DOTTORATO DI RICERCA IN ETOLOGIA ED ECOLOGIA ANIMALE
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Savi's pine vole (*Microtus savii*) ecology in agroecosystems

Tesi di

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ABSTRACT

The research I conducted during this PhD focused on the Savi's pine vole (*Microtus savii)*. It presents the state-of-the-art knowledge about this species which still has numerous gaps; these gaps should however be filled in order to conceive an effective management system of a species that can affect human agriculture.
Moreover this thesis provides a new effective method that can be adopted by future researchers intending to study this species or any other fossorial small mammal.
The information here provided includes data on reproductive period, which is far more complex than what previously thought, monthly fluctuations and survival and dispersal. These information were acquired during an intensive and detailed one-year-long study carried out in antrophic environment.
Lastly I make use of innovative genetic techniques to prove that even though this species' diet is composed by various plants, it displays marked preferences or avoidances for specific kinds of food.
This work might lay the groundwork for future studies on this relatively unknown Italian species.

Chapter 1

INTRODUCTION

Rodents, one of the three groups of small mammals, are the members of the order Rodentia, which is the largest within the class Mammalia. To date, 2.277 are the species recognised (Carleton and Musser 2005). This order is considered cosmopolitan because of rodents' colonization ability. The remarkable variety of species enables rodents to inhabit many and various habitats and ecological niches, in which each species plays different roles in shaping biotic or abiotic materials within ecosystems. The direct and indirect influence of these ecosystem engineers modulate the availability of resources, such as shelters, to other species (e.g. small mammals, amphibians, reptiles) (Jones et al. 1994), which can take advantage of the activity of rodents. They also represent an important food source for many vertebrates and actively contribute to the forest renovation by seed dispersal (Amori 2008). On the other hand, most rodents are r-selected species and the population densities can vary considerably in relation to the local conditions. Indeed, cyclic populations of some Arvicolid species reach extremely high densities during the population outbreaks, in some cases up to 1.000 individuals per hectare (Taitt and Krebs 1985), causing severe damages to cultivations, woodlands and foodstuffs. Furthermore, some of the deadliest human diseases can be transmitted by rodents, being relevant vectors of micro and macro parasites (Amori 2008).

Several ecological studies of small mammal communities were carried out in natural and wild ecosystems, such as coniferous forest (see e.g. Maser et al. 1978), beach wood and perennial grass fields (see e.g. Wegner and Merriam 1979). These studies are in connection with ones carried out in agroecosystems and anthropogenic habitats, which are of increasingly concern in studying the structure and dynamics of small mammal populations (Utrera et al. 2000; Andreo et al. 2008). Agroecosystems are mosaic landscapes, heterogeneous in space and time, which may cause significant changes in the small mammal assemblage that occupies the area in relation to diversity, demography and habitat use (Utrera et al. 2000). Furthermore, rural environments are of special interest to public health because of the transmission of several zoonosis from wildlife to humans. Moreover, small mammal populations may reach high densities, making them agricultural pests responsible for heavy economic losses (Capizzi et al. 2014). On the other hand, the high fragmentation and alteration level of these anthropogenic environment could threaten some specialist rodent species' survival or even lead them to local extinction (Amori 2008). For the reasons explained above, the investigation of ecological and biological traits of small mammal

populations in agroecosystem is still of major importance in terms of public health risks, novel methodologies for pest control and safeguard of endangered species.

Among rodents, voles belong to the Cricetidae family, subfamily Arvicolinae, which includes 28 genera and 151 species (Carleton and Musser 2005). Of the Arvicolinae, the genus Microtus is the richest in term of species (62). Such species are widely distributed in the Holarctic region (Asia, North America and Europe), with the exception of arid areas.

In Italy the most widespread vole species is the Savi's pine vole (*Microtus savii*, De Sélys Longchamps 1838), with a more southern distribution than that of its congeners (Fig 1). *M. savii* is distributed in most of the Italian Peninsula, except for Sardinia and north-eastern regions (Trentino Alto Adige, Friuli Venezia Giulia and the northern part of the Veneto), from sea level up to 2,000 m a.s.l. (Santini 1983).

Figure 1 - Distribution of *Microtus* species in Italy.

The taxonomic status of Savi's pine vole is already debated. To far subspecies that have been described are: *M. s. savii* in central and northern Italy (De Sélys Longchamps 1838) and *M. s. nebrodensis* (Minà Palumbo 1868) in Sicily (Contoli 1999; Contoli 2003; Le Louarn and Quéré 2003; Castiglia et al. 2008; Contoli 2008). Other two subspecies, *M. s. tolfetanus* (Contoli 2003) and *M. s. niethammericus* (Contoli 2008) have currently been described in Tolfa Hills, a little area of the central Italy near Rome, and in Gargano (Contoli 2003; Contoli 2008; Castiglia et al. 2008).

In the last years, genetical researches have been carried out to study the phylogenetic relationships among *M. savii* subspecies (Jaarola et al. 2004; Acosta et al. 2010; Gornung et al. 2011). The evidence coming from these researches was a separation between the Calabrian populations and the other Italian ones (Jaarola et al. 2004). This separation is now confirmed by genetical and molecular data (Castiglia et al. 2008) that elevate the *M. s. brachycercus* (von Lehmann 1961), earlier considered a subspecies of *M. savii*, to rank of a species.

Despite its large distribution its ecology and biology are poorly known (Ranchelli et al. 2016). Savi's pine vole occurs in fallow fields, ecotonal areas, banks of ditches and canals, cereals, orchards and forage crops (Osella and Montolli 1986; Cagnin and Grasso 1999; Capizzi and Santini 2007) and wherever there is an abundant herbaceous cover up to 2500 m above sea level. Only occasionally is it found in forests (Cagnin and Grasso 1999). On the contrary, it avoids too hard, dry and rocky soil (Contoli 2008), preferring deep, moist and well-drained soil (Cagnin et al. 1998; Sarà 1998; Cagnin and Grasso 1999).

Its presence is characterized by a well-defined trail system and burrows (Amori et al. 2008). The burrow system consists of several chambers, stores and primary and secondary entrance holes connected by a network of corridors up to 40–50 cm deep (Sarà 1998). Exits are close to the burrow entrance holes that allow voles to feed above ground, mainly on grasses (Capizzi and Santini 2007). Savi's pine vole has a polyphasic daily activity rhythm, alternating short rest and activity periods during both day and night. The body weight varies between 18.7 and 26 grams (D'Errico et al., 1981).

Reproductive activity of the Savi's pine vole extends throughout the year. In the Mediterranean area, breeding season extends from March to November (Salvioni 1983), but it is concentrated especially in spring and summer (Sarà 1998), sometimes with breaks during the summer months, possibly owing to dryness (Salvioni 1983). The small litter size and the prolonged gestation time suggest that the Savi's pine vole could be considered a k strategist within the Microtinae (Caroli et al. 2000).

Few surveys were carried out on wild populations and they concern mainly population abundance studies (Osella and Montolli 1986; Bertolino et al. 2015) and the assessment of seasonal and annual fluctuations within populations (Petretti 1977; Krapp 1982; Massa and Sarà 1982; Contoli et al. 1983; Santini 1983; Sarà and Massa 1985; Siracusa and Ciaccio 1985; Boldreghini et al. 1988; Mangaro et al. 1990; Bon et al. 1993; Capizzi and Santini 2007; Contoli 2008), in order to develop better control strategies of this species.

For this reason, the PhD research herein presented investigated ecological and biological aspects of *M. savii* in anthropic habitats, to lay a useful groundwork for

further field-based studies and novel methodologies, there being a need to explore different control methods and to follow up on the impacts and the interface with trophic relations, as well as the role as prey of this ecosystem engineer small mammal.

Chapter overview

In the second chapter, I present a review of all of ecological and biological aspects of *M. savii* described and studied so far.

As it is considered to be the main cause of rodent-attributed damage in Italy, detailed knowledge of this species is needed, in order to achieve an effective management. However, the available information about this species is fragmentary and incomplete. This review aims to organise available information about Savi's pine vole taxonomy, reproduction, population dynamics, habitat and food preferences, and identify priority areas of future research.

In the third chapter, I describe the development of a successful protocol that maximises trapping success for this species. As the Savi's pine vole is quite difficult to capture with standard trapping procedures, this requires the identification of the active tunnel holes and the placement of the traps directly in front of the exits. I additionally compared capture and recapture rates of Savi´s pine voles in three different trap types in order to assess the best suitable trap for studying this deeply elusive species. Our results may have implications for planning and implementing management strategies based on traps rather than rodenticides, as well as field studies on other fossorial small mammals.

The Ugglan trap, being the most successful in capturing this species, was used for the dynamic population study presented in the fourth chapter. Data on demographic parameters of *M. savii* are not available, thus ecologically-based management strategy are difficult to plan. In this study Savi's pine voles were trapped once a month for one year in two study areas in central Italy in order to assess density, turnover rates, time of residency and survival of the two studied populations.

These results could clarify some of the ecological and biological traits of the target species and could represent a reliable basis for further long-term studies. The evolutionary origin of the *M. savii* dynamics it will be increasingly clear.

In the last chapter, the fifth, I present preliminary results on the diet of Savi's pine vole through analysis of stomach samples collected in Imola, Emilia Romagna (central Italy). By assessing, for the first time, a quantitative approach in studying diet composition of this species, this study opened questions on the food preferences of this ecosystem engineer in an anthropic habitat.

Although recent studies have used DNA-based techniques to determining diet composition by the analysis of stomach samples, the results remain limited to qualitative determination of plant species. Our quantitative technique could a valid alternative for studies of species-level food selection.

Appendix overview

In the Appendix I I present the results of a secondary research carried out during my PhD. The aim of this study is to propose a sampling strategy ("vantage point counts") that allows to monitor roe deer populations and estimate relative abundance. In particular, we investigated the possibility to use the abundance indexes achieved from vantage point counts performed on all the open areas of a study region (complete surveys) for monitoring population trend. Results discourage the use of vantage point counts for monitoring purposes.

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Chapter 2

FROM BIOLOGY TO MANAGEMENT OF SAVI'S PINE VOLE (*MICROTUS SAVII*)

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Abstract
Savi's pine vole (*Microtus savii*) is a rodent species of the *Cricetidae* family, inhabiting southern European agroecosystems. It is considered to be the main cause of rodent-attributed damage in Italy. To achieve an effective management, detailed knowledge of this species is needed. However, the available information about this species is fragmentary and incomplete. In this paper, the existing knowledge of Savi's pine vole taxonomy, reproduction, population dynamics, habitat and food preferences is reviewed in order to organise available information and identify priority areas of future research. Some of the changes in farming practices that have occurred in recent decades may have increased the impact of Savi's pine vole populations in crop fields. To manage this pest species effectively, an integrated strategy is recommended (involving habitat management, trapping and, when appropriate, the use of rodenticides). The apparent lack of cyclical population outbreaks and the relatively small litter size and long gestation and interpartum period of this species suggest that

it could be more manageable than other vole species, while its strict herbivorous diet, stable population size in open habitats and wide distribution seem to indicate it as an ideal model species for risk assessment studies.
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Keywords: *Microtus savii*; crop damage; small mammals; agriculture; pest species; Savi's pine vole

1. INTRODUCTION

Fossorial and semi-fossorial species of rodents are important agricultural pests, and voles are especially relevant pests in Asia, Europe and North America.[1] In fact, in past centuries, voles gave rise to famine in Hungary, the Soviet Union and France.[2]

In Italy, the most damage to agricultural activities is caused by *Microtus* voles. Among the Arvicolinae, the genus *Microtus* is the richest in terms of species (62); these species are widespread throughout the holarctic region (Asia, North America and Europe), the only exception being arid areas. Following the definition by Contreras andMcNab,[3] voles are considered to be fossorial species
that may spend a certain amount of time above ground. Their different degrees of aptitude for underground life may allow a rough distinction among fossorial voles (subgenus *Terricola*, e.g. *M. savii*, *M. multiplex*, *M. subterraneus*) and above-ground-active voles (subgenus *Microtus*, e.g. *M. agrestis*, *M. arvalis*). Voles have a herbivorous diet based mainly on grass, roots and bark, although some species may also consume seeds and invertebrates.[4] Owing to their small size and intense activity levels, voles have a high food intake rate. The common vole (*Microtus arvalis*) is the most abundant wild mammal species in Europe,[5,6] while in Italy it occurs only in the north-east regions. In the other parts of the Italian peninsula and in Sicily, the most widespread species is Savi's pine vole (*Microtus savii*). Savi's pine vole is a fossorial species that nests in a network of tunnels; it is usually found in rural areas, edge clearings of forests, uncultivated fields, meadows and orchards and is held responsible for most of the rodent-attributed damage to agricultural crops.[7] To date, knowledge about this species has been scattered and often based on local studies, despite these voles being considered one of the most important agricultural pest species of the Mediterranean region.

The aim of this review is to summarise the available knowledge about Savi's pine vole. We reviewed taxonomy, distribution, habitat and feeding preferences, breeding biology and social organisation, population dynamics and main predators. We screened the main scientific databases, articles, conference proceedings, books and chapters for publications about this species written in English, French and Italian. We

compared the information about Savi's pine vole with that about other vole species, especially regarding breeding biology, fossorial activity and population dynamics. Furthermore, specific attention was paid to the management of Savi's pine vole in agriculture and control strategies.

This review provides a basis for direct further research on optimising plant protection and risk assessment.

2 SAVI'S PINE VOLE

2.1 Taxonomy and morphology

The taxonomic status of *Microtus savii* is still debated. Several subspecies have been described: *M. s. savii* in northern and central Italy (De Sèlys Longchamps, 1838), *M. s. brachycercus* (von Lehmann, 1961) in southern Italy and *M. s. nebrodensis* (Minà Palumbo, 1868) in Sicily.[8–12] Two other subspecies, *M. s. tolfetanus* (Contoli, 2003) and *M. s. niethammericus* (Contoli, 2008), have recently been described in a small area of central Italy (Tolfa Hills, near Rome) and in Gargano (Apulia) respectively.[8,10,12] Genetic studies recently addressed the phylogenetic relationships between *M. savii* subspecies.[13–15] There is evidence of a separation between Calabrian populations and the other Italian populations,[12,13] but whether *M. s. brachycercus* should be elevated to the rank of a species is not yet clear. According to Galleni et al.,[16,17] morphological, karyological and hybridisation studies suggest that Savi's pine vole is a heterogeneous group at the first stages of speciation, consisting of two different species:[18] *M. savii* in northern and central Italy, and *M. brachycercus* in the south-west. In addition, chromosomal distances revealed differences also between *M. savii nebrodensis* and the group *M. savii savii–M. brachycercus*.[12] These data would be in agreement with the genetic divergence of the clade *brachycercus*, which departed from *M. s. savii* only in the Middle Pleistocene (0.3 – 0.5 million years ago).[12,15]

Regarding Savi's pine vole morphology, some authors argued that the pattern of teeth morphology among the populations of north-central and southern Italy reflects the interaction between morphological and environmental variability.[19] The variability of tooth morphology indeed shows two geographical scales: at a regional level, morphological variability seems to be related to the pressure of climatic conditions, while at a more local scale it may be linked to the fragmentation of areas where small populations undergo ecological isolation, thus developing different morphotypes.[19] At an intraspecific level, north-central and southern populations may be discriminated by the morphology of the first lower molar. Interestingly, the same pattern is also known at an interspecific level within the subgenus *Microtus (Terricola)* between northern and southern species in western Europe.[19]

2.2 Distribution and habitat

Savi's pine vole is widespread in the Italian peninsula, and it is also present in a small portion of southern France and Switzerland. The southern part of the Canton Ticino is the northern limit of its distribution range, and the presence of the species is reported at altitudes between 250 and 700 m.[20] In Italy, Savi's pine vole has been found up to an altitude of 2800 m in Gran Paradiso National Park, Valle d'Aosta.[21] It is present in Sicily, but seems to be missing from the slopes of Etna.[8] The north-eastern boundary of the species' distribution range is the Tagliamento river, between Veneto and Friuli Venezia Giulia.

Savi's pine vole occurs in fallow fields, banks of ditches and canals, ecotonal areas, cereals, forage crops and orchards.[22–24] Only occasionally is it found in forests.[23]

Canova[25] reported the presence of Savi's pine vole in reed beds, hedgerows and poplar plantations. In Sicily, the species is found mainly in open hills and plains, steppes, meadows, pastures and cereal crops.[26] In Switzerland, in the southern Ticino, it lives in meadows with deep and permeable soils, in vineyards and orchards of the lowland areas.[20]

Savi's pine vole also occurs in urban areas, but it avoids too hard,[8] dry and rocky soil, preferring deep, moist and well-drained soil.[23,26,27] However, it may also inhabit sandy soils, peat, clay and soils subject to water logging.[22] Savi's pine vole has a polyphasic daily activity rhythm, alternating short rest and activity periods during both day and night. Home range size ranges from 300 m^2 (females) to 450 m^2 (males).[28] The burrow system consists of several chambers, stores and primary and secondary entrance holes connected by a network of corridors up to 40 - 50 cm deep.[26] Exits are close to the burrow entrance holes that allow voles to feed above ground, mainly on grasses.[22] Nests are often built by modifying pre-existing mole burrows (*Talpa* spp.). Each burrow system consists of several nests that are occupied either simultaneously or successively by several individuals.[28] Individuals who occupy the same tunnels and the same nests do not seem to show a synchronisation of daily activity rhythm.[20] Up to 15 voles use the same nests and share the same area in a colony of closely related individuals.

2.3 Reproductive behaviour and population dynamics

In the Mediterranean area, the Savi's pine vole's breeding season extends from March to November,[20] but it is concentrated especially in spring and summer,[26] sometimes with breaks during the summer months, possibly owing to dryness.[20]

Data from laboratory studies show that females reach sexual maturity at an average age of 50 days, and the first parturition is on average at 73 ± 12 days; the

gestation period is 22 – 24 days,[29] while the interpartum time is on average 30 days. This interval is shorter in spring and winter and longer in summer and autumn, and is not affected by age. The litter size of captive-bred females is on average 2.5 ± 1.1, and is similar to that of females captured in the wild (2.49 ± 0.7).[29]

Furthermore, laboratory data show a reproductive potential of females of 11.8 ± 3.4 litters per year under optimal conditions. The average life expectancy in captivity is less than 2 years (Table 1).[29]

Wild populations consist of promiscuous groups usually formed by 1 – 3 sexually active females, generally an adult male (in summer), some subadults and several juveniles, but composition may vary.[28] Two or more females can nurse their pups in communal nests, taking parental care in turns. Studies carried out in a population of Viterbo in central Italy have shown that, if the mother leaves the nest,[30] it is immediately replaced by a male, possibly the father, or another adult. This behaviour probably relates to the pups' inability to thermoregulate during the first hours of their life. In the case of danger, females carry the newborns in their mouth to safer places.[30] Female co-nesting is, according to Salvioni,[20] one of the main factors causing the highest population densities.

Numerical fluctuations in Savi's pine vole populations are less significant than in other *Microtus* species. Voles species living at northern latitudes (e.g. field vole) usually show large seasonal fluctuations in population size. In northern and central Europe, the common vole is subject to periodic outbreaks, with cycles of 3–5 years.[31] Savi's pine vole, instead, shows both multiannual and seasonal peaks, but no extreme cyclical population outbreaks.

The availability of food is an important limiting factor. The seasonal population pattern has been studied in Switzerland (Canton Ticino), where the population density has minimum values at the end of the summer (<50 individuals ha^{-1}) and a maximum in spring (>100 individuals ha^{-1}).[20]

After the studies by Salvioni in the 1980s and early 1990s, little research effort has been devoted to this rodent. Recently, a study of population dynamics in Italy has been conducted by our group, and results show population density peaks between August and November.[32] Contoli[8] reported density values ranging from 10 to 100 individuals ha^{-1}, which may sometimes rise up to 1000 individuals ha^{-1}.[8]

Table 1 - Activity and reproductive behaviour of captive-bred vole colonies (modified from Caroli *et al.*[29]).

Species	*Activity*	*Mating system*	*Litter size (range)*	*Litter size (mean)*	*Age at puberty*[a] *(days)*
M. agrestis	Above ground	Polygamy	2–8	5[74]	21 (F),[74] 42 (M)[76]
M. arvalis	Above ground	Polygamy	1–9	5[29]	13 (F), 21 (M)[29]
M. californicus	Above ground	Polygamy	1–9	5[74]	21 (F), 42 (M)[77]
M. duodecimcostatus	Fossorial	?	1–4	2.5[29]	60 (F)[78]
M. montanus	Above ground	Polygamy	3–9	6[74]	28 (F)[79]
M. multiplex	Fossorial	Monogamy	1–4	2.5[29]	?
M. ochrogaster	Above ground	Monogamy	1–8	4.5[74]	35 (M),[74] 40 (F)
M. oregoni	Above ground	?	1–6	3.5[74]	27 (F), 45 (M)[29]
M. pennsylvanicus	Above ground	Polygamy	2–8	5[74]	26 (F), 35 (M)[74]
M. pinetorum	Fossorial	Monogamy	1–6	3.5[74]	51 (M), 77 (F)[74]
M. savii	Fossorial	?	1–4	2.5[29]	47 (M), 50 (F)[29]
M. subterraneus	Fossorial	Polygamy	1–5	3[29]	60 (F), 90 (M)[29]
Myodes glareolus	Above ground	Polygamy	2–8	5[75]	32 (F), 49 (M)[74]

[a] M – male; F – female.

