it was low for the pair pine marten-badger (Tab. 2). Nonetheless the activity of the pine marten reached a peak when those of the other two mesocarnivores were the lowest (Fig. 3).

Table 2. Coefficient of overlap and Watson's two-sample test for all species pairs in the two study areas (TI: River Ticino; SCL: San Colombano al Lambro).

Area	Species pairs	Δ	CI_{low}	CI_{up}	U^2	P-value
TI	Fox-Badger	0.70	0.61	0.79	0.466	< 0,001
	Fox-Pine marten	0.76	0.62	0.89	0.182	n.s.
	Pine-marten-Badger	0.54	0.41	0.69	0.591	< 0,001
SCL	Fox-Badger	0.69	0.58	0.81	0.597	< 0,001
	Fox-Stone marten	0.72	0.57	0.86	0.242	< 0,05
	Stone-marten-Badger	0.87	0.73	0.98	0.056	n.s.

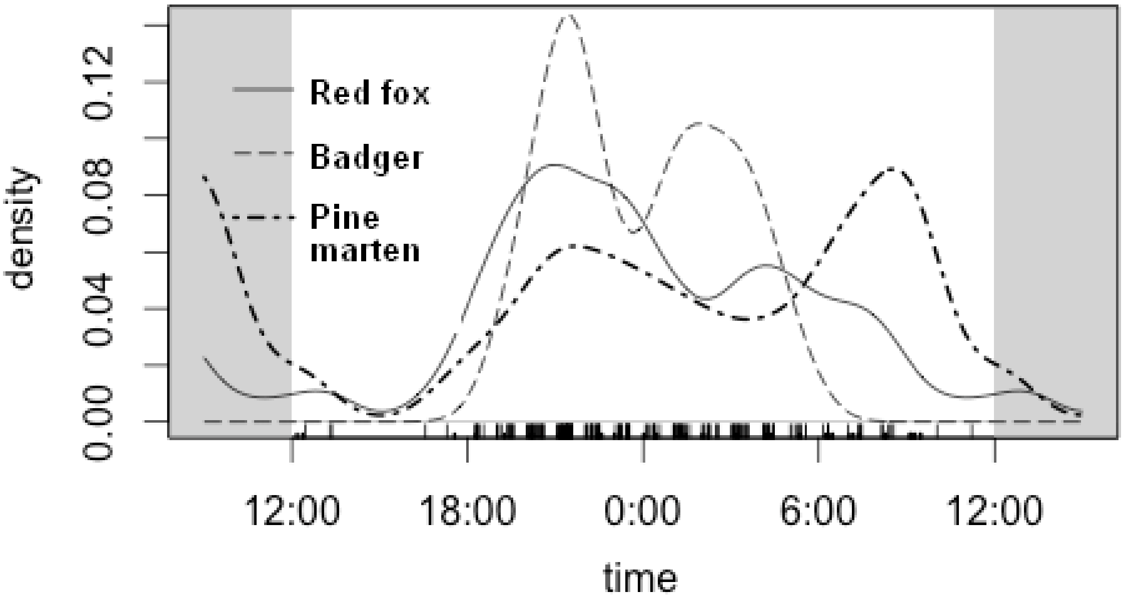

Figure 3. Activity patterns of mesocarnivores in the River Ticino study area.

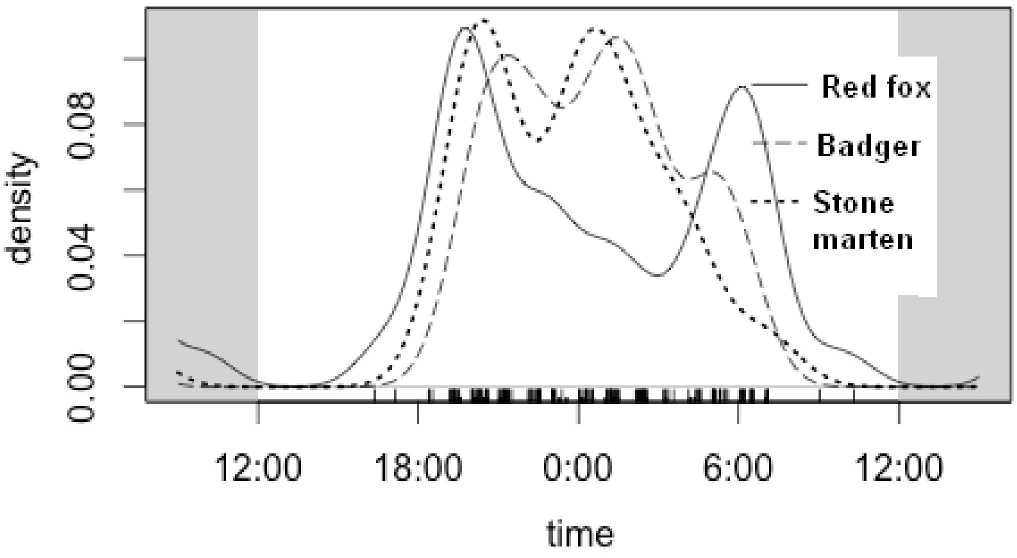

Figure 4. Activity patterns of mesocarnivores in the hilly area (San Colombano al Lambro).

