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TECHNOLOGICAL TOOLS IN WILDLIFE RESEARCH AND MANAGEMENT: ANALYSES OF BEHAVIOUR AND DAMAGE PREVENTION METHODS BY WOLF, IN A HUMAN-DOMINATED LANDSCAPE.

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‘…Then the she-wolf sat down, pointed her nose at a star, and began to howl. One by one the wolves joined her, till the whole pack, on haunches, with noses pointed skyward, was howling its hunger cry...’

‘…poi la lupa si accucciò, puntò il muso verso una stella e prese ad ululare. Ad uno ad uno, tutti i lupi si unirono finché tutto il branco, anch'esso seduto, con i nasi volti verso il cielo, emise il proprio grido affamato…’

Jack London, *white fang,* 1906

KEYWORDS

- *Canis lupus* - Predation - Kill rate - Livestock damage - Alps

Index

Summary

No longer used to a high degree of naturalness, developed countries find are unprepared for the return of many animal species that disappeared following harsh persecution. In particular, the return of large carnivores to human-dominated landscapes opens up new scenarios and new challenges for coexistence. Investigating the ecology of the wolf with a consideration of its temporal and spatial dimensions, harnessing the industry's advanced technology as GPS telemetry or camera traps, would allow for a better understanding of the ecology of this predator even in the most anthropised and least studied contexts, providing useful management implications for different situations, with the aim of improving conflict mitigation measures. In Chapters 1 and 2 I studied the recolonisation of a wolf population and the spatial ecology on a large and fine scale, considering environmental factors such as the degree of anthropisation and the level of naturalness. In Chapters 3 and 4, I studied the wolf's predatory behaviour and the dissuasion of a particularly bold individual using rubber bullets. An overall interpretation of the results showed that the wolf tends to avoid spatial overlap with humans, but that once it has reached high densities and can no longer avoid them, it lives with them by exploiting human derived resources. It is likely that the mitigation measures promoted and implemented so far are no longer sufficient or suitable to reduce the human-wolf conflict, which is shifting from the rural environment to large human settlements. This evidence should be considered if the conflict is to be mitigated effectively. The finding that anthropogenic resources in some contexts have influenced the ecological role of this opportunistic, generalist predator, suggests that we are not doing enough to prevent human-wolf interactions from increasing over time, compromising the conservation of this species in the future. The results also suggest that management and conflict resolution should not be generalized but must be site specific as the wolf is so plastic in its behaviour. In conclusion, the study of the spatial and trophic ecology of the wolf with an adequate consideration of its quickly evolving distribution has increased knowledge of this species, opening up interesting prospects for its management on a large and small scale.

INTRODUCTION

Over the past decade, several mammal predators have recovered large portions of their previous distribution both in North America and Europe (Chapron et al., 2014; Enserink and Vogel, 2006; Mech, 2017; Ripple et al., 2014). One of the causes has certainly been the abandonment of the rural environment by humans (Cimatti et al., 2021), which has encouraged the return of many prey species (Apollonio et al., 2010), in addition to efficient protection (e.g., in Europe the 1979 Bern Convention and the 1992 Habitat Directive on animal species and environments protection). The increase in suitable habitat and increased densities of primary consumers has encouraged the recovery of secondary consumers, such as cougar, bear, and wolf (Bruskotter and Shelby, 2010; Chapron et al., 2014; Gippoliti et al., 2018; Ripple et al., 2014). The return of large carnivores to the European and American continents has reconstituted trophic cascades in ecosystems, influencing the number and behavior of their prey, which changed vegetation dynamics and/or influenced mesopredators, and their prey (David Mech, 2012; Kuijper et al., 2016; Ray et al., 2005).

However, large carnivores are recovering not only in natural ecosystems but also to human modified landscapes. Even if there are several evidences that large carnivores avoid human disturbance in space and time (Rogala et al., 2011), an habituation is to be foreseen. As hypothesized by Kijuper et al. (2016), the current recolonization by large carnivores of anthropogenic landscapes indicates that they may have more flexible habitat requirements than previously thought. The future distribution and density of these species in these landscapes may therefore be driven more by human acceptance than by other factors (Carter and Linnell, 2016; Oriol-Cotterill et al., 2015).

Despite the obvious benefits of the return of large carnivores, they are not always accepted by the public (Bisi et al., 2010; Dressel et al., 2015; Johansson et al., 2017; Støen et al., 2018). Rural populations around the world see them as nuisance species for the economic damage caused to domestic livestock (Naughton-Treves et al., 2003; Treves et al., 2009) and for the threat to personal safety (Linnell et al., 2002, 2021; Linnell and Alleau, 2016; Treves and Karanth, 2003). This has been the main reason why these species have been at risk of extinction on both the American and European continents in the past decades, carrying out real legal hunting campaigns but also poaching (Liberg et al., 2012; Nowak and Mysłajek, 2016; Ogada et al., 2003; Woodroffe and Ginsberg, 1998). Indeed, the protection of large carnivores is a difficult challenge.

The Europeans brought this attitude in the American continent as they colonized them, however some differences both in the environment and the legislation are to be found between these two areas. One of these is human density (United Nations statistics). On the European continent, human population density of the different states is on average higher than in North America, and this, in the near future, may increase human-predator conflict jeopardizing the conservation effort (Bisi et al., 2010; Lescureux, 2018). One of the species that is currently recolonizing habitats in Europe increasing human conflict is the wolf (Dressel et al., 2015). For example, it has been shown that in a context where the wolf population had increased, the attitudes of a selected group of citizens became more negative and fearful over time, regardless of whether they were subjected to wolf impacts (Treves et al., 2013).

Currently, the wolf population on the European continent is recovering and colonizing areas in which it was absent for more than half a century or more (Boitani et al., 2022; Chapron et al., 2014; Enserink and Vogel, 2006; Kuijper et al., 2016), thanks to its ability to adapt to very different ecological context (Ahmadi et al., 2014; Blanco and Cortés, 2007; Eggermann et al., 2011; Llaneza et al., 2012).

The wolf is difficult to study as it is often present at wide ranges and low densities (Boitani, 2003). Moreover, its plasticity (Mech and Boitani, 2007) and elusiveness (Thompson, 2004), which allows it to colonize the most diverse habitats (Newton et al., 2017), may lead to a very different behaviors in the different environments occupied. However, the wolf is intensively studied (Ripple et al., 2014), both from a conservation and management perspective being one of the most widespread carnivores on the planet (Ripple et al., 2014).

Despite many studies most knowledge of this species is limited to portions of its distribution where its population growth is less intense like some portion of Europe or North America (Chapron et al., 2014). Furthermore, knowledge is highly skewed for habitats characterized by a strong degree of naturalness and low level of human population density, as colonization of human dominated landscape is recent.

Increased knowledge of wolf ecology in these contexts could help to gain a better understanding of wolf trade-offs in an anthropized environment reducing conflicts with humans. In fact, people who were initially insensitive to wolves developed a more positive attitudes as their knowledge of wolf biology increased, (Bisi et al., 2007; Ericsson and Heberlein, 2003).

In order to promote an effective coexistence, it will be necessary both to raise awareness among human populations by increasing knowledge of their biology and to implement effective damage management. Predator damage management is a conservation issue, and the intersections between carnivores, people, and livestock should be effectively focused. Several studies show how the implementation of prevention practices has dramatically lowered damage-related costs (Berzi et al., 2021; Treves et al., 2016). However, even if prevention systems are considered of outmost importance their effectiveness was tested often relying on an anecdotical evidence. So, a better understanding of the ecology of wolves will also allow us to develop a more effective prevention systems for different situations with the aim to improve conflict mitigation measures (Shivik, 2006).

Southern Europe, despite having a quickly increasing wolf population living in an anthropized wolf environment is poorly represented by wolf population studies focusing on conflicts and their solutions.

In my thesis first I investigated aspects of large- and fine-scale spatial ecology by comparing different colonization phases: in particular, how the wolf recolonized mountains, hills and plains in one of the European regions with the highest density of ungulates and a high human presence (chapter 1).

Then, I investigated the small-scale trophic ecology in a recently colonized area with a focus on both kill sites and their selection (chapter 2), and prey species selection and relative kill-rate (chapter 3).

Finally, I evaluated modern and technologically advanced prevention systems exploiting GPS technology both to implement them and to study their effectiveness. Specifically, I analyzed the effectiveness of aversive conditioning on a confidant wolf toward a shepherd and his flock of sheep using rubber pallets (Chapter 4).

In Chapter 1 I assessed the presence of wolves in an area densely inhabited by humans and many ungulate species and how recolonization of this area has occurred over time.

Many studies highlight how wolves avoid anthropogenic disturbance and the environment modified by it (e.g. Bassi et al., 2015; Benson et al., 2015a, 2015b; Carricondo-Sanchez et al., 2020). Despite this, in recent years we are witnessing an unexpected effect that is going against all predictions and models made so far, in which wolves are increasingly present in habitats with a strong anthropogenic footprint (Chapron et al., 2014). However, it is unclear when and where wolves will recover to significant densities, what effects they may have in more anthropized landscapes (Kuijper et al., 2016). Using historical data from a region with high anthropogenic densities and where wolves were never exterminated, we monitored the recovery and predict the evolution of recolonization of this species.

It is not easy to assess the presence of wolves because of their strong elusiveness. To address this limitation, it is advisable to use a multiple methods approach (as recommended by Ausband et al., 2014; Duchamp et al., 2011; Merli et al., 2023 among others). This approach would allow for a minimum number of wolves and with greater effort also the actual pack density (Mattioli et al., 2018).

Finally, using these estimates of wolf presence in relation to distance to urban settlements with a range of environmental variables will allow us to better understand their ability to use human dominated environments.

In Chapter 2, I studied kill sites in a newly colonized multi-prey system within an area where humans are seasonally quite abundant.

Many studies, as already anticipated, confirm that wolves are returning to occupy territories that over time humans have modified (Chapron et al., 2014). The presence of humans could condition prey-predator dynamics (Kuijper et al., 2016; Wittmer et al., 2007), indeed humans for some prey species can act as a "human shield" (Berger, 2007; Rogala et al., 2011) by modify rates and spatial patterns of predation (Kunkel and Pletscher, 2000).

Large carnivores often suffer from disturbances related to human activities as forestry (Nielsen et al., 2008). However, predators may benefit from higher prey densities in more densely populated areas (Basille et al., 2009; Carter et al., 2012) or exploit anthropogenic infrastructure to increase killing success (Newton et al., 2017). So, the presence of humans may reduce or increase the impact of carnivores on prey populations through effects on carnivore behavior. We currently have little understanding of what drives variation in these effects. The suitability of landscapes for large carnivores is generally predicted on their habitat requirements in their current ranges (Huck et al., 2010; Niedziałkowska et al., 2006). However, the current recolonization by large carnivores of anthropogenic landscapes in parts of Europe indicates that carnivores may have more flexible habitat requirements than previously thought, even at fine scales (3rd and 4th order selection). In particular, the wolf is recognized to have great plasticity (e.g. Bassi et al., 2012; Muhly et al., 2019). GPS technology could be used to increase this knowledge, as has already been used in other studies to investigate predatory behavior and kill-site selection (Atwood et al., 2007; Hebblewhite et al., 2005; McPhee et al., 2012). This would lead to increased knowledge for effective wolf conservation and management.

In Chapter 3, I studied the killing rate and prey selection of the wolf at different levels in a complex food webs.

Kill rate and prey selection are a key aspects of wolf ecology and the balance of ecosystems where wolves occur. Although this is a much-studied aspect of wolf ecology, most of these studies have studied simple food webs (e.g., islands: Vucetich et al., 2002) in which the main prey species was clearly identified (North America: Lake et al., 2013; Smith et al., 2004; Vucetich et al., 2012; Northern Europe: Sand et al., 2008; Zimmermann et al., 2007). We have little knowledge about the behaviour of the wolf in complex food webs, in particular in context anthropized.

It is know the predatory behavior requires careful analysis of the trade-off between costs and benefits, thus leading to prey selection (Mattioli et al., 2011). If in nature this can be this can be implemented based on abundance or vulnerability of preys, we do not yet know what happens in a anthropised environment. In nature the predator can chooses the prey most available in the environment, this results in a higher probability of encountering the prey by lowering the costs of predation (Fritts and Mech, 1981) or chooses the weakest prey, which therefore requires less effort during the predatory act (Mattioli et al., 1995). The anthropogenic environment could affect these dynamics by compromising the wolf's ecological role (Ciucci et al., 2020) and altering predator-prey dynamics.

In these contexts, it would be important to know the number of prey that a predator kills over time so that we can better evaluate the functional response of preys that are often harvested by humans.

In Chapter 4, I studied and monitored the effectiveness of aversive conditioning on a GPS-collared confidant wolf.

Unlike other carnivores, direct persecution has been the actual cause of wolf decline and extinction in several areas, more so than environmental changes or sharp reductions in prey availability (Boitani, 2000; Breitenmoser, 1998). Indeed, the extinction of wolves in many areas has been the result of a deliberate and sustained effort to eliminate a major nuisance cause for livestock husbandry (Goldman and Young, 1944) and sometimes a danger to humans themselves (Bisi et al., 2007; Cayuela, 2004; Kusak et al., 2005).

For rare and large predators, reserves will probably never be large enough, and transgression beyond their boundaries will continue to lead to the death of individuals and sometimes the decline of species (Woodroffe, 2000; Woodroffe and Ginsberg, 1998). So, conflict mitigation measures are increasingly necessary as it becomes evident that reserves and legal status are not sufficient to ensure predator survival.

It has been shown that lethal culling does not always work and often has counterproductive effects (McManus et al., 2015; Moreira-Arce et al., 2018; Santiago-Avila et al., 2018), moreover, in many European countries large carnivores are protected by law making lethal management techniques of those problematic individuals inapplicable, as is the case in North America.

As a consequence, in accordance with a public opinion increasingly opposed to culling, one solution will be nonlethal prevention systems (Berzi et al., 2021; Blackwell et al., 2016; McManus et al., 2015; Shivik, 2006). While it is true that there is extensive knowledge of some nonlethal disruptive stimulus techniques (e.g., electronic guard: Linhart et al., 1992; guard dogs: Fritts et al., 2003; fladry: Musiani et al., 2003; hard plastic collars: King, 2004), on the contrary a lack of knowledge is evident for techniques using aversive conditioning (e.g., electric shock collars: Shivik et al., 2002; rubber bullets: Beckmann et al., 2004). For example, the use of rubber bullets to scare off the predator is poorly documented, although several European countries, such as France, Italy, and Sweden, use them for management purposes. Extensive anecdotal knowledge exists on this topic, and there is very little scientific literature (Rauer et al., 2003). One of the few published studies tested rubber bullets in different situations on brown bears, with some radiocollared individuals being studied after the aversive conditioning event (Rauer et al., 2003). The little literature that does exist has tested rubber bullets on species with different behaviour to the wolf (e.g. brown bear; Rauer et al. 2003). Despite the fact that on the bear the results do not look very promising on durability, the effect of aversive conditioning could work much more efficiently on canids as has been demonstrated in the case of the coyote (Andelt et al., 1999).

Therefore, as suggested by Linnell et al. (2021) it would be useful to use intensive GPS data to increase knowledge of fine-scale movements near dwellings by studying how wolves respond to deliberate approaches from people.

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Chapter 1

The wolf and the city: insights on wolves conservation in the anthropocene

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The wolf and the city: insights on wolves conservation in the anthropocene

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Canis lupus; large carnivores; human-wildlife conflict; wildlife management; urban wildlife syndrome; recolonization; human-dominated landscapes.

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Abstract

After a long period of human persecution that led it to extinction in most of its distribution range, the wolf is undergoing a fast recovery. Despite being described as an elusive species only living in remote areas, wolves are recently occupying also human-dominated landscapes, increasing the frequency of direct contacts with humans. Nevertheless, it is unclear whether this situation is only caused by a numerical increase or partially facilitated by a higher tolerance of wolves with respect to human proximity. We focused on a European region offering an abundant, widespread and long-term monitored wolf population to analyse wolf pack distribution and its relationship with human presence across areas recolonized across four different time periods (1972, 1996, 2005 and 2016). Throughout areas recolonized during different periods, wolves were initially located in mountains and hills, occupying plains only in the recent past. Although they consistently tended to be located as far as possible from urban settlements, especially from those with over 5000 inhabitants, in 2016, 70% of packs included at least one urban settlement within the expected home range. Moreover, the distance kept by wolves from the nearest urban settlement was more constrained in areas of recent recolonization (2005 and 2016) and in the mountainous altitudinal range, likely due to a reduced availability of territories. We showed that wolves tend to keep as far away as possible from humans, but they can also permanently occupy human-dominated landscapes to cope with the lower availability of space induced by their remarkably successful recolonization. Our results shed light on an upcoming scenario for the conservation of large carnivores.

Introduction

Wolves *Canis lupus* experienced historically one of the strongest persecutions ever recorded on a large carnivore by mankind. They were completely wiped out from a large portion of their original distribution by a steady and intense hunting, trapping and poisoning, in Western Europe and in North America (Boitani, 2000; Ripple *et al*., 2014). Differently from other carnivores, direct persecution was the actual cause of wolves' decline and extinction in several areas, rather than environmental modifications or strong reduction in prey availability (Breitenmoser, 1998; Boitani, 2000). Indeed, differently from large cats or bears, wolves could rely on their well-known ecological plasticity to survive in many different environmental contexts, even in those strongly modified by humans (Blanco & Cortés, 2007; Eggermann *et al*., 2011; Llaneza, López-Bao, & Sazatornil, 2012; Ahmadi, López-Bao, & Kaboli, 2014; Kuijper *et al*., 2016). Thus, their extinction was, in most cases, the outcome of a deliberate and constant effort aimed at removing a cause of strong nuisance for livestock breeding and sometimes a danger for humans themselves (Cayuela, 2004; Treves *et al*., 2004; Kusak, Skrbinšek, & Huber, 2005; Bisi *et al*., 2007). Even nowadays, human-induced mortality is the main source of mortality for wolves in large portions of their range, mainly in Western countries with variable human population density (Álvares, Pereira, & Petrucci-Fonseca, 2000; Carreira & Petrucci-Fonseca, 2000; Lovari *et al*., 2007; Treves *et al*., 2017; Musto *et al*., 2021). As reported for other large carnivores (Klees van Bommel *et al*., 2020), the proximity to urban settlements may be dangerous for wolves, which may reduce movements (Ferreiro-Arias and Llaneza, submitted) or approach human settlements only at night (Kojola *et al*., 2016) to avoid contacts with humans. This is consistent with the history of the species in Western Europe and North America where wolves were eradicated from all environments with the exception of forested remote areas characterized by limited human presence and scarcity of urban settlements, eventually represented by scattered farms or tiny villages (Promberger &

Hofer, 1994; Boitani & Ciucci, 1995) making the wolf a typical representative of ecological refugees (Kerley, Kowalczyk, & Cromsigt, 2012). This phenomenon biased the perception of wolf habitat preferences, convincing researchers that wolf was a typical forest-dwelling species with a strong aversion for all human-related infrastructures, like roads and cities, and human-modified environments such as agricultural landscapes (Ciucci *et al.*, 1997; Theuerkauf, Rouys, & Jedrzejewski, 2003; Cayuela, 2004; Włodzimierz Jędrzejewski *et al.*, 2005; Oakleaf *et al.*, 2006; Lesmerises, Dussault, & St-Laurent, 2012).