2.4 Main predators

The barn owl (*Tyto alba*) is considered to be the main predator of Savi's pine vole in some cultivated areas of central Italy, and it has been hypothesised to be an important factor for biological control of populations.[33] The same conclusion was drawn by Contoli and Sammuri,[34] who studied the diet of barn owls and tawny owls (*Strix aluco*) in the Farma valley (Tuscany). Their study revealed that both species prey upon the same small mammals (representing more than 95% of their diet), although the barn owl was more specialised in the predation of Savi's pine vole, preying on this species at a significantly higher rate (12.5% versus 2.4% for tawny owl). Other studies on barn owl diet showed percentages from 64.2 to 79.4% of rodents in pellets,[35,36] with Savi's pine vole remains accounting for 3.9 – 39.8%.[35,37]

Other important predators of Savi's pine voles are diurnal raptors, such as kestrel (*Falco tinnunculus*) and common buzzard (*Buteo buteo*),[38,39] and mammals such as weasel (*Mustela nivalis*) and red fox (*Vulpes vulpes*).[22,40,41] Predation by snakes is considered to be rare for Savi's pine vole.[37,42]

2.5 Impact of Savi's pine vole in agroecosystems

Savi's pine vole plays an important role in natural ecosystems. Its burrowing activity can change the soil structure, bringing to the surface the soil collected in depth and mixing up the layers. This can affect the composition of the herbaceous plant communities near the burrows. Moreover, the material that these small rodents carry

in and out of their burrows for the construction of nests and as food stocks makes them important agents of seed dispersal of arboreal and herbaceous plant species.[22] However, voles of the genus *Microtus* cause substantial damage to agricultural activities.[22] The strictly herbivorous diet of Savi's pine vole leads to extensive damage to arable crops, in particular in vegetable fields and orchards.[43]

Savi's pine vole feeds on annual and perennial herbaceous plants, both wild and cultivated, preferring the *Graminaceae*, *Leguminosae*, *Chenopodiaceae* and *Compositae*,[21,22,44] mostly within a radius of about 10 cm from the burrow exit hole.[20] During winter, when the grass cover is strongly reduced or covered by snow, Savi's pine voles exploit alternative food sources.[22] In this season, the species causes debarking of trunks and roots just above and below ground level.[45] The reason for this behaviour is probably the search for nutrients present in the phloem. This stripping can remove an entire circular band of bark. Potentially, a single adult vole is able to cause a lethal laceration of a tree in 24 h.[46]

The grass collected at the surface is dragged and eaten in underground tunnels. Thus, during the outbreak periods, Savi's pine vole may cause severe damage to the taproots of sugar beets, and to cereals in their growth and maturation phase.[21] However, the most serious damage is caused to valuable horticultural species such as artichokes (*Cynara cardunculus*), fennel (*Foeniculum vulgare*), lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*), radicchio (*Cichorium intybus*), parsley (*Petroselinum crispum*), garlic (*Allium sativum*), onion (*Allium cepa*) and chard (*Beta vulgaris*).[21] Santini[21] describes the typical way the artichoke plants are attacked: the vole reaches the base of the plant with a tunnel and then eats the soft and juicy inner tissues, rising inside along the axis of the plant. Within 2 or 3 days, the plant wilts and withers.

The most damaged trees are apple, citrus and, to a lesser extent, olive.[22] In Sicily, the problem of vole infestation in citrus orchards has become more and more relevant, causing a significant decrease in profits.[45] Some damage in Sicilian vineyards was recently reported.[39] In an area of approximately 2 ha, 10 – 15% of the grapevines were damaged. Probably the lack of herbaceous cover, weeded out since 2006 to avoid drifts, shifted the food selection to the roots of grapevines.

In Monti Cimini (province of Viterbo), in the winter of 1986 – 1987, in a plot of 4 ha, the species caused the death of 1500 four-year-old apple trees in only 6 days.[46] Infestation of Savi's pine vole also affected northern Italy, with substantial damage on fruit farms.[47] In central Italy, damage caused a remarkable decrease in agricultural production, especially in the province of Pisa. In these regions the most serious damage was detected in horticultural crops, especially in the artichoke. In the province

of Latina, artichokes and melons were severely damaged during outbreak years in 1967 – 1968 and 1970 – 1971.[43]

The most detrimental infestation of this species ever recorded in Italian history occurred in some areas of the Abruzzo, Molise Apulia and Basilicata regions. The crops damaged were artichokes, grass and cereals.[43]

Santini[48] also mentions other areas affected by this phenomenon, such as citrus orchards of Calabria, Basilicata (Gioia Tauro, Rosarno, Lamezia Terme, the Plain of Sybaris and the Ionian coast) and south-eastern Sicily (between the provinces of Catania and Ragusa). Latian apple orchards were damaged by this species (100 ha),[48] helped by the permanent grass cover maintained within plant rows according to new agricultural practices.

D'Errico *et al*.[49] report damage to asparagus, artichoke, iris, hyacinth and tulip cultivation in Campania that caused total yield loss. Furthermore, the environmental changes produced by modern farming techniques (see below) played an important role in this scenario, creating optimal conditions for feeding and reproduction and encouraging population growth.[45]

2.6 Impact of agriculture on Savi's pine vole

Recent changes in the agricultural environment and farming practices have favoured fluctuations in rodent populations in crop fields.[47,50] The permanent grass cover maintained within orchard rows provides food throughout the year. In particular, it allows voles to extend their reproductive activity beyond the usual seasonal limit.[46] In a study conducted in apple orchards of the Cimini hills (Viterbo), about 20% of adult females continued to breed even in autumn and winter.[46] Herbaceous vegetation provides optimal habitat for this species in forage crops, horticulture and orchards.[22] In citrus and apple orchards,[46,50] the replacement of the traditional surface irrigation method (application of water by flooding the entire field) with drip irrigation (sprinklers above canopy or microsprinklers under canopy) leads to a growth of herbaceous cover throughout the year that supports Savi's pine vole population growth. In fact, given the burrowing habits of Savi's pine vole, periodic flooding by surface irrigation is a limiting factor for population growth.[22,50]

Conversely, the mechanisation of agricultural practices in orchards and arable fields that occurred in the second half of the twentieth century has reduced vole damage, because deep tillage prevents the establishment of stable populations. Ecotonal, marginal environments, not subjected to tillage and thus providing shelter for voles, are nowadays greatly reduced.[22]

2.7 Savi's pine vole management

Although Savi's pine vole populations do not go through cycles like other voles (e.g. common vole, field vole), the densities reached and the damage they can cause to vegetable crops and fruit trees may often require an appropriate control strategy. Many studies have been carried out worldwide to identify the best methods to manage vole populations,[1] such as tunnel fumigation,[51] use of repellents in both agriculture and forestry,[52–56] use of natural predators, parasites and pathogens,[57] diversionary feeding[58,59] and barriers and fences.[60,61] However, rodenticides[62] and traps are still the most used techniques.[61,63,64] Both anticoagulants and acute rodenticides (e.g. aluminium phosphide) have been used against voles in Europe. In general, control strategies adopted against other European pest voles are also suitable for implementation against Savi's pine voles, despite the latter being more fossorial. However, in Italy, Savi's pine vole management has been carried out mainly via trapping and poisoning, but the latter method was the most popular, and the most used compound was chlorophacinone.[22] The common practice was to deploy the bait with the rodenticide inside the tunnel entrances, avoiding scattering it on the soil and thus reducing the risk for non-target species.[48] However, in 2007, EU registration of chlorophacinone for use in plant protection ceased. In 2008, a new Italian ministerial regulation prohibited the use of any rodenticide bait outside special containers, strongly limiting (if not actually prohibiting) vole control by rodenticides.

Traps may reduce the number of individuals during outbreaks to supplement the use of toxic baits.[46,48] Capizzi and Santini[22] reported that good control of a Savi's pine vole outbreak was achieved by massive trapping only. Traps should be placed close to the tunnel exits. Santini[46] reported the removal of 90% of voles in 3 – 4 days. Galliano *et al*.[47] integrated the use of traps with rodenticide baits on two fruit farms in the province of Cuneo. Firstly, they used traps to identify the species responsible for the damage and its distribution. Secondly, they applied chlorophacinone at bait points in two separate treatments. This led to a 95% reduction in vole density. In addition, the reduction of potential habitat is useful. Farmers should cut the grass frequently and mow between the trees. Tillage is also important for the destruction of underground tunnels.[7] Moreover, using barn owls and other biological methods has been suggested as profitable, especially where the use of rodenticide is forbidden or strongly limited legally.[65] As there is no single technique that is efficient, economical and safe for non-target species, it is necessary to adopt both preventive (limiting the availability of food resources and burrowing activity) and control strategies (i.e. trapping and/or application of rodenticides).[22,46,48]

3 DISCUSSION

The literature on Savi's pine vole provides an overview of the knowledge about this species. The ongoing taxonomic debate poses problems for assessing the species' distribution. For the time being, it is appropriate to refer to the 'Savii group'. If some taxa are to be elevated to species level, the economic importance of each of them should be reassessed (e.g. see the damage by *M. s. nebrodensis* to orchards in Sicily, as well as damage by *M. s. brachycercus* in Calabria).[22]

Given Savi's pine vole's widespread distribution in Italy and its opportunistic habitat preferences,[8,23] it can be stated that the species can flexibly adapt to cultivated areas. It is not a species of conservation concern, being listed as LC (least concern) in the IUCN Italian Red List.[66] There is no evidence for an increasing relevance as pest, even though Savi's pine vole is actually considered to be a serious local pest in specific crops.

Although this species is no longer subject to control interventions as in past decades, it could be relevant as a target species for the analysis of ecological processes. In fact, stable populations in open habitats (including those intensively cultivated) with a strictly herbivorous diet seem to be an ideal target for this kind of study, with the potential for almost immediate response to experimental treatments.

Compared with other voles, fossorial species such as Savi's pine vole are less prolific than species that are active above ground (Table 2) such as the common vole. Litter size is comparable with that of other fossorial voles such as Mediterranean pine vole, Woodland vole and European pine vole. The small litter size and the long mean gestation time and interpartum period suggest that Savi's pine vole is less an r-strategist than other *Microtus* species (i.e. *M. arvalis*).[29] This might suggest that this species could be more manageable than other species of voles characterised by population outbreaks.

Table 2 - Mating systems of European fossorial voles

Species	*Activity*	*Mating system*
Microtus cabrerae	Semi-fossorial	Monogamy[80]
Microtus duodecimcostatus	Fossorial	Monogamy[81]
Microtus lusitanicus	Semi-fossorial	Monogamy[82]
Microtus multiplex	Fossorial	Monogamy[83]
Microtus oeconomus	?	Polygamy[84]
Microtus subterraneus	Fossorial	Polygamy[28]
Microtus tatricus	?	Polygamy[85]

In reviewing the work published to date on this species, and trying to provide useful information for a more effective management, a gap in the current knowledge

of Savi's pine vole emerged. This gap regards the behaviour of the species. Such knowledge may be of importance for planning monitoring and control programmes, as the most appropriate control programmes imply integrated strategies rather than a single technique.[67]

Adopting the behavioural approach is not easy, especially in cases of fossorial organisms that cannot be observed directly in the field. However, a wider knowledge of Savi's pine vole behaviour may prove essential in order to gain a better understanding of its biology and perform effective control actions.

Savi's pine voles live in groups,[28] but group composition may vary between populations or depending on season. Knowing the group composition would make it possible to estimate the number of animals present in an area, based on the number of tunnels and holes, using for example an active burrow index.[68] This could help to understand whether interventions are needed. Furthermore, the Savi's pine vole's mating system is still unknown. Comparison with other European fossorial species of the genus *Microtus* does not help to make predictions because the majority are monogamous, but there are exceptions (Table 2). Mating systems influence effective population size (N_e): in the case of a promiscuous species, members of the more abundant sex might have a greater opportunity to breed and this would lead to a larger N_e than at monogamy (used here as a basis for comparison). In the case of polygyny or polyandry, instead, N_e declines because fewer males (polygyny) or females (polyandry) contribute genetically to the next generation.[69] Given the species' status as a recognised pest, ascertaining its reproductive strategies would be important and could help to make predictions about population fluctuations.

Models show that the removal of certain age/sex classes from a population may affect population growth rates.[69] Thus, in those species that exhibit infanticide, the removal of males results in a reduction in population size, because immigrating males kill the offspring of deceased or ousted males.[69] Infanticide occurs in some microtine species,[70] but there are no data for Savi's pine vole. Acquiring this information may allow the control of whole populations, targeting only specific demographic classes.[71]

Overall, studies aimed specifically at understanding the behavioural perspective would integrate the purely ecological approach, which is usually adopted in the study of the pests, and may be advantageous to understanding how the target species reacts to habitat manipulations.[72,73]

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Chapter 3

TRAP TYPE AND POSITIONING: HOW TO TRAP SAVI'S PINE VOLES USING THE TUNNEL SYSTEM

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Abstract

Savi's pine vole (*Microtus savii*) is the most widespread Italian vole species, an important rodent pest in agriculture, and yet one of the least studied species. One of the reasons for this gap in knowledge is that members of this species are quite difficult to capture with standard trapping procedures, being fossorial and rarely active above ground. For this reason, we developed a successful protocol that maximises trapping success. This requires the identification of the active tunnel holes and the placement of the traps directly in front of the exits. We additionally compared capture and recapture rates of Savi´s pine voles in three different trap types: INRA, Longworth and Ugglan. If properly equipped with food and nesting material, INRA, Longworth and Ugglan traps showed similar capture rates, whereas Ugglan traps´ recapture rate was the highest of the three kind of traps. These results, in combination with the species´ fossorial and social habits, lead us to conclude that Ugglan traps are the best suited for studies on Savi´s pine voles. Our results may have implications for planning and implementing management strategies based on traps rather than rodenticides, as well as field studies on other fossorial small mammals.

Keywords: Savi's pine vole, Trap success, INRA traps, Longworth traps, Ugglan traps, Small mammals

Introduction

Population monitoring techniques to assess the density, ecology and distribution of small mammals in the wild are largely based on live-trapping (Flowerdew et al. 2004). When planning field studies, particular attention should be devoted to the choice of the most suitable type of trap in relation to the aims of the study. As a matter of fact, good capture rates are the first step for the success of a research, because they provide a representative sample of the examined population.

Considering studies on the relationship between species and habitats, the most common topics of interest regard the composition of communities and the population dynamics of species; both require an efficient live-trapping protocol. In the first case, the aim is to trap the largest possible number of species, and it is commonly accepted that the most efficient solution is to use different trap types (Anthony et al. 2005; Dizney et al. 2008). In the second case, the focus is directed on one or a few target species, and high capture and recapture rates are required for investigating long-term demographic trends. Therefore, it may be useful to use a single type of trap with higher efficiency and lower mortality rates. However, when studying complex communities, including semi-fossorial and fossorial species, more effective systems for above-ground-active species are used, whereas little is known about capturing markedly fossorial species.

The Savi's pine vole (*Microtus savii*) is both the most widespread Italian vole species and, at the same time, one of the least studied from a behavioural and ecological point of view (Bertolino et al. 2015a; Ranchelli et al. 2016). This is mainly because its fossorial habits make it extremely difficult to observe in the wild, as well as to capture (Ranchelli et al. 2016). Savi´s pine voles are strictly fossorial, and spend most of their time in underground tunnels, which they leave mainly to forage (Contreras and McNab 1990). This member of the subfamily *Arvicolinae,* however, can be of great interest, since it is endemic to Italy, inhabits the whole country (Bertolino et al. 2014; Ranchelli et al. 2016), is a serious rodent pest in agriculture (Capizzi et al. 2014), and plays an important role in the trophic and ecological dynamics of many habitats, being a prey for many raptors and small- and medium-sized mammal predators (Amori et al. 2002; Capizzi and Santini 2007; Magrini et al. 2009). From a behavioural and ecological perspective, however, it is very little known, because the few studies conducted on this species so far focused on other aspects, such as phylogenetics, reproductive physiology and impact on crops (Caroli et al. 2000; Castiglia et al. 2008). Increasing the knowledge of Savi's pine voles behaviour and dynamics, however, would prove especially important from a management point of view. Savi´s pine vole, in fact, is considered a pest species because of the damages it can cause to crops when densities are high. During winter, when food availability is

low, Savi's pine voles feed on plants' roots, and may cause extensive debarking, especially to peach, apple, cherry and citrus orchards, as well as artichokes and potatoes (Capizzi and Santini 2007; Bertolino et al. 2015b; Ranchelli et al. 2016).

Field studies on Savi´s pine voles have either employed signs of presence (Bertolino et al. 2015b), or pitfall or snap traps (Osella and Contoli 1986; Cagnin and Grasso 1999). Live-traps, commonly employed in population dynamics studies, were used only occasionally and for short periods, and information on the cost/effectiveness of different trap types are not available for this species. In this article, we present the trapping method that proved most effective to capture this extremely elusive species using the tunnel system dug by the voles.

Materials and methods

In the year 2013 we started a live-trapping research on Savi's pine vole in agroecosystems. A first pilot trial in orchards (kiwi, peach, cherry and apricot) located in Emilia-Romagna where the species was known to be present, due to the high density of holes in the ground. We established a trapping grid with 40 Ugglan and 40 Longworth traps, baited with peanut butter, carrots, apples and oatmeal. We trapped for 10 consecutive nights in each of the 4 orchards, for a total of 40 trapping nights. However, our overall catch was of only two Savi´s pine voles.

We therefore started a new preliminary study in an apple orchard (42.327463 N, 11.977942 E) the following year, to determine the most effective protocol for trapping Savi's pine voles, gathering information on positioning and capture efficiency of three types of trap: INRA (Institut National de la Recherche Agronomique), Longworth and Ugglan. Since we registered high mortality rates for INRA traps – with 4 dead individuals out of 15 trapped animals in two nights-, we decided to activate them only during the day.

Using the information thus acquired, we carried out the study from April to May 2014 in apple and peach orchards of three Italian regions: Tuscany (hereafter A, coordinates: 43.254467 N, 11.835570 E), Emilia-Romagna (hereafter B, 44.370779 N, 11.753046 E) and Piedmont (hereafter C, 44.581134 N, 7.497749 E). We used 120 traps: 40 for each of the three models, INRA, Longworth and Ugglan.

The INRA trap measures 15 x 5 x 5 cm. The lodging has a rectangular shape and is made of zinc; internal parts are made of galvanized sheet metal: these consist in two axes and a bracket. Animals enter the trap via an oscillating trap-door. A plastic rear door allows the extraction of the captured animal (details in Aubry 1950, Girardoux et al. 1998).

The Longworth trap consists of a nesting chamber made of aluminium, measuring 14 × 6.5 × 9 cm, connected to a tunnel (13 × 4.5 × 4.5 cm), equipped at the

other end with a treadle that is triggered by the entrance of the animal (details in Chitty and Kempson 1949).

The Ugglan trap is a multiple-capture wire trap (24 × 8 × 6 cm), with bottom plastic plates and an aluminium lid which covers the sides of the trap, protecting it from rain but allowing partial air circulation through it. A "tramp" tip plate treadle (gravity controlled) allows the animal to reach the bait. A 5 g counterweight repositions the door, locking the animal in and resetting the trap (details in Lambin and McKinnon 1997).

We placed the traps only in front of active holes, that is the exits from the tunnel currently in use (Fig 1, created with Paint). We placed the traps horizontally, directly inside the first section of the tunnel, so that no light passed through; this created an artificial extension of the tunnel itself that left to the vole leaving the nest no choice but to enter the traps. If necessary, we dug out some earth to better position the traps.

In order to identify these active holes, on day 0 we proceeded to close with earth all the holes found in trapping areas, and we marked them with a red stick. After 24 hours, we identified the re-opened holes as active holes, and we placed traps only in front of these (EPPO 1975, Tkadlec and Stenseth 2001). This method is commonly used in Europe to evaluate common voles' abundance indexes (Lisická et al. 2007); Bertolino et al. (2015b) recently used it for Savi's pine voles. The three types of traps were set alternating between them. Each capture session lasted four days. We equipped traps with hay, to provide thermoregulation and nesting material, as well as apples and sunflower seeds as bait. When a vole was caught in a trap, we replaced hay and food. In order to assess recapture rates, we marked all captured individuals with fur clipping. We checked the traps approximately every 8 hours. Since voles of the genus Microtus are polyphasic, and it was our intention to gather information on night and day capture rates, Ugglan and Longworth traps were left open continuously 24 hours/day, whereas INRA traps were de-activated at night to reduce mortality.