Discussion

Most mammal species are nocturnal (69%), nocturnality probably being an ancestral characteristic of this group (Bennie et al., 2014).

In contrast, only 8.5% of mammal species is cathemeral, this activity pattern occurring more frequently at mid to high latitudes in the Northern Hemisphere, including the Alps and Pyrenees (Bennie et al., 2014). Cathemeral activity in mammals has been reported to increase with habitat fragmentation: nine-banded armadillos (*Dasypus novemcinctus*) were strictly nocturnal in forest areas > 1,000 ha, whereas their activity followed a cathemeral pattern (up to 60% of records in daylight) in smaller forest patches (Norris et al., 2010).

While stone martens were strictly nocturnal, as previously reported for other study areas (Posillico et al., 1995; Herr, 2008; López-Martin et al., 2008; Wilson and Mittermeier, 2009; Monterroso et al., 2014), on the River Ticino, pine martens showed a cathemeral pattern.

Although considered to be mainly nocturnal (Bennie et al., 2014), the pine marten has shown to be more flexible than the stone marten with respect to activity patterns (Monterroso et al., 2014) and, in central Italy, diurnal activity has been reported to be high (up to 50% of records) in spring-summer (del Fante, 2012).

Moreover, its sister species, the sable (*Martes zibellina*), is considered cathemeral (Bennie et al., 2014). Being potentially active within a broad period, cathemeral species may be more capable than strictly nocturnal species to adjust their activity pattern to local conditions, increasing their fitness and allowing their coexistence with competitors (see Di Bitetti et al., 2009 about two south-American fox species).

Whether this higher plasticity of the pine marten with respect to the stone marten depends on the geographical origin of the two species (northern Europe and Middle East, respectively) or different responses to human disturbance, daytime activity may help the pine marten to reduce the frequency of its encounters with the two larger mesopredators – red foxes and badgers -, occurring in the study areas, and also the overlap of resource use (Kronfeld-Schor and Dayan, 1999).

The fox confirmed to be primarily nocturnal (Artois, 1989), with activity peaks at nightfall and sunrise, as several other canids (Bennie et al., 2014). This pattern of activity has been related to both physiological periodicity (Ables, 1969) and accessibility to prey (Boitani and Vinditti, 1988). As expected, the badger was active from dusk to sunrise, when it regularly returns to its underground burrows (Mori et al., 2015; Balestrieri et al., subm. b).

Within the night-time, the differential intensity of activity potentially reduced competition (Tokeshi, 1999; Kronfeld-Schor and Dayan, 2003), red fox activity being concentrated in about two-hour long peaks, while badgers being active more uniformly throughout the night.

If we assume that trapping rate is correlated to density (O'Brien et al., 2003), the abundance of foxes and martens were similar in the two study areas, while badgers were more numerous in the hilly area, consistently with the higher density previously recorded there with respect to other lowland areas of northern Italy (Balestrieri et al. subm. b).

The highest similarity index recorded for the pair red fox – badger was consistent with previous results, indicating that, although badgers can dominate foxes at feeding sites, encounters between these two species are generally unaggressive (Macdonald et al., 2004). In contrast, index's values suggested that both marten species tended to avoid the other mesocarnivores, with the lowest index recorded for the pair pine-marten – red fox. Accordingly, Barrull et al. (2014) reported that stone martens tend to avoid interactions when detecting the odour paths from the other two predators.

Combining space and time pattern of use, the smallest species, the pine marten (Marchesi, 1989), was the most efficient in avoiding encounters with the two larger mesocarnivores, particularly with the red fox. We suggest that the behavioural plasticity of the pine marten is playing a major role in its expansion in lowland areas. In particular, the replacement of the stone marten by the pine marten in residual woodland could be the consequence of the latter higher ability to avoid interference competition within the local guild of mammalian predators.

As different guild compositions and structures result into different interspecific relations among coexisting species (Monterroso et al., 2014), our hypothesis needs to be further tested with analyses of the activity patterns of carnivorous mammals living in different communities and across different study areas.

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8. Conclusions

Based on the results of the non invasive survey the pine marten can be considered to have colonised the overall northern section of the study area, at least wherever wood patches still occur. The current pine marten distribution in the western Po plain suggests that expansion has mainly followed a north-to-south direction, with the River Po, which crosses the study area from the south-west to the east, acting, till now, as a natural barrier.

The relatively high percentage of martens belonging to the Central-Northern European phylogroup, which currently represents an *unicum* in southern Europe, is consistent with the hypothesis of an ongoing expansion of Alpine and trans-Alpine pine marten populations. Unfortunately, information about the status of the pine marten in Italy is insufficient to determine the causes of its expansion, although, on the Alps, increasing forest cover may have favoured the growth of pine marten populations, as occurred for other forest species.