However, the recent history of this species in Western Europe is characterized by a strong change in the aforementioned patterns, as wolves are impressively increasing (Chapron *et al.*, 2014; Mech, 2017). The decrease in direct persecution by man, which was partially linked to the decline of free-ranging livestock breeding as a major cause of conflict with humans, played a relevant role in allowing population recovery (Treves *et al.*, 2004; Bisi *et al.*, 2007; Ripple *et al.*, 2014), together with array of national and European laws protecting large carnivores (Boitani, 2000). Moreover, rewilded areas increased due to the abandonment of mountain and hilly areas by people that moved to urban areas (Navarro & Pereira, 2015) mainly after Second World War. This allowed natural vegetation to increase (Navarro & Pereira, 2015) resulting in a cascade effect on the abundance of wild ungulates (Apollonio, Andersen, & Putman, 2010), which in turn led their predators to recover their former abundance and distribution (Bruskotter & Shelby, 2010; Chapron *et al.*, 2014; Gippoliti *et al.*, 2018).

Many studies modelled and predicted favourable habitats for wolves (Whittington, St. Clair, & Mercer, 2005; Lesmerises, Dussault, & St-Laurent, 2012; Ahmadi, López-Bao, & Kaboli, 2014; Bassi *et al.*, 2015; Benson, Mahoney, & Patterson, 2015; Benson, Mills, & Patterson, 2015; Muhly *et al.*, 2019; Carricondo-Sanchez *et al.*, 2020). Nonetheless, most studies considered only sparsely populated areas, just because these areas were the first to be recolonized by wolves (Massolo & Meriggi, 1998; Theuerkauf *et al.*, 2003; Jędrzejewski *et al.*, 2008; Eggermann *et al.*, 2011; Llaneza, López-Bao, & Sazatornil, 2012; Ahmadi, López-Bao, & Kaboli, 2014; Carricondo-Sanchez *et al.*, 2020; Fernández-Gil *et al.*, 2020). As an outcome, the resulting models emphasized natural factors, as the forest cover, as the most important predictor of wolf presence or future settlement. On the contrary, Avgar, Betini, & Fryxell (2020) showed that habitat selection is highly context-dependent on availability of prey and consumer density, suggesting that non-human factors can act as main drivers of wolf presence.

However, several studies showed that wolves can survive in human-dominated landscapes (Theuerkauf *et al.*, 2003; Chavez & Gese, 2006; Blanco & Cortés, 2007; Eggermann *et al.*, 2011; Llaneza, López-Bao, & Sazatornil, 2012; Ahmadi, López-Bao, & Kaboli, 2014; Kuijper *et al.*, 2016) or even take advantage of them (Kittle *et al.*, 2017; Muhly *et al.*, 2019), more likely thanks to a high ecological and behavioural plasticity and to the ability to cope with mortality risks due to human proximity (Newton *et al.*, 2017). On the contrary, it was observed that wolves tend to avoid humans and their activities (Ordiz *et al.*, 2011; Benson, Mills, & Patterson, 2015; Kaartinen, Antikainen, & Kojola, 2015; Sazatornil *et al.*, 2016; Filla *et al.*, 2017), showing a positive selection only when a substantial payoff is available (Newsome *et al.*, 2015; Kittle *et al.*, 2017). Wolves indeed exploit anthropogenic features only when the risk of mortality due to human presence becomes a background noise and the human environment becomes favourable from a feeding opportunity perspective (Muhly *et al.*, 2019).

In the face of this quickly changing situation and because of the ongoing expansion of this species in Europe, increasing knowledge on wolf presence and site selection in anthropogenic contexts is currently needed to improve effectiveness of conservation and management of this species.

To investigate this issue, we considered the proximity of wolf packs to urban settlements to test whether wolves still actively avoid human disturbance by settling as far as possible from urban settlements or if, as an alternative, wolves are increasing their tolerance towards humans and do settle without avoiding towns and cities.

We used data from pack distribution of a densely wolf-populated region in Europe, Tuscany (Central Italy), that shows a widespread and long-term monitored wolf population as well as three well-characterized environments (mountain, hill and plain) differing in both magnitude of human presence and availability of natural habitats. To test the hypothesis that wolves avoid areas with higher density of urban settlements, we formulated the following predictions:

1. packs distribution would be unequally subdivided among the three macro environments present in the region following the density of urban settlements that are less abundant in mountains and more abundant in plains; moreover, the process of recolonization would be gradual, from least human-inhabited environments to most human-dominated ones;
2. packs would not be randomly distributed with respect to urban settlements as they prefer occupying locations as far as possible from humans.

To test the hypothesis that pack distribution is constrained by both the human presence and the intra-specific competition for spatial availability, we predicted that:

3. the distance of packs from the nearest urban settlement would be constrained towards stable values by higher wolf density (i.e. across the recolonization process) and by environmental and human factors (i.e. across the three altitudinal ranges).

Materials and methods

Study area

Tuscany is a region of Central Italy (Lat. 43° 25′ N; Long. 11° 00′ E) that extends for 18,513 km^2 excluding the islands (where the wolf is absent).

Tuscany habitats range from mountain to Mediterranean ones, as its elevation on sea level varies from 0 to 2054 m a.s.l. Woods are mostly composed of beech *Fagus sylvatica* and white fir *Abies alba* at higher elevation followed by deciduous oak *Quercus* sp., and chestnut *Castanea sativa* and Mediterranean woods characterized by holm oak *Quercus ilex* and domestic pine *Pinus pinea*. Altogether, woods occupy almost 9000 km^2 and are progressively increasing due to abandonment by farmers of vast areas once cultivated. About 52% of the region is occupied by forests and semi-natural environment, 38% by agricultural areas, 9% by human-dominated areas and 1% by water bodies and wetlands (Tuscany Region, 2022). The protected areas cover almost 10% of the regional territory, for a total area of 2,270 km^2. Human activity, as well as the dense road network (292 km/100 km^2), is widespread throughout the territory with a greater urban concentration along the Arno valley. The average human density of the study area is 181 inhabitants/km^2 and specifically of 15 inhabitants/km^2 in the mountain areas, 59 inhabitants/km^2 in hill areas and 328 inhabitants/km^2 in plain areas (Tuscany Region, 2022).

Agriculture and livestock farming are still relevant to the region's economy, free-ranging livestock breeding is practised, with 401,151 sheep and 20,165 goats raised within the region (Berzi, 2018).

Wolves never got extinct in Tuscany. Even during their minimal historical distribution in the 1970s, wolves were reported along the Thyrrenian coast on the metalliferous hills (between the provinces of Pisa, Livorno and Grosseto) and along the Apennine in the Casentino and Mugello areas (northernmost part of the province of Arezzo and Florence) and in a very small area of Massa Carrara province (Cagnolaro *et al.*, 1974). Boitani & Ciucci (1996) confirmed the presence of wolves in these areas, with an expansion to the neighbouring territories, which included a large part of the Apennine chain and the province of Grosseto. Subsequent investigations (Gazzola & Viviani, 2006; Apollonio, 2013, 2014, 2015, 2016) have been monitoring wolves in this region since year 2005. In this time, the distribution of wolves increased from mountains to hilly and flat areas (Fig. 1) as wolf presence was reported in 29 and 220 municipalities in 1974 and 2016, respectively.

Data collection

We identified wolf pack locations during a survey conducted between 2014 and 2016 in the whole region, by means of a multiple-method approach (as recommended, inter alia, by Duchamp *et al.*, 2011 and Ausband *et al.*, 2014).

We used a combination of camera trapping (Canu *et al.*, 2017; Mattioli *et al.*, 2018), wolf-howling recording and sonogram analysis (Gazzola *et al.*, 2002;Passilongo *et al.*, 2015; Palacios *et al.*, 2016); direct observation and filming, taking advantage of a previous knowledge of the spatial habits of most packs and a network of local collaborators (i.e. hunters, shepherds and other volunteers). Each pack known in previous years or reported by the citizen science network was investigated using the techniques mentioned above, to ensure presence and current location of the territorial pair and the presence of offspring (Table S1).

Camera trapping was conducted on a year-round basis to detect wolf presence within opportunistically selected locations and to ascertain eventual reproduction. Territorial pairs were recognized by observation of marking behaviours (Llaneza, García, & López-Bao, 2014). In cases of uncertainties on pack identity, this approach was integrated by genetic analyses through sampling of dead wolves collected (Scandura, Iacolina, & Apollonio, 2011; Canu *et al.*, 2017). A detailed description of the camera trapping methodology and wolf individual recognition is reported in Canu *et al.* (2017) and Mattioli *et al.* (2018).

Wolf-howling surveys were conducted yearly from June to October, focusing on the previously known or supposed pack's home sites (through the collaborative group's prior knowledge), according to the methodology described by Gazzola *et al.* (2002) and Passilongo *et al.* (2010). For discriminating different packs based on howling responses, we adopted the criteria described in Apollonio *et al.* (2004). Wolf howling provided information on pack reproduction by sonographic analysis of chorus howls (Passilongo *et al.*, 2015; Palacios *et al.*, 2016).

We considered 'pack' each social unit constituted by, at least, a territorial pair. When reproduction was ascertained, we identified the pack location with the rendezvous site, that approximately coincided with the site where pups were detected (i.e. the camera trapping site they were filmed or the site they emitted the recorded chorus howl). Conversely, when reproduction was not ascertained, we considered as pack location the site with the maximum number of detections of adults (i.e. the camera trapping site where a certain pack was filmed more times).

To analyse the differences in wolf distribution across recolonized areas during different periods, we assigned each pack location to a recolonization step of the Tuscan wolf population, by using the occupancy data at municipal level in 1972 (Cagnolaro *et al.*, 1974), 1996 (Boitani & Ciucci, 1996) and 2006 (Gazzola & Viviani, 2006). These historical occupancy data were based on records of wolf presence reported by citizens and stakeholders. Only in the case of the 2006 survey, wolf occupancy was further verified by means of wolf howling, snow-tracking and genetic analyses (Gazzola & Viviani, 2006). Analogously to our approach, these methods were only used to verify wolf presence where it had been reported by the citizen science network. Thus, the higher reliability and precision of the more modern methods made the results of 2006 (Gazzola & Viviani, 2006) and 2016 (this study) more conservative than the previous surveys. Based on the municipality in which each pack location fell, we assigned to each pack the reference year of the earliest record of occupancy of its municipality. The year of our monitoring (2016) was then assigned to those packs located in areas not previously reported as occupied by wolves (recent recolonization).

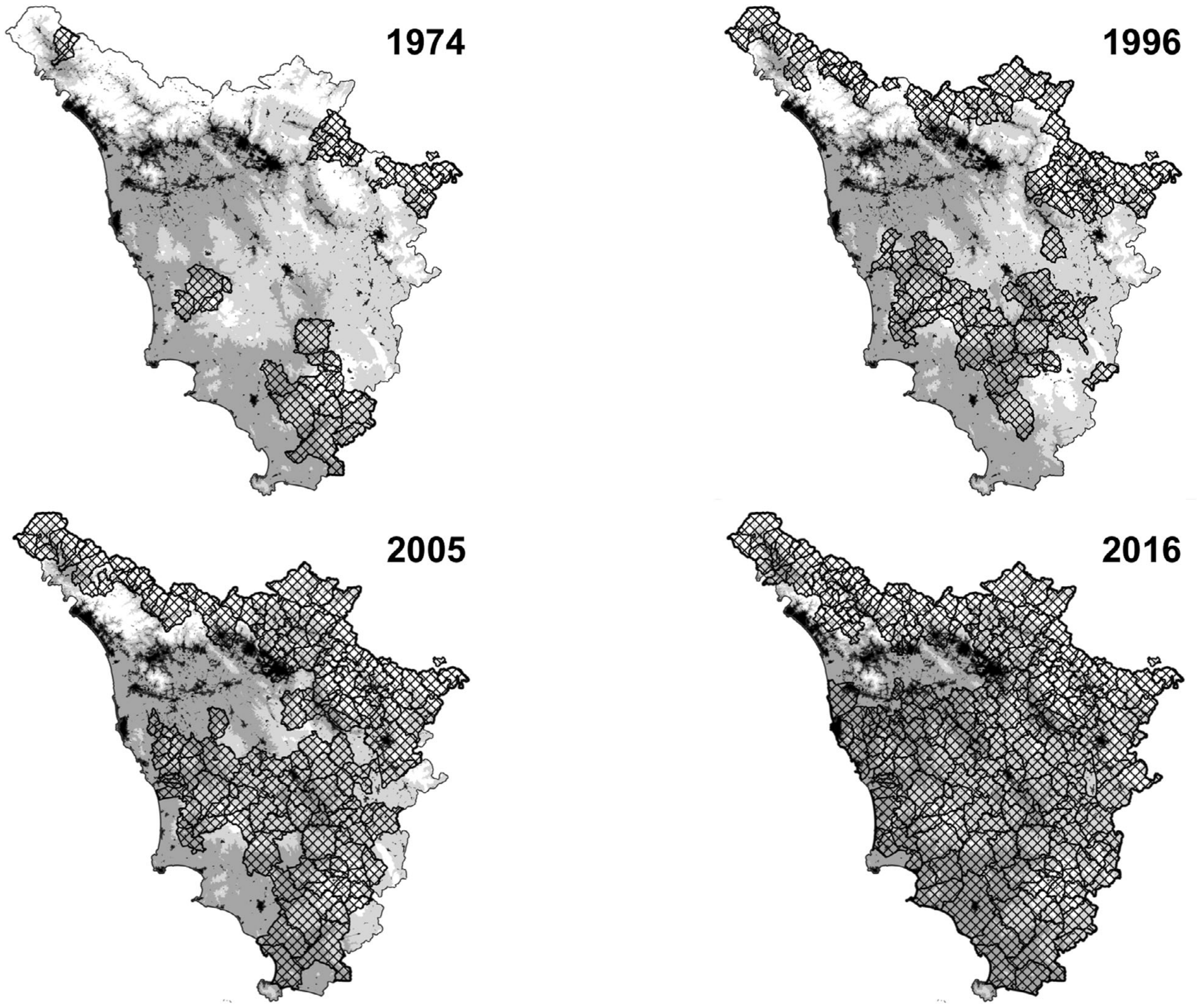

Figure 1 Wolf presence in Tuscany based on municipality, between 1974 and 2016. In white the mountains, in light grey the hills, and in grey the plain. Black dots represent urban settlements, while the black grid shows the municipalities occupied by wolves in the reference year.

Data analysis

Following our predictions, analyses were performed by a three steps approach.

Distribution of observed pack locations

Within the first step, we aimed to evaluate pack distribution among three macro-habitats (with different urban settlements presence; Table S2) defined at municipal level by altitudinal ranges (mountain, hill and plain) by comparing through a chi-squared test the observed frequency of pack locations in each habitat, with respect to a frequency distribution proportional to habitat availability in Tuscany. We considered as mountainous area those ranges higher than 600 m a.s.l.; as hills those located between 200 and 600 m a.s.l., and as plain those lower than 200 m a.s.l. Mountains covered 19% of the region, while hills and plains cover 46% and 35%, respectively (Tuscany Region, 2022). We then assigned each observed pack location to its altitudinal range. This procedure was performed both on the whole set of pack locations and separately for each recolonization step. The available surface for each recolonization step area was calculated by subtracting the surface with the stable presence of wolves of the previous steps from that of the whole region (Fig. 1).

Effect of urban settlements on pack locations

In the second step, we tested whether pack location distribution was affected by the distance from urban settlements by comparing observed pack locations with randomly generated ones. We used a GIS software (QGIS 3.10 A CORUÑA) to generate 240 random points (from now on control pack locations) distributed among altitudinal ranges in the same proportions as the observed pack locations. The optimal amount of control pack locations was defined as the number of points needed to stabilize the variance of the distance from urban settlements. The value was found by a visual inspection of a line plot relating distance variance to the number of locations considered. We classified each control pack location by altitudinal range and recolonization step areas, as already described for observed pack location. We then generated the variable 'presence', assuming 0 and 1 in control and observed pack locations, respectively. 'Presence' was considered as the response variable of a generalized linear model (GLM) with a Logit link function and the predictors described in Table 1 (see Table S5 for their mean and standard error in the observed and control pack locations).

All those independent variables were tested for collinearity. First, we calculated the Pearson correlation coefficient between all possible pairs within the predictor variables considered (Zuur, Ieno, & Smith, 2007). We considered as non-negligible correlations those with an r coefficient higher than 0.6 or lower than −0.6 (Zuur *et al.*, 2009a). The surface covered by the nearest urban settlement was collinear with its number of inhabitants (for both any size- and large urban settlements). As the settlement surface had lower significance and score in a Random Forest rank (*varImpPlot* function from the 'randomForest' R package), we retained as predictors the number of inhabitants of the nearest urban settlement of any size and that of the nearest large urban settlement.

Then, once the least significant variables were removed, we recalculated Person's correlation coefficient and repeated this process until no residual correlated pairs remained. Subsequently, we performed a multicollinearity test using the *corvif* function of the 'AED' package (Zuur *et al.*, 2009b) in R software to confirm the absence of multicollinearity among the remaining variables. All VIF values were less than three (see Chapter 26 of Zuur, Ieno, & Smith, 2007) indicating that there was no multicollinearity in the variables tested. All the residual variables were entered as predictors in the GLM stepwise models with backward elimination procedure. Different models were evaluated by combining unrelated predictors in all additive and multiplicative (interactions) ways. The best model was then selected according to Akaike's information criterion (Burnham & Anderson, 2004; AIC; Burnham, Anderson, & Huyvaert, 2011). We evaluated the relative importance of each predictor included in the best model by computing the Akaike weights from all the models whose AIC differed less than two points from the best one (Burnham & Anderson, 2004; Symonds & Moussalli, 2011). In order to avoid the issues arising from model averaging (see Banner & Higgs, 2017; Dormann *et al.*, 2018), only the results of the best model were discussed. This approach was particularly appropriate in this analysis since our main goal was to describe the possible effect of urban settlements on the observed pack locations and not to predict their distribution. Nonetheless, we still performed a model averaging analysis and included the results in Appendix (Table S6).