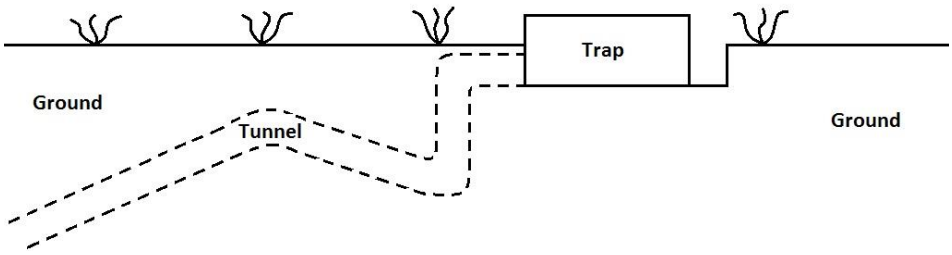

Figure 1 - Scheme of the positioning of the traps.

Data analysis

Considering our protocol, we compared the three trap types using only diurnal first captures, and recaptures. Ugglan and Longworth traps were compared using 24-hours trapping data. The null hypothesis that the efficiency of the three kind of traps was the same among study areas was verified using the Kruskal-Wallis test. When the null hypothesis was accepted, data were grouped among areas, and Chi-square tests were used to compare trapping data. Statistical analyses were performed using the software R (version 3.3.1).

Results

We recorded 73, 39 and 7 capture events of Savi´s pine voles in A, B and C respectively (Tab 1). The diurnal efficiency of traps was similar among study areas, for both first-time captures (Kruskal-Wallis H=0.29, P=0.86) and recaptures (Kruskal-Wallis H=1.09, P=0.58). Then we grouped data of the study areas and we compared the efficiencies of the different trap types. Considering diurnal first captures only, we found no significant difference in the capture rates of the three trap types ($\chi^2 = 0.95$, P = 0.62), but recapture rates differed significantly ($\chi^2 = 7.82$, $P < 0.02$), with Ugglan traps having the higher success. Similarly, 24-hours trapping data were comparable between Ugglan and Longworth for first captures (yates $\chi^2 = 0.69$, P = 0.49), but a higher recapture rate was found for Ugglan traps (yates $\chi^2 = 5.90$, $P < 0.02$).

Table 1 – First captures and recaptures of Savi´s pine voles during day and night.

Study area	Trap Type	First Captures		Recaptures	
		Day	Night	Day	Night
A	INRA	8	/	6	/
	Longworth	10	8	7	8
	Ugglan	6	4	11	5
B	INRA	3	/	1	/
	Longworth	5	3	1	0
	Ugglan	5	4	8	9
C	INRA	0	/	0	/
	Longworth	1	2	0	0
	Ugglan	2	2	0	0

Mortality rates were similar for Longworth (8.33%) and Ugglan (9.37%) traps (Tab 2). During the preliminary study, we verified a mortality rate of 26.67% for

INRA traps when active 24h/24; this rate decreased to 11.11% when INRA traps were deactivated during the night.

Table 2 - Mortality rates of the three trap types for Savi´s pine voles.

Trap type	Captures (M. savii)	Dead	Mortality rate (%)
INRA	15	4	0.27 Before nocturnal deactivation
	18	2	0.11 After nocturnal deactivation
Longworth	24	2	0.08
Ugglan	32	3	0.09

Discussion and conclusions

This is the first study that provides detailed information on trapping procedures for Savi´s pine voles, and on how to maximise trapping success. In order to successfully trap Savi´s pine voles, it is essential to use a protocol that allows the animals to actually enter the traps, of whatever type these may be. In our experience, for the capture of Savi´s pine voles, the trap-positioning protocol could be even considered more important than the trap type itself. Placing the traps only in front of active holes maximises the trapping success. Savi´s pine voles, in fact, dig numerous tunnels and even more exit holes, but often abandon them rather quickly; tunnels can moreover collapse or fall out of use. A noteworthy consequence is that regularly-spaced trapping configurations (i.e. grids, transects, or mixed designs, e.g. Flowerdew et al. 2004) are not suitable for this species, which led to an extremely low number of captures in our preliminary study.

INRA traps have been used for presence/absence studies of small mammals (Giraudoux 1998), but to our knowledge, have never before been included in a comparison of efficiency; Longworth and Ugglan traps are commonly used in field studies on small mammals worldwide (Jacob et al. 2002). They can perform with comparable success (Lambin and MacKinnon 1997), or with very different outcomes, depending on both target species and environmental conditions (Jacob et al. 2002; Ylönen et al. 2003); Anthony et al. (2005), found that Longworth traps were unsuitable for the capture of microtine rodents. For Jacob et al. (2002), it was the Ugglan type that proved much less successful, both for the target species and the environmental conditions in which it was employed. In our case however, all three models of traps have achieved similar capture efficiency during first trapping of animals.

Despite the fact that the statistical analyses did not show a significant difference between INRA's efficiency and the other trap types', we encountered several difficulties in their use that suggest that they are best suited for studies where animals' survival is not fundamental. INRA traps' high mortality rate when left active 24h/24 is probably due to their dimensions. Being so narrow, they can contain little food, and even less hay, to allow the vole space to enter and breathe, and allow very little movement. Little food and no nest material cause thermoregulation problems for the night, and high stress levels that will naturally conduce to an increased mortality, especially if traps are not frequently checked. If INRA traps are deactivated when temperature drops or controls are more frequent, however, they have mortality rates comparable to those of the other types considered. Therefore, since the aim is to capture live Savi's pine voles causing as little stress as possible, these traps need to be checked every few hours, or deactivated in case of harsh weather conditions.

Longworth and INRA traps present the further disadvantage of saturation; once the first animal is captured, they remain ineffective until they are checked (Andrzejewski et al. 1966). We have also noticed that, the efficiency of Longworth traps could decrease in conditions of bad weather because the mud obstructs the trap trigger mechanism, and therefore prevents the closure of the traps.

Ugglan traps have efficiency at first captures and mortality rates comparable to the Longworth traps; this result is in accordance with previous studies in other small mammals species (Lambin and McKinnon 1997). Ugglan traps, however, present fewer problems of thermal insulation, both in hot and cold weather, provided that they are equipped with nesting material. They have moreover a significantly higher recapture rate, making this model more efficient for studies involving the application of capture and recapture demographic models.

This set of considerations lead us to conclude that the more suitable trap type for the Savi´s pine vole is the Ugglan trap. It is equally efficient in every season, has the highest recapture rate, requires little maintenance, besides being quite inexpensive. Moreover, it allows multiple captures of live animals, which represent an advantage in the case of a species both social and little known from the behavioural point of view as the Savi´s pine vole. Finally, recent trends in European policy concerning biocides (i.e. EU Regulations 1107/2009 on plant protection products and 528/2012 on Biocides), make it urgent to identify an alternative strategy for rodent control based on techniques that do not involve the use of rodenticides. Our results may be useful for planning and implementing management programs relying on trapping, thus avoiding rodenticides and, as a consequence, the risks for non-target species.

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Supplementary pictures

Picture of study area A.

Picture of study area B.

Picture of study area C.

Pictures of the three different trap types: Ugglan (1), Longworth (2), INRA (3).

Chapter 4

DEMOGRAPHIC PARAMETERS OF TWO SAVI'S PINE VOLE (*MICROTUS SAVII*) POPULATIONS IN AGROECOSYSTEMS

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Keywords: small mammals, live trapping, orchards, mark-recapture, rodents.

Abstract

The Savi's pine vole (*Microtus savii*) is an Italian species living in grasslands and agroecosystems, where it is generally considered a pest because it may damage crops and orchards. The extent of the damage depends on population density and temporal food availability. However, data on demographic parameters are not available, making ecologically-based management strategies difficult to plan. Therefore, we conducted a study on Savi's pine voles demography for one year in two study areas in central Italy performing the capture-mark-recapture method. Density values ranged from 3 to 32 ind./ha, the highest population densities occurred in October, while the lowest occurred in February-April in both study areas. Turnover rates of both populations were very high, with time of residency usually no longer than two months. Juveniles´ survival was constant in both study areas, but it differed between the two populations regarding adults.

The two populations showed relatively small intra-annual fluctuations, but whether these should be attributed to a phase of a multi-annual cycle is still to be ascertained. Moreover, the obtained results could be explained by an adaptation of these

populations to anthropic environments, and need to be compared with data from populations living in more natural habitats.

Introduction

Savi's pine vole (*Microtus savii*) is the most widespread vole species of Italy. However, despite its large distribution, its ecology and population dynamics are still poorly known (Ranchelli et al. 2016). This fossorial rodent belongs to the *Cricetidae* family (subfamily *Arvicolinae*) (Carleton and Musser 2005), though its taxonomic status is still under debate due to the presence of subspecies (*M. s. savii*, *M. s. nebrodensis*, *M. s. tolfetanus* and *M. s. niethammericus*), some of which highly genetically differentiated (Bertolino et al. 2015a; Bezerra et al. 2016; Castiglia et al. 2008; Contoli 2003; Galleni et al. 1991, 1998; Gornung 2011; Jaarola 2004). The habitat of Savi pine voles is mainly represented by grasslands, ecotonal areas, fallow fields, banks of ditches and canals, as well as agricultural crops and orchards (Cagnin and Grasso 1999; Capizzi and Santini 2007). Savi's pine voles lives in a system of underground burrows, and feeds on annual and perennial herbaceous plants, both wild and cultivated (Capizzi and Santini 2007; Caroli 1992). During late autumn and winter, it may cause extensive debarking of tree roots and stems, leading ultimately to plant death, being therefore considered as a pest species (Capizzi et al. 2014). Thus, as for many other rodents (Bertolino et al. 2015a), the management of this species should be considered where damages risk to become too severe.

Anecdotal observations (e.g. Contoli 2008) suggested that Savi's pine vole populations may exhibit both multiannual and seasonal fluctuations, although extreme cyclical population outbreaks are not reported as it happens for common voles, (e.g Jacob and Tkadlec 2010).

To date, few studies have investigated the biology of the Savi's pine vole focusing on demography, and no information is available regarding demographic structures (as reviewed by Ranchelli et al. 2016). To partly fill this gap, we carried out a study on demography of two populations living in farmed areas, to evaluate density fluctuations and patterns of survival and recruitment along the year.

Materials and methods

Study area

This study was carried out in two peach orchards of 2 ha each. These areas are in two regions of central Italy, Emilia-Romagna and Tuscany, where climatic conditions are similar.

The study area in Emilia Romagna is located near the town of Imola (44°21′ N, 11°42′ E), in a highly-fragmented, predominantly rural area, with average annual

rainfalls of 750 mm and mean annual temperatures between +2.6°C and +23.7°C. Savi's pine voles here coexist with common voles (Microtus arvalis), with the latter attaining very low densities. The study area in Tuscany is located near the town of Foiano della Chiana (43°15′ N, 11°48′ E), hereafter Foiano, an area relatively far away from urban settlements, with average annual rainfalls of 700 mm and mean annual temperatures between +5.8°C and +23.0°C.

The only vole species to be found in this area is the Savi's pine vole. Both areas consist of peach orchards, similarly managed, with trees ageing from 5 to 15 years, with a between-trees distance ranging from 1.5 to 3 meters (depending on age), a between-rows distance of 4.5 meters, and permanent grass-cover among rows. Both areas are treated with insecticides, fungicides, and herbicides, but not rodenticides.

Trapping and handling

This study was performed by the capture-mark-recapture method (Nichols and Pollock 1983). Sampling took place monthly, from July 2014 to June 2015 in Imola, and from August 2014 to July 2015 in Foiano. Each sampling session lasted 8 days, with traps kept active continuously for 6 trapping days, for a total of 144 hours of sampling, which remained constant throughout the year. Multiple-capture live-traps (Ugglan Special Traps n. 2, Grahnab AB, Hillerstorp, Sweden) were used, whose number (N = 183) remained constant both through sessions and study areas. Traps were baited with apples and provided with cotton for thermoregulation and lowering of stress response.

A preliminary study showed that Savi's pine voles do not enter traps scattered in the field, and the only way to effectively trap them is to place the traps horizontally, directly inside the first section of the tunnel, so that no light passes through (unpublished data). For this reason, it was crucial to pinpoint 'active' holes, that is holes belonging to currently-used tunnels. To do this, on the first day of every sampling session, the study area was walked through in all its width, and every tunnel holes were closed (Bertolino et al. 2015b). After 24 hours, re-opened (active) tunnel holes were counted and traps were placed only in these, to optimize catching success. Traps were checked every 8 hours in summer, and every 4-5 hours in winter, to reduce trapping mortality.

Captured animals were marked using a syringe-injected, 1.4 mm x 9 mm ISO FDX-B glass transponder (Planet ID GmbH, Essen, Germany). Animals weighing less than 10 grams were marked by fur clipping, and with PIT tags when recaptured once over this weight threshold. Animals were assessed for weight, sex, age and breeding condition. Males with scrotal testes and females with apparent nipples, open vagina, vaginal plug, or evident pregnancy were considered as reproductively active individuals. Age classes were defined in relation to weight: we considered subjects

with body weight ≤15g as juveniles, and subjects >15g as adults. Data were recorded on the transponder reader at every capture.

Data analysis

Data on population abundance and demographic parameters were analysed using the program MARK version 8.0 (White and Burnham 1999). We used an information-theoretic approach to select models that were most informative using the Akaike Information Criterion corrected for small sample size (AICc) (Pradel 1996). Candidate models were ranked based on the AICc score and we used delta AICc (ΔAICc) and the Akaike weights (w_i) to select models with the best support. A ΔAICc < 2 suggests substantial evidence for the model. The Akaike weights indicate the probability that the model is the best among the whole set of candidate models.

We first tried to fit a Closed Robust Design Multistate Model, because we were interested in separating male and female groups and the two conditions of animals as juveniles and adults. However, the results were not reliable, especially for the very huge number of juveniles recorded in some months. Therefore, we used a Robust Design approach to estimate the population densities, and a Multistate Recapture Model to evaluate survival. The Pollock's Robust Design can be used when the trapping scheme is composed by primary periods between which the population is open to immigration and emigration, and secondary periods that are close together temporally when populations may be considered closed. In our cases, the population was considered closed during the 6-days monthly sessions, while it was considered open from one month to the other. The Robust Design allows for estimating the probability of first capture (p), the probability of recapture (c) and the population size (N); it allows also to estimate temporary emigration factors here not considered. The Multistate Recapture Model was used to evaluate the survival (S) of juveniles, and of adult males and females between monthly periods.

Turnover, defined as the rate of renewal of the population, was calculated using this formula: $[(N_r+N_l)/N_{rd}]x100$ (Bertolino el al. 2001), where N_r is the number of recruits (animals caught during session *t* that were not present before); N_l is the number of losses (animals trapped during session *t* - 1 and not caught in session *t* or after); N_{rd} is the number of residents (animals present in session *t* - 1 that were recaptured at time *t* or after).

Time of residency was defined as the time during which a vole can be considered present in the population (Briner et al. 2007). It was computed as the time passed between the first and the last capture event, plus two weeks (half of inter-session time). Only adult individuals captured at least twice, in different sessions, were considered for this analysis. A χ^2 test was used to compare time of residency of

males and females, and between study areas. The comparison of body weight of adult voles was done pooling data in 4 periods of 3 months using three-way ANOVA and LSD test for post hoc comparisons.

Results

Population densities ranged from 8 ind./ha to 32 ind./ha in Imola and from 3 ind./ha to 30.5 ind./ha in Foiano. Density values followed a similar trend in the two areas, with an increase from July to October, a slow decrease till March-April followed by a rapid increase (Fig 1).

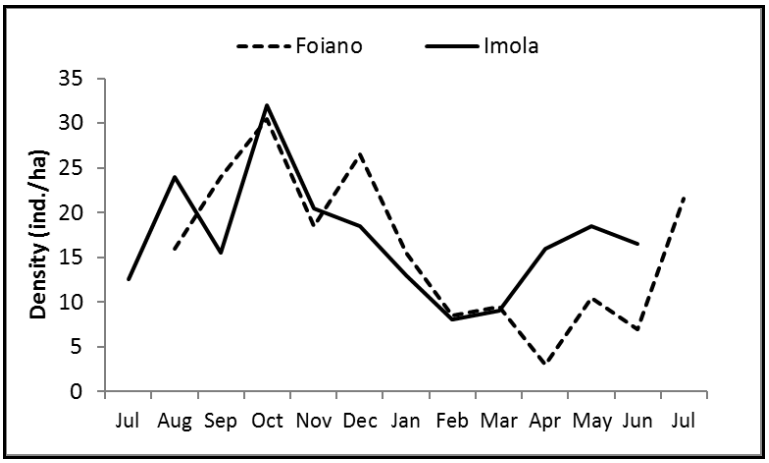

Figure 1 – Population densities (n° of ind./ha) trends.

The juveniles/adults ratio, showed an increase in the percentage of juveniles in both populations in the late spring, with a peak in July, where juveniles made up 54% of all captures (Fig 2). Even the number of reproductively-active individuals varied over time for both populations, with maximum values in in late winter and spring and lowest values in summer (Fig 3).

The best supported multistate recapture model for Imola indicated a constant survival probability in juveniles (0.56±0.14), and a temporal effect in adults (Fig 4). In Foiano a first model indicated a constant survival probability both in juveniles (0.39±0.11) and adults (0.49±0.04) (AICc=350, w_i=0.63). There was, however, a substantial evidence for the support of a second model as well, with the same constant survival probability in juveniles (0.39±0.11), and a sex-effect in adults with a slightly higher survival in males (0.51±0.06) compared to females (0.47±0.06) (ΔAICc=1.88, w_i=0.24) (Tab 1).

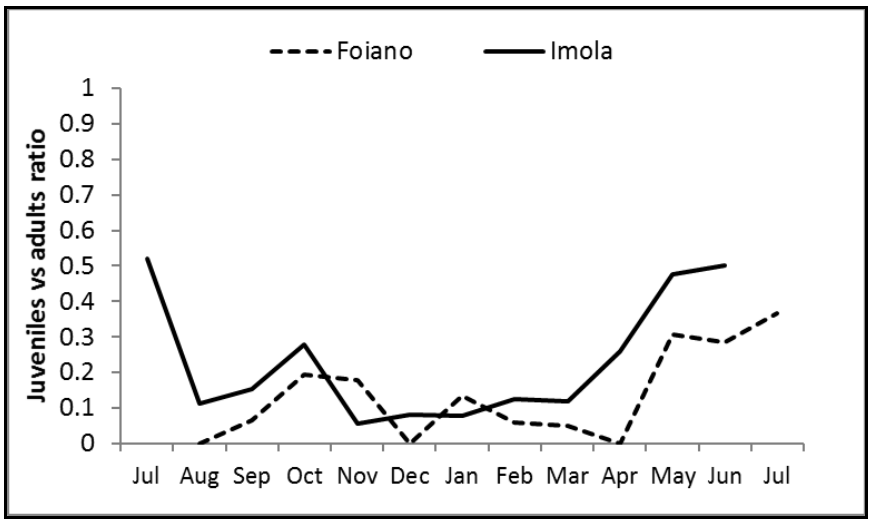

Figure 2 – Monthly juveniles/adults ratio in the study areas.

Turnover rates (Fig 5) of both populations were conspicuously high: from 60% to 900% in Imola, from 30% to 500% in Foiano; in nearly all capture session, the number of recruits and losses (i.e. turnover rate) was more than twice the number of residents (mean turnover rate of 230±245 in Imola; 246±144 in Foiano). Time of residency, consequently, was quite short in both study areas, being on average 88±7.9 days in Imola, 73±5.4 days in Foiano.

The average time of residency was not significantly different neither between males and females (Imola: χ^2=1.8; df=1; p=0.17. Foiano: χ^2=0.6; df=1; p=0.43) nor between study areas (χ^2=1.5; df=1; p=0.22). No statistical differences were observed in the sex ratios among seasons (Foiano: χ^2=0.2; df=3; p=0.98. Imola χ^2=2.9; df=3; p=0.41) and study areas (χ^2=0.6; df=1; p=0.43). Differences in body weight were investigated with sex and season as fixed factors, and study area as random factor. Test of between-subjects' effects revealed the presence of statistically significant differences for sex ($F_{1,3,219}$=438.4, p=0.03; marginal mean, male = 21.53 female = 21.04), but not for study area (p > 0.27) and season (p > 0.38). The analysis did not reveal any two- (at least p > 0.31) or three-way interaction (p > 0.18).