Species distribution models suggest that the potential for pine marten expansion is high only for the western part of the River Po plain (i.e. Piedmont region) and, secondarily, for the Adriatic coast (Veneto region). As expected, the distribution of residual wood patches and availability of suitable riparian corridors plays a major role in shaping the potential expansion area of the pine marten in agricultural lowlands.

As recorded in neighbouring France, the hypothesis that lowland woods may represent marginal habitats for the pine marten was not supported by the data collected about both its density and macronutrient balance. In the valley of the River Ticino, microsatellite analysis of faecal DNA allowed to point out one of the highest densities ever recorded in continental Europe. The high genetic relatedness of identified individuals support the “habitat saturation” hypothesis, i.e. that the agricultural matrix may act as a barrier to the dispersal of juveniles.

Meanwhile, genotyping allowed to test for the effectiveness of the Random Encounter Model, a camera-trapping-based method for assessing the density of elusive species that does not depend on individual recognition. The REM proved to be unreliable for estimating pine marten population size, and we suggest that this may be the case for all elusive species for which the assessment of average daily movements cannot be achieved without the use of invasive methods.

The meta-analyses of pine marten diet in its European distribution range showed that despite a high variety of foods comprising the diet, the macronutrient energy ratios of the pine marten were limited to a range of 50−55% of protein, 38−42% of lipids and 5−10% of carbohydrates. Although being a generalist predator, in terms of energy contributed by protein the pine marten is equivalent to obligate carnivores. In the western Po plain, dietary macronutrient ratios of pine marten differed from the target to an almost negligible extent. The poor prey base of the study area in terms of small rodents was compensated by the pine marten by relying on introduced Eastern cottontail *Sylvilagus floridanus*, confirming that plasticity in feeding behaviour plays a major role in pine marten ability to colonise agricultural areas.

Within Mustelidae, there are several sympatric species with similar ecological requirements that have developed mechanisms to coexist and pine- and stone martens are sympatric across a large part of their respective ranges. Nonetheless, the negligible occurrence of stone marten recorded north of the River Po and, particularly, for the valley of the River Ticino confirmed that pine marten expansion is coinciding with a reduction in stone marten range and/or abundance. The relatively large availability of suitable areas for pine marten expansion allows to predict that stone marten range in the western Po plain may further decline in the near future.

Where the two species are syntopic, stone marten exclusion from woodland by the smaller pine marten is a common output, which has been reported to depend on the higher competitive ability of the latter species, although the mechanisms underlying it are still unclear.

Based on camera-trapping data, we suggest that the replacement of the stone marten by the pine marten in residual woodland could be the consequence of the higher ability of the latter to avoid interference competition within the local guild of mammalian predators. In other words, pine marten higher competitive ability with respect to the stone marten would be the indirect consequence of its cathemerality and consequent greater plasticity in activity patterns.

The combined use of two non-invasive census methods, i.e. camera-trapping and feacal DNA-based genotyping, allowed to gain actual insights in the distribution and behavioural ecology of such elusive species as pine- and stone martens. With respect to such a powerful but invasive methods as radio-tracking, the adopted non invasive methods were both more cost-effective and admissible from the ethic point of view.

As guild composition and structure affect interspecific relations among coexisting species, the findings of this study need to be further tested on different communities and across different study areas. As a first step, it would be worth of applying species distribution models to compare pine- and stone marten habitat selection in a sympatric area, to assess how environmental characteristics shape the relative distribution of the two species. Secondly, the activity patterns of the two martens should be analysed for different habitats and guilds to confirm the greater plasticity of the pine marten and strict nocturnality of the stone marten. Finally, knowledge on the distribution and abundance of *Martes* spp. in Alpine and sub-Alpine areas is still negligible and offers several opportunities for future field research.

List of papers (2013-2015)

1. **Balestrieri A.**, Remonti L., Vezza P., Prigioni C., Copp G.H. (2013). Do non-native fish as prey favour the conservation of the threatened indigenous Eurasian otter? Freshwater Biology, 58: 995–1007
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Congress reports (2013-2015)

1. Remonti L., Ruiz-González A., **Balestrieri A**. 2013. The colonisation of the River Po plain (NW Italy) by the pine marten: influence of habitat constraints and interspecific competition. IMC 11, Small Carnivores in Space and Time. Belfast, 11-16th August 2013
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4. Loy A., **Balestrieri A**., Bartolomei R. et al. 2015. The Eurasian otter (*Lutra lutra*) in Italy: distribution, trend and threats. European Otter Workshop, Stockholm, 8-11/06/2015.
5. Gariano P., **Balestrieri A**., Prigioni C. 2015. Modello di idoneità ambientale del massiccio della Sila per la lontra *Lutra lutra* . III Convegno Italiano sulla Riqualificazione Fluviale, Reggio Calabria, 27-30/10/2015.

Workshops (2013-2015)

1. La recente espansione della martora (*Martes martes*) nella Pianura Padana: una forest-specialist tra le risaie. Dip. di Scienze della Terra e del'Ampbiente, Università di Pavia, 02 April 2014.
2. I mustelidi del Parco del Ticino: monitoraggio e fototrappolaggio. Parco Ticino, Magenta, 16 December 2015

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