Constraints of the variability of pack location distance from urban settlements

The limits to the free choice of pack location were tested by a comparison of the variability of the distance from the nearest urban settlement. We evaluated whether the coefficient of

Table 1 Predictor variable acronyms and description

Name	Description
Distance from the nearest urban settlement (dU)	The distance (m) from the nearest urban settlement of any size. We considered as urban settlement any cluster of houses with a number of residents equal or greater than that of the smallest municipality in Tuscany (65 inhabitants)
Distance from the nearest large urban settlement (dLU)	The distance (m) from the nearest large urban settlement. We considered as large urban settlements the biggest urban areas that together accounted for 60% of region inhabitants (ISTAT, 2017). The smallest settlement of this category had 5046 inhabitants (ISTAT, 2017)
Altitude (A)	The altitude (m a.s.l.) of the pack location
Altitudinal range (Ar)	The altitudinal range (categorical): mountains (>600 m a.s.l.), hills (>200 m and <600 m a.s.l.) and plain (<200 m a.s.l.)
Recolonization step (Cs)	The reference year (1972, 1996, 2006 or 2016) of the earliest record of occupancy, based on the municipality the pack was located in
Inhabitants of the nearest urban settlement (pU)	The number of inhabitants of the nearest urban settlement of any size
Inhabitants of the nearest large urban settlement (pLU)	The number of inhabitants of the nearest large urban settlement
Surface of the nearest urban settlement (SU)	The surface (km^2) covered by the nearest urban settlement of any size
Surface of the nearest large urban settlement (SLU)	The surface (km^2) covered by the nearest large urban settlement
Human density of the nearest urban settlement (DpU)	The density of inhabitants (inhabitants/km^2) of the nearest urban settlement of any size
Human density of the nearest large urban settlement (DpLU)	The density of inhabitants (inhabitants/km^2) of the nearest large urban settlement

All variables were obtained from the spatial layers freely available at https://www.regione.toscana.it/-/geoscopio.

variation (CV) significantly differed among the four recolonization step areas (as proxies of the number of wolves and their regional density) and the three altitudinal ranges (as example of three different human densities). We computed CV via bootstrapping (with 100 resampling) by using the Boot package (Canty & Ripley, 2014) in R. The estimated means were then compared by ANOVA and Tukey honest significant difference post hoc test (R Core Team, 2022) to find actual differences among paired groups.

Results

Distribution of observed pack locations

During the monitoring conducted between 2014 and 2016, 110 pack locations were identified. Distribution of pack location sites in the three altitudinal ranges was not proportional to their availability ($\chi^2 = 71.747$; $P < 0.001$) showing a preference for the mountains, where 54 pack location sites were observed over 21 expected. On the hills, 45 pack locations were observed compared with 51 expected. Finally, in the plain we observed 11 pack location instead of the 38 expected (Fig. 2). Accordingly, in the different recolonization step areas the number of packs for each area differed significantly from expectations (Table S4). The number of packs on the mountains was always higher than expected, while those in the plain were always lower than expected. Conversely, the number of packs in the hilly area until 1996 was less than expected, while after 1996, it was higher than expected.

With respect to the recolonization process, packs initially tended to occupy mountains and hills and later started to occupy plains (Table S4).

Effect of urban settlements on pack location

The best logistic GLM explaining ‘presence’ probability included as predictors: (1) the distance from the nearest urban settlement of any size, (2) the number of inhabitants of the nearest urban settlement of any size and (3) that of the nearest large urban settlement, (4) the altitude and (5) the interaction between the altitudinal range and distance from the nearest urban settlement of any size (Table 2). Considering the set of models with ΔAIC <2 (Table 2), the Akaike weight of the selected predictors averaged 1, 1, 0.72, 0.78 and 1, respectively (Table 3). Conversely, the predictors not included in the best model had much lower weights: 0.30, 0.62 and 0.62 for the distance from the nearest large settlement, the population density of the nearest settlement of any size and that of the nearest large settlement, respectively. The effects described by the best model were consistent with the outcome of the model averaging (Table 3, Table S6).

The probability of pack presence was positively affected by increasing the distance from human settlements and by increasing altitude, while the number of inhabitants of the nearest urban settlement had a negative effect (Table 3). The intensity of the effect of the distance from the nearest urban

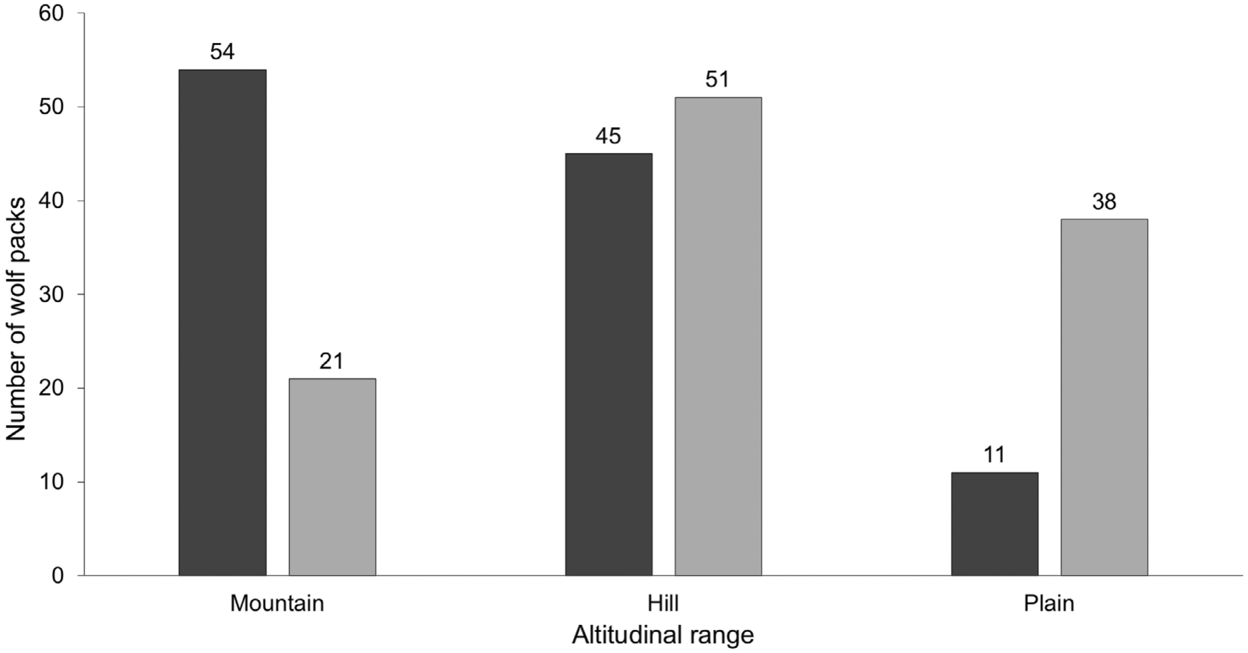

Figure 2 Number of wolf pack in the three altitudinal ranges. Black and grey bars represent the number of observed and expected wolf packs within each altitudinal range, respectively. Distribution of the observed pack locations in the three altitudinal ranges significantly differed from the expected ($\chi^2 = 71.747$; P-value < 0.001).

Table 2 Multimodel inference of logistic Generalized Linear Models on wolf pack location

Model	d.f.	R^2	loglik	AIC	ΔAIC	weight
Presence = dU + A + pU + pLU + dU:Ar	7	0.11	197.758	409.52	0.00	0.097
Presence = dU + A + pU + pLU + DpLU+dU:Ar	8	0.11	196.812	409.63	0.11	0.092
Presence = dU + A + pU + pLU + DpU + DpLU+dU:Ar	9	0.12	196.039	410.08	0.56	0.073
Presence = dU + pU + pLU + dU:Ar	6	0.10	−99.060	410.12	0.60	0.072
Presence = dU + dLU + A + pU + DpLU+dU:Ar	8	0.11	197.096	410.20	0.68	0.069
Presence = dU + A + pU + pLU + DpU + dU:Ar	8	0.11	−197.123	410.25	0.73	0.067
Presence = dU + A + pU + DpLU+dU:Ar	7	0.11	−198.143	410.29	0.77	0.066
Presence = dU + dLU + A + pU + pLU + DpLU+dU:Ar	9	0.12	−196.184	410.37	0.85	0.063
Presence = dU + pU + pLU + DpU + dU:Ar	7	0.11	−198.213	410.43	0.91	0.061
Presence = dU + dLU + A + pU + DpU + DpLU+dU:Ar	9	0.12	−196.234	410.47	0.95	0.06
Presence = dU+ dLU + A + pU + pLU + DpU + DpLU+dU:Ar	10	0.12	−195.274	410.55	1.03	0.058
Presence = dU + A + pU + DpU + DpLU+dU:Ar	8	0.11	−197.476	410.96	1.44	0.047
Presence = dU + dLU + A + pU + pLU + dU:Ar	8	0.11	−197.489	410.98	1.46	0.047
Presence = dU + pU + pLU + DpU + DpLU+dU:Ar	8	0.11	−197.554	411.11	1.59	0.044
Presence = dU + pU + pLU + DpLU+dU:Ar	7	0.10	−198.562	411.13	1.61	0.043
Presence = dU + A + pU + dU:Ar	6	0.10	−199.586	411.18	1.66	0.042

dU = distance of the wolf pack location from the nearest urban settlement; dLU = distance of the wolf pack location from the nearest large urban settlement; pU = number of inhabitants of the nearest urban settlement; pLU = number of inhabitants of the nearest large urban settlement; A = altitude of the pack location; DpU = density population of the nearest urban settlement; DpLU = density population of the nearest large urban settlement; Ar = altitudinal range (for more details on each predictor see Table 1); df = degrees of freedom; R^2 = coefficient of determination, the proportion of the dependent variable variability predictable by the model; loglik = log likelihood; AIC = Akaike's information criterion; ΔAIC = the difference between AIC values for two nested models; weight = Akaike weight.

Table 3 Results of the best logistic generalized linear model on wolf pack location

Predictor	Coefficient estimate	Akaike weight	se	z	*P*
(Intercept)	−1.687		0.383	−4.403	<0.001
dU	0.0004	1.00	0.0001	3.840	<0.001
pU	−0.0003	1.00	0.0001	−2.302	0.021
pLU	0.000002	0.72	0.0000009	1.888	0.059
A	0.0009	0.78	0.0005	1.612	0.107
dU:Ar (Hill vs. Mountain)	−0.0003	1.00	0.0001	−2.362	0.018
(Hill vs. Plain)	−0.0003		0.0001	−2.005	0.044

dU = distance of wolf pack location from the nearest urban settlement; pU = number of inhabitants of the nearest urban settlement; pLU = number of inhabitants of the nearest large urban settlement; A = altitude of pack location; Ar = altitudinal range (for more details on each predictor see Table 1); Coefficient estimate = estimated β coefficient of the predictor within the best model; Akaike weights = average Akaike weights of each predictor among the models with ΔAIC <2 (Table 2); se = standard error of estimated coefficient; z = z-ratio; *P* = *P*-value.

settlement was lower in the hills with respect to mountain and plain.

Wolf packs were located at an average distance of 2590 ± 128 m (mean ± standard error) and 1961 ± 103 m from urban settlements of any size, for observed and control pack locations, respectively. Analogously, observed packs were located at an average distance of 10913 ± 598 m from large settlements, while control points were at 9802 ± 442 m from them (Fig. S1 and Table S5). Observed pack locations were located near to settlements with 565 ± 85 inhabitants, while control points were located near settlements with 14492 ± 5352 inhabitants. The 93% of packs were located at less than 5 km from urban settlements of any size. As for large urban settlements, the 18% of the observed pack locations were within 5 km from them (Fig. 3).

Constraints of the variability of pack location distance from urban settlements

The variability of the distance from the nearest urban settlement was significantly lower in areas recolonized recently (2016) in comparison with those recolonized in previous years (*F* = 138.6; d.f. = 3/396; *P* < 0.001; Fig. 4a and Table S7).

The comparison of the CV among the three macro-habitats (identified by the three altitudinal ranges) showed a statistically significant difference (*F* = 23.61; d.f. = 2/297; *P* < 0.001), with the distance from the nearest urban settlement being significantly less variable in mountains than in the hilly and plain ranges of the Region (Fig. 4b and Table S7).

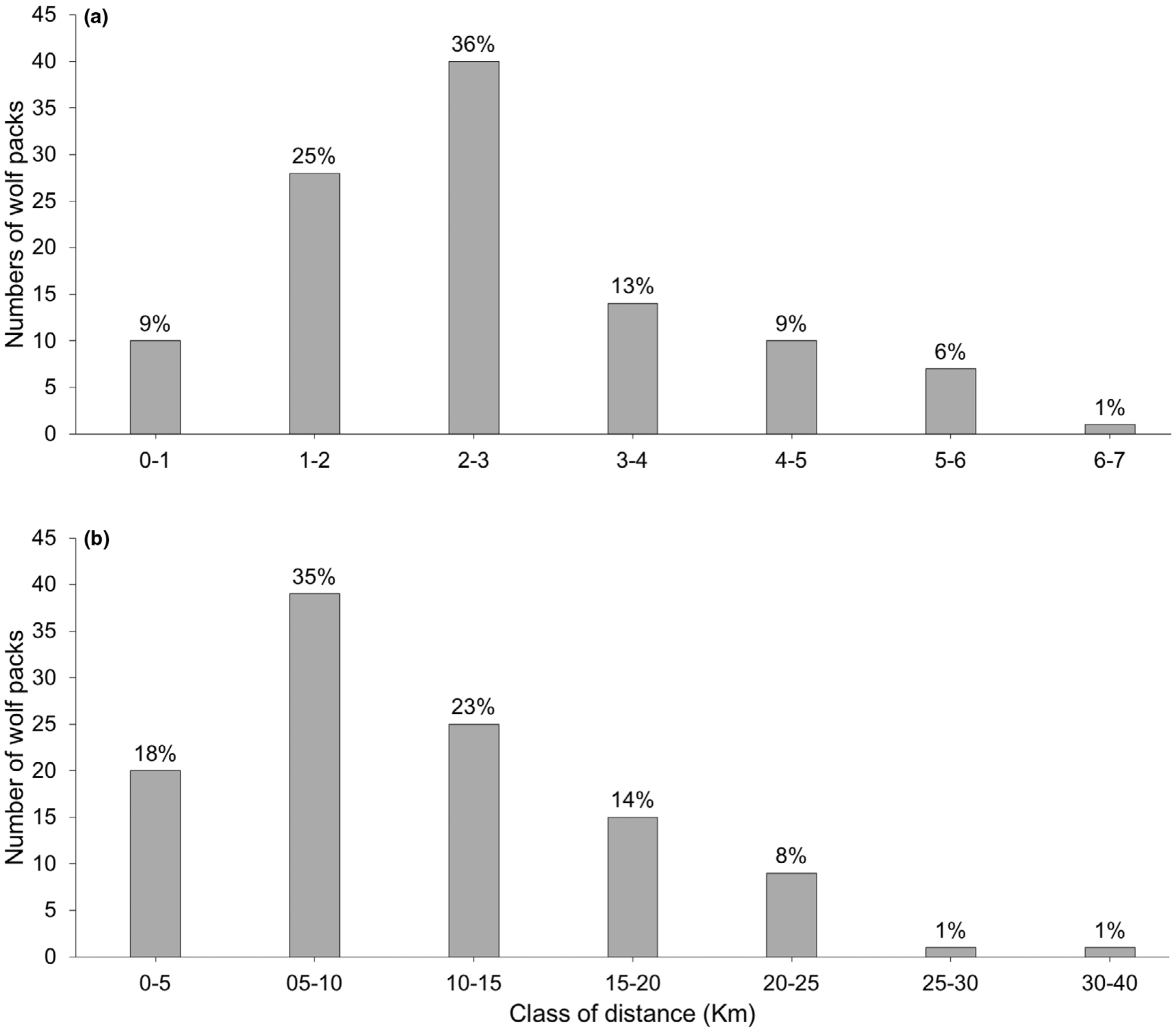

Figure 3 Number of wolf pack locations per class of distance, (a) from the nearest urban settlement of any size; (b) from the nearest large urban settlement.

Discussion

Wolves preferred mountains and tended to avoid plains across all the recolonization steps, while hills were avoided during the first recolonization step and selected during later steps. Proximity with an urban settlement and the number of its inhabitants reduced the likelihood of a wolf pack location. The distance of wolves to the nearest urban settlement was less variable in areas more recently recolonized, where the density of packs was the highest, and the same was true on the mountains with respect to plains and hills.

Distribution of observed pack locations

The results were in accordance with our first prediction. Mountainous areas were more occupied by wolf packs in comparison with plain areas, although their availability was lower in all the four recolonization steps. Moreover, the development of recolonization suggested that wolves preferred mountains and, once the latter were mostly occupied, started to locate at lower altitudes as well. Interestingly, this effect concerned hill areas until 1996 and then, as the pack density grew, started to spread in plain as well. Such phenomenon was likely mediated by a density-dependent dispersal, with young wolves being forced to move to lower altitude, more anthropized areas in order to establish new territories. The occupation of plains being lower than expected (particularly evident during the earlier recolonization steps) may be accounted to bottom-up and/or top-down processes. The low prey densities characterizing plains during 70s–90s (Apollonio, Andersen, & Putman, 2010) could have actually limited the potential expansion of wolves. Additionally, the

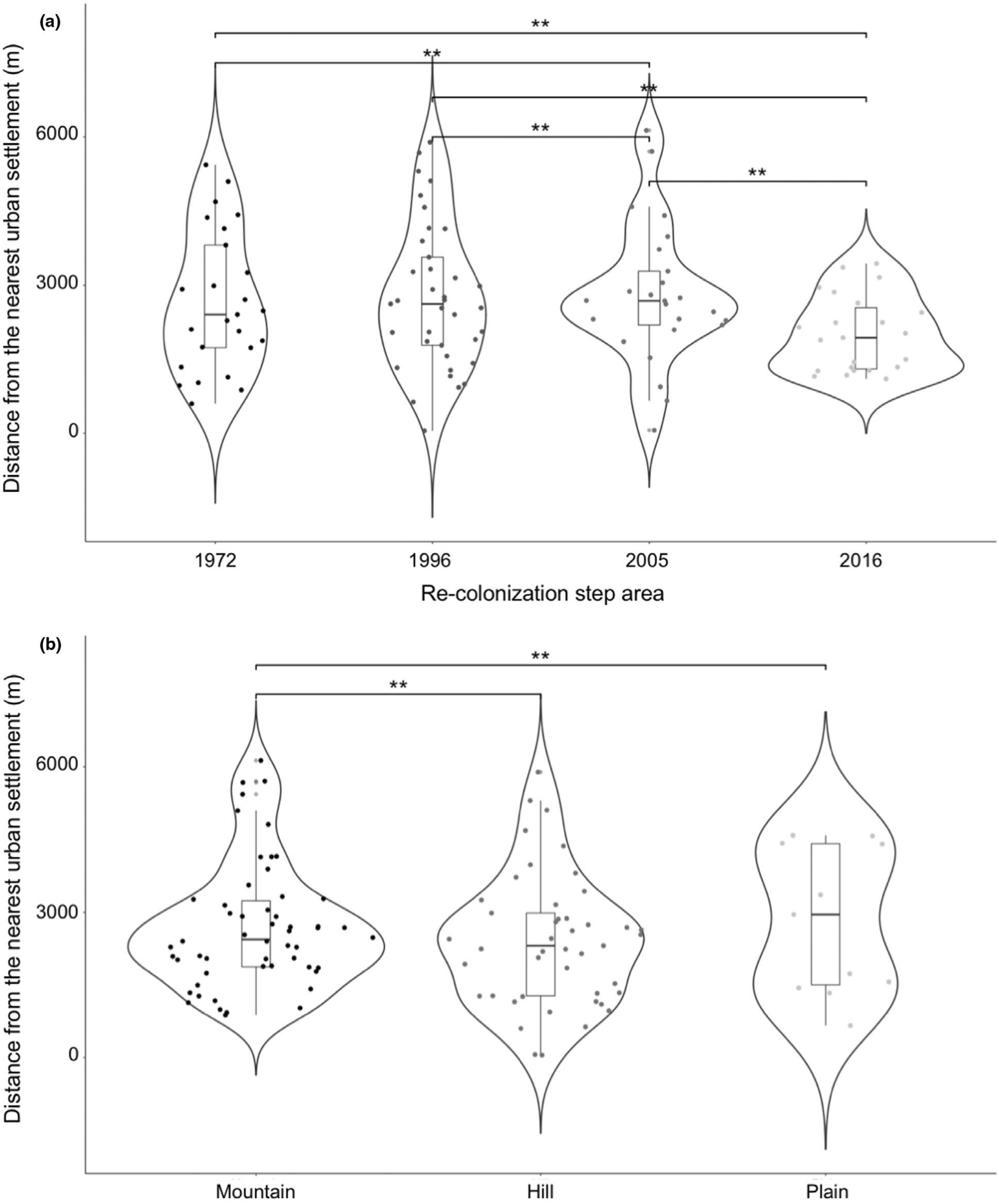

Figure 4 Violin plot representation of the distances of wolf pack locations from the nearest urban settlement considering: (a) the four recolonization steps and (b) the three altitudinal ranges. **The coefficients of variation of the two distributions are significantly different, with a *P*-value < 0.05.

potential of human-caused mortality to strongly hinder wolf population recovery (Quevedo *et al.*, 2019) may indeed be expected to be higher in the areas more densely inhabited by humans (i.e. plains). Nonetheless, the recent overall increase in ungulate communities even in low-altitude environments (Apollonio, Andersen, & Putman, 2010) and the human-caused mortality rates being nowadays unrelated to human density in Tuscany (Musto *et al.*, 2021) may suggest that human-dominated plains are gradually losing their limiting effect on wolf populations.