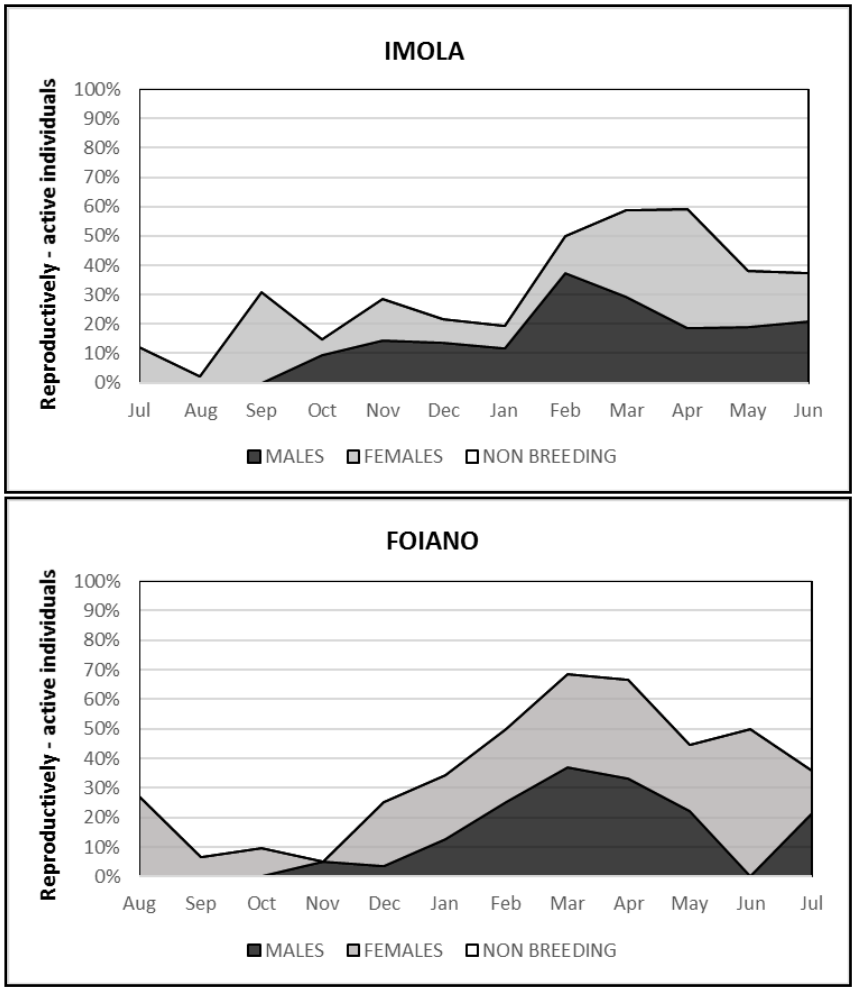

Figure 3 – Proportion of reproductively – active males and females.

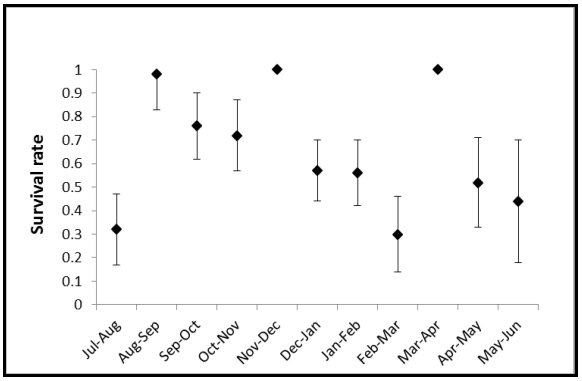

Figure 4 – Adults survival rate in Imola.

Table 1 – Outputs of the first Multistate Recapture Models for the two study areas of Foiano and Imola. Juv = juveniles; Ad = adults.

Model	AICc	Δ AICc	AICc weight	Model likelihood	No. Parameters	Deviance
Foiano						
Survival Juv (.) Survival Ad (.) p Juv (.) p Ad (.)...	350.675	0.000	0.626	1.000	6	196.820
Survival Juv (.) Survival Ad (sex) p Juv (.) p Ad (.)...	352.562	1.887	0.244	0.389	7	196.548
Survival Juv (.) Survival Ad (t) p Juv (.) p Ad (.)...	353.817	3.142	0.130	0.208	16	177.229
Survival Juv (.) Survival Ad (sex*t) p Juv (.) p Ad (.)...	355.016	4.341	0.079	0.173	17	175.841
Imola						
Survival Juv (.) Survival Ad (t) p Juv (sex) p Ad (.)...	627.578	0.000	0.350	1.000	16	305.153
Survival Juv (.) Survival Ad (t) p Juv (.) p Ad (.)...	628.199	0.621	0.257	0.733	15	307.774
Survival Juv (.) Survival Ad (t) p Juv (sex) p Ad (sex)...	629.326	1.748	0.146	0.417	17	304.901
Survival Juv (.) Survival Ad (t) p Juv (.) p Ad (sex)...	629.983	2.405	0.105	0.300	16	307.558
Survival Juv (.) Survival Ad (t) p Juv (sex) p Ad (t)...	630.973	3.395	0.064	0.183	21	298.546
Survival Juv (.) Survival Ad (t) p Juv (.) p Ad (t)...	631.663	4.085	0.045	0.130	20	301.236

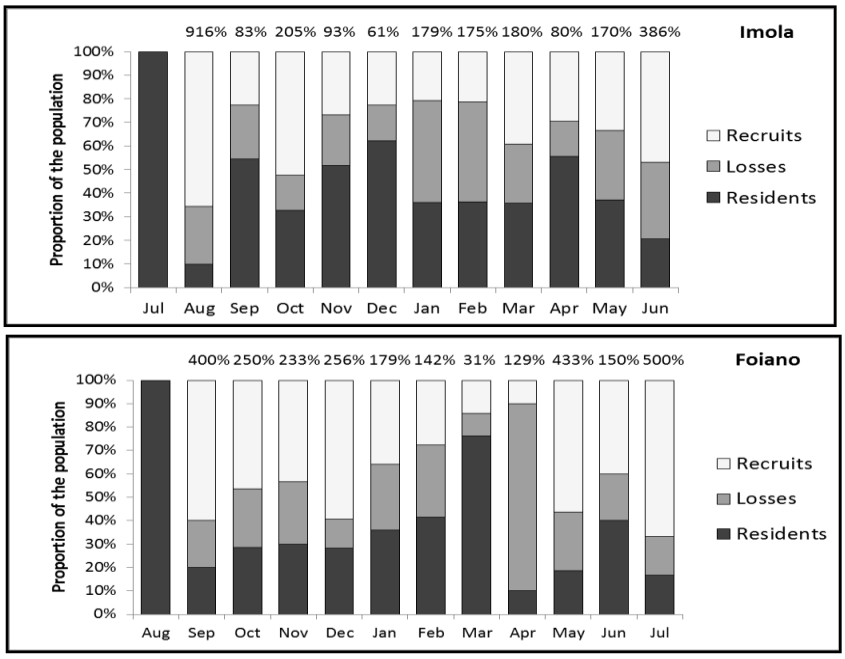

Figure 5 – Turnover rates in Imola and Foiano.

Discussion

Density values recorded for Savi's pine vole in our study areas (3-32 ind./ha) were much lower than those reported for other species of the genus *Microtus* in Europe, e.g, *Microtus arvalis* (Briner et al. 2007) and *Microtus agrestis* (Burthe et al. 2010). These values appear rather low even when compared to previous studies of the same species. Salvioni (1995) reported a minimum of 50 ind./ha and a maximum of 100 ind./ha in Switzerland, while Contoli (2008) reported density values ranging from 10 to 100 ind./ha, which could rise to 1000 ind./ha. None of these studies, however, was

conducted in orchards, so there is no real basis for comparison for this type of environment. In our study, densities in both areas did not exceed 30-32 ind./ha, attaining minimal values in winter, rising in spring, and reaching peak values in autumn. Density peaks in late spring and mid-autumn occur in the months in which most births are concentrated.

Monthly fluctuations were rather small, compared to those reported for other European species (Jacob and Tkadlek 2010; Korpimäki et al. 2004). However, our research considered only one year, and further long-term studies are therefore needed to better understand the presence of regular cycles and outbreaks in this species.

Our data regarding reproductively-active individuals show how the breeding period of this species extends throughout the year. Salvioni (1995) indicated a breeding season extending from March to November, with maximal concentration in spring and falls in August. Conversely, our studied populations showed no interruption of the reproductive period, with a peak of reproductively-active individuals between the end of winter and spring.

The juvenile/adult ratio reaches the maximum values in June and July. This can be explained considering that the average gestation period ranges from 22 to 24 days (Caroli et al. 2000), and new-borns reach independence around 24–25 days of age (Santini 1983). The presence of new-borns during the winter month could depend on the availability of food resources, which in our study areas remained relatively abundant throughout the year. Neither population appeared completely stable, though, because they both exhibited very high turnover rates and quite a short time of residency.

In general, survival rates were much lower than those of other fossorial microtine species of Mediterranean Europe (e.g. *Microtus duodecimcostatus*, Paradis & Guédon 1993). Populations were totally renewed in a very short time, and this cannot be attributed only to the normal generational renewal (see survival rates). One possible explanation may be a high predatory pressure, since this species is the prey of choice of raptors as the kestrel (*Falco tinnunculus*) and the barn owl (*Tyto alba*), but also of Tawny owl (*Strix aluco*) and Long-eared owl (*Asio otus*) (Capizzi & Luiselli 1998) both present in the study areas. Moreover, Savi's pine voles are an important part of the diet of terrestrial predators like the red fox (*Vulpes vulpes*) and the weasel (*Mustela nivalis*) (Ranchelli et al. 2016).

Population models indicate that change in survival are more important than reproductive output in determining voles' population cycles (Norrdahl and Korpimäki 2002; Korpimäki et al. 2004). For instance, early studies in the seventies (e.g. Krebs and Myers 1974) showed that juvenile survival is a major factor in determining the population growth in some American *Microtus* species. Since our results show that

juvenile survival did not vary between seasons in both study areas, they may partly explain the absence of population cycles in Savi's pine voles. However, a sampling conducted over a longer time should corroborate these findings, confirming (or not) the stability in the juvenile survival in both populations.

Density values reported here were unexpectedly low; however, due to the temporal limit of the study we could not say if these were the bottoms of synchronized cycles, or the mean values in these orchards. Our data on turnover and time of residency indicate a high renewal rate in both populations. Ascertaining if Savi's pine vole represent a threat for these kinds of crops even at such low densities through damage assessment would certainly be important. However, only a more specific study focused on space use, and dispersal during the year, could help to improve the understanding of Savi's pine vole's population dynamics.

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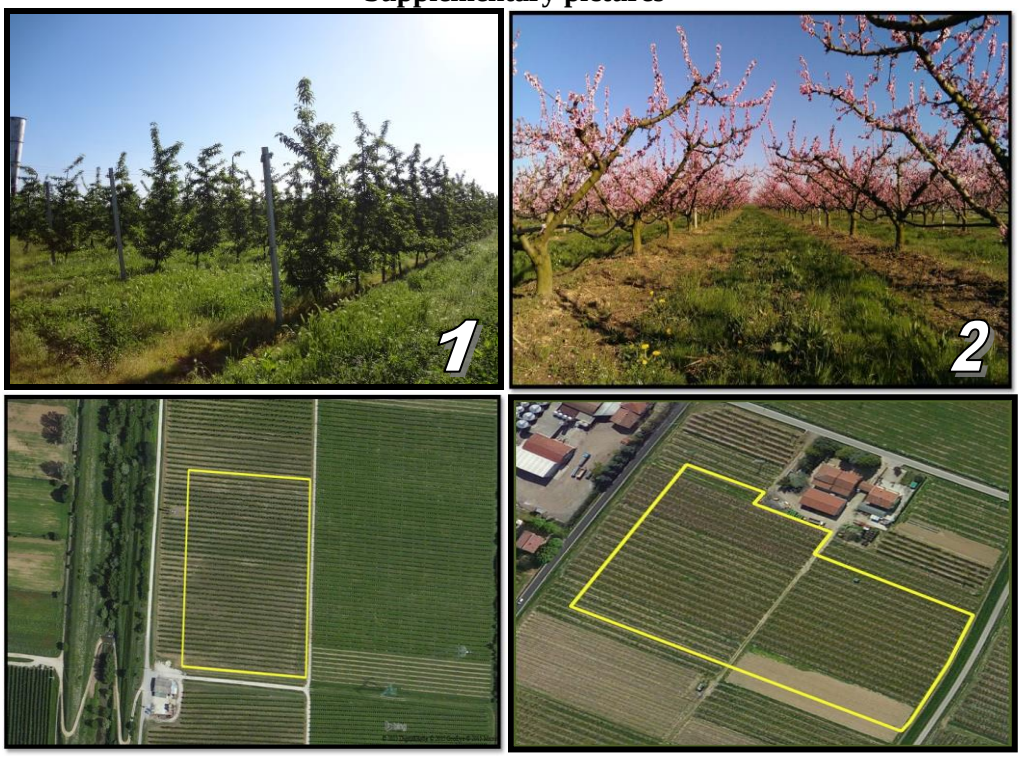
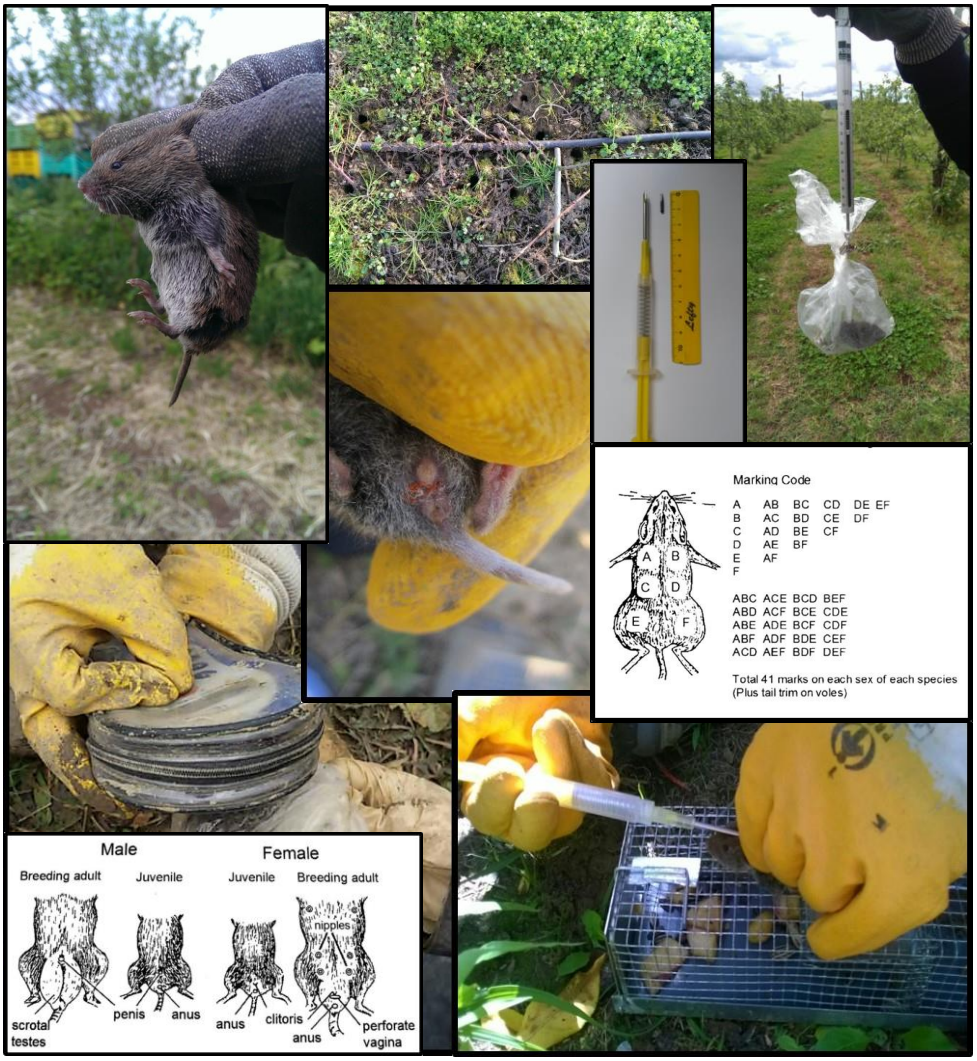
Supplementary pictures



Pictures of the two study areas: Foiano della Chiana (1) and Imola (2).



Pictures about the data collection carried out during the study.

Chapter 5

THE DIET OF SAVI'S PINE VOLE: A NEW QUANTITATIVE APPROACH

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Manuscript in preparation

Introduction

The complex mosaic of ecosystems and land use forms that characterize arable lands, represent a broad variety of habitats for plant and animal communities. Crops, meadows, fallow lands, trenches and canals constitute a miscellaneous vegetation cover that represent an important food resource for many animals. The most numerous vertebrates that inhabit these different habitats are small rodents (Janova et al. 2016).

Small rodents, like all synanthropic species, benefit greatly from their association with human settlement (Francis & Chadwick 2012). Spatial distribution of these mammals, such as voles and mice, and their abundances in agricultural landscapes are largely influenced by their food preferences and the distribution of preferred crops (Heroldová et al. 2008). It has been shown that small rodents increase the use of a food item if its availability is increased, although it is unclear what regulates this relationship (Batzli et al. 1981; Lundberg 1988; Gross et al. 1993; Hobbs et al. 2003) and what influence has the availability of an alternative food item (Pusenius et al. 2003). Anyway, even though scarcely investigated for most species, the variability of small rodent diets among different habitats reveals that food availability often influences diets (Batzli and Pitelka 1983; Batzli and Henttonen 1990; Tast 1991), population density and reproduction of small rodents (Ylonen et al. 2003; Htwe & Singleton 2014). Furthermore, predation risk affects both the time invested on feeding and small rodent habitat selection, choosing for more sheltered habitats (Hamback et al. 1998; Ylonen & Brown 2008).

Recently this kind of study focused on pest species, where accurate knowledge of which items they are eating is fundamental to determine crop damages and to plan new management strategies (Khanam et al. 2016; Huang et al. 2016). Voles are important rodent pest species in European agriculture; they are folivorous species

preferring green foliage, although some species may also consume seeds roots and invertebrates (Abt and Bock 1998) and they rely on abundant and quickly renewable food resources (Heroldová et al. 2008).

The Savi's pine vole is the most widespread vole species in Italy. This fossorial rodent belongs to the *Cricetidae* family (subfamily *Arvicolinae*) (Carleton and Musser 2005). Savi's pine voles lives in a system of underground burrows. The burrow system consists of several chambers, stores and primary and secondary entrance holes connected by a network of corridors up to 40 - 50 cm deep (Sarà 1998). The habitat of Savi pine voles is mainly represented by grasslands, ecotonal areas, fallow fields, banks of ditches and canals, as well as agricultural crops and orchards (Cagnin and Grasso 1999; Capizzi and Santini 2007), where it is generally considered a pest because of it damages crops and orchards (Capizzi et al. 2014).

Anecdotal observations (e.g. Contoli 2008) suggested that Savi's pine vole diet mostly consists on annual and perennial herbaceous plants, both wild and cultivated and the species mainly prefers *Graminaceae*, *Leguminosae*, *Chenopodiaceae* and *Compositae* (Santini 1983; Capizzi and Santini 2007), that the animal consumes mostly within a radius about 10 cm from the barrow exit holes (Salvioni 1995). These Data about its diet and food preferences in agroecosystem are scares.

Diet analysis is a challenge in this species with a fossorial and elusive behavior. In fact, knowledge on the food preference are lacking and based on the evidence of damages on crops (Santini 1977, Capizzi 2007). Recent diet analysis studies have stated that DNA-based techniques are useful in diet determination when the food is a mixture of plant cuticle fragments in faecal or stomach samples and is not identifiable by morphological criteria, or when the diet cannot be deduced by feeding behavior (Soininen et al. 2009). It has been shown by Valentini et al. (2009a, b) that a short chloroplast DNA fragment, the P6 loop of the *trn*L (UAA) intron, can act as a minimalist barcode. Thus, several authors have recently been able to analyze the diet of various herbivore species by combining this method and next-generation sequencing (NGS) (Rayé et al. 2011; Hibert et al. 2013; Pegard et al. 2009; Baamrane et al. 2012).

Using DNA barcoding and qPCR we analyzed stomach content of Savi's pine voles, caught in a peach orchard, in order to recognize plant species and to evaluate their ratio in the stomach samples. This is the first study that analyzed Savi's pine vole diet and food preferences. However, despite its large distribution, its ecology and population dynamics are still poorly known; therefore, this study is useful both to gain knowledge of feeding ecology and impact in croplands of a strictly fossorial rodent.

Material and methods

Vole trapping

The research site (44°21′ N, 11°42′ E) was located near the town of Imola, in Emilia Romagna, northern Italy. It is a highly-fragmented, predominantly rural area, with average annual rainfalls of 750 mm and mean annual temperatures between +2.6°C and +23.7°C. The study was carried out in a peach orchard of 1 ha, with trees ageing from 5 to 15 years, with a between-trees distance ranging from 1.5 to 3 meters (depending on age), a distance between-rows of 4.5 meters, and permanent grass-cover between rows. This area is cultivated according to traditional farming methods, periodically treated with insecticides, fungicides, herbicides but not rodenticides.

Vole sampling was conducted by snap trapping every two months, from November 2014 to September 2015. In each sampling session traps were kept active 24h for 6 trapping nights, for a total of 144 hours of sampling. Traps, placed closed to the holes, were checked every 8 hours. Voles were dissected and their stomachs were stored at -18°C. We analysed the stomach contents of 84 individuals.

Plant availability

Food availability was evaluated by sampling vegetation in order to determine species composition and richness. We carried out the vegetation sampling every trapping session following the following steps: partition of the study area into 2500 quadrats of size 2x2 m; random selection with replacements of 40 quadrats; partition of the selected quadrats into 100 sub-quadrats of size 20x20 cm; random selection without replacements of 10 sub quadrats; identification and quantification of the coverage of each plant species in the selected sub-quadrat. Statistical estimation of the percentages of coverage (availability).

Diet Analysis

Real-Time PCR vs. conventional PCR

Real-time PCR, also called quantitative PCR or qPCR, provides a straightforward and elegant method for determining the amount of a target sequence or gene that is present in a sample.

In conventional PCR, the amplified product, or amplicon, is detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. DNA is detected in the gel by adding an intercalating agent which binds to the PCR product. When exposed to ultraviolet light the agent fluoresce with a light colour, intensifying almost 20-fold after binding to DNA. Fluoresce is therefore detected after the amplification reaction has been completed, usually after 30–40 cycles, and this final

fluorescence can be used to back-calculate the amount of template present prior to PCR. This method of quantification, however, can give somewhat inconsistent results.

A typical PCR has, in fact, two phases, an exponential phase followed by a non-exponential phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. This is the non-exponential, plateau phase where the reaction efficiency decreases and the amount of DNA produced at each cycle does not double anymore and quickly decreases.

In a conventional PCR amplification plot, fluorescence initially remains at background levels, and increases in fluorescence are not detectable even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. As PCR reagents are consumed the reaction slows and enters the plateau phase. These effects can vary from sample to sample, which will result in differences in final fluorescence values that are not related to the starting template concentrations. The data collected at the reaction endpoint are not uniform even when identical samples are being amplified. The data spread of endpoint values demonstrates that data measured following canonical PCR amplification are not uniform or reproducible enough to be useful for the precise measurements required for quantitative analysis. For applications that do not require a great deal of copy number discrimination, such as qualitative studies which seek to determine whether a target sequence of interest is present or not, end- point measurements are generally sufficient. For this reason, conventional end-point PCR is also known as end-point semi-quantitative PCR.

In contrast to conventional PCR, real-time PCR or quantitative PCR (qPCR) allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”. This allows quantification of the template to be based on the fluorescence signal during the exponential phase of amplification, before limiting reagents or inactivation of the polymerase have started to have an effect on the efficiency of amplification. Fluorescence readings at these earlier cycles of the reaction will measure the amplified template quantity where the reaction is much more reproducible from sample to sample than at the endpoint.

Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labelled sequence-specific primers or *probes*. A Specialized thermal cycler equipped with fluorescence detection

modules is used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each PCR cycle.

The main advantage of real-time PCR over conventional PCR is that real-time PCR allows the operator to determine the starting template copy number with accuracy and high sensitivity over a wide range of DNA samples. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). Additionally, real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a close-tube system, opportunities for contamination are reduced and the need for post-amplification manipulation is eliminated.

In qPCR, the cycle number at which enough amplified product has accumulated to yield a detectable fluorescent signal is called the threshold cycle, or C_T. When a number of samples are tested for relative DNA concentrations, the resulting plots of fluorescence vs. cycle number for all the samples are set with their background fluorescence at a common starting point (a process known as baseline correction). Then, the threshold cycle number, or C_T value is set above the background but still in the exponential phase of amplification for all the plots. Since the C_T value is measured in the exponential phase when reagents are not limited, the C_T value can be used to reliably and accurately calculate the amount of template present in the reaction and therefore directly correlated to the initial DNA concentration of each sample. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early, C_T. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, C_T.

Real-Time PCR efficiency parameters

A powerful way to determine whether a qPCR assay is optimized is to run serial dilutions of a DNA template of known concentration and use the results to generate a standard curve. Following amplification of the dilution series, the standard curve is generated by plotting the log of the starting quantity of DNA template against the C_T value obtained during amplification of each dilution. The plot of these points should generate a linear regression line. This line represents the standard curve. If the aliquotting is accurate and the efficiency of the amplification does not change over the range of template concentrations being used, the dilution series will produce amplification curves that are evenly spaced. Ideally, the amount of PCR product will

perfectly double during each cycle of exponential amplification. The spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (i.e. the difference between the C_T values of the curves). For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, n = 3.32, and the CT values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce a linear standard curve. The linearity is denoted by the R^2 value (or Pearson Correlation Coefficient) and should be very close to 1 (> 0.985). The R^2 value of a standard curve represents how well the experimental data fit the regression line, that is, how linear the data are. Linearity, in turn, gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template DNA copy numbers. A linear standard curve implies that the efficiency of amplification is consistent at varying template concentrations. When the R^2 value is very close to 1 (> 0.985) the value of the y-axis (C_T) in the standard curve plot can be used to accurately predict the value of X (initial DNA concentration). Thus, comparing the C_T values of the samples of unknown concentration to the standard curve allows the quantification of starting DNA concentration.

The slope of the regression line in the standard curve provides an index of the PCR amplification efficiency. This is the rate at which a PCR amplicon is generated, commonly expressed as a percentage value. If a particular PCR amplicon doubles in quantity during the exponential phase of its PCR amplification then the PCR assay has 100% efficiency. The amplification efficiency is calculated using the slope of the regression line in the standard curve. With a 10-fold serial dilution of DNA, a 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification. Amplification efficiency, E, is calculated from the slope of the standard curve using the following formula:

$$E = 10^{-1/\text{slope}}$$

The amount of PCR should double during each amplification cycle, that is, there will be a 2-fold increase in the number of DNA copies with each cycle. This translate to a reaction efficiency of 2. E is converted into a percentage as follows:

$$\% \text{ Efficiency} = (E-1) \times 100\%$$

For example, if at the end of each cycle the amplicon number increases 1.954-fold, the amplification efficiency is 95.4%, that is 95.4% of the template has been amplified. Using the efficiency equal to 2 in the equation above, $2 = 10^{-1/\text{slope}}$, indicates that the

optimal slope of the standard curve will be -3.32. The absolute value of the slope is the same as the ideal spacing of the fluorescent traces described above. A standard curve slope of -3.32 therefore indicates optimal, 100% PCR amplification efficiency. Slopes more negative than -3.32 (e.g. -3.9) indicate reactions that are less than 100% efficient. Slopes more positive than -3.32 (e.g. -2.5) may indicate low sample quality, pipetting problems, probe degradation (see below) or coamplification of nonspecific products, such as primer-dimers. The presence of inhibitors can also result in an apparent increase of efficiency. This is because samples with the highest concentration of template also have the highest level of inhibitors, which cause a delayed CT, whereas samples with lower template concentrations have lower levels of inhibitors, so the CT is minimally delayed. As a result, the absolute value of the slope decreases and the calculated efficiency appears to increase. If the reaction efficiency is > 105%, PCR primers and/or probes (see below) should be redesigned.

Real Time PCR chemistries

The most commonly used chemistries to monitor the amplification of the target sequence are the DNA-binding dye SYBR Green I and sequence-specific Taqman hydrolysis oligonucleotide probes. The Taqman probe takes advantage of fluorescence resonance energy transfer (FRET) to ensure that specific fluorescence is detected only in the presence of amplified product. A target-specific oligonucleotide probe is designed and synthetized so that its sequence is complementary to the portion of DNA sequence specific of the target being tested (e.g. a plant species). The probe is labelled with a reporter fluorophore at the 5' end and a quencher at the 3' end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step of the amplification reaction, the probe hybridizes to the target sequence. As the thermostable polymerase (e.g. Taq) reach the site where the probe ha hybridized to the template, it cleaves off the reporter thanks to its 5' $\rightarrow$ 3' exonuclease activity. As a result, the reported is separated from the quencher, and the resulting fluorescence signal is proportional to the amount of amplified product in the sample. A Taqman assay, also known as 5'-nuclease assay include two primers for PCR amplification of the target sequence and target-specific probe. The main advantage of using Taqman probes include high specificity. The disadvantage are that the initial cost of the probe is relatively high and the assay design may be not trivial.

A Taqman assay can be used to assess both presence/absence and concentration of a particular target in a sample. In our study, the target is a specific plant species which presence and concentration need to be tested in the stomach content (sample) of *Microtus savii*. If the target species which the probe has been designed for is present in

the sample, then the probe find its complementary sequence and hybridize to the target. The Taq polymerase cleaves off the reporter and fluorescence is emitted. The presence of the target is determined and its concentration in the sample quantified in real time. If the target is a species of plant other than the one the probe has been designed for, then the probe finds no complementary sequence and will not hybridize to the target. The reporter is not cleaved off and no fluorescence is emitted during the amplification process.

Presence/absence and quantitation of plasmid DNA in *M. savii* stomach contents

The real-time PCR amplification profile of our study was design to test presence/absence and DNA copy number of 34 candidate plant species (targets) in 84 *M. savii* stomach contents (samples).

Genes sequenced for all 34 candidate plant species were searched for in the Barcode of Life Data (BOLD) System. The sequence of the plastid DNA gene coding for the large subunit of the Ribulose-1,5-bisphosphate carboxylase/oxygenase, commonly known by the abbreviations RuBisCO large subunit or *rcb*L, was available in the BOLD System for all candidate species and was therefore chosen as target sequence for our study. The RuBisCO is an enzyme involved in the first major step of carbon fixation by plants. The 34 *rcb*L sequences were aligned using CodonCode Aligner (CodonCode Corporation) and species-specific polymorphic sites were identified in the sequence of target species. A Taqman assay was designed to include target-specific probes of approximately 20 bp, which included the segregating sites, and PCR primers for amplification of a fragment of less than 200 bp. Taqman probes were labelled with flourescin (FAM) reporter and nonflouorescent quencher (NQF). They also included a minor groove-binging (MGB) moiety on the 3´ end that acted to stabilize annealing to the template.

Whole DNA was extracted from the entire stomach content using a custom protocol modified from Sambrook and Russell (2001).

For each of the 34 target species, a total of 114 qPCRs were performed using species-specific Taqman assays. Amplification reactions included 84 samples, one negative control and a standard dilution series made of five 10-fold serial dilutions of target DNA extracted from an herbarium sample and quantified using a Qubit dsDNA BR assay kit in a Qubit 3.0 fluorometer. Three replicates of each dilution point in the standard curve were performed to ensure statistical significance.

Real-time PCR experiments were performed in a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) equipped with a Fast 96-well block. Amplification reactions were conducted in 20 µl total volume containing 10 µl Taqman Fast Advanced Master Mix, 1 µl Taqman assay and 2 µl of sample DNA.

Thermal-cycling profiles consisted of a denaturation step at 95 °C for 20 s, followed by 40 cycles of 1 s at 95 °C and annealing / extension of 20 s at 60 °C.

Performance of Real-Time PCR reactions were evaluated by the Pearson Correlation Coefficient and the slope of the regression line of the standard curve. Average R^2 was 0.997±0.001SE (range: 0.964-1.000). Average slope values of the standard curve was -3.314±0.046SE (range: -2.478-3.676; three values only below above -3.211) resulting in an average PCR reaction efficiency of 100.7%±2.5SE (range: 87.1%-102.9%).

Statistical Analysis

To determine selectivity we used the sign test to compare proportion of use versus availability, where food-plant selection is assessed separately for each plant type. The resulting p-values are combined in an overall test statistic whose significance is determined permuting sample observations. The "phuassess" package for the R software, available from Comprehensive R Archive Network (CRAN), allows to straightforwardly perform the assessment (Fattorini et al. 2014). The H_0 hypothesis consist in food use proportional to its availability; if H_0 is rejected the combination of the sign test individuates the set of preferred and avoided plant species.

Results

Diet

During the whole year we created a floristic list made up of 34 species. Plants' DNA was successfully amplified from all 84 stomach contents. Comparison with GenBank database allowed identification of 31 (91%) of the 34 sequences to species level and 3 (9%) to family level.

Each stomach sample contained an average of 17.5 species (SD 4.0, range 8-28). Field surveys of our peach orchard showed that food availability varies between species and periods. The main grass species are represented by *Elytrigia repens*, *Plantago lanceolata*, *Plantago major* and *Taraxacum officinale* that made up about 70% of the available food for the voles (Tab. 1).

DNA analysis

Using real-time PCR to quantify each plant species in the stomach contents of voles collected in different periods we showed that Savi's pine voles' diet is composed mostly of groundsel (*Senecio vulgaris*). This species makes up approximately 55% of the food intake of *M. savii*, but can sometimes rise to 80-85%. The remaining species, like *Elytrigia repens*, *Erigeron sp.*, *Sonchus sp.*, *Portulaca oleracea*, do not usually get above an average of the 10% of the intake (Tab. 2).

Food preference analysis

Sign test analysis showed that plant species are not used proportionally to their availability ($P < 0.001$) (Tab. 3). In particular there are 7 species that voles always avoid (*Bellis perennis*, *Geranium pusillum*, *Plantago lanceolate*, *Plantago major*, *Setaria verticillata*, *Taraxacum officinale* and *Trifolium pratense*) and one species that is always used proportionally to its availability (*Geranium rotundifolium*). Regarding to other species the selection or avoidance changes during the time.

Table 1 – Food availability based on percent cover of each plant species.

Species	Nov.	Jan.	Mar.	May	Jul.	Sep.
Amaranthus retroflexus	0.0%	0.0%	0.0%	0.0%	2.2%	2.5%
Avena barbata	0.0%	0.0%	0.0%	0.0%	2.9%	1.9%
Bellis perennis	0.9%	12.0%	0.0%	1.1%	0.6%	0.4%
Cardamina hirsuta	0.3%	0.0%	0.0%	0.0%	0.0%	0.0%
Capsella bursa pastoris	1.3%	0.0%	0.0%	0.5%	0.0%	0.0%
Cirsium arvensis	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Cynodon dactylon	0.0%	0.0%	0.0%	0.0%	3.2%	5.5%
Echinochloa crus-gallii	0.0%	0.0%	0.0%	0.1%	0.0%	0.3%
Elytrigia repens	38.2%	14.4%	50.7%	21.8%	26.0%	25.9%
Erigeron sp.	0.6%	1.1%	0.0%	3.3%	1.8%	1.9%
Geranium dissectum	0.0%	0.0%	0.0%	0.3%	0.0%	0.0%
Geranium pusillum	0.5%	1.6%	0.0%	1.8%	0.0%	0.0%
Geranium rotundifolium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Hordeum bulbosum	0.0%	0.0%	0.0%	3.9%	2.2%	0.5%
Lolium sp.	0.0%	0.0%	0.0%	0.1%	2.9%	2.4%
Matricaria chamomilla	0.0%	0.0%	0.0%	0.4%	0.3%	0.2%
Malva neglecta	4.1%	1.2%	0.0%	1.2%	0.2%	0.7%
Medicago lupulina	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
Plantago lanceolata	11.1%	26.0%	0.0%	14.6%	14.1%	12.6%
Plantago major	5.1%	1.6%	3.3%	4.2%	12.1%	12.7%
Poa annua	0.0%	0.0%	0.0%	7.4%	6.7%	3.2%
Poa trivialis	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Portulaca oleracea	0.0%	0.0%	0.0%	0.4%	0.0%	0.6%
Prunus persica	-	-	-	-	-	-
Rumex conglomeratus	0.0%	0.0%	0.0%	0.0%	0.6%	0.3%
Rumex crispus	0.0%	0.0%	0.0%	0.9%	1.4%	0.3%
Senecio vulgaris	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%
Setaria verticillata	0.0%	0.0%	0.0%	0.0%	2.3%	9.2%
Sonchus sp.	1.3%	0.0%	0.0%	0.4%	0.0%	0.0%
Stachys arvensis	5.0%	0.8%	6.7%	0.1%	0.0%	0.0%
Taraxacum officinale	24.0%	34.1%	37.3%	34.9%	18.1%	17.2%
Trifolium pratense	0.0%	0.0%	0.0%	0.8%	1.0%	0.6%
Trifolium repens	7.7%	6.7%	0.0%	1.6%	1.4%	0.9%
Veronica persica	0.0%	0.5%	2.0%	0.2%	0.0%	0.0%
TOTAL	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table 2 – Diet composition based on food intake of each plant species, determined by real-time PCR.

Species	Nov.	Jan.	Mar.	May	Jul.	Sep.
Amaranthus retroflexus	1.8%	1.0%	0.4%	0.8%	2.5%	0.1%
Avena barbata	1.5%	4.7%	7.4%	2.9%	0.3%	0.0%
Bellis perennis	0.1%	0.1%	0.0%	0.8%	0.1%	0.0%
Cardamina hirsuta	0.1%	0.0%	0.0%	0.2%	0.0%	0.0%
Capsella bursa pastoris	0.1%	0.0%	0.0%	0.2%	0.0%	0.0%
Cirsium arvensis	0.1%	0.1%	0.0%	4.8%	0.1%	0.0%
Cynodon dactylon	1.1%	0.0%	0.0%	0.3%	0.1%	0.0%
Echinochloa crus-gallii	0.6%	0.1%	0.0%	1.0%	0.0%	0.0%
Elytrigia repens	6.0%	12.0%	18.6%	0.6%	3.1%	3.5%
Erigeron sp.	4.3%	2.8%	17.9%	16.5%	2.4%	0.7%
Geranium dissectum	0.3%	0.1%	0.1%	0.2%	0.1%	0.0%
Geranium pusillum	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
Geranium rotundifolium	0.4%	2.5%	1.2%	1.0%	0.1%	0.0%
Hordeum bulbosum	0.0%	0.3%	0.2%	0.3%	0.0%	0.0%
Lolium sp.	0.1%	0.7%	1.1%	0.7%	0.1%	0.0%
Matricaria chamomilla	0.4%	0.4%	0.4%	0.4%	0.1%	0.0%
Malva neglecta	1.1%	2.7%	1.1%	0.5%	0.4%	0.1%
Medicago lupulina	0.2%	0.4%	0.1%	0.3%	0.1%	0.0%
Plantago lanceolata	3.0%	0.3%	2.7%	5.3%	0.1%	0.0%
Plantago major	0.5%	0.1%	0.6%	0.4%	0.0%	0.0%
Poa annua	0.0%	3.2%	2.1%	0.0%	0.3%	0.0%
Poa trivialis	0.2%	0.5%	0.9%	0.3%	0.1%	0.0%
Portulaca oleracea	0.2%	0.0%	0.0%	0.5%	7.7%	11.8%
Prunus persica	1.9%	5.5%	2.0%	5.5%	1.6%	0.1%
Rumex conglomeratus	0.3%	0.6%	0.0%	0.4%	0.0%	0.0%
Rumex crispus	1.6%	3.8%	0.1%	2.0%	0.0%	0.0%
Senecio vulgaris	66.9%	38.4%	32.7%	17.0%	71.6%	82.9%
Setaria verticillata	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%
Sonchus sp.	5.1%	10.6%	4.8%	16.1%	6.3%	0.3%
Stachys arvensis	0.1%	0.0%	0.1%	0.0%	0.0%	0.0%
Taraxacum officinale	0.7%	2.8%	1.2%	0.5%	0.1%	0.1%
Trifolium pratense	0.1%	0.1%	0.0%	4.4%	0.0%	0.0%
Trifolium repens	1.1%	5.7%	2.5%	6.8%	2.7%	0.2%
Veronica persica	0.3%	0.1%	1.7%	0.2%	0.0%	0.0%
TOTAL	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table 3 – Food preferences of Savi's pine vole. Species with "red" background are avoided, "yellow" are proportionally used and "green" are selected. Plant species on the top constitute a higher percentage of stomach content than species at the bottom of the table.