The tendency to avoid humans may have delayed the recolonization of human-dominated landscapes, leading many researchers to define suitable wolf habitats only forested areas with a low human impact (Mladenoff *et al.*, 1995; Massolo & Meriggi, 1998; Mladenoff & Sickley, 1998; Salvatori *et al.*, 2002; Gehring & Potter, 2005; Potvin *et al.*, 2005; Włodzimierz Jędrzejewski *et al.*, 2005; Karlsson & Sjöström, 2007; Jędrzejewski *et al.*, 2008; Ahmadi, López-Bao, & Kaboli, 2014), where they were in fact confined as ecological refugees (Kerley, Kowalczyk, & Cromsigt, 2012). On the contrary, our data show that after 1996, wolves were increasingly present in anthropized environments (Table S3), which can thus be considered as suitable habitats for wolf presence. It is worth noting that these results describe the actual distribution of stable packs (i.e. social units composed by at least two individuals owing and defending a territory) in relation to human presence. Since our analytical approach did not distinguish between packs with and without ascertained reproduction, further studies are needed to investigate possible differences in resource selection patterns between reproductive and not reproductive packs. We are also aware that our results may have been influenced by not considering the human density changes from 1972 to 2016. Nonetheless, during this period the human density slightly increased in plains but decreased in mountains (Reynaud *et al.*, 2020). This may not only represent a further explanation for the fast recolonization of mountains, but also highlight that wolves expanded in lowland, human-dominated environments when the human density was growing. As habitat selection depends on consumer density and/or resource availability (Avgar, Betini, & Fryxell, 2020), the approach of wild boar to urban areas (Cahill, Llimona, & Gràcia, 2003; Podgórski *et al.*, 2013; Stillfried *et al.*, 2017; Banti *et al.*, 2021) may have favoured wolf expansion in that areas being this species its major prey item in the region (Bassi *et al.*, 2012, 2017).

Effect of urban settlements on pack location

Among the wolf packs identified in 2016, 70% included at least one small and 18% at least one large urban settlement, respectively, within a 5 km radius, that is the radius of a hypothetical home range of 85–110 km^2 (Ciucci *et al.*, 1997; Corsi, Dupre, & Boitani, 1999; Apollonio *et al.*, 2004; Mattioli *et al.*, 2018).

Although our control points were not of ascertained absence, but rather of undetected presence, the distances of observed pack locations to the nearest urban settlement differed significantly from the control, in contrast with Theuerkauf et al. (2003) but in accordance with our second prediction. The tendency of wolves to locate farther from the nearest urban settlement in comparison with control points is consistent with previous studies showing that human infrastructure distribution negatively affects the likelihood of packs locating in a certain area (Capitani *et al.*, 2006; W. Jędrzejewski *et al.*, 2008; Bassi *et al.*, 2015).

Moreover, the effect of the distance from the nearest urban settlement was lighter in the hilly range. This is probably the outcome of the presence of many wolves in an intensively anthropized environment with a greater availability of refuge areas (small wooded or bushy patches) than in the plain; however, they were highly localized in a patchy landscape (Table S2). This may have forced the wolf to settle where these specific conditions were found, with limited care for human presence. Indeed, the hillside is the range where human density is relatively high (see Section 2.1 and Table S2), but also the second altitude range most occupied by wolves with respect to its availability (Fig. 2).

The number of inhabitants in the nearest urban settlement also significantly influenced wolves' choices on where to locate, indicating that wolves preferred the outskirts of urban settlements with low population numbers (see also Table S5).

Constraints of the variability of pack location distance from urban settlements

As predicted, the variability of the distance from the nearest urban settlement differed significantly both across areas recolonized in different periods and across macro-habitats (i.e. Altitudinal ranges). Between 1972 and 2016, there was a gradual decrease in the possibility of choosing suitable locations due to the increase in pack density. Since there was a gradual increase in the amount of surface occupied by wolves and thus in the number of wolves themselves along the considered period (Tables S2 and S3), this further supports our hypothesis. That is, as the number of wolves increased, the availability of suitable areas decreased, leading wolves to select suboptimal areas. In contrast to what was predicted for the three altitudinal ranges, we found the least variability of the distance from the nearest urban settlement in the mountain range, considered as the most suitable macro-habitat. This is likely due to a higher density of wolves in this range, which was in fact more occupied than expected (Fig. 2). Thus, packs, having to keep a safe distance from humans, are forced to rearrange their territories to cope also with other packs and prey availability (density-dependent habitat selection, as explained by O'Neil *et al.*, 2020).

Conclusions

In conclusion, we showed that wolves preferred locating as far as possible from humans but that they occupied locations relatively close to urban settlements in a densely inhabited region, likely to cope with the intra-specific spatial competition. As a consequence, the presence of wolves, even if conditioned by the presence of man, is spreading into the most anthropized areas without a saturation point being foreseen. It should be noticed that in the plain, we found only 10% of the total pack locations observed; therefore, a further expansion is probable in face of the increase in wolf packs.

Karlsson & Sjöström (2007) showed how attitudes and perceptions of wolves varied with the distance of wolves from respondents. Probably, the impression that wolves are now closer to urban settlements than in the 1990 s is also because there are more and more packs in lowland areas, which are also the most anthropized. The increase in pack distribution and the expansion of their range can raise the possibility of encounters between wolves and humans; therefore, this progressive closeness between human beings and wolves, which it is already a major issue for large carnivore conservation, may increase over time. This is true both in Tuscany, where there is a ubiquitous presence of wolves, and in other European areas that are being quickly recolonized by wolves, as this species is able to live both in densely populated plains and in almost abandoned mountains, confirming its adaptability (Muhly *et al.*, 2019). In such context, a proper management of human-wolf conflicts is pivotal in ensuring the conservation of wolves and in preventing a new wave of persecution of the species (Lute *et al.*, 2018). Since risk-enhancing human behaviours are often the main cause of human-predator conflicts (Penteriani *et al.*, 2016), where wolves are recovering after decades of absence it is necessary to encourage an appropriate behaviour of citizens, including a proper management of possible food items as garbage (Mohammadi *et al.*, 2019) and domestic pets (Bassi *et al.*, 2021).

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Conflict of interest

The authors declare no competing interests.

Author contributions

MA originally formulated the idea. EM, MZ and RB collaborated in imaging statistical analysis. MZ and EM performed statistical analyses. MZ and RB wrote the manuscript. MA and EM provided editorial advice. MA provided materials and analysis tools.

Data availability statement

Since they include sensitive information on reproduction sites of wolves, raw data are available from the corresponding author upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Detailed effort and regional results of each pack known in years or reported by the citizen science network integrated survey.

Table S2. Indicators of human presence within the considered altitudinal ranges.

Table S3. Indicators of human presence within the areas available during each re-colonization step (see the text for more details).

Table S4. Chi-square test results of the comparison between expected and observed wolf pack location frequency distribution in the three altitudinal ranges and in the four re-colonization step areas.

Table S5. Means and standard errors of all continuous variables used in the Generalized Linear Models used on wolf pack location.

Table S6. Results of the model averaging among the set of equivalent Generalized Linear Models (ΔAIC <2) on wolf pack location.

Table S7. Post hoc tests between the Coefficients of Variation of the distance from the nearest urban settlement, among the three different altitudinal ranges and the four different re-colonization steps.

Figure S1. Violin plot representation of the distances of wolf pack locations from the nearest urban settlement; in black the observed wolf pack location and in grey the control wolf pack location.

Chapter 2

Kill site selection in multi-prey system by wolves in an anthropogenic context.

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Abstract

To establish a coexistence between man and wolf and an effective conservation of the species, it is necessary to expand our knowledge on the selection of the habitat by the wolf and its social and predatory behaviour. That habitat characteristics can affect prey-predator interactions and may influence a predator's hunting success.

We investigated wolf kill-site selection in a multi-prey context characterized by different anthropogenic activities (livestock farming, tourism, hunting) and different levels of anthropogenic disturbance during different times of the year, where the wolf has recently recolonized the area and it is in gradual recovery. To accurately identify the kill sites of a wolf pack and define their habitat characteristics and selection, we used GPS collars.

Kill site selection analysis showed that wolves in the pre-alpine environment select given areas where they may periodically return to prey. Specifically, wolves were more likely to prey at higher altitudes, on south-facing slopes, near roads or trails and human structures. The selection probability for covered habitats increased with decreasing distance from both roads and human infrastructures. The environmental characteristics of predation sites selected by wolves were different depending on the prey species. When preying livestock, wolves selected areas at higher elevation, when preying chamois, wolves selected areas at higher elevation and steeper terrain. In contrast, wolves selected sites at lower elevation when preying other wild ungulates, particularly mouflons.

Introduction

Habitat selection is a hierarchical process in which four orders of selection are identified: the geographic area, the home range, the core area, and the foraging sites (Johnson, 1980) being represented by kill site for predators (DeCesare, 2012). Indeed, large territorial carnivores, select kill-site within a habitat that reflects the highest prey density (3th-order habitat selection) and the environmental features that increase the probability of killing prey (4th-order habitat selection Davidson et al., 2012). These two orders of habitat selection are not exclusive but may both contribute to explain habitat selection within the home range, as in the case of an opportunistic predator as the wolf. Indeed, despite the tendency of wolves to select the most abundant prey (McPhee et al., 2012; Sand et al., 2016; Smith et al., 2004), there are evidences showing that wolves can select the most vulnerable one (Bergman et al., 2006; Mattioli et al., 2011).

Empirical studies have suggested that habitat characteristics can affect prey-predator interactions and may influence a predator's hunting success (Gorini et al., 2012; Hebblewhite et al., 2005). In Yellowstone Park it has been shown that landscape structure and features are the main determinants of wolves predation sites (Kauffman et al., 2007). Most predation events occur near the ecotone between different environments and near streams or fences that can hinder prey escape; the presence of underbrush, forest density, and terrain irregularities can also contribute to predation success (Bojarska et al., 2017;. Moreover, Torretta et al. (2018) showed that steep slope areas or large areas such as pastures and grasslands promote predation success. Instead, Gula (2004) observed that wolves killed most of their prey in deep creeks and ravines, where wild ungulates can be more easily cornered.

Selection of predation site characteristics also depends on prey type, as habitat use by different prey species can influence predator land use (Barbosa and Castellanos, 2005). Bojarska et al. (2017) showed that the characteristics of wolf predation sites differed between the two main prey species: red deer were killed more frequently near the boundaries between different habitats, while roe deer were preyed more frequently near fences as were fallow deer in costal Italy (Del Frate et al., 2023).

The selection of kill-site features within the home range may vary with wolf activity rhythms, which in turn may be influenced by several variables such as human activity, wolf reproductive state, activity and body condition of prey (Ballard et al., 1991; Hebblewhite and Merrill, 2007; Milakovic et al., 2011; Sand et al., 2005; Theuerkauf et al., 2003). Generally, wolves begin to be active at twilights and reach maximum activity during the nighttime hours (Theuerkauf et al., 2003) avoiding human activity (Mori et al., 2020). As a consequence, predation occurs most often from dawn to dusk (Ciucci et al., 1997; Sand et al., 2005), when there are both activity overlap between wolf and its preys and limited overlap between men and wolves (Mori et al., 2020). In so doing wolves can select hunting site that during the day are often used by humans as in the case of roads that are generally selected when the human presence is limited (Muhly et al., 2019; Newton et al., 2017). Some phases of the biological cycle of the wolf (such as reproduction or birth) could modify the selection of kill-sites. In summer, the presence of less mobile and more vulnerable pups, reduced adults' movements affecting their kill-site selection (Jedrzejewski et al., 2001; Merrill and Mech, 2003). Prey species throughout the year have different body conditions and more vulnerable biological periods, such as the breeding season for males of many ungulate species (Owen-Smith, 2008). Moreover, also changing weather conditions should be considered as they may change prey encounter mode as in the case of snow (Ciucci et al., 2003) by increasing search costs and facilitating predation immediately after the encounter because snow hinders ungulate locomotion (Huggard, 1993; Post et al., 1999).

Numerous studies on wolf kill-site selection illustrated the plasticity of this predator (e.g. Atwood et al., 2007; Gervasi et al., 2013; Gula, 2004; McPhee et al., 2012). Milakovic et al. (2011) showed that wolves from different packs selected different environmental kill-site characteristics, depending on the location of the home range in the landscape: wolves occupying the boreal flats and muskeg selected coniferous forests to prey, while the pack inhabiting rolling foothills terrain used south aspects.

However, most of these studies were conducted in natural protected areas with very limited human activities, low human density and consequently quite .limited human footprint (Collard and Foley, 2002). To the best of our knowledges, there are few or no studies conducted at lower latitudes, in subalpine or Mediterranean habitats characterized by a significant anthropogenic footprint. In environments where human presence is important, wolves face additional demands as avoiding humans. Therefore, in choosing space (or simply predation sites) they face the trade-off between maximizing predation success and avoiding human disturbance.

We investigated wolf kill-site selection in areas of recent wolf recolonization and a multi-prey context characterized by many anthropogenic activities (livestock farming, tourism, hunting) and consequent different levels of disturbance along the year. Most parts of the pre-Alpine regions where the recovery took place are heavily influenced by human activities, especially for agriculture and dairy farming (Ramanzin et al., 2016), that increase the fragmentation of natural habitats (Sanderson et al., 2002). The positive growth trend of the wolf and its trophic plasticity have led to an increase in the probability of contact, and therefore of conflict with humans, especially in respect with livestock husbandry and hunting (Menzano et al., 2018; Woodruff and Jimenez, 2019).

In order to establish a coexistence between man and wolf and an effective conservation of the species, it is necessary to expand our knowledge on the selection of the habitat by the wolf and its social and predatory behaviour in this pre-Alpine and Alpine context and disseminate the results. .

People initially insensitive to wolf presence generally develop more positive attitudes as their knowledge about wolf biology and behaviour increases (Bisi et al., 2007; Ericsson and Heberlein, 2003). Thus, increasing our knowledge of the drivers of wolf behavioral patterns in human-dominated landscapes may help to develop effective management and conservation strategies, which could mitigate conflicts with local human populations (Kuijper et al., 2016).

Study area

The study area was in the north-eastern Italian Pre-Alps, in an area surrounding the Massif of Mount Grappa (45°52’24 N, 11°47’57 E). The study area (35,008 ha, altitudinal range 110-1,775 m a.s.l.) is characterized by a rich floristic diversity including about 1,400 plant species (Busnardo, 1991; Busnardo and Lasen, 1994). It is 68% forested and is composed of mixed forest (14%), dominated by spruce (*Picea abies*) and beech (*Fagus sylvatica*), deciduous forest (54%) with a prevalence of downy oak (*Quercus pubescens*), black hornbeam (*Ostrya carpinifolia*), field maple

(*Acer campestre*), and beech at different elevations. The open areas occupied about 30% of the study area and are represented by meadows, pastures, and arable land. Meadows are widespread with varied associations, from xerophilous pasture at the higher altitudes and on the steepest south-facing slopes, to the high-quality meadows and pastures used by livestock breeders. Typically, agricultural landscape and human settlements decrease with increasing altitude being concentrated in the bottom valley and only sparse over 500 asl. Despite this there is a dense network of roads and trails (3.42 km/km^2), mainly built in WWI.

Livestock breeding is still a relevant economic activity in the area. The study area is characterized by a greater presence of humans in the spring-summer period, both because of the presence of livestock grazing and of tourists. In winter the massif is mostly uninhabited.

The Grappa massif represents a territory recently colonized by wolves. The first reproduction was documented in 2017, when a pack consisting of two adults and six pups (5 male and 1 female: Avanzinelli et al., 2018) was confirmed. Roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and chamois (*Rupicapra rupicapra*) are the most abundant ungulate species, but also mouflon (*Ovis aries musimon*) and wild boar (*Sus scrofa*) are present in low densities. Other species present in the study area are fox (*Vulpes vulpes*), badger (*Meles meles*), brown and mountain hare (*Lepus europaeus and L. timidus*), and mole (*Talpa europaea*), which may represent occasional prey for wolves (Bassi et al., 2017, 2012; Marucco et al., 2008).

Data Collection

In the study area, four wolves were captured between 2019 and 2021 by using a foothold trap (FremontTM Humane Foot Snare Wolf/Cougar 1/8 7x7 and FremontTM Humane Foot Snare Fox/Coyote 3/32 7x7). Once captured, the wolves were immobilized with a mixture of drugs (medetomidine-ketamine-acepromazine) using a syringe blowpipe (Telinject). Biometric data were collected, and biological samples were taken; wolves were weighed and fitted with GPS-collars (VERTEX Plus Vectronic Aerospace GmbH). At the end, in order to reverse the effect of anesthetic drugs atipamezole was injected and the wolves were monitored during recovery. Animal care activities and study procedures were carried out by a veterinarian adhering to the regulations and guidelines on animal welfare, any damage to wolves was ever recorded.

The collars were programmed to record a localization every 2 hours during the day, from 6:00 am to 6:00 pm UTC, and every 30 min during the night. The collars were set to transmit data twice a day (IRIDIUM transmission), at specific times, precisely at 3:00 and 6:00 am.

From September 2020 to January 2022, we remotely tracked 3 out of 4 GPS collared wolves, since one of the GPS collars was no more working during data collection. In total we followed a breeding female (September 2020 - June 2021), an adult male (April 2021 - January 2022) and a young female (October 2021 - January 2022), all belonged to the same pack in different years. By using their locations, we identified clusters as a minimum number of two fix no more than 200 meters apart (Sand et al., 2005). All the cluster sites with a radius not exceeding 50-m were attentively checked by means of direct field surveys to look for signs of wolf predation (presence of a carcass or gut contents of prey). Great effort was made to qualify whether the prey was killed by the wolf or died by other causes. A suite of supporting indicators was used with this aim including wolf tracks, hair and faeces, indications of a struggle visible in broken and trampled vegetation, signs of fresh blood, the type of consumption of the carcass, prey remains, the condition of prey hide bearing bite marks typical of wolf handling, and the estimated time of death in relation to wolf presence according to GPS positions. Prey phenotypic characteristics (hairs, antlers/horns, and eruption/wear of the teeth) were used to classify species and their age. Moreover, we identified the exact place where the animal was killed primarily on the amount of blood and the remains of stomach contents, particularly where the rumen was left. We filled a form (see supplementary materials) containing all relevant information on kill site orography and structure together with the presence of natural or artificial barriers. In case the kill site was located in the forest we took 3 measurements of the basimeter area of it, using the Bitterlich application that in our case replaced the relascope with the only difference that with the application the distance between the phone and the eyes does not matter.

Finally, when fresh wolf droppings were present, a sample was collected in order to perform genetic analysis. These data combined with satellite telemetry made it possible to confirm that the killed prey was in a single territorial family unit.

Data Analysis

We first analyzed the spatial distribution of predation sites that were determined looking at clusters, of a minimum number of two locations in one hour no more than 500m distant, by DBSCAN clustering function in GIS (QGIS 3.10.6). Then we investigated whether predation sites were distributed randomly, dispersed, or in clusters, by using nearest neighbor index method by means of the *nearest neighbor* function.

Next, to investigate the environmental characteristics of wolf predation locations and whether the monitored wolves selected specific environmental features, we adopted a "use availability design" (Manly et al., 2007). Accordingly, we matched locations where wolf predation was found (hereafter referred to as "used" locations) to randomly selected locations (hereafter referred to as "available" locations). The locations that represented the available were randomly generated within monthly home-range of the monitored wolves. The home-range was calculated by using the traditional 100% minimum convex polygon (MCP) method (Mohr, 1947), constructed using all locations of wolves that had GPS collars at the monthly level. As for some months we had two individuals of the same pack monitored simultaneously, the monthly home range was calculated by using the locations of both wolves. For home-range computations we used the GIS software (QGIS 3.10.6). We used a use-available ratio of 1:25 considering it sufficient given the typical environment of the study area.

The individual attributes of each observed predation site (wolf identity, prey species, date of predation) were also assigned to the corresponding available locations. Subsequently by using GIS software, we assigned the following spatial covariates to all locations (both used and available), based on their spatial coordinates:

- “distance to anthropic structures”: a continuous variable measuring the linear distance (m) from the nearest human infrastructure (isolated house, farm, shepherd's cottage, cemetery, school, hospital, urban areas to different degrees of urbanization, industrial areas, quarrying area, sports area, military areas)
- “Distance to roads or trails”: a continuous variable measuring the linear distance (m) from the nearest paved road, dirt road, trail.
- “Habitat type”: classified in open environments (i.e. meadows, pastures, arable land) and closed environments (i.e. mixed and deciduous forest and shrubland)

Finally, we used a Digital Elevation Model (DEM, web site of Regione Veneto) to assign to all locations four further covariates describing the terrain morphology: altitude (m a.s.l.), terrain slope, and surface aspect (cosine-transformed: 0=East and West, 1=North, -1=South), and terrain ruggedness. This last feature was calculated as a quantitative measure of terrain heterogeneity by computing the elevation change within the 3x3 pixel grid, described by Riley et al. (1999).

We built resource selection functions (RSFs) by fitting generalized linear mixed models (GLMMs) with a binary response variable (used = 1, available = 0). GLMMs were fitted by using the *glmer* function of the *lme4* package. We used the distance to anthropic structures, distance to roads, habitat type (open/closed), altitude, slope, aspect, asperity, prey species (chamois, roe deer, red deer, mouflon and livestock), and Julian date as predictor variables in the model. Wolf identity was included as a random effect in the model. All numerical predictors were scaled [(x-mean)/SD] before running any model to improve *glmer* convergence (Bates et al., 2015). All predictors were screened for collinearity (Pearson coefficient |rp| < 0.6) and multicollinearity (Variance Inflation Factor, VIF < 3 Zuur et al., 2009). As asperity was collinear with slope, we retained the latter because was found to be a best predictor by means of a machine learning method (*randomForest* package, n = 500 decision trees).

We first created a GLMM with a full model structure, based on our expectation of the effect of the predictors in driving wolf selection of predation sites. In addition to the single variables, we included some interactions among them. Habitat type (covered or open) was included in interaction with altitude, slope, surface aspect, distance to roads or trails, distance to anthropic structures to evaluate whether selection of open and closed habitats varied with changes terrain morphology and human disturbance. We evaluated if the different environmental features were selected differently throughout the year by including them in interaction with Julian date. We included the interaction between "altitude" and "distance to roads or trails" and "distance to anthropogenic structures", to evaluate the effect of human disturbance in the altitudinal gradient, as anthropization decreases with the increase of altitude. Moreover, to evaluate an additive effect of human disturbance by its presence on both roads and anthropic structures, we included the interaction between "distance from roads or trails" and "distance from anthropic structures". Finally, to better describe wolves' predatory behavior, we included the variable "prey species" (livestock, chamois, roe deer, red deer, mouflon) in interaction with the variables describing the environmental characteristics (altitude, slope, surface aspect, ruggedness, distance to roads or trails, distance to anthropic structures, habitat type).

We then chose the best model by applying a manual step AIC procedure, iteratively removing the worse predictor (that with the higher P-value) and re-running the model until achieving a model with the lowest AIC. In so doing, all the predictors that contributed to increase the model AIC were removed from the best model (see TableS1 for the best model structure).

Finally, the beta coefficients estimated by the most parsimonious model were entered into the resource selection function to obtain RSF scores, which are proportional to the probability of selection. The RSF scores were used to represent the scenarios predicted by the model.

Results

In 514 days of monitoring, we checked a total of 1705 clusters finding 202 predation sites (14 diurnal, 41 twilight and 147 nocturnal). Of these, in 52% of cases the prey was roe deer (n=104), in 19% mouflon (n=38), in 12% chamois (n=25), in 3% red deer (n=7), 9% were domestic ungulates (sheep and goat; n=18) and 3% was on other species (hare, fox, duck; n=6). In 4 cases (2%), we were not able to determine the prey species, so these kill sites were excluded from the subsequent analysis. The kill-sites used by the monitored wolves showed a specific clustered spatial pattern showing a highly significant nearest neighbor index of 0.669 ($z = -8.99$; P-value < 0.01). We identified a total of 34 clusters in which wolves returned to the same cluster to kill prey on average 4.0 ± 3.6 occasions (mean ± SD, range= 2-21 times/year).

In 74% of the kill sites, we identified within a buffer of 50 m at least one natural and/or artificial obstacle, which was likely used by wolves to enhance the predation success. 68% of predation events occurred in a closed environment with an average forest density of 24.99 ± 13.40 m^2/ha (mean ± SD).

For the SRF analysis we used 192 predation sites out of 198 available, because we excluded fox (n=3), hare (n=2), and duck (n=1) kill sites from the analysis, being numerically unrepresentative. The best GLMM to explain selection on wolf kill sites within the study area, included species, altitude, slope, distance to roads or trails, distance to anthropic structures, habitat type and the interactions between species * Altitude, species * slope, species * distance to roads or trails, habitat type * distance to roads or trails and habitat type * distance to anthropic structures as predictor variables (Table S1).

Predictions of the most parsimonious RSF (Table1) showed that in our study area, wolves were more likely to prey at higher altitudes, on south-facing slopes, near roads or trails and human structures. The selection probability for covered habitats increased with decreasing distance from both roads and human infrastructures (Fig.1d and 1e).

Table 1 Results of the best logistic GLMM one the wolf kill-site.

Coefficients	**Estimate**	**es**	**z**	**p**
(Intercept)	-3.822	0.311	-12.289	< 0.001
Species (Chamois vs Roe deer)	0.389	0.327	1.191	0.234
(Chamois vs Red deer)	0.306	0.558	0.548	0.584
(Chamois vs Livestock)	-0.552	0.603	-0.916	0.360
(Chamois vs Mouflon)	0.382	0.362	1.055	0.292
Altitude	0.784	0.277	2.835	0.005
Aspect	-0.206	0.080	-2.589	0.010
Slope	0.214	0.241	0.885	0.376
Distance_roads	-0.355	0.111	-3.197	0.001
Distance_anthr_struct.	-0.847	0.176	-4.800	<0.001
habitat type (Covered vs Open)	-0.026	0.200	-0.128	0.898
Species*Altitude (Chamois vs Roe deer)	-0.952	0.288	-3.303	0.001
(Chamois vs Red deer)	-0.971	0.444	-2.186	0.029
(Chamois vs Livestock)	0.316	0.554	0.570	0.569
(Chamois vs Mouflon)	-1.394	0.319	-4.369	<0.001
Species*Slope (Chamois vs Roe deer)	-0.748	0.263	-2.841	0.005
(Chamois vs Red deer)	-0.722	0.500	-1.445	0.148
(Chamois vs Livestock)	-0.398	0.389	-1.023	0.306
(Chamois vs Mouflon)	-0.313	0.291	-1.077	0.282
Species*Distance_roads (Chamois vs Roe deer)	0.598	0.191	3.125	0.002
(Chamois vs Red deer)	0.374	0.395	0.946	0.344
(Chamois vs Livestock)	1.970	0.436	4.517	<0.001
(Chamois vs Mouflon)	0.420	0.219	1.923	0.054
habitat type*Distance_roads	0.365	0.156	2.339	0.019
habitat type*Distance_anthr_struct.	0.452	0.189	2.395	0.017

Distance_roads= Kill site distance from roads and trails; Distance_anthr_struct.= Kill site distance from the nearest human infrastructure (isolated house, factory, shepherd's cottage, cemetery, school, hospital, urban areas to different degrees of urbanization, industrial areas, quarrying area, sports area, military areas); Estimate= estimated coefficients; es= standard errors of estimated coefficients; z= z-ratio; p= p-value.

The environmental characteristics of predation sites selected by wolves were different depending on the prey species. When preying livestock, wolves selected areas at higher elevation (Fig. 1A). The probability of selection of livestock predation sites increased with decreasing terrain slope and increasing distance from roads and trails (Fig. 1b, c). When preying chamois, wolves selected areas at higher elevation and steeper terrain (Fig. 1a, b). In contrast, wolves selected sites at lower elevation when preying other wild ungulates, particularly mouflons (Fig. 1a). When preying roe deer and red deer, the probability of selection by wolves increased steeply with decreasing terrain slope. This pattern of selection was weaker when wolves preyed mouflon (Fig. 1b). The relative probability of selection of predation sites increased steeply with decreasing distance to roads and trails, when wolves preyed chamois, while this relationship was weaker when wolves preyed other wild ungulates.

Discussion

Our study showed that wolves in the pre-Alpine environment selected specific areas where they periodically returned to prey. Wolves selected kill sites on south-facing slopes regardless of the prey species, selecting sites close to roads and anthropogenic features only when located in a closed environment. Wolves selected different environmental characteristics depending on the prey species. When preying livestock, wolves selected flat terrain at higher elevation far away from roads. Flat terrains were also selected when preying roe deer, red deer, and mouflon, but these sites were located at lower altitudes and in proximity to roads and trails. Finally, when preying chamois, wolves selected steep areas at high altitudes but in proximity to trails. All these results were consistent on the habitat preferences of European ungulates (Corlatti and Zachos, 2022).

The findings of this study confirmed the plasticity of this predator in adapting to different environmental characteristics, as shown in other studies (Atwood et al., 2007; McPhee et al., 2012; Milakovic et al., 2011). We identified 4 main prey species of wild ungulates (roe deer, mouflon, chamois, red deer), which were supplemented in the summer period with domestic prey (sheep and goats).

The selection of kill sites, in our study area, seems to reflect prey space use. Previous studies have shown that the wolves generally select hunting sites where the species is most abundant and, within these environments, they tend to select favorable features to increase predation success (Hebblewhite et al., 2005), such as natural or artificial barriers (Bergman et al., 2006; Bojarska et al., 2017). In our case, in 74% of the kill sites there was at least one natural and/or artificial obstacles within 50m that were likely used by wolves during predation.

The selection for steep or flat terrains by the monitored wolves depended on the prey species. When preying roe deed, red deer, mouflon and livestock wolves selected flat environments where they were probably advantaged in chasing and killing the prey, confirming the results found by Kaufman et al. (2007). Differently Torretta et al. (2018) showed that wolves in their study area selected steep areas to kill roe deer, fallow deer, wild boar, chamois. In our study area, only when preying on chamois wolves selected steeper slopes. This difference in different studies could be explained by the habitat preference of different prey species. Indeed, it has been shown that predators in general, and especially wolves, generally select areas where the prey species are most abundant (McPhee et al., 2012).

Interestingly, the kill sites selected by wolves were more likely located close to anthropogenic linear infrastructures such as roads and trails. In many studies, it has been shown that wolves use linear environmental features (both natural such as streams and anthropogenic such as roads) to move better and faster in their territory (Kittle et al., 2017) but also to increase the encounter rate with prey species (McKenzie et al., 2012; Newton et al., 2017). Our results are in accordance with these previous findings: the very high density of roads and trails (3.42 km/km^2) could have contributed to this result. Wolves in our study area probably selected kill-sites near roads because they increased the encounter probability with prey species (McKenzie et al., 2012). Furthermore, when wolves selected kill sites closed to roads and to anthropogenic structures, they preferentially selected closed environment such as forests (Fig. 1a). This confirms that kill site selection by wolves was driven by the trade-off between predation success and the probability to be detected by humans, which are perceived as a threat. Accordingly, selection probability of open environments by wolves increased with increasing distances from anthropogenic elements, such as houses and roads, because in these areas they are more exposed to human disturbance.

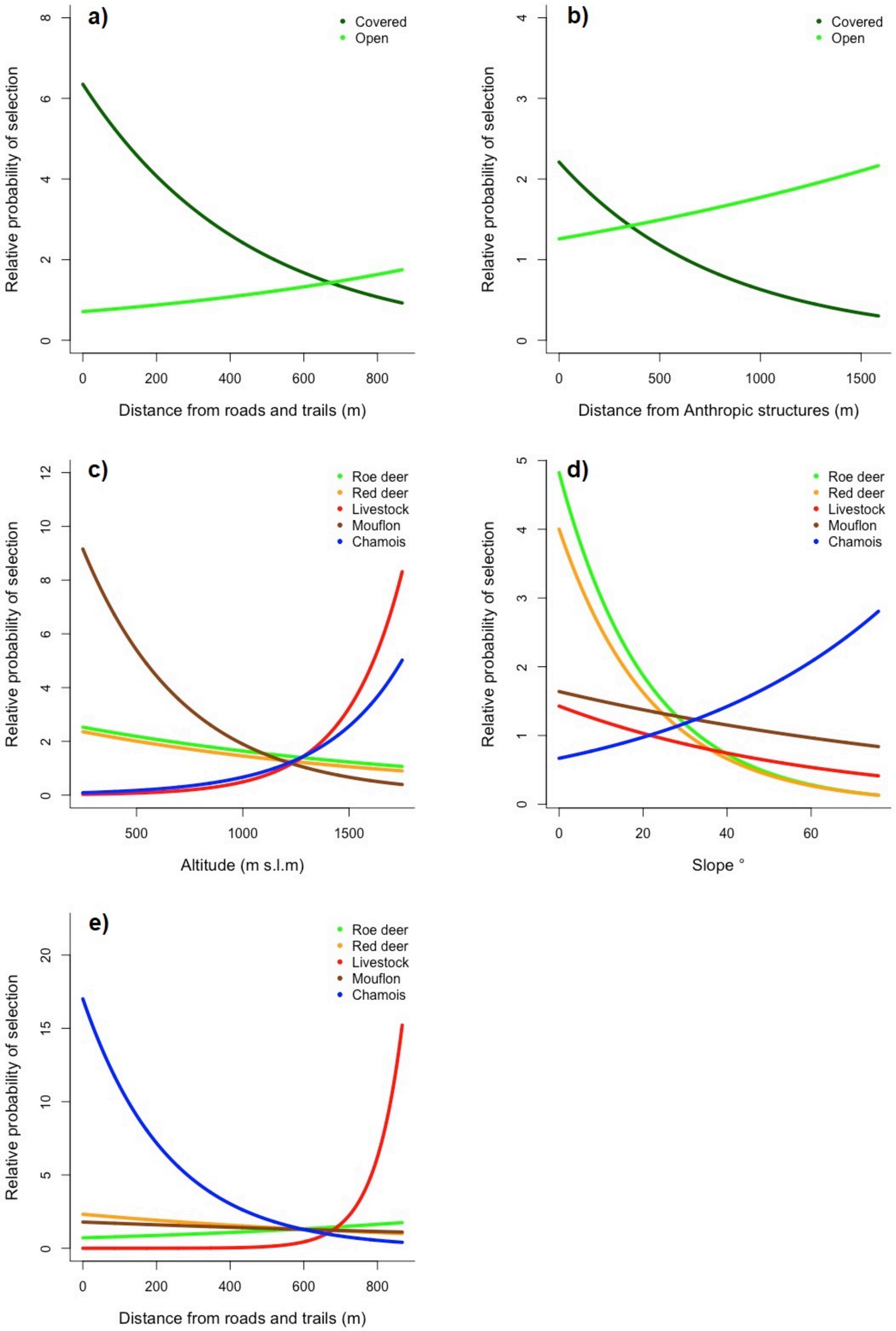

Figura 1 Relative probability of selection for the environment type (Covered or Open) in interaction with a) Distance from Roads and trails and b) Distance from anthropic structures. Relative probability of selection for the five species (Chamois, Roe deer, Red deer, Livestock and Mouflon) in interaction with c) Altitude; d) Slope and e) Distance from roads and trails.

In contrast to what reported in the literature, we found that wolves positively selected anthropogenic structures to kill their prey. We conjectured that this was driven by the higher concentration in these environments of prey species, which are more tolerant of anthropogenic disturbance than carnivores (Rogala et al., 2011) and take advantage of the "human shield effect." contrary to carnivores that avoid human activity by implementing space-time segregation (Rogala et al., 2011).

Presumably, the environmental characteristics selected by wolves to prey on livestock mostly mirrored the herding practices adopted by humans, which generally moved livestock to more favorable foraging areas. Indeed, wolves in our study area preyed on livestock at high elevations predominantly in flat terrains but away from roads, where the best pastures in summer are found. The open environment, typical of pastures, induced wolves to prey away from roads, as in these environments they may perceive a higher risk by human (shepherds and tourists).