RANK	November (n = 20)	January (n = 15)	March (n = 10)	May (n = 15)	July (n = 13)	September (n = 11)
	P=0.00003	P=0.00098	P=0.02148	P=0.00134	P=0.00366	P=0.00879
	H_0: Rejected	H_0: Rejected	H_0: Rejected	H_0: Rejected	H_0: Rejected	H_0: Rejected
1	*S. vulgaris*	*S. vulgaris*	*S. vulgaris*	*S. vulgaris*	*S. vulgaris*	*S. vulgaris*
2	*E. repens*	*E. repens*	*E. repens*	*Erigeron sp.*	*P. oleracea*	*P. oleracea*
3	*Sonchus sp.*	*Sonchus sp.*	*Erigeron sp.*	*Sonchus sp.*	*Sonchus sp.*	*E. repens*
4	*Erigeron sp.*	*T. repens*	*A. barbata*	*A. retroflexus*	*E. repens*	*Erigeron sp.*
5	*P. lanceolata*	*A. barbata*	*Sonchus sp.*	*T. repens*	*T. repens*	*Sonchus sp.*
6	*A. retroflexus*	*R. crispus*	*P. lanceolata*	*P. lanceolata*	*A. retroflexus*	*T. repens*
7	*R. crispus*	*P. annua*	*T. repens*	*C. arvensis*	*Erigeron sp.*	*T. officinale*
8	*A. barbata*	*T. officinale*	*P. annua*	*T. pratense*	*M. neglecta*	*M. neglecta*
9	*C. dactylon*	*Erigeron sp.*	*V. persica*	*A. barbata*	*P. annua*	*A. retroflexus*
10	*M. neglecta*	*M. neglecta*	*G. rotundifolium*	*R. crispus*	*A. barbata*	*M. chamomilla*
11	*T. repens*	*G. rotundifolium*	*T. officinale*	*E. crus-gallii*	*C. arvensis*	*G. rotundifolium*
12	*T. officinale*	*A. retroflexus*	*M. neglecta*	*G. rotundifolium*	*M. lupolina*	*A. barbata*
13	*E. crus-gallii*	*Lolium sp.*	*Lolium sp.*	*S. verticillata*	*M. chamomilla*	*S. verticillata*
14	*P. major*	*R. conglomeratus*	*P. trivialis*	*B. perennis*	*T. officinale*	*P. lanceolata*
15	*M. chamomilla*	*P. trivialis*	*P. major*	*Lolium sp.*	*B. perennis*	*E. crus-gallii*
16	*G. rotundifolium*	*M. lupolina*	*A. retroflexus*	*E. repens*	*Lolium sp.*	*V. persica*
17	*G. dissectum*	*M. chamomilla*	*M. chamomilla*	*P. oleracea*	*C. dactylon*	*P. major*
18	*R. conglomeratus*	*P. lanceolata*	*H. bulbosum*	*T. officinale*	*P. trivialis*	*M. lupolina*
19	*V. persica*	*H. bulbosum*	*M. lupolina*	*M. neglecta*	*G. dissectum*	*P. trivialis*
20	*P. trivialis*	*V. persica*	*S. arvensis*	*P. major*	*P. lanceolata*	*B. perennis*
21	*P. oleracea*	*G. dissectum*	*R. crispus*	*M. chamomilla*	*G. rotundifolium*	*C. dactylon*
22	*M. lupolina*	*C. arvensis*	*G. dissectum*	*R. conglomeratus*	*S. verticillata*	*G. dissectum*
23	*B. perennis*	*B. perennis*	*R. conglomeratus*	*C. dactylon*	*E. crus-gallii*	*T. pratense*
24	*C. arvensis*	*E. crus-gallii*	*B. perennis*	*M. lupolina*	*G. pusillum*	*C. hirsuta*
25	*C. bursa pastoris*	*P. major*	*T. pratense*	*H. bulbosum*	*H. bulbosum*	*C. arvensis*
26	*C. hirsuta*	*T. pratense*	*E. crus-gallii*	*P. trivialis*	*S. arvensis*	*C. bursa pastoris*
27	*S. arvensis*	*S. arvensis*	*C. arvensis*	*C. bursa pastoris*	*P. major*	*S. arvensis*
28	*Lolium sp.*	*P. oleracea*	*P. oleracea*	*C. hirsuta*	*T. pratense*	*Lolium sp.*
29	*T. pratense*	*C. hirsuta*	*C. bursa pastoris*	*V. persica*	*C. bursa pastoris*	*H. bulbosum*
30	*H. bulbosum*	*C. dactylon*	*C. hirsuta*	*G. dissectum*	*V. persica*	*G. pusillum*
31	*G. pusillum*	*C. bursa pastoris*	*C. dactylon*	*G. pusillum*	*C. hirsuta*	*P. annua*
32	*P. annua*	*S. verticillata*	*G. pusillum*	*P. annua*	*R. crispus*	*R. conglomeratus*
33	*S. verticillata*	*G. pusillum*	*S. verticillata*	*S. arvensis*	*R. conglomeratus*	*R. crispus*

Discussion

The DNA-barcoding of stomach contents collected in the wild clearly appears to be a powerful method (Soininen et al. 2009; Valentini et al. 2009a, b), allowing such a resolution that both temporal and inter-individual variability (as well as intra-individual variability when such data can be collected) can be investigated. The resolution of the *trn*L approach is therefore much higher than plant cuticle identification using microhistology (Pegard et al. 2009; Soininen et al. 2009). This technique in association with real-time PCR represent a useful method for quantifying the amount of DNA in a sample (Wong & Medrano 2005). In our study, we demonstrate that real-time PCR on individual voles can be used for evidencing food preference and feeding patterns. The advantage of real-time PCR is that it allows to evaluate food intake for individual voles where food intake is in a small amount. Using specific primer targeting each plant present in the study area, we could ascertain both composition and quantity of food-plants in the vole stomachs. We successfully reconstructed the diets of Savi's pine vole in an agroecosystem where it could be considered a pest.

This is the first study that analyse the diet of Savi's pine vole at the plant species level. Our results showed that there is a high variability in the diet composition, with a range of species that changes among individuals and periods. The plants availability varied across seasons, but the highly anthropogenic environment and the frequent mowing carried out in the study area, may alter the natural phenological cycle of plants, so that seasonal plants become annual and can be available throughout the year.

Voles usually are selective feeders with regard to plant species, genders and genotypes (Virjamo et al. 2013); our results regarding food preferences showed that Savi's pine vole do not use plant species proportionally to their availability. In particular, we found a strong selectivity of Savi's pine vole for some herbaceous species like the *Senecio vulgaris*, the *Sonchus sp.* and the *Portulaca oleracea*, almost always selected during the year. Despite this, many plant species that are present in the vole's diet, are avoided.

Furthermore, looking at the ten species most frequently found in the stomachs, we noticed that between March and May, when there is a reduction in the groundsel (*Senecio vulgaris*) amounts, other species are more frequently eaten. This could be due to the fact that, in spring, there is an increase in both diversity and abundance of plant species. However, in the course of the whole year, these species never exceed the 20% of the food intake. A different set of considerations should be made, however, for the *Prunus persica*. Since Savi's pine vole feeds only on roots of peach tree, we did not compare the stomach contents of this plant with a specific availability, because we

considered the availability of this species as ad libitum. Even though this plant is never represented above the 5% in the voles' stomachs, except for some individuals with intakes amounting to approximately 20% in their stomachs, we found the *Prunus persica* present in all samples.

These results seem to contradict some existing literature reporting that Savi's pine vole feeds only in a 10cm radius around its tunnel exit holes. It is evident, however, that to achieve such high percentages in the consumption of a plant species like groundsel, which was registered at availabilities lower than 2-3%, requires a great deal of exploration. Furthermore, this study highlighted a selective feeding behaviour of Savi's pine vole confirming the same result found for others vole species (e.g. *Microtus arvalis*, Jacob et al. 2014). To now we are not able to explain the reasons of the obtained food preferences, and the nutritional value of some species over others. Thus, this results open new questions that should be deeper investigated.

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Supplementary pictures

Picture of the study area and random vegetation sampling.

Pictures of the floristic list created during the study.

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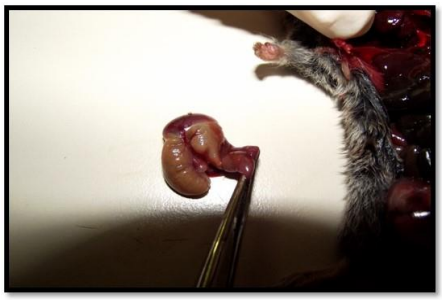

Picture of the samples collection.

Chapter 6

CONCLUSIONS

The research presented in this dissertation was initiated to investigate the ecology of the Savi's pine vole in agroecosystems, with a specific focus on their demographic parameters and diet. The Savi's pine vole is the most widespread vole species in the Italian peninsula; it is a fossorial vole that lives in rural areas, edge clearings of forests, uncultivated fields, meadows and orchards. To date, knowledge about this species has been scattered and often based on local studies, despite these voles being considered one of the most important agricultural pest species in the Mediterranean region. To achieve an effective management, detailed knowledge of this species is needed.

In order to obtain accurate estimates of population density in agroecosystems, it was necessary to first evaluate the catchability of this species, difficult to achieve due to its fossorial behaviour. The results of my first research indicated that Ugglan traps are the best suited for studies on Savi's pine vole, but above all that the trap-positioning protocol is more important than the trap type itself (Dell'Agnello et al. 2016). This method was consequently adopted in my research of demographic parameters of two populations in central Italy.

Based on a capture-mark-recapture protocol, my research showed that the two populations recorded density values lower than those of other species of the genus *Microtus* in Europe. Monthly fluctuations were quite contained, with the absence of those major outbreaks reported for other European species (Jacob and Tkadlek, 2010). Both populations exhibited very high turnover rates and quite a short time of residency that indicate a high renewal rate. However, these results may be hardly generalised, as our research consisted of a single-year study. We investigated demographic parameters that contribute to fill the gap in the current knowledge of Savi's pine vole, but clearly further long-term studies are needed to better understand the presence of regular cycles and outbreaks in this species. Furthermore, Savi's pine vole's population dynamics could be better understood only through more specific studies focused on space use and dispersal during the year.

By combining the real-time PCR method and the DNA barcoding it has been possible to analyse the diet structure both qualitatively and quantitatively. These innovative molecular techniques showed a composite diet (with an average of 17.5 different species) both across individuals and periods. According to results of real-time PCR and estimated food availability, we could evaluate more accurately food preference of Savi's pine vole, including information on both individual intake and plant community structure. Our results indicated that Savi's pine vole did not use plant

species proportionally to their availability. We found a strong selectivity of Savi's pine vole for some herbaceous species like the *Senecio vulgaris*, the *Sonchus sp.* and the *Portulaca oleracea*, almost always selected during the year. Furthermore, seven species were always avoided while selection or avoidance of the remaining species changed during the year. Moreover, we found the *Prunus persica* present in all samples making up approximately 5% of the diet of the voles. This plant species therefore is scarcely consumed by Savi's pine voles, and even if the consumption takes place throughout the year, the amounts should not be considered a threat to the orchards at any time.

These results open many questions about why they prefer or avoid a given species, or which are the cost-and-benefit trade-offs in less appetizing species consumption. Moreover, the result that the peach species is always present in almost all stomachs, could represent a starting point for studies about the impact of Savi's pine vole populations on agriculture. In conclusion, examining further Savi's pine vole ecology from a demographic and feeding behaviour point of view, my research sets the groundwork for improving the management and control strategies of this species.

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Appendix I

ARE VANTAGE POINT COUNTS A RELIABLE METHOD FOR MONITORING ROE DEER POPULATIONS?

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Abstract

Vantage point counts are primarily adopted to estimate the densities of roe deer populations for harvest planning. To this purpose, counts should be performed only within those blocks where it is possible to relate counts to block extents. If vantage point counts are simply carried out on all the open blocks in a study region, the expectations of total counts could alternatively be used as relative abundance indexes. In most cases, surveying all the open blocks is too demanding in terms of operators, time and organization. Therefore, vantage point counts are performed only on a portion of the open blocks, and total count expectations are estimated from these counts. If the blocks are selected by means of probabilistic sampling schemes and statistically sound estimators of total count expectations are adopted, then the estimation of the sampling errors, construction of confidence intervals and assessment of change are possible, together with a post hoc power analysis for evaluating the probability of failing to detect a change in the expectations. The aim of this study is i) to consider some sampling strategies that allow the performance of all these statistical steps and ii) to check the performance of these strategies on a hunting district located in Tuscany (Central Italy), in which all the open blocks were surveyed in 2013 and 2014. The results provide evidence of the imprecision of the estimators. Even for large sampling fractions of 40-50%, the relative standard errors never decrease below 20%, while the corresponding powers in detecting a change of 30% at a level $\alpha = 0.05$ are always smaller than 0.65. The results highlight the need to adopt alternative strategies

that are simple, efficient and robust. In this context, the use of mark-resighting combined with the Bowden estimator may constitute a suitable alternative.

Keywords: *Capreolus capreolus*, relative abundance indexes, area sampling, Horvitz-Thompson estimation, change detection, statistical power.

Introduction

In the last century, cervids showed a significant increase in terms of abundance and distribution in North America and Europe (Gill 1990, Putman et al. 2011) due to the increase of wooded areas, the reduction of extensive grasslands, changes in wildlife management techniques and reintroductions (Gill et al. 1996, Cederlund et al. 1998, Carnevali et al. 2009). In Europe, the roe deer (*Capreolus capreolus*) is the most widespread wild ungulate (Apollonio et al. 2010), which is adapted to a wide variety of environments and habitats and shows a high level of flexibility and success (Linnell et al.1998).

The roe deer is important from an ecological point of view as prey for large carnivores and as a species of hunting interest (Putman et al. 2011). However, population growth has resulted in numerous social conflicts, political and economic, because of damage to crops and to forestry operations (Cederlund et al. 1998, Putman and Moore 1998). The estimate of the size and structure of populations of deer is the base knowledge on which to resolve conflicts among forest managers, wildlife managers, and hunters by modifying harvest levels or silvicultural systems (Radeloff et al. 1999, Vospernik and Reimoser 2008).

The choice of a method for population monitoring depends on the habitat characteristics, management objectives, cost and practical constraints (Mayle and Staines 1998). In Europe, several approaches are used to estimate the abundance of deer populations. Most of them are considered by Morellet et al. (2011).

So-called *vantage point counts* (VPCs) are used in many European countries: in Belgium (Flanders), Netherlands and Portugal to estimate roe deer density, in Belgium (Vallonia), Scotland, Hungary, Slovakia and Portugal to estimate red deer density, and in Portugal to estimate the density of the Iberian goat, wild boar and mouflon as well (Morellet et al. 2011). VPCs are the most popular census method in Italy to estimate roe deer densities in areas where culling is allowed and the wood percentage is lower than 50% (Meriggi et al. 2008, Raganella Pelliccioni et al. 2013).

In accordance with Mayle et al. (1999: 24-25), VPCs should be adopted within well-delineated blocks that are sufficiently open to be completely viewed from one or several vantage points for a pre-fixed time interval. All deer observed within the block

are recorded together with their movements. Deer moving into the block from an outer area are recorded (to prevent double counting) but not included in the count (see Figure 1). At the end of the count period, the number of deer seen within the block (discarding the double counts) is related to the block area and expressed as a density. In accordance with the same authors, a sample of blocks should be "*randomly selected*" among those judged as "*representative*" of the study region and suitable to be surveyed by VPCs. Then, the mean of the densities recorded within each sampled block is taken as the estimate of density over the study region.

The procedure suggested by Mayle et al. (1999) raises several methodological doubts about the effectiveness of using VPCs to estimate densities. The first drawback is the subjectivity in compiling the list of the "*representative*" blocks to be subsequently sampled. Moreover, as shown in Figures 1 and 2, these blocks should allow counts to be related to block areas. Therefore, many open areas not considered as "*representative*" or not suitable for VPCs are discarded, entailing a strong dependence of the density estimate on the set of blocks that has been initially selected. In addition, counts are liable to be influenced by many factors, and as such, they are specific to the day of the count. On this point, Mayle et al. (1999) suggest repeating the VPCs three or four times for the same blocks, taking the maximum density as the final estimate. Finally, similar to any method based on the direct observation of animals, VPCs may be influenced by the sighting conditions and observers' experience. Trials in lowland broadleaf forests prove that VPCs are an unreliable way to estimate density due to poor sighting capabilities (Mayle and Staines, 1998).

Because of the many arbitrary steps involved in the use of VPCs to estimate absolute densities, we consider an alternative and more straightforward use of VPCs to achieve relative abundance indexes. We propose to perform VPCs on all the open blocks of the study region simply by counting the number of roe deer detected within the blocks, irrespective of the fact that these counts can be related or not to the block extents. In principle, by means of VPCs carried out simultaneously on all the open blocks, it is possible to determine the total number of roe deer observed in the open habitat of the study region at the time of the survey. Obviously, this quantity does not constitute the true abundance due to some of the reasons discussed above; mainly, the total count is influenced by the visibility and does not account for the animals settled in the wooded areas at the time of the survey, and it is specific to the day of counts and, as such, constitutes a random variable that varies from day to day. However, it is quite logical to presume a strong direct relationship between the total counts and the actual roe deer abundance, and hence, a similar direct relationship should also hold between the expectation of total counts and the abundance. In principle, expectations of total counts can be used for monitoring purposes as indexes of relative abundance.

Henceforth, these indexes are referred to as vantage point count abundance indexes (VPCAIs).

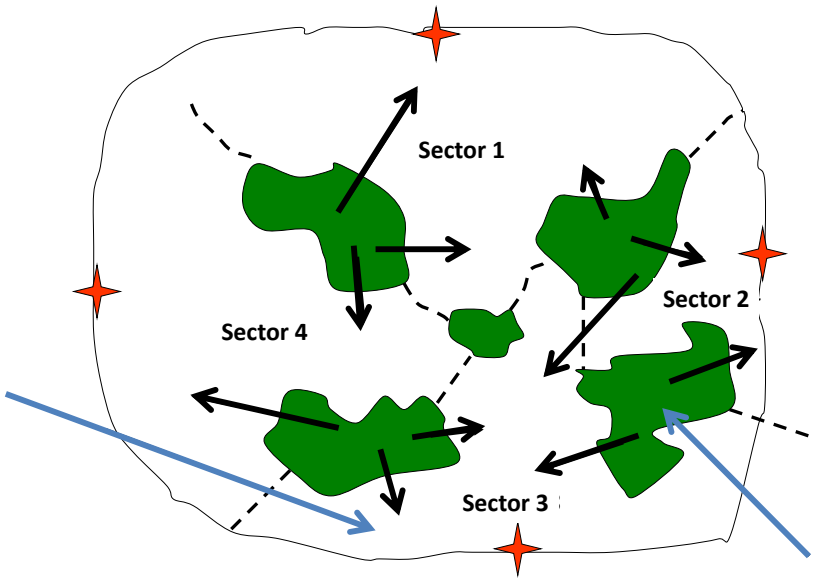

Figure 1 - A correct planning of vantage point counts to estimate densities. The delineated block (continuous line) is open with small woodlots within. The red stars identify the vantage points from which the observers watch the block. Each vantage point has a sector of observation (dotted lines). The black arrows denote the detected animals and their movements for the animals settled within the block. The blue arrows denote the detected animals coming into the block from the outer zone that are discarded from counts. In this way, it is possible to relate the counted deer to the block area and determine the density.

From a practical point of view, covering all the open blocks of a study region may be too expensive in terms of operators and very complex in terms of planning and organization, especially for large study regions. Thus, VPCs are usually performed on a portion of the open areas, and VPCAIs are estimated from these partial counts. If the blocks are selected by means of opportunistic criteria, the resulting estimates strictly depend on the selected blocks, and no objective conclusion about the accuracy and the precision of these estimates may be drawn. This fact also precludes the use of rigorous statistical testing to compare estimates achieved at different times for detecting changes, as recommended in statistically sound monitoring protocols (e.g., Elzinga et al. 2001).

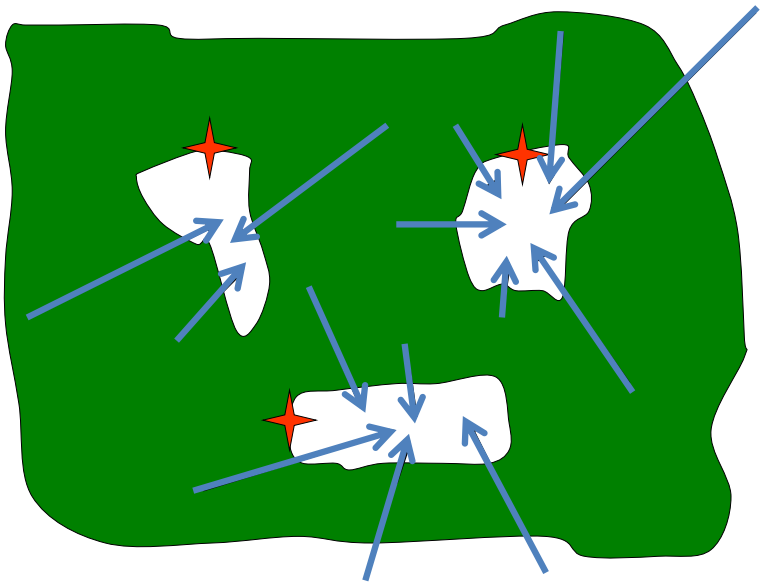

Figure 2 - An incorrect planning of vantage point counts to estimate densities. The three delineated blocks (continuous line) are small open areas surrounded by forested areas. The red stars identify the vantage points from which the observers watch the blocks. Each vantage point has a block of observation. The blue arrows denote the detected animals coming into the blocks from the outer zone that should be discarded from counts. In this way, it is not possible to relate the counted deer to the block areas and determine the densities.

A way to achieve objective evaluations of the accuracy and precision of the VPCAI estimates based on a portion of the open blocks is to select the blocks by means of probabilistic sampling schemes and then adopt estimators that share suitable statistical properties. If these estimators are unbiased and allow for unbiased and conservative variance estimators, then estimation of the sampling errors, construction of confidence intervals and assessment of change are possible. Moreover, a post hoc power analysis is possible for evaluating the probability of the adopted test failing to detect a true change.

In accordance with these considerations, we propose some sampling strategies that are suitable for dealing with populations of spatial units and, as such, are suitable to estimate VPCAIs with statistical rigor. We also evaluate the performance of the proposed strategies on the basis of the results achieved in a hunting district of Tuscany (Central Italy). Because all the open blocks of the district were surveyed, we have the possibility of checking the proposed strategies using real data.