Understanding why animals use the landscape and spaces is critical to the management and conservation of wildlife populations. Particularly in a context with a major anthropogenic footprint that varies at different times of the year, ranging from tourists to herders to hunters. In this study, we have shown that in a multi-prey environment the wolf predatory behavior was driven by the maximization of prey encounter probability, that is, wolves preyed in the environments most used by the prey species, while taking advantage of the environmental features that could increase predation success, as shown in other studies conducted in other study areas (Huggard, 1993; Milakovic et al., 2011). The wolf did not only prey by exploiting landforms but whenever possible used anthropogenic elements to its advantage. Our study highlights how anthropic elements are very important not only for increasing encounter success with prey species but also to catch them.

The findings of this study could have important implications on the future management of this species as its densities are increasing throughout Europe (Chapron et al., 2014). The importance of closed habitat, as a key element which may facilitate wolves in taking advantage of anthropogenic features, could in the future increase the presence of this species near large cities, as more and more rural areas are being lost in Europe and forests are becoming increasingly close to urban settlement (Navarro and Pereira, 2015).

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Supplementary materials

Table S1 Multi-model inference of general mix linear model of family binomial to predict resource selection the wolf kill-site. Variable binary dependent was "used" with locations where wolf predation was actually found (1), to randomly selected locations (0). Top ten models.

	Model									
Variable	**16**	**15**	**13**	**12**	**11**	**14**	**17**	**18**	**10**	**9**
Sp	x	x	x	x	x	x	x	x	x	x
Jday										x
A	x	x	x	x	x	x	x	x	x	x
S	x	x	x	x	x	x	x	x	x	x
SA	x	x	x	x	x	x	x	x	x	x
dR	x	x	x	x	x	x	x	x	x	x
dA	x	x	x	x	x	x	x	x	x	x
Htype	x	x	x	x	x	x	x	x	x	x
Sp*A	x	x	x	x	x	x	x	x	x	x
Sp*S	x	x	x	x	x	x	x		x	x
Sp*SA									x	x
Sp*dR	x	x	x	x	x	x	x	x	x	x
Sp*dA			x	x	x	x			x	x
Sp*Htype		x	x	x	x	x			x	x
Htype*S				x	x				x	x
Htype*SA			x	x	x				x	x
Htype*dR	x	x	x	x	x	x	x	x	x	x
Htype*dA	x	x	x	x	x	x			x	x
A*dR					x				x	x
df	26	30	35	36	37	34	25	21	41	42
AIC	1537.31	1537.96	1538.78	1539.08	1539.86	1540.6	1540.7	1543.77	1545.2	1547.23
Delta	0	0.64	1.47	1.77	2.55	3.29	3.41	6.45	7.89	9.92
Weight	0.3	0.22	0.14	0.12	0.08	0.06	0.05	0.01	0.01	0
Loglik	-742.51	-738.79	-734.14	-733.27	-732.65	-736.06	-745.23	-750.79	-731.3	-731.25

EType= environment type (Covered or Open); Jday= Julian day; A=Altitude of locations; S=Slope of locations; SA=Surface aspect of locations; dR= Distance from nearest roads or trails; dA=Distance from nearest anthropic structures; Sp=Prey species (Chamois, Roe deer, Red deer, Muflon, Livestock); df= degrees of freedom; loglik= log likelihood; AIC= Akaike's Information Criterion; delta= the difference between AIC values for two nested models; weight= Akaike weight.

Chapter 3

Prey selection and kill-rate estimation of wolves in the eastern Alps (Italy).

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Abstract

The wolf's trophic plasticity allows it to exploit and survive in many ecosystems even with a high human presence. The study of wolf's predatory behaviour is an important to develop a better understanding its ecological role in these contexts. We exploited satellite technology and the ecological conditions of our study area, characterized by different levels of human presence, to investigate the prey selection and kill rate of 3 wolf packs. We surveyed 2655 location clusters, which allowed us to identify 306 kill sites and 212 scavenging sites. Our results confirm the high plasticity of this predator which, within a multi-prey system, selected different species according to the best cost-benefit trade-off and selected individuals with worse physical condition. Finally, kill rates were significantly different depending on the study area and wolves did not always meet their hypothetical daily energy requirements, which, however, was reached most probably through scavenging. In more anthropized environments the energy requirements were met by kitchen scraps and incorrectly disposed of carrion, compromising the wolf's ecological role. Our results extend knowledge on the trophic ecology of the wolf and open several points on the future management of this predator in human-dominated landscapes. In conclusion, soon it will be important to promote proper human behavior in areas affected by the urbanisation of the wolf in order to eliminate alternative food sources to wild species. Furthermore, it will be important to increase studies and monitoring of the urbanization phenomenon of this species to mitigate human-wolf conflicts.

Introduction

Wolves, as predators, have a fundamental importance in different ecosystems and have long been extensively studied. They are flexible and highly adaptable predators intensively investigated in their relationships with preys (Mech and Boitani, 2003). Predatory behavior requires careful analysis of the trade-off between costs and benefits leading to prey selection (Mattioli et al., 2011).

This can be implemented based on abundance or vulnerability. In the first case the predator chooses the prey most available in the environment resulting in a higher probability of encounter of the prey lowering the costs of predation (Fritts and Mech, 1981). Sand et al. (2016), observed that wolves in Scandinavia mainly prey on roe deer, which is a small prey, and that predation on the larger moose is closely correlated with roe deer density in the area. These results are explained by the increasing density of the roe deer population which results in a higher rate of prey-predator encounters; in addition, an increasing number of individuals results in increased visibility of the roe deer which is cause for easier detection by the wolf. Thus, in this case there is selection based on abundance.

In the second case, the weakest prey is chosen, which therefore requires less effort during the predatory act (Mattioli et al., 1995). In the northeastern Apennines, Mattioli et al. (2011), evaluated wolf selection in a high-density multi-species system in which roe deer represented the most prevalent species but wild boar represented the preferred prey. The strong selection exerted on this species could be explained by its higher productivity leading to a higher presence of the most vulnerable age classes like young and subadults. Knowing how, when, and how often carnivores feed is of strong interest to many biologists because their regulatory effect on different species communities (Taylor, 1984) and the conflicts with humans in more anthropized landscapes (Mech and Boitani, 2003).

Often the diet of wolves have been extensively studied by collecting and analyzing wolf scats, this is because the sampling effort is relatively limited allowing to analyze and compare the diets of different wolf populations living in different areas. However, scat analysis is an indirect technique that has limitations that are often not easily resolved. For example, estimating the number of preys killed requires several assumptions about both energy requirements and prey size and digestibility (Bassi et al., 2012; Newsome et al., 2016). Moreover, it must be assumed that all prey consumed are from direct predation, and it is not possible to distinguish active predation from simple scavenging on a carcass. This leads to an approximate estimation of kill rates (Gable and Windels, 2018; Wachter et al., 2012). Otherwise, obtaining more reliable estimates of active predation and kill rates requires direct monitoring of predation. Since the early 2000s, thanks to satellite technology (Global Positioning System), studies on direct detection of predation have increasingly occurred (e.g., Anderson and Lindzey, 2003; Metz et al., 2012; Sand et al., 2005; Webb et al., 2008). In these studies, prey was sought where there were clusters of GPS locations (clusters), i.e., where the predator had stopped for sometimes. If a carcass is found short after predation occurred it is possible to distinguish predation from scavenging to increases the reliability and accuracy of studies of wolf trophic ecology such as kill rates (e.g., Gable and Windels, 2018; Sand et al., 2008; Vucetich et al., 2011).

Kill rate is a key aspect of wolf ecology and the balance of ecosystems where wolves occur. Several studies have been done on wolf kill rates at different times of the year and in different study areas (e.g., Smith et al., 2004; Vucetich et al., 2012; Zimmermann et al., 2014). In these studies, kill rates vary greatly depending on the study area and prey species. In addition, kill rates can also fluctuate throughout the year in those climates where there is strong seasonality (Metz et al., 2011), either because of the structure of prey populations throughout the year or because of the health status of prey (e.g., Metz et al., 2012). For example, Sand et al. (2008) showed that kill rates per wolf increased during the summer period due to the higher presence of the small prey represented by young classes of ungulates. In contrast, Smith et al. (2004) showed how wolves significantly increased prey kills in winter, particularly on more debilitated animals, during colder winters, due to the greater difficulty of the prey to escape predation.

Although this is a much-studied aspect of wolf ecology, most of these studies considered simple food webs (e.g., islands: Vucetich et al., 2002) in which the main prey species was clearly identified (e.g. North America: Lake et al., 2013; Smith et al., 2004; Vucetich et al., 2012; North Europe: Sand et al., 2008; Zimmermann et al., 2007) in ecosystems with a relatively high naturalness and a low human footprint.

However, the wolf is a very plastic species and is returning to many highly anthropized environment (Zanni et al., 2023), where they had disappeared because of human persecution

(Chapron et al., 2014) and where hunting pressure on its prey species is strong. These environments often have a very diverse trophic availability with different prey species and a complex food webs (Packard, 2010). Until now, no study has investigated the kill rate in these context they were inferred from diet analyses (Bassi et al., 2017; Marucco et al., 2008) or carcasses use (Ciucci et al., 2020).

In these contexts, it would be important to know the number of prey that a predator kills over time to better evaluate the impact of the combined effect of human and wolf predation.

In order to fill this gap we studied kill rates and prey selection by wolf, in an area of the northeastern Alps in a multi-prey context where the wolf has returned recently.

With this research we test the hypotheses

- that the monthly kill rate is influenced by the size of the main prey species, the number of wolves per pack and scavenging rate.
- that there is strong selection based on the characteristics of different species, on body condition of individuals.

Study area

Two of the three areas were located predominantly on the Monte Grappa Massif (45°52'24 N, 11°47'57 E; altitudinal range 110-1,775 m a.s.l.), in Veneto Prealpine area. The third study area was in the northernmost dolomites near M. Antelao (46°27’03 N, 12°16’02 E; altitudinal range 525-2,407 m a.s.l.). The north-eastern Italian Pre-Alps are characterized by a Cool temperate (Cf) based on the Köppen–Geiger scheme (Fratianni and Acquaotta, 2017). Winter is the coldest season, with average temperature raging between 0 and −3 °C. Summer is the warmest season with temperature averaging between 17 °C and 21 °C. Differently, the third study area, falling in the Alpine region, is characterized by a cold temperate climate (Dw). The winter is coldest season and average temperature of the -3 °C; average temperature of the summer, warmest season, ranging between 10 to 14.9 °C. Increasing in altitude, two types of climate can be recognized; a cold climate due to altitude (H), affecting the highest sectors of the Alps and the summit areas of the highest Apennine groups and a nival climate (EF), affecting the Alpine zone above 3500 m, with perennial snowfall. In both study areas the annual precipitation amounts to 1200 – 1500 mm, with no significant differences among seasons (Winter, Spring, Summer, and Autumn).

In all three study areas, vegetation is strongly influenced by topography, exposure, and elevation. Above 200 m a.s.l., forest occupies 65% to 78% of the total area depending on the study area and is composed of coniferous forest (11% to 69%), dominated by spruce (*Picea abies*) and larch (*Larix decidua*), deciduous forest (89% to 31% depending on the study area) dominated by downy oak (*Quercus pubescens*), hornbeam (*Ostrya carpinifolia*), field maple (*Acer campestre*) and beech (*Fagus sylvatica*). Open areas occupy 12 percent to 35 percent depending on the study area and are divided into meadows, pastures, and arable land. Grasslands are widespread and have the most varied associations, ranging from xerophilous pasture formations at higher elevations and on steeper south-facing slopes to high quality meadows and pastures of shepherds' hut. In general, the arable landscape and anthropized areas decrease with increasing elevation. Anthropogenic structures from 500 m above sea level are sparse but widespread throughout the area, and only in the valley bottom there are urban settlements. Nevertheless, there is a dense network of roads and trails (3.42 km/km2), built largely during the wartime period of World War I. In contrast, below 200 m a.s.l., only 41% is occupied by forests, the remaining 58% are open environments and mostly 37% are anthropogenic ecosystems.

Livestock breeding is still a relevant economic activity in the different areas. The different study areas are characterized by a greater presence of humans in the spring-summer period, both because of tourism and because of the presence of livestock grazing. In winter at higher altitudes are mostly uninhabited.

The north-eastern Italian Alps were recently colonized by wolves. The first reproduction was documented in 2012, when a reproductive couple consisting of a Dinaric male wolf and Italic female wolf was confirmed (Marucco et al., 2014). Roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and chamois (*Rupicapra rupicapra*) were the most abundant ungulate species, but also mouflon (*Ovis aries musimon*) and wild boar (*Sus scrofa*) were present in low densities. Other species present in the study area were fox (*Vulpes vulpes*), badger (*Meles meles*), brown and mountain hare (*Lepus europaeus, Lepus timidus*), which may represent occasional prey for wolves (Bassi et al., 2017, 2012).

Data Collection

From September 2020 to January 2022, we remotely tracked 3 GPS collared wolves. We followed a breeding female (September 2020 - June 2021), an adult male (April 2021 - January 2023) and a young female (October 2021 - August 2022), originally all belonged to the same pack in different years (Grappa Pack). Later two of them created two different packs in different areas. The young female from January 2022 after a short period of dispersion from the original pack formed her own pack adjacent to her former home territory (Monfenera pack). The adult male from March 2022, after a dispersal, settled in a new area by mating with a female (Dubiea pack). Both wolves had pups in spring 2022.

By using their locations, we identified clusters as a minimum number of two fix no more than 200 meters apart (Sand et al., 2005). All the cluster sites with a radius not exceeding 50-m were attentively checked by means of direct field surveys to look for signs of wolf predation (presence of a carcass or gut contents of prey). Great effort was made to qualify whether the prey was killed by the wolf or died by other causes. A suite of supporting indicators was used with this aim including wolf tracks, hair and scats, indications of a struggle visible in broken and trampled vegetation, signs of fresh blood, the type of consumption of the carcass, prey remains, the condition of prey hide bearing bite marks typical of wolf handling, and the estimated time of death in relation to wolf presence according to GPS positions. We identified the exact place where the animal was killed primarily on the amount of blood and the remains of digestive tracts, particularly where the rumen was left. Differently, prey phenotypic characteristics (hairs, antlers/horns, and eruption/wear of the teeth) were used to classify species and their age. Moreover, with each predation found, we determined the percentage of carcass consumed by wolves following the classification of Wilmers et al. (2003). We filled a form containing all relevant information on kill site orography and structure together with the presence of natural or artificial barriers. In case the kill site was in the forest we took 3 measurements of the basimeter area of it, using the Bitterlich application that in our case replaced the relascope with the only difference that with the application the distance between the phone and the eyes does not matter.

Finally, when fresh wolf droppings were present, a sample was collected to perform genetic analysis. These data combined with satellite telemetry made it possible to confirm that the killed prey was in a single territorial family unit.

Prey body condition

To estimate the body condition of the preyed animal, when possible, we collected the metatarsal or metacarpal of the preyed animal to later perform bone marrow fat analyses of the killed prey. These analyses were useful to determine body condition as fat stores, are consumed sequentially; subcutaneous fat stores are used first, followed by those in the mesenteric, then those in the kidney, and finally the fat stores in the bone marrow, where the last ones used are those in the metatarsal (Okarma, 1984). Several studies have concluded that the kidney fat index (Riney, 1955) and the amount of fat in the bone marrow constitute the best indices for assessing the body condition of an animal (Anderson et al., 1972; Bear, 1971; Ransom, 1965).

Moreover, in different research it has been pointed out that marrow fat value in ungulates is a direct indicator of total body fat (Holand, 1992; Huot and Goudreault, 1985; Watkins et al., 1991).

For our study, Nailand's method was used, which estimates the percentage of fat within the bone marrow by calculating the dry weight of the sample, then the weight of fat and residue in the wet marrow sample (Neiland, 1970). Since water and fat content are inversely correlated, the dry weight of the marrow provides a convenient and accurate method for estimating fat content.

In addition to metatarsals and metacarpals of wolf prey we also collected samples from animals taken by hunters. This sample we used as a control in subsequent analyses.

Finally, the samples were processed still frozen to avoid deterioration (Ratcliffe, 1980).

Data analysis

As a first step, we obtained abundance indices of the 4 most abundant prey species in the study areas. The raw data were provided by the Veneto Regional Wildlife Service (Italy) and were collected from visual counts from vantage points, carried out in the spring of each year, by hunting groups in the study areas concerned. To assess a possible selection process on wild ungulates by wolves, we compared use and availability using W-index (forage ratio), calculated for multispecific prey complexes with constant populations (Krebs, 1998). We used the Wides I function of the 'AdehabitatHS' package of the R software (R Core Team, 2022).

Next, we estimated the monthly kill rates of the three monitored wolf packs in the north-eastern Italian Alps. For the Grappa pack we considered 15 months of monitoring (from 10/2023 to 12/2021), for the Monfenera pack 11 months of monitoring (from 02/2022 to 12/2022). Finally, for the Dubiea pack, monitoring lasted 4 months (05/2022 to 08/2022). We calculated the kill rates in three different ways to increase the comparison with studies that used different methods. We calculated the kill rate as kill/pack/day, as kill/wolf/day, and as kg/wolf/day. In the first case we divided the number of packs kills by the number of days that pack was monitored; in the second case we divided the number of kills by the estimated wolf days for a given pack (Becker et al., 2008). Wolf days were the product of the average pack size at the monthly level. The average number of wolves was obtained by combining information from different monitoring techniques. Opportunistic video trapping (2-15 video-traps), snow tracking, direct observations and when possible genetic sampling. Otherwise, in the third case we divided the kg of meat by the estimated wolf days for a given pack.

Because sometimes the GPS collars would fail to send data we decided to exclude these days from analysis. That is, we performed a t.test for paired data between the kill rates obtained by considering a different number of uncollared hours to decree the exclusion of days. We considered it sufficient to eliminate all days when nighttime clusters (including from 18:00 to 08:00) were not controlled for more than 4h. We decided to exclude daytime clusters because wolves in this time interval predominantly made resting sites; in fact, we found only 2% of predations during daytime hours.

Finally, we studied the variation in monthly kill rates by pack using the procedure of general linear models. The variable "kill rate" (kills/pack/day) was considered as the response variable of a generalized linear model (GLM). The variables considered were: study area (Grappa, Monfenera and Dubiea), season (Summer and Winter), mean number of wolves in the pack, scavenging rate, and mean weight of predated species, all being important factors that can regulate kill rate (Gable and Windels, 2018; Metz et al., 2012; Sand et al., 2012; Vucetich et al., 2002). We also added the interaction between the average number of wolves and the scavenging rate, since both are variables that can affect food intake and thus increase and decrease the kill rate.

Prey body condition analysis

We estimated the bone marrow fat percentage of field-collected metacarpals and metatarsals. To assess the physical condition of the prey, the following formula was applied to determine the percentage of fat within the marrow:

$$\boxed{\%\, Marrow\ fat = \frac{dry\ marrow\ weight}{fresh\ marrow\ weight} \times 100}$$

This allowed animals to be classified as being in good or poor physical condition based on the percentage of fat present. The threshold considered to evaluate the animal in good physical condition is 70/75%, below this threshold the animal is gradually classified in decreasing physical and nutritional condition and therefore debilitated and more likely to be preyed upon (Mech, 1995; Smith et al., 2004).

Having a non-uniform sample, consisting of both metacarpals and metatarsals, it was necessary to evaluate whether there was a correlation between the values of fat present in the bone marrow of these two samples. Some studies state that there is a good correlation between different long bone samples, and they can be used indiscriminately for bone marrow analysis (Fuller et al., 1986; Gazzola et al., 2007). Using a Spearman Correlation analysis and a Student's t-test we confirmed that no statistically significant difference occurred between metacarpal and metatarsal medullary fat percentage (Fig. S1).

To study seasonal variations in the percentage of marrow fat in wolf-killed animals we used beta regression, since our dependent variable was a proportion of a continuous variable (MarrowFat = percent marrow fat /100). Julian day, year, pack name and species were used as covariates in the model.

Finally, using a smaller dataset, we compared the marrow fat of prey killed by wolves with a control sample collected by hunters. We used only the wolf samples included in the period when control samples were available (mid-September to mid-December each year). We then created the variable "used" by assuming 0 and 1 for control and observed marrow fat, respectively. The "used" variable was considered as a response variable of a generalized linear model (GLM) with a logit link function with the following predictors:

- MarrowFat
- Year (2020, 2021, 2022)
- Species (Roe deer, Chamois and Red deer).

From this model, we excluded the Dubiea pack and mouflon species samples as the missing control data.

Results

We obtained 33024 localizations in 1266 days in which the 3 wolves were monitored with GPS collars. In this monitoring period, we classified 3192 clusters of which 2655 we surveyed (83%) Of the clusters reached in the field, 1069 were resting sites (40%), 1068 other sites (e.g. no kill site, no resting site, no scavenging site; 40%), 306 kill sites (12%) and 212 scavenging sites (8%; Table 1). The three different study areas had different proportion of the 4 most abundant prey species (roe deer, chamois, mouflon and red deer; Table. S1).

Table 1 Cluster type number of the different wolf packs monitored during the study period.

Wolves Pack	**Monitoring Period**	**Cluster type**	**n**	**%**
GRAPPA	From 15/10/2020 to 31/12/2021	Scavenging site	101	7%
		Other site	606	41%
		Kill site	202	14%
		Rest site	566	38%
		Total	1475	100%
MONFENERA	From 01/02/2022 to 31/12/2022	Scavenging site	91	9%
		Other site	418	41%
		Kill site	73	7%
		Rest site	435	43%
		Total	1017	100%
DUBIEA	From 06/05/2022 to 12/08/2022	Scavenging site	20	12%
		Other site	44	27%
		Kill site	31	19%
		Rest site	68	42%
		Total	163	100%
Total	From 15/10/2020 to 31/12/2022	Scavenging site	212	8%
		Other site	1068	40%
		Kill site	306	12%
		Rest site	1069	40%
		Total	2655	100%

Wolves pack= pack of wolves monitoring during our study; Monitoring period= Monitoring period of three wolves' pack; Cluster type= Type of cluster; n= Numbers of cluster type; %= Percentage of cluster type.

In the 306 killing sites we found 310 carcasses. Wild species accounted for 77.74% of the sample (Table. S2). The most preyed upon wild species was roe deer (42.26%), followed by mouflon (18.71%), chamois (7.74%) and red deer (5.81%). Conversely, livestock accounted for 19.03% of the sample with prevalence of sheep (16.13%) and to a lesser extent goat (2.26%) and birds (0.65%). Finally, in 3.23% of the sample the species could not be determined (Table.S2).

In the Grappa and Monfenera packs, the most preyed upon wildlife species was roe deer, 49.01% and 33.33% respectively (Table.S2). If for the Grappa pack, roe deer was the most preyed species, in the Monfenera pack it was sheep (Table.S2). In contrast, the most preyed species in the Dubiea pack was found to be red deer (37.04%). However, using Manley's selection index we showed that all three wolf packs positively selected mouflon and negatively selected chamois (Fig.1). Roe deer was selected by the Monfenera pack and used as available by the Grappa and Dubiea packs. Finally, red deer was avoided by the Grappa and Monfenera packs and selected by the Dubiea pack (Fig.1).

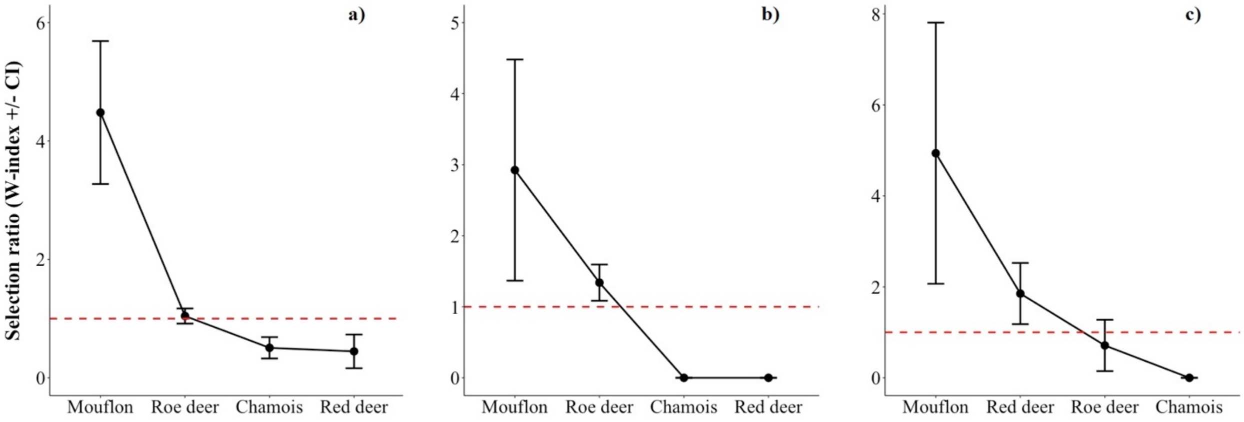

Figure 1 Manley's selection index of wild ungulates (chamois, roe deer, red deer and mouflon) by monitored wolves in eastern alps from 15/10/2020 to 31/12/2022. a) Grappa pack; b) Monfenera pack and c) Dubiea Pack. Red line indicates the level of absence of selection and that the utilization of a given resource reflects availability (W-index=1). Values above the red line indicate resource selection (W-index>1), values below the red line indicate avoidance (W-index<1).

Kill rate

The Grappa pack was the pack with the highest average monthly kill rate 0.49 ± 0.03 kill/pack/day. In contrast, the Monfenera pack was the pack with the lowest average monthly kill rate 0.25 ± 0.04 kill/pack/day. Instead, the Dubie pack had a kill rate of 0.43 ± 0.06 kill/pack/day. Differently, if considering the kill/wolf/day the Dubiea pack that highest 0.18 + 0.02, the Grappa pack showed lowest averange 0.07+0.03 and the Monfenera pack showed the average with the highest month variation during the monitoring period 0.11 + 0.06.

The best model that explained the monthly difference in kill rates included study area, average pack wolf number, scavenging rate, season and the interaction average pack wolf number x scavenging rate as predictors. The remaining variables explained 66% of the variance of the dependent variable (R^2=0.66). Monthly kill rates were significantly different by study area particularly between the Grappa and Monfenera packs (t=-2.116; p=0.045) and between the Dubiea and Monfenera packs (t=-3.506; p=0.002). In addition, in the winter seasons, the kill rates were significantly higher than the summer season (t=2.403; p=0.025), and as the kill rate decreased, the scavenging rate increased significantly (t=-2.318; p=0.030). Finally, as the kill rate and the average number of wolves in the pack increased, the scavenging rate increased significantly (t=2.310; p=0.030).

We estimated that each wolf obtained from the kills an average of 1.68 kg/wolf/day. If the hypothetical energy requirements calculated in Peterson & Ciucci (2003), of 0.09 kg/kg wolf/day, is also correct for wolves in our latitudes and ecosystems, then the wolves in our study obtained 57% of their energy requirements from kills if we consider that the average weight of wolves captured in this study area is 31.7 kg. In particular, the Grappa Pack was the pack with the lowest per capita amount obtained from kills, 1.29 ± 0.11 kg/wolf/day (45% of its energy requirements). Differently, the Dubiea Pack was the pack with the highest amount per capita 3.08 ± 0.66 kg/wolf/day (108% of its energy requirements). Finally, the wolves of the Monfenera Pack obtained from the kills 1.70 ± 0.29 kg/wolf/day (60% of its own energy requirements).

Prey body condition

We collected a total of 194 samples from the found wolf prey. 28% of this sample were a double sample of the same prey (metacarpal and metatarsal) which we used for Spearman correlation and t.test for paired data (Fig. S1). The correlation between the metatarsus and metacarpus of the same animal was positive and significant (R=0.94; p<0.001), and the t-test pr paired data indicated that there was no significant difference between the percentage of fat in the metacarpal marrow compared to that of the metatarsus (t=0.27; p=0.79). This allowed us to use either the metacarpal or the metatarsal of the prey for the subsequent analysis.

Of the 140 samples from different species, 54% were roe deer, 11% chamois, 11% red deer and 23% mouflon. Only in one case did we collect a wild boar metatarsus, which we removed from the analysis sample.

In 34% of cases, the species preyed upon by wolves had a low body condition, i.e., less than 75% marrow fat (Table S3). Red deer prey had the highest percentage of poor body condition (53.33%). In contrast, mouflon was the species with the lowest percentage of prey in poor body condition (3%). Roe deer and chamois had 43.42% and 25% respectively in poor body condition, respectively (Fig. 3).

The best model explaining the variation in marrow fat percentage was the one that included the covariates pack name and species. We showed that the Dubiea pack preys on animals with significantly lower body condition than the Grappa pack (z=2.301; p=0.021). Instead, wolves in all the monitored packs preyed on red deer and roe deer with significantly worse body condition than mouflon (z=-3.188; p=0.001 and z=-3.263; p=0.001).

Finally, we showed that wolves positively selected roe deer, chamois and red deer with worse health status than available (z=-2.850; p=0.004).

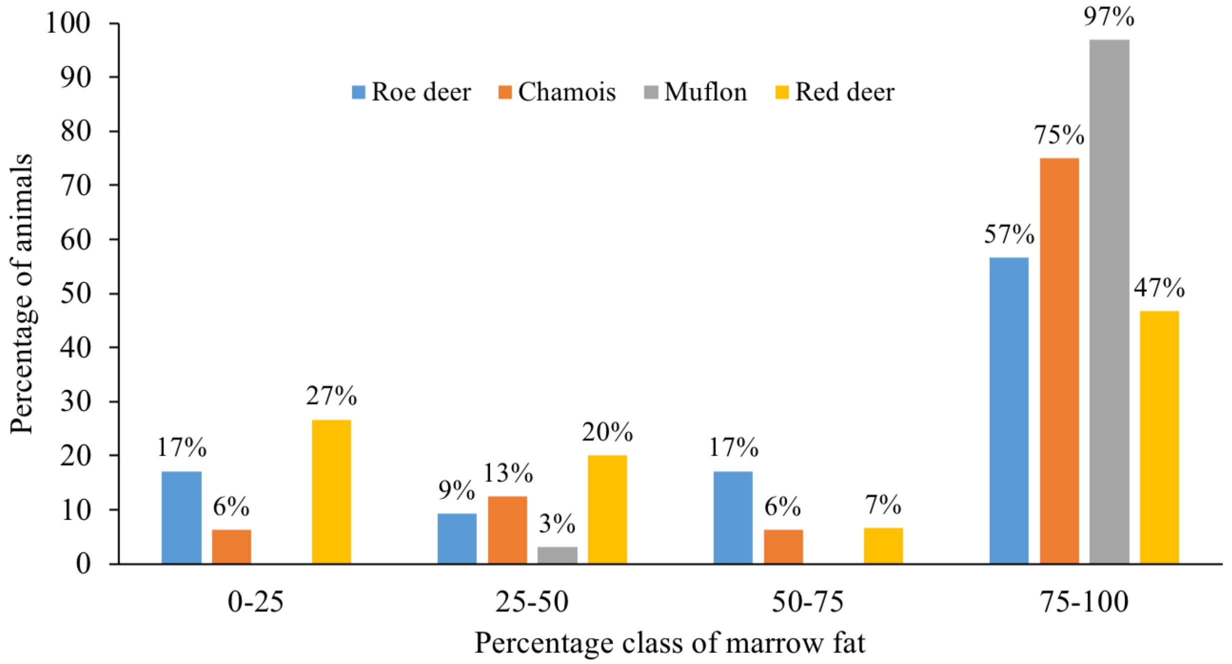

Figure 3 Marrow fat percentage classes of the four main prey species killed by the monitored wolves. Below 70/75% threshold the animal is in poor physical and nutritional condition and therefore debilitated.

Discussion

In this multi-prey system, wolves killed little more than once every other day and did not always meet their hypothetical energy requirements from the animals they killed. In our study, wolf packs preyed upon several ungulate species they positively selected mouflon in all study areas despite the fact that it was not the most abundant and most frequently found species in the prey. Kill rates were significantly different according to the study area and did not always meet the hypothetical energy requirements that they might have achieved most probably through scavenging. Finally, the health status of prey showed that the ungulate species preyed upon by wolves differed in physical condition. Roe deer, red deer and chamois preyed upon were in worse physical conditions than the reference population.

In our study, wolves proved once again to be efficient opportunistic generalist predators. Although roe deer was often the wild species most used by wolves, only the Monfenera pack selected it. From the Grappa and Dubiea packs it was not selected but used according to availability. This could be because the Monfenera pack had at disposal less dense populations of other prey species and therefore selected the most abundant and smallest species. In contrast, we showed how the mouflon, despite being the least present species in the three study areas, was the most selected one. This could be due to the better tradeoff by wolves in preying on this species, which in these areas is an introduced species unfamiliar with Alpine environment (IUCN, 2020; Loy et al., 2019). Despite its limite presence mouflons were probably still profitable for the wolf to prey upon. This would explain why in our study areas the mouflon, was the species with the lowest percentage of individuals preyed with poor body condition (3%) compared to the other species.

In contrast to the mouflon, the chamois was avoided in all three study areas despite often being the most abundant species. Of the four species, the chamois mostly uses environments characterized by steep slopes and rock faces the most (Corlatti et al., 2021). Selecting impervious and hard-to-reach areas makes it less likely to be attacked by predators and particularly wolves (Baruzzi et al., 2017). Indeed, as we assumed, wolves predominantly killed chamois in worse body condition than the population average that probably are mostly accessible to predators.

Finally, the red deer, the largest wild prey species in our study areas, was only positively selected by the Dubiea pack, although the founder wolf came from the Grappa pack where the red deer was avoided. In this study area, red deer is the second most abundant species after chamois. It is likely that the low density of roe deer changed the tradeoff in preying on this species which, in addition to being selected, is also the species most used by the wolf. This mirrors the findings of Sand et al. (2016), i.e. in Sweden where the increase in predation on a large prey such as elk by wolves depended on a decrease in roe deer density. Moreover, in our study the red deer were also the species with the highest proportion of individuals in poor body condition killed by wolves. Probably being a larger species, the selection of individuals on this species is more intense.

kill-rate

During the monitoring we found differences in the monthly kill rate which varied both among packs and season. As demonstrated in other studies, the rate of predation was found to be significantly higher during the cold season (Smith et al., 2004; Vucetich et al., 2002). When there are harsh winters, wild species worsen their health by decreasing their body condition. This happens especially when there are cold and snowy winters (Metz et al., 2012). Under such circumstances kill rates increase due to an increased ease for wolves to prey on debilitated individuals (Smith et al., 2004). Our results support this hypothesis as 34% of the animals killed by wolves were in poor body condition: for roe deer, chamois and red deer the preyed individuals were in worst condition than in autumn. Otherwise, this seasonal difference could be also due to an increased availability in the summer period of small prey such as ungulate young (Sand et al., 2008). Being very small prey species, wolves probably did not always form clusters at the kill sites. This may have prevented the finding of wolf kills. Moreover, wolves change their behaviour during this period by taking food to their pups. This may have further hampered finding prey in the case that the prey was not consumed at the kill site but in the rendezvous site area. The seasonal difference could be also due to the presence of domesticated animals in the wild on alpine pastures during summer and thus to more remunerative prey from the point of view of energy and trade-off, which would lead to a decrease in the kill rate of wild ungulates. A decrease in anti-predator behavior may be expected in domestic species because of a high human protection, making livestock more vulnerable (Brokordt et al., 2006; Mignon-Grasteau et al., 2005). Thus, when livestock are not adequately protected and managed by farmers, they represent a more advantageous resource than wild prey in terms of cost and benefits trade-off. These hypotheses are supported by the result of the Monfenera pack that showed the lowest rate of kill on wildlife. The Monfenera pack is also the pack that had the most anthropized territory (TableS1) and that presented the highest percentage of domestic animals killed during all seasons of year (Table S2).

Also, the presence of carrion coming from farms may have affected kill rates. Indeed, in our study areas, the wolves scavenged mainly on dead animals and slaughter waste left near the farms. This may have limited the ecological role of the wolf, as already showed in other studies (Ciucci et al., 2020), by influencing killing rates. Indeed, it is interesting to highlight in our study area, as the killing rates decrease, the scavenging rates increase.

Contrary to predictions and to what has been seen in other studies (see Post et al., 1999; Schmitz et al., 1997), the number of wolves per pack did not reflect changes in kill rate. The trend was not significant, and this could be since the increase in kill rate depended on the positive interaction between number of wolves per pack and scavenging rate. To better understand this effect unexpected, we will need more data. A possible alternative explanation could be that as the number of wolves per pack increases, the pack is more likely to split into several units in predation activity, as has already been documented in other studies (Metz et al., 2011; Vucetich et al., 2012). Thus, at a threshold kill rate the increase in wolves per pack would seem to affect either an increase in scavenging or the building of predatory units consisting of fewer individuals from the pack. In the case of the Grappa pack, i.e. the larger pack, the pack was likely to split in smaller units which could have led to an underestimation of the kill rate recorded. This would also explain the lower kg of meat/wolf/day recorded by this pack compared to the other two (see results). A last alternative hypothesis could be that packs we studied require a lower daily per capita requirement than indicated by Peterson & Ciucci (2003), 0.09 kg/kg wolf/day.

Conclusion

Our results show the great plasticity of wolves, particularly in their trophic ecology and their importance in ecosystems. The wolves we studied were able to select different species in different contexts according to the best trade-off. The mouflon only, regardless of its abundance in relation to the other species, remained selected. This result confirms the value of the wolf's ecological role in positively selecting an exotic species that is not adapted to a specific environmental context.

The important ecological role of the wolf is also confirmed by the results that show selection of the preys based on their body condition. A result in agreement with other case studies in different ecosystems (Metz et al., 2012; Smith et al., 2004).

Finally, we highlight how in our study areas wolves were able to adapt to different environmental and seasonal conditions by taking advantage of both wild ungulates and human related food source like domestic ungulates and garbage, Ciucci et al. (2020) discuss, with justified concern, whether this does not affect the ecological role of this predator. From our results, scavenging would seem to influence the kill rate developing an inverse correlation.