Materials and methods

Study area

The study was conducted in Tuscany (Italy), in the Province of Siena in the ATC 19 hunting area of 124,540 hectares divided into 9 ungulate hunting districts. For this study, we selected the hunting district of Val d'Orcia, which showed the highest percentage of open areas (43°03'N, 11°49'E). It extends for 8,215 ha (see Figure 3). The climate is Mediterranean, with a rainfall range from a minimum of 40 mm in July to a maximum of 111 mm in November. The mean temperature range is from a minimum of 1°C in January to a maximum of 31°C in August. Land use is partitioned as follows: 57% agricultural areas characterized by wheat, oats, vineyards, and olive groves, 38% forest and scrub areas, 4% urban areas corresponding to two small villages, and 1% water bodies. The only form of deer hunting used in this district is stalking. In the last few years, the hunting period was from June 1 to July 15 and from August 15 to September 20 for males and from January 1 to March 15 for females and young. In 2013, the end of the hunting season was moved up to March 12.

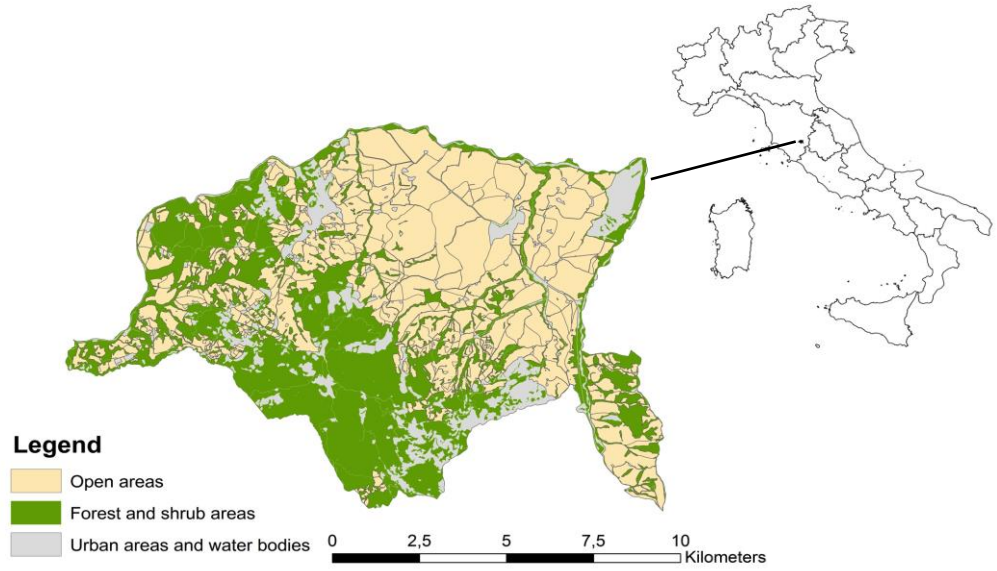

Figure 3 - The Val d'Orcia hunting district and its position in the administrative region of Tuscany (Central Italy). The green color represents forest and scrub areas; the yellow color represents the open areas surveyed during the vantage point count, and the grey color represents urban areas and water bodies.

Vantage point counts

We analyzed aerial photos to identify those points from which it was presumably possible to have complete visibility of one or more open blocks nearby. These points were taken as vantage points. Then, each vantage point was checked in the field to verify the visibility, and when necessary, it was changed to a different position. For each vantage point, we drew a map showing the open blocks and their boundaries to be controlled from the points. Because more than one open block may be surveyed from a single point, at the end of the procedure, a set (population) $\cup$ of $N = 454$ open blocks was delineated to be surveyed by means of a total of 120 vantage points.

The complete surveys were conducted on March 13, 2013 and on April 13, 2014 by 120 trained hunters randomly assigned to vantage points. Animal observations were performed from 18:00 to 20:00. To avoid any disturbance to the animal, observers went to the vantage points one hour before the scheduled time of the beginning of observation. Observers had a map of the vantage point assigned and a record form on which to report data about the time and the species observed, the position of entrance and exit from the area under control, the number of animals observed, the sex and the age. At the end of the survey, record forms and maps were collected, and double counts were removed when possible by the analysis of age, sex, and movement direction of animals observed in neighboring blocks. In 2013, the survey of all the open blocks was repeated on March 23 and March 30. In the year 2014 replications occurred on April 14 and April 24. In both years, all the survey dates were chosen outside of the hunting period, when the spring vegetative regrowth was limited to open areas (cfr. Mayle et al. 1999), on weekends, and on the basis of the availability of the observers.

Sampling and estimation

The number of animals observed in the open block *j* during a complete survey of all the open blocks was denoted by y_j in such a way that the population total

$$T = \sum_{j \in \cup} y_j$$

constitutes the total number of deer counted in the open area. From the complete surveys of the open blocks performed on March 13, 2013 and on April 13, 2014, the true values of *T* were known on these days. Thus, they were exploited to check the performance of some sampling strategies that could be adopted to estimate *T* if only a sample of open blocks was selected and surveyed.

Once a sample $S \subset U$ of $n < N$ blocks had been selected by a probabilistic sampling scheme, VPCs were performed on the n selected blocks to record y_j for $j \in S$. Any sampling scheme, i.e., any randomized protocol adopted to select the sample *S* from the population *U*, determines the inclusion probabilities, i.e., the probability π_j that block $j \in U$ enters the sample. The inclusion probabilities are crucial in estimation because the most common estimation criterion, referred to as the Horvitz-Thompson (HT) criterion, is established to estimate population totals by means of the sum of the sample data divided by the respective inclusion probabilities (see, e.g., Thompson, 2002). Accordingly, if π_j are the inclusion probabilities determined by the scheme adopted to select the blocks and y_j, $j \in S$ are the counts performed within the sampled blocks, the HT estimator is given by

$$\hat{T} = \sum_{j \in S} \frac{y_j}{\pi_j} \tag{1}$$

It is a well-known result of the basic sampling theory that the HT estimator is an unbiased (accurate) estimator of *T*, i.e., $E_S(\hat{T} \mid y_1, \ldots, y_N) = T$ with variance $\mathrm{Var}_S(\hat{T} \mid y_1, \ldots, y_N)$, which depends on the population values as well as on the sampling scheme through the probabilities of the areas to be selected and the probabilities of the pairs of areas to be selected jointly (e.g., Thompson 2002: 53-54). It is worth noting that $E_S(\hat{T} \mid y_1, \ldots, y_N)$ and $\mathrm{Var}_S(\hat{T} \mid y_1, \ldots, y_N)$ denote expectation and variance with respect to the sample selection, conditional on the y_j values, which would be recorded if all the open blocks were surveyed. Moreover, even if we do not dwell further on this issue, the HT estimators have unbiased or conservative estimators of their variances (e.g., Thompson 2002:54-55).

Spatial sampling strategies

When populations are constituted by spatial units (blocks in our case) scattered over a study area, there is a wide variety of schemes available to select a sample of these units. The achievement of a *spatially balanced sample* (SBS), in which the sampled units are well spread throughout the study region, has been the main target for a long time. SBSs can be achieved by using spatial versions of the traditional sampling schemes, such as stratified or systematic sampling (e.g., Thompson 2002: 117-141), or by schemes explicitly constructed to avoid or reduce the selection of neighboring units such as the generalized random-tessellation stratified sampling by Stevens and Olsen

(2004), the drawn-by-drawn sampling excluding the selection of contiguous units by Fattorini (2006), the local pivotal method of first and second type by Grafström et al. (2012), the spatially correlated Poisson sampling by Grafström (2012) and the doubly balanced spatial sampling by Grafström and Tillé (2013). Due to the stated wide variety of spatial schemes available, choosing one of them is challenging. In this paper, we based on a comparison study recently performed by Fattorini et al. (2015), in which the so-called *one-per stratum stratified sampling* (OPSS) revealed a suitable spatial scheme that was simple and efficient. It is worth noting that OPSS is a very simple scheme of long standing in the statistical literature (e.g., Breidt 1995). The results from that study demonstrate that OPSS has a performance similar to the more complex explicitly constructed spatial schemes, but contrary to these schemes, it provides SBSs in a straightforward way and can be well understood and readily planned, even by people who are not statisticians.

OPSS is straightforward to implement by partitioning the population U of N units into n strata $\mathsf{U}_1,\ldots,\mathsf{U}_n$ of equal or approximately equal sizes $N_1,\ldots,N_n$ constituted by neighboring units and then selecting a unit in each stratum. Under OPSS, the probability of unit $j \in \mathsf{U}_k$ to be selected is $\pi_j = 1/N_k$. Then, denoting by $j_1,\ldots,j_n$ the labels of the units selected within each stratum, the HT estimator of T reduces in this case to

$$\hat{T} = \sum_{k=1}^{n} N_k y_{j_k} \tag{2}$$

with variance

$$\mathrm{Var}_{\mathrm{S}}(\hat{T} \mid y_1,\ldots,y_N) = \sum_{k=1}^{n} N_k (N_k - 1) S_k^2 \tag{3}$$

where S_k^2 is the variance of the y_js within the stratum k.

However, the random selection of areas from the strata does not take into account the unit extent. As noted by Skalski (1994), large differences in extents may increase the variances within strata over the natural spatial variability of the phenomenon, thus inflating equation (3). To handle this problem, the author proposes to select units within strata with probabilities proportional to their sizes. In this case, the probability of the unit $j \in \mathsf{U}_k$ to be selected is $\pi_j = a_j / A_k$, where a_j and A_k are the extents of unit j and stratum k, respectively. Then, the HT estimator of T reduces in this case to

$$\hat{T} = \sum_{k=1}^{n} \frac{A_k y_{j_k}}{a_{j_k}} \tag{4}$$

with variance

$$\mathrm{Var}_S(\hat{T} \mid y_1, \ldots, y_N) = \sum_{k=1}^{n} \sum_{h>j \in \cup_k} a_j a_h \left(\frac{y_j}{a_j} - \frac{y_h}{a_h} \right)^2 \tag{5}$$

The simplest way to select a sample of spatial units is the simple random sampling without replacement (SRSWOR), i.e., the consecutive selection of n units each time while removing the selected unit from the population. Therefore, SRSWOR is usually considered as a benchmark to determine the improvement provided by more complex spatial schemes. Under SRSWOR the HT estimator of *T* reduces to

$$\hat{T} = N\bar{y} \tag{6}$$

with variance

$$\mathrm{Var}_S(\hat{T} \mid y_1, \ldots, y_N) = N(N-n)\frac{S_y^2}{n} \tag{7}$$

where $\bar{y}$ is the sample mean and S_y^2 is the variance of the y_js in the whole population $\cup$.

Based on the 454 values of y_j recorded on March 13, 2013 and on April, 13, 2014, we determined the variances of the three sampling strategies by means of equations (3), (5) and (7) for sample sizes $n = 23,45,68,91,136,182,227$ corresponding to sampling fractions of 5%, 10%, 15%, 20%, 30%, 40% and 50%. Regarding OPSS, the 454 open blocks were partitioned into 23, 45, 68, 91, 136, 182, and 227 strata, which constituted an approximately equal number of neighboring blocks.

The partitions were automatically performed using the program PAM (Partition Around Medoids) in the package Cluster of the statistical software R 3.0.2 (Maechler et al. 2016), available at http://cran.rproject.org/web/packages/cluster/cluster.pdf). Figures 1-7 reported in Appendix D of the Supplementary Material show the resulting strata (represented in different colours) for each sample size.

Non-sampling uncertainty

As previously stated, the y_j values arising from VPCs are not fixed in the sense that, due to visibility, animal movements and other factors, the value y_j recorded in block *j* during a survey may attain a very different value if the survey was performed on

another day. Thus, even under complete surveys of the open blocks, the total of counts *T* is not fixed but varies from day to day. This variability is not present when performing complete surveys of immotile objects. The number of trees in a forest remains the same irrespective of the day in which the survey is carried out. Even if these considerations appear obvious, the variability that exists among counts performed on different days is neglected in most cases.

From a probabilistic point of view, the y_j values are not fixed values, but rather, they can be viewed as determinations of *N* random variables from a joint probability distribution $F(y_1,\ldots,y_N)$. Accordingly, the total of counts *T* achieved from a complete survey of all the open blocks is itself a random variable that varies in accordance with a probability distribution derived from *F*. Hence, what actually should be taken as VPCAI is the expectation of *T*, i.e.,

$$\tau = \mathrm{E}_F(T) = \sum_{j\in U} \mu_j$$

where $\mu_j = \mathrm{E}_F(y_j)$ is the expectation of y_j with respect to *F*. Henceforth E_F and Var_F will denote the expectation and variance with respect to *F*, i.e., with respect to the variation of the y_j values between different survey occasions.

From the results on conditional expectation and variance, it can be proven that the HT estimator of *T* achieved from a partial survey by means of equation (1) is also an unbiased (accurate) estimator of τ , , i.e., $\mathrm{E}(\hat{T}) = \tau$ with variance

$$\mathrm{Var}(\hat{T}) = \mathrm{E}_F\left\{\mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N)\right\} + \mathrm{Var}_F(T) \qquad (8)$$

where the first term is the variance due to the sampling of blocks, while the second term is the variance due to the variability of *T* between different surveys, and $\mathrm{E}(\hat{T})$ and $\mathrm{Var}(\hat{T})$ denote the expectation and variance with respect to both sources of variability, respectively (see Appendix A in the Supplementary Material for the proofs of these results).

However, equation (8) cannot be determined from the complete data acquired on March 13, 2013 and April 13, 2014 while the probability distribution *F* is left unspecified. Actually, finding a realistic, parsimonious model for *F* is a very challenging task. Fortunately, an unbiased estimate of equation (8) was achieved from

$$\hat{\mathrm{Var}}(\hat{T}) = \mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N) + s_T^2 \qquad (9)$$

where the first term was the variance determined from the complete surveys of March 13, 2013 and April 13, 2014 based on equations (3), (5) and (7), depending on the

sampling scheme adopted, while s_T^2 was the variance of the results achieved from the three surveys performed in 2013 and 2014, which constituted an unbiased estimator of $\mathrm{Var}_F(T)$ if the three surveys were viewed as independent replications of the same experiment (see Appendix B in the Supplementary Material for the proof of the unbiasedness of equation 9). Because variances are squared quantities, we preferred to report the relative standard error

$$\hat{\mathrm{RSE}}(\hat{T}) = \hat{\mathrm{Var}}(\hat{T}) / \overline{T}$$

where $\overline{T}$ is the average of the results achieved from the three surveys performed in 2013 and 2014. For each sampling scheme and each sample size, Tables 1 and 2 report the RSE estimates for 2013 and 2014, respectively.

Power analysis

The detection of change is one of the most important goals of any monitoring program. In our framework, the objective is to determine whether there has been a change in the VPCAIs between two times. Suppose that two estimates $\hat{T}_1$ and $\hat{T}_2$ are obtained for times 1 and 2, respectively, from the same sample of open blocks S. From the results of the previous section, $\hat{T}_1$ and $\hat{T}_2$ are realizations of unbiased estimators of the VPCAIs τ_1 and τ_2. Accordingly, a very natural estimate of the change $\Delta = \tau_2 - \tau_1$ is simply given by $D = \hat{T}_2 - \hat{T}_1$. Because any estimate differs from the true parameter value, *D* may be different from 0 even in the case of no change between the two periods, i.e., even if $\Delta = 0$. Thus a significance test must be conducted to determine if no true change has occurred and the observed difference is simply due to the uncertainty jointly induced by the variability of counts and by the area sampling.

Because the estimators $\hat{T}_1$ and $\hat{T}_2$ are unbiased and for sufficiently large *n* and *N* they tend to be normally distributed, the difference $\hat{D}$ is a normal random variable with expectation Δ. Thus, if an unbiased (or conservative) estimator for the variance of $\hat{D}$ is available, the familiar two-sided t-test can be performed to assess the hypothesis of no change at a pre-fixed significance level α (the false positive probability or type 1 error).

In this framework, it is crucial to evaluate the probability of refusing the hypothesis of no change when a change of size Δ has actually occurred (the power of the test, usually denoted by 1–β). To this purpose, presume that the two estimators $\hat{T}_1$ and $\hat{T}_2$ have the same RSE ν and that they are correlated with correlation coefficient

ρ. These assumptions should be quite realistic because the two estimates are achieved by surveying the same sample of blocks, so they should share similar precision and should be correlated. In this case, if the t-test is performed at a level α, the power of the test in detecting a change of 100r % turns out to be

$$1-\beta = 2-\Phi\left(z_{1-\alpha/2}+\frac{r-1}{\nu K}\right)-\Phi\left(z_{1-\alpha/2}-\frac{r-1}{\nu K}\right) \quad (10)$$

where z_p is the p-quantile of the standard normal distribution, Φ is its distribution function and $K=\sqrt{1+(r-1)^2-2\rho(r-1)}$ (see Appendix C in the Supplementary Material). Being a probability, the power ranges from 0 to 1. It is 0 when the change remains undetected with certainty, while it is 1 when the change is detected with certainty.

For each RSE of Tables 1 and 2, the power of detecting changes was computed presuming a type 1 error $\alpha=0.05$, a correlation coefficient $\rho=0.9$ and a change of 30%. The resulting power values are reported in parentheses in Tables 1 and 2.

Table 1 - Survey of March 13, 2013 performed on all the 454 open blocks of the Val d'Orcia hunting district (Tuscany, Central Italy). Percent values of the relative standard error (RSE) estimates(*) of the vantage point count abundance index estimators achieved under simple random sampling without replacement (SRSWOR), one-per-stratum stratified sampling (OPSS) and OPSS with probability proportional to the size for sampling fractions of 5%, 10%, 15%, 20%, 30%, 40% and 50% are shown. Values in parentheses are the powers of detecting a change of 30% under type 1 error $\alpha=0.05$ and a correlation coefficient between the two occasions of $\rho=0.9$.

(*) To achieve errors expressed in terms of the number of animals, the RSEs must be divided by 100 and multiplied by 503, i.e., the average of the total counts achieved from the three surveys repeated in 2013.

Sampling Fraction	SRSWOR	OPSS	OPSS with probability proportional to size
5%	53.1 (0.12)	51.5 (0.13)	55.2 (0.12)
10%	38.6 (0.19)	36.7 (0.21)	40.5 (0.18)
15%	32.0 (0.26)	29.9 (0.29)	31.5 (0.27)
20%	28.1 (0.32)	26.2 (0.37)	27.4 (0.34)
30%	23.7 (0.43)	21.7 (0.50)	25.9 (0.37)
40%	21.1 (0.52)	19.5 (0.58)	19.4 (0.58)
50%	19.4 (0.58)	18.4 (0.63)	18.3 (0.64)

Table 2 - Survey of April 13, 2014 performed on all the 454 open blocks of the Val d'Orcia hunting district (Tuscany, Central Italy). Percent values of the relative standard error (RSE) estimates(*) of the vantage point count abundance index estimators achieved under simple random sampling without replacement (SRSWOR), one-per-stratum stratified sampling (OPSS) and OPSS with probability proportional to the size for sampling fractions of 5%, 10%, 15%, 20%, 30%, 40% and 50% are shown. Values in parentheses are the powers of detecting a change of 30% under type 1 error $\alpha = 0.05$ and a correlation coefficient between the two occasions of $\rho = 0.9$.

(*) To achieve errors expressed in terms of the number of animals, the RSEs must be divided by 100 and multiplied by 311, i.e., the average of the total counts achieved from the three surveys repeated in 2014.

Sampling Fraction	SRSWOR	OPSS	OPSS with probability proportional to size
5%	36.8 (0.21)	36.7 (0.21)	47.3 (0.15)
10%	29.6 (0.30)	29.6 (0.30)	35.6 (0.22)
15%	26.6 (0.36)	26.4 (0.36)	31.4 (0.27)
20%	25.0 (0.39)	24.9 (0.40)	28.1 (0.32)
30%	23.3 (0.44)	23.8 (0.43)	27.7 (0.33)
40%	22.4 (0.47)	22.8 (0.46)	25.9 (0.37)
50%	21.8 (0.49)	22.2 (0.48)	24.7 (0.40)

Results

The 454 open blocks identified within the study area covered a total area of 5,894.55 ha, with an average size of 12.98 ha (coefficient of variation 180%). In 2013, a complete survey was performed on March 13, giving rise to a total of $T = 559$ detected animals, with an average number per open block of 1.23 animals (coefficient of variation 225%). The correlation coefficient between the number of detected animals and the size of the blocks was 0.42 (p=0.0017). The complete survey was repeated on March 23 and March 30, giving rise to $T = 414$ and $T = 536$ detected animals, respectively. On average, the number of detected animals was $\bar{T} = 503$, while the variance of complete counts across occasions was $s_T^2 = 6{,}073$ (coefficient of variation 15.7%). In 2014, the complete survey was performed on April 13, giving rise to a total of $T = 380$ detected animals, with an average number per open block of 0.84 animals (coefficient of variation 184%). The correlation coefficient between the number of detected animals and the size of the blocks was 0.15 (p<0.0001). The complete survey was repeated on April 14 and April 26, giving rise to $T = 253$ and

$T = 300$ detected animals, respectively. On average, the number of detected animals was $\bar{T} = 311$, while the variance of complete counts between occasions was $s_T^2 = 4{,}123$ (coefficient of variation 20.6%).

Regarding the RSEs of Tables 1 and 2, they invariably decreased as the sampling fraction increased. For 2013, the three sampling schemes provided very similar performances, with RSEs decreasing from approximately 50% for a sampling fraction of 5% to approximately 20% for a sampling fraction of 50%. OPSS with probabilities proportional to the size performed the worst for small sampling fractions. Analogous results were achieved for the year 2014. In this case, the RSEs were smaller than those achieved in 2013 for small sampling fractions but became greater for the sampling fraction of 50%. They decreased to approximately 35% for a sampling fraction of 5% and were approximately 20% for a sampling fraction of 50%. OPSS with probabilities proportional to the size performed the worst for any sampling fraction. Regarding the powers corresponding to these RSE values, they invariably increased as the sampling fraction increased (and RSE decreased subsequently). For 2013, the three sampling schemes provided very similar powers, increasing from approximately 0.10 for a sampling fraction of 5% to approximately 0.6 for a sampling fraction of 50%. OPSS with probabilities proportional to the size showed the smallest powers for the small sampling fraction.

Analogous results were achieved for the year 2014. In this case, powers were greater than those achieved in 2013 for small sampling fractions but decreased for the sampling fraction of 50%. They increased to approximately 0.20 for a sampling fraction of 5% and were approximately 0.50 for a sampling fraction of 50%. OPSS with probabilities proportional to size showed the smallest powers for any sampling fraction.

Discussion

The RSEs achieved from the case study are excessively high (30-50%) for small sampling fractions (5%) and remain unsatisfactory (approximately 20%) even for very large sampling fractions (40-50%). The spatial stratifications (OPSS) do not provide improvement with respect to SRSWOR. This result means that there are no identifiable spatial trends in the counts, in such a way that the spatial stratification does not provide homogeneous strata that are able to split the overall variability of the population into a large portion due to between-strata variability and a smaller portion due to the variability within the strata. Even the selection proportional to the block sizes does not provide any improvements due to the weak relationships between sizes and counts. For these reasons, the sampling variability is similar to that provided by

SRSWOR. In addition to the sampling variability, there is the variability of counts (estimated to be approximately 15-20%), which inflated the RSEs.

The large uncertainty in the estimates reduces the power of the monitoring process. The power of detecting changes of 30% at a level $\alpha = 0.05$ are always smaller than 0.65, even for sampling fractions of 40-50%. Because the power of the t-test is an increasing function of both *r* and ρ (see equation 10), even smaller powers are achieved in more realistic situations in which changes are smaller than 30% and correlations are smaller than 0.9. Practically speaking, at least for the situation observed in our study region, it would be similar to monitor by means of tossing a coin rather than by means of a t-test based on vantage point counts.

Management implications

Monitoring the population of roe deer using VPCs (as suggested by Cicognani et al. 2000) does not seem effective. The estimation of absolute densities by means of the procedure suggested by Mayle et al. (1999) involves several arbitrary steps, and even the simpler use of VPCs to estimate relative abundance indexes, as proposed in this paper, has proven to be ineffective, providing unacceptable powers for the monitoring process.

These conclusions highlight the need for determining alternative strategies to estimate and monitor roe deer abundance. The alternatives should be suitable in terms of the required time and resources and at the same time should be statistically sound. Most strategies, such as those based on distance sampling, are highly model dependent. They should be avoided because model assumptions are usually difficult to assess from disposable data and the failure of these assumptions usually involves bias in the resulting estimators.

Among the few strategies requiring a minimal set of assumptions, the mark-resighting method based on the Bowden estimator (Bowden and Kufeld, 1995) seems the most suitable. It requires a closed population, and in addition to this assumption, it only requires that the set of radio-collared deer is selected from the population by means of SRSWOR. No other requirement is needed regarding the resighting experiments. Resightings can be replications of the same experiment (e.g., repeated counts along the same trail) or different experiments (e.g., counts on different trails), irrespectively. It is worth noting that the use of SRSWOR to choose the animals to be radio-collared is actually an assumption because deer cannot be selected from the population just like balls from an urn (as supposed in many studies). However, Fattorini et al. (2007) prove that if marks are evenly distributed among individuals and groups, the Bowden estimator is effective, offering computational simplicity and robustness. Recently, the Bowden estimator has proven to be the most reliable method

for estimating the abundance of chamois populations in more extreme situations than those related to roe deer (Corlatti et al. 2015). Accordingly, the mark-resighting method based on the Bowden estimator could provide a suitable alternative for estimating and monitoring roe deer populations in Italy, as well as in other European countries where VPCs are adopted.

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SUPPLEMENTARY MATERIAL

for

Are vantage point count a reliable method for monitoring roe deer populations?

Marco Zaccaroni, Filippo Dell'Agnello, Giulia Ponti, Francesco Riga, Chiara Vescovini and Lorenzo Fattorini

Appendix A

Regarding whether $\hat{T}$ is unbiased as an estimator of τ, because $\hat{T}$ is an HT estimator, it is unbiased for *T*, i.e.

$$\mathrm{E}_S(\hat{T} \mid y_1,\ldots,y_N) = T \tag{A.1}$$

Then, from the result on the conditional expectation, it follows that

$$\mathrm{E}(\hat{T}) = \mathrm{E}_F\left\{\mathrm{E}_S(\hat{T} \mid y_1,\ldots,y_N)\right\} = \mathrm{E}_F(T) = \tau$$

Regarding the variance, from (A.1) and the result on the conditional variance, it follows that

$$\mathrm{Var}(\hat{T}) = \mathrm{E}_F\left\{\mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N)\right\} + \mathrm{Var}_F\left\{\mathrm{E}_S(\hat{T} \mid y_1,\ldots,y_N)\right\}$$

$$= \mathrm{E}_F\left\{\mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N)\right\} + \mathrm{Var}_F(T)$$

Appendix B

The variance estimator (9) is obviously a constant with respect to the uncertainty due to sampling, i.e.

$$\mathrm{E}_S\left\{\hat{\mathrm{V}}\mathrm{ar}(\hat{T}) \mid y_1,\ldots,y_N\right\} = \hat{\mathrm{V}}\mathrm{ar}(\hat{T})$$

Then, from the result on the conditional expectation, it follows that

$$\mathrm{E}\left\{\hat{\mathrm{V}}\mathrm{ar}(\hat{T})\right\} = \mathrm{E}_F\left[\mathrm{E}_S\left\{\hat{\mathrm{V}}\mathrm{ar}(\hat{T}) \mid y_1,\ldots,y_N\right\}\right] = \mathrm{E}_F\left\{\hat{\mathrm{V}}\mathrm{ar}(\hat{T})\right\}$$

$$= \mathrm{E}_F\left\{\mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N) + s_T^2\right\} = \mathrm{E}_F\left\{\mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N)\right\} + \mathrm{E}_F(s_T^2)$$

$$= \mathrm{E}_F\left\{\mathrm{Var}_S(\hat{T} \mid y_1,\ldots,y_N)\right\} + \mathrm{Var}_F(T) = \mathrm{Var}(\hat{T})$$

providing that s_T^2 is an unbiased estimator of $\mathrm{Var}_F(T)$.

Appendix C

The power of the t-test, i.e. the probability of rejecting the hypothesis of no change, is given by

$$1-\beta = \Pr(t \le -z_{1-\alpha/2}) + \Pr(t \ge -z_{1-\alpha/2}) \tag{C.1}$$

where $t = \hat{D}/\sqrt{\hat{V}_D}$ is the familiar t-test statistic, and $\hat{V}_D$ is a suitable estimator for $\mathrm{Var}(\hat{D})$. If a true change Δ has occurred and if $\hat{V}_D$ is a consistent estimator for $\mathrm{Var}(\hat{D})$, then for large samples *t* is asymptotically distributed as a normal random variable with expectation $\Delta/\sqrt{\mathrm{Var}(\hat{D})}$ and variance 1. Accordingly, (C.1) reduces to

$$1-\beta = \Pr\left\{z \le -z_{1-\alpha/2} - \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\} + \Pr\left\{z \ge z_{1-\alpha/2} - \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\}$$

$$= \Phi\left\{-z_{1-\alpha/2} - \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\} + 1 - \Phi\left\{{}_{1-\alpha/2} - \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\}$$

$$= 1 - \Phi\left\{z_{1-\alpha/2} + \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\} + 1 - \Phi\left\{{}_{1-\alpha/2} - \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\}$$

$$= 2 - \Phi\left\{z_{1-\alpha/2} + \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\} - \Phi\left\{{}_{1-\alpha/2} - \frac{\Delta}{\sqrt{\mathrm{Var}(\hat{D})}}\right\} \tag{C.2}$$

Now suppose that the same precision is achieved at the two monitoring times, i.e.

$$\frac{\sqrt{\mathrm{Var}(\hat{T}_1)}}{\tau_1} = \frac{\sqrt{\mathrm{Var}(\hat{T}_2)}}{\tau_2} = \nu$$

from which

$$\mathrm{Var}(\hat{T}_1) = \nu^2\tau_1^2 \ , \ \mathrm{Var}(\hat{T}_1) = \nu^2\tau_1^2 \tag{C.3}$$

Moreover, suppose a correlation coefficient ρ between the two estimator, i.e.

$$\frac{\mathrm{Cov}(\hat{T}_1, \hat{T}_2)}{\sqrt{\mathrm{Var}(\hat{T}_1)\mathrm{Var}(\hat{T}_2)}} = \rho$$

from which

$$\mathrm{Cov}(\hat{T}_1, \hat{T}_2) = \rho\sqrt{\mathrm{Var}(\hat{T}_1)\mathrm{Var}(\hat{T}_2)} \tag{C.4}$$

Due to (C.3) and (C.4), the variance of $\hat{D}$ can be rewritten as

$$\mathrm{Var}(\hat{D}) = \mathrm{Var}(\hat{T}_1) + \mathrm{Var}(\hat{T}_2) - 2\mathrm{Cov}(\hat{T}_1, \hat{T}_2) = \nu^2\tau_1^2 + \nu^2\tau_2^2 - 2\rho\nu^2\tau_1\tau_2 \tag{C.5}$$

Finally, without loss of generality, τ_2 can be expressed in terms of τ_1 as $\tau_2 = r\tau_1$, in such a way that

$$\Delta = \tau_2 - \tau_1 = (r-1)\tau_1 \tag{C.6}$$

By substituting equation (C.6) into equation (C.5), it follows that

$$\mathrm{Var}(\hat{D}) = \nu^2\tau_1^2\left\{1 + (r-1)^2 - 2\rho(r-1)\right\} \tag{C.7}$$

Substituting equations (C.6) and (C.7) into equation (C.2), the power of the t-test turns out to be

$$1-\beta = 2 - \Phi\left\{z_{1-\alpha/2} + \frac{r-1}{\nu\sqrt{1+(r-1)^2 - 2\rho(r-1)}}\right\} - \Phi\left\{_{1-\alpha/2} - \frac{\Delta}{\nu\sqrt{1+(r-1)^2 - 2\rho(r-1)}}\right\}$$

Appendix D

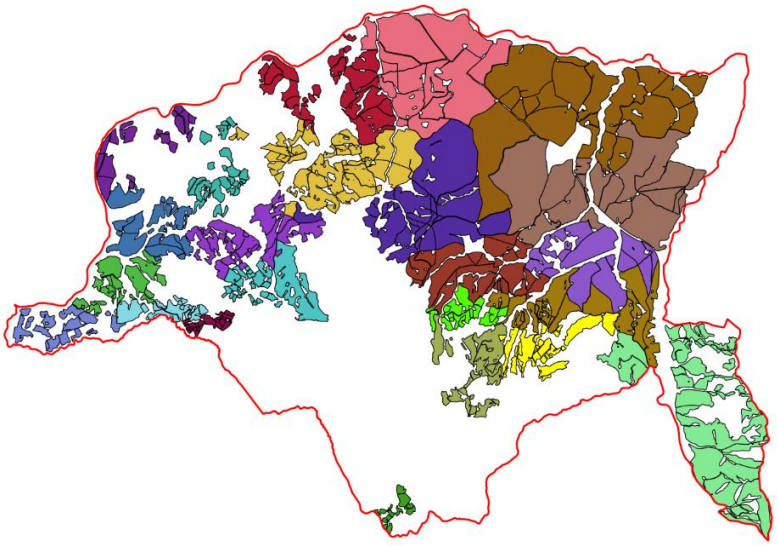

Figure 1 - Partition of the population of open areas into 23 strata of neighbouring areas

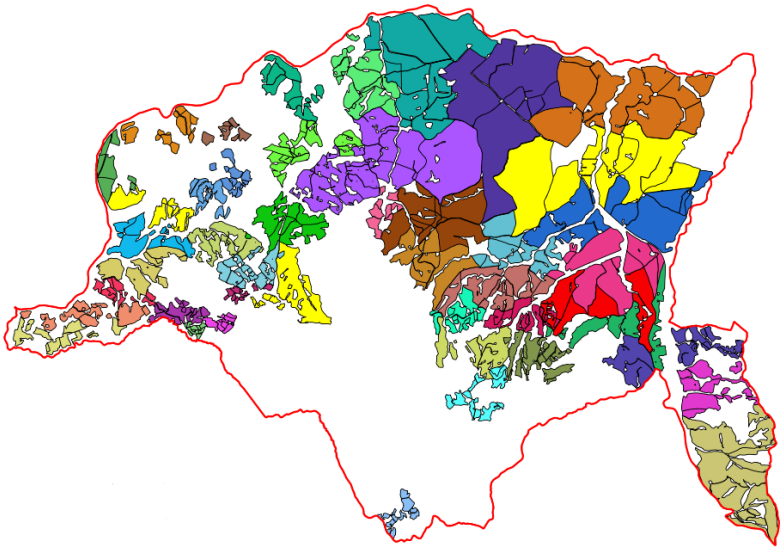

Figure 2 - Partition of the population of open areas into 45 strata of neighbouring areas

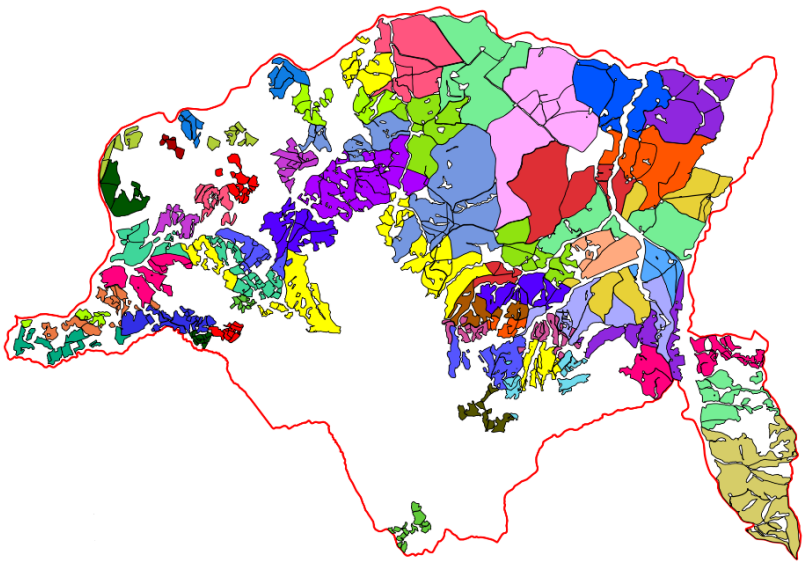

Figure 3 - Partition of the population of open areas into 68 strata of neighbouring areas

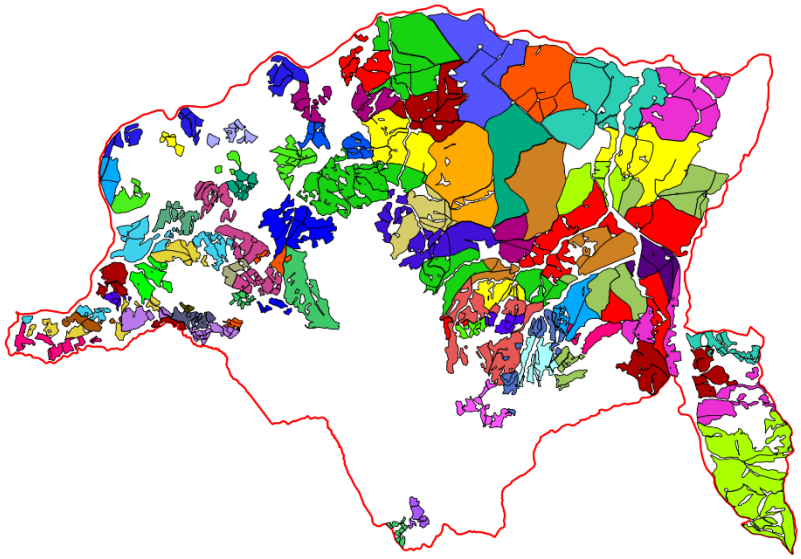

Figure 4 - Partition of the population of open areas into 91 strata of neighbouring areas

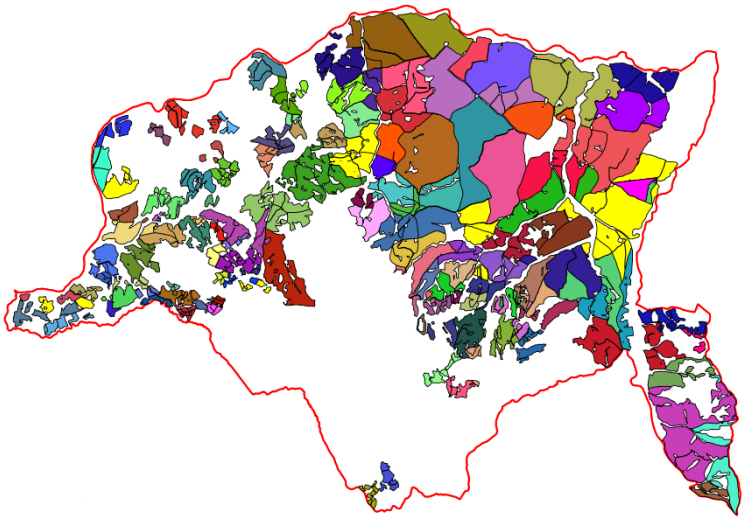

Figure 5 - Partition of the population of open areas into 136 strata of neighbouring areas

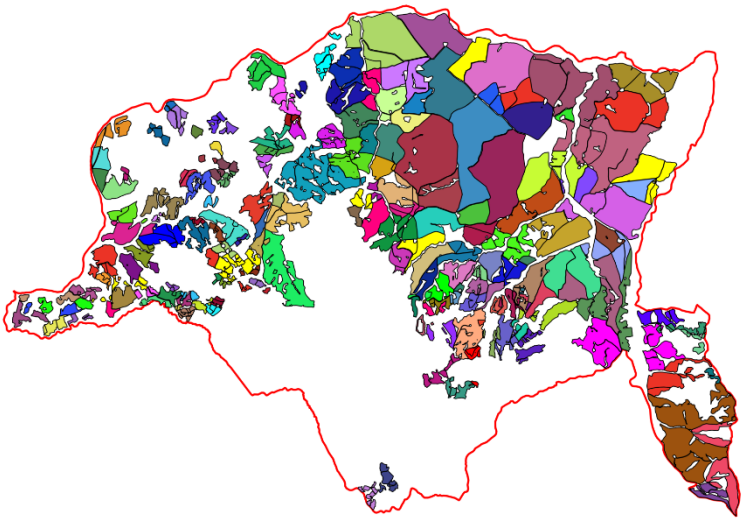

Figure 6 - Partition of the population of open areas into 182 strata of neighbouring areas

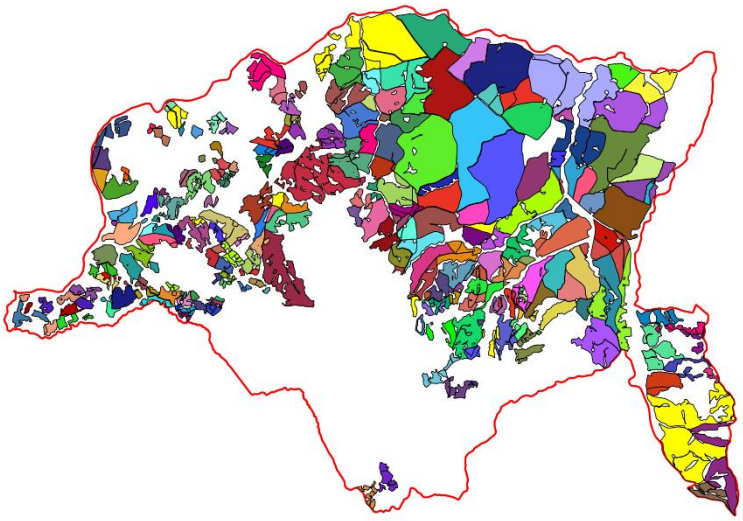

Figure 7 - Partition of the population of open areas into 227 strata of neighbouring areas.

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