Furthermore, our results suggest that the wolves we studied, rarely fulfilled their energetic requirements from the animals they killed (Peterson and Ciucci, 2003). This difference would be worth investigating to see whether at our latitudes in different environmental contexts the daily energy requirements of wolves could be lower and may be closer to our results.

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Supplementary materials

Table S1 Indicators of available resources for the three different wolf pack monitored (see text for details).

Wolves pack	Monitoring period	Availability	%	Species	n	Prop.
Grappa	From 15/10/2020 to 31/12/2021	Anthropic	2	Roe deer	577.67	0.54
		Forest	76	Mouflon	54.51	0.05
		Open	21	Chamois	288.52	0.31
		Shrubland	1	Red deer	114.49	0.11
Monfenera	From 01/02/2022 to 31/12/2022	Anthropic	24	Roe deer	271.76	0.55
		Forest	49	Mouflon	46.18	0.09
		Open	23	Chamois	90.18	0.18
		Shrubland	3	Red deer	88.78	0.18
Dubiea	From 06/05/2022 to 12/08/2022	Anthropic	3	Roe deer	139.14	0.26
		Forest	79	Mouflon	32.06	0.06
		Open	10	Chamois	219.25	0.40
		Shrubland	7	Red deer	151.75	0.28

Wolves pack= pack of wolves monitoring during our study; Monitoring period= Monitoring period of three wolves' pack; Availibility= Environment type availability; %= Percentage of environment type availability; Species= main prey species of study area; n= Numbers of main prey species availability; Prop.= proportion of main prey available.

Table S2 Number of individuals killed by wolves monitored during the study period, for each individual species.

Wolves pack	**Monitoring period**	**Species**	**n°**	**%**
Grappa	From 15/10/2020 to 31/12/2021	Chamois	24	11.88
		Goat	3	1.49
		Roe deer	99	49.01
		Red deer	8	3.96
		Indeterminate	6	2.97
		Hare	2	0.99
		Mouflon	40	19.80
		Sheep	18	8.91
		Fox	2	0.99
		TOT	**202**	**100.00**
Monfenera	From 01/02/2022 to 31/12/2022	Goat	3	3.70
		Roe deer	27	33.33
		Wild boar	2	2.47
		chicken	2	2.47
		Indeterminate	1	1.23
		Mouflon	10	12.35
		Nutria	2	2.47
		Sheep	32	39.51
		Fox	2	2.47
		TOT	**81**	**100.00**
Dubiea	From 06/05/2022 to 12/08/2022	Chamois	1	3.70
		Roe deer	5	18.52
		Red deer	10	37.04
		Indeterminate	3	11.11
		Mouflon	8	29.63
		TOT	**27**	**100.00**
Total	From 15/10/2020 to 31/12/2022	Chamois	24	7.74
		Goat	7	2.26
		Roe deer	131	42.26
		Red deer	18	5.81
		Wild boar	2	0.65
		Chicken	2	0.65
		Indeterminate	10	3.23
		Hare	2	0.65
		Mouflon	58	18.71
		Nutria	2	0.65
		Sheep	50	16.13
		Fox	4	1.29
		TOT	**310**	100.00

Wolves pack= pack of wolves monitoring during our study; Monitoring period= Monitoring period of three wolves' pack; Species= different prey species killed by monitoring wolf; n= Numbers of animals killed by monitoring wolf of the different prey species availability; %= Percentage of animals killed by monitoring wolf of the different prey species.

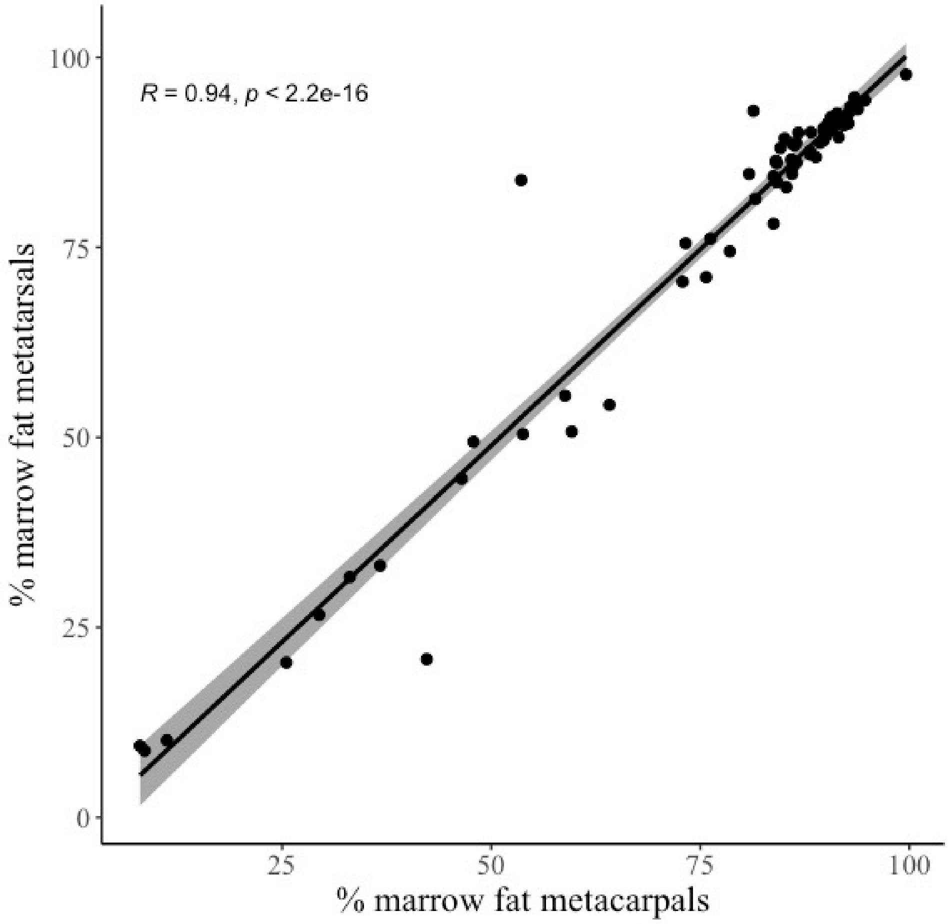

Figure S1 Spearman Correlation Model between percentage marrow fat of metacarpal and percentage marrow fat of metatarsal.

Table S3 Body condition of prey killed by monitored wolves expressed in marrow fat percentage classes.

Wolves Pack	**Marrow fat percentage class**	**n**	**%**
Grappa	0-25	6	6
	25-50	9	9
	50-75	12	12
	75-100	73	73
Monfenera	0-25	8	32
	25-50	2	8
	50-75	2	8
	75-100	13	52
Dubiea	0-25	4	27
	25-50	2	13
	50-75	2	13
	75-100	7	47
Total	**0-25**	**18**	**13**
	25-50	**13**	**9**
	50-75	**16**	**11**
	75-100	**93**	**66**

Wolves pack= pack of wolves monitoring during our study; Marrow fat percentage class= Percentage of marrow fat divided into classes; n= Number of samples analyzed; %= Percentage of samples analyzed, for each percentage class.

Chapter 4

A report of short-term aversive conditioning on a wolf documented through telemetry.

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RESEARCH

A report of short-term aversive conditioning on a wolf documented through telemetry

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Abstract

Predation by large predators on livestock is one of the main concerns in species conservation as it elicits prompt and effective retaliations. Therefore, conflict mitigation is essential to ensure long-term coexistence of predators with humans. We performed aversive conditioning (AC) with rubber bullets on one collared wolf that had become particularly bold toward a transhumant shepherd and had preyed on livestock. By exploiting the unique fine-resolution location data available before and after the AC event, alongside careful retrospective field investigations, we were able to analyse the effects of AC on wolf behaviour. Our study revealed that after just a single AC event, the wolf modified its spatial and predatory behaviour: the wolf changed its use of space by increasing distance from humans and ceased to attack farms in the following 2 months; actually, the only livestock preyed after AC was represented by a sheep and two goats lost by shepherds that had left alpine pastures. This study represents a first step to increase knowledge on AC effect on the wolf. Additional researchers are encouraged to conduct and publish findings on this topic in order to provide a useful and widely tested array of tools to promote wolf conservation in human-dominated landscapes.

Keywords Aversive conditioning · *Canis lupus* · GPS collars · Livestock · Predation

Introduction

An increasing number of large carnivores in some areas of the world has been reported over the past years (Chapron et al. 2014) as well as human-predator conflicts have been increasingly mentioned (Lute et al. 2018; Hoffmann et al. 2019; Pettersson et al. 2021; Bogezi et al. 2021). Effective strategies to mitigate these conflicts should involve decisions balancing the efficiency and costs of management actions, while taking also into account animal welfare, social and ethical acceptability (Breck et al. 2017; Moreira-Arce et al. 2018; Sampson and Van Patter 2020). Where possible, non-lethal interventions should be preferable to lethal control, as it is increasingly being advocated by the conservation community (Dubois et al. 2017). Moreover, in many European countries, large carnivores are protected by law, making unapplicable lethal management techniques for those problematic individuals, as it is instead commonly practiced in North America.

The debate on alternative non-lethal methods to manage and mitigate the conflict between large carnivores and humans is growing (Shivik 2006; McManus et al. 2015; Blackwell et al. 2016; Berzi et al. 2021), largely due to a public opinion increasingly opposed to lethal interventions. There are

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several non-lethal deterrent methods to avoid conflicts between predators and humans (Shivik 2006; Blackwell et al. 2016; Sampson and Van Patter 2020). These methods are based on two main approaches (Shivik and Martin 2000): disruptive-stimulus approaches and aversive-stimulus approaches. Former approaches act by disrupting predator access to resource and scaring the predator away (e.g. community-level hazing, Bonnell and Breck 2017). The second method aims to modify predator behaviour through conditioning: such methodology aims to make the predator associate a physical pain and/or discomfort feelings (e.g. illness, nausea, vomiting) with an environmental element such as scent, taste and human presence. As a result of this negative reinforcement, the animal should avoid the previously attractive stimulus (McCarthy and Seavoy 1994; Linnell et al. 1996; Tobajas et al. 2020) re-creating the landscape of fear (Gaynor et al. 2019). While there is an extensive knowledge on some non-lethal techniques (electronic guard: Linhart et al. 1992; guard dogs: Fritts et al. 2003; fladry: Musiani et al. 2003; hard plastic collars: King 2004; community-level hazing: Bonnell and Breck 2017), a lack of knowledge is evident for techniques that use aversive conditioning (e.g. electric shock collars: Shivik et al. 2002; rubber bullets: Beckmann et al. 2004; conditioned food aversion: Tobajas et al. 2020). For instance, the use of rubber bullets to scare the predator is poorly documented even though several European countries, such as France, Italy and Sweden, can use them for management purposes. On this topic, there is a wide anecdotal knowledge while scientific literature is very scarce (Rauer et al. 2003). One of the few published studies tested rubber bullets in different situations on brown bear, with some radiomarked individuals that were studied after the aversing conditioning event (Rauer et al. 2003).

On several other problematic species, such as the wolf, the scientific literature is very scarce. Through this study, we aim to fill these gaps by publishing the first results obtained from an aversive conditioning experiment performed on a problematic wolf accustomed to the presence of shepherds. We predicted that after the aversive conditioning intervention the wolf increased human avoidance and decreased its predatory activity upon livestock. Our goal is also to spur other authors who have comparable data to replicate the experience in other contexts and to publish and share their results.

Study area

The study was conducted during summer 2021 in the North-Eastern Italian Pre-Alps (45°52′24 N, 11°47′57 E; Fig. 1) on the Grappa Massif. The study area (35,008 ha, altitudinal range: 110–1775 m a.s.l.) is characterized by an oceanic climate according to Köppen's classification (Pinna 1978), also referred to as Cool temperate (Cf) based on the Köppen–Geiger scheme (Fratianni and Acquaotta 2017). Winter is the coldest season, with the mean temperature of the coldest month ranging

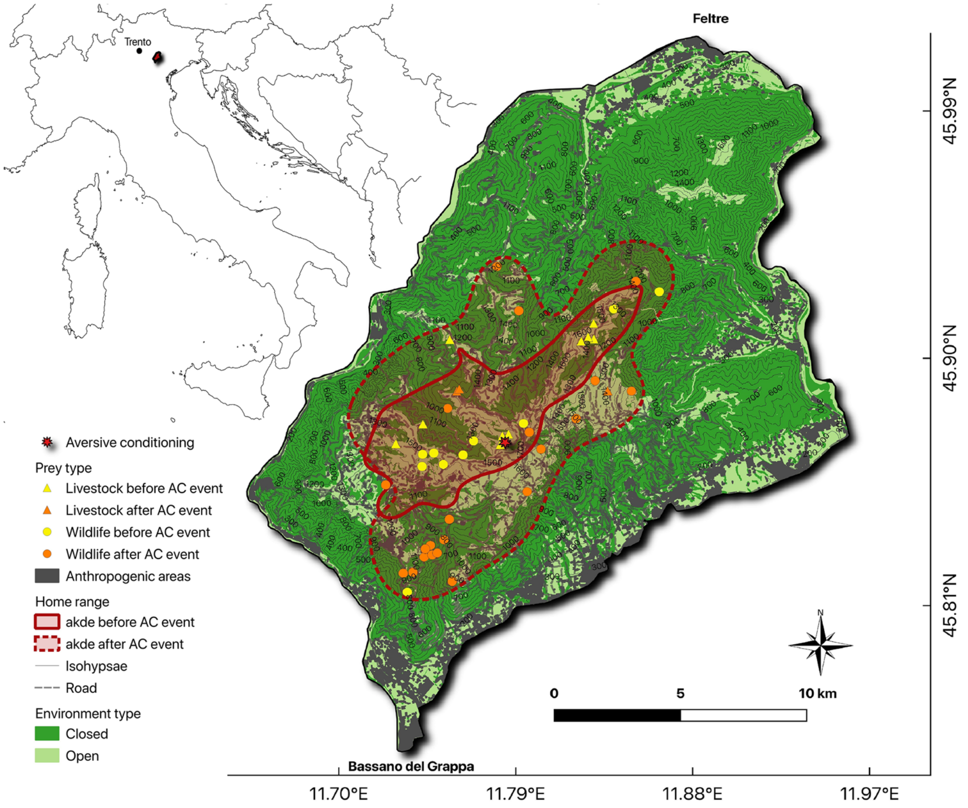

Fig. 1 Map of Italy (top-left) showing the localisation of the study area, located in the north-eastern Italian Pre-Alps, and an enlargement of the map displaying the localisation of the aversive condition event (AC), the home range of the wolf before (from 07/06/2021 to 19/08/2021) and after the AC event (from 19/08/2021 to 17/10/2021), and the positions of the wolf's preys observed in the period before and after AC. The map was generated in Qgis

from 0 to – 3 °C. Summer is the warmest season, with mean temperature of the hottest month ranging from 15 to 19.9 °C (Fratianni and Acquaotta 2017). Annual precipitation amounts to 1200–1500 mm, with no significant differences among seasons. The study area is mainly covered with forest (deciduous forest 54%; mixed forest 14%). The open areas occupy about 30% of the study area and included meadows, pastures, arable land and anthropized areas. Livestock breeding is a relevant economic activity, with some thousands of sheep spending late spring and summer on the Grappa massif. The time spent in high-elevation pastures by sheep flocks starts in June and might last over a long time, with conclusion ranging from the end of August till mid-October, depending on environmental conditions in different feeding areas. The study area is characterized by a considerable human presence in the spring–summer period both due to tourism and to livestock grazing. The area has been recently occupied by wolves: the first reproduction was documented in 2017 with a pack consisting of two adults and six pups (Avanzinelli et al. 2018). Roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*) are the most abundant ungulate species, but also Mediterranean mouflon (*Ovis aries*) and wild boar (*Sus scrofa*) can be found in low densities.

Methods

Data collection

We captured five wolves between 2019 and 2021 by using foothold traps (FremontTM Humane Foot Snare Wolf/Cougar 1/8 7 × 7 and FremontTM Humane Foot Snare Fox/Coyote 3/32 7 × 7). Once captured, the wolves were immobilized with a mixture of anaesthetic drugs (medetomidine-ketamine) using a syringe blowpipe (Telinject). We collected biometric data and we took biological samples; we weighted animals and fitted them with GPS collars (VERTEX Plus Vectronic Aerospace GmbH). At the end, we injected atipamezole in order to reverse the effect of anaesthetic drugs and we monitored the wolves during recovery. We programmed the collars to record a localization every 2 h during the day, from 6:00 am to 6:00 pm UTC, and every 30 min during the night. We set the collars to transmit data twice a day (IRIDIUM transmission).

We tracked all collared wolves remotely and by using their location data we identified cluster sites (i.e. a minimum number of two locations no more than 200 m apart; Sand et al. 2005) to be checked by means of direct field surveys to identify wolf predation. We distinguished livestock and wildlife prey using prey phenotypic characteristics (hairs, antlers/horns). One of the aims of the study was to test aversive conditioning methods that can be helpful in reducing predation on livestock and therefore conflicts with humans. To this purpose, we used virtual fences traced around the sheep farms that are warned if the wolves entered the virtual perimeter around the farm; in addition, we implemented a dissuasion system consisting of a proximity sensor combined with a sound and light emitter (commonly known as the radio-activated guard “RAG”; Breck et al. 2002).

During summer 2021, one of the monitored wolves (an adult male) became particularly confident towards a transhumant shepherd in the alpine pastures (henceforth referred to as reference shepherd). The wolf, together with other members of its pack, visited the enclosure trying to get the sheep out of the fence, despite the presence of the shepherd with 4 dogs (breed Pastore Bergamasco), and an electrified fence. The formerly mentioned dissuasive and warning systems were effective to signal the wolves’ presence and to deter the entry in the sheep corral but were ineffective in achieving the result of wolves leaving the area. From May 25 to July 4, we observed 18 cases of wolf approaches up to 10 m from the shepherd with reduced escape distances. Their presence induced panic within the sheep herd in the corral causing some sheep to die crushed by the others and some to jump out of the corral being promptly preyed upon by waiting wolves. To dissuade the wolf from approaching the area used by the shepherd, we planned to implement an aversive conditioning action (AC). This was anticipated by a legal procedure involving the preparation of a report on the case and on the aversive conditioning action planned for the regional Government that approved it and sent it to the national Ministry of Environment; the Ministry approved the dissuasive action after a positive evaluation from the Italian Agency for Nature Protection (ISPRA). Following the project approval, from August 19 to September 24, two rangers from the provincial police stayed from 7 p.m. to 12 p.m. near the electric fence that protected the sheep observing the surroundings by means of an infrared-camera (Flir Scout II-640 9 Hz) with the aim to shoot the approaching wolf with rubber bullets (Fiocchi 12 bore single bullet) by using a shotgun (Franchi, Alcione model). In a first period (19–29 August), the survey was conducted every day, while in the second period (30 August–24 September), the survey was conducted with a random frequency ($N = 16$). The survey protocol was to shot targeting the wolf’s thigh, only when the wolf was less than 30 m away from the sheep fence displaying a predator-like behaviour. After the AC event, we monitored the wolf intensively by using telemetry and 13 camera traps (Browning spec ops advantage) opportunistically distributed inside the study area on the main transit points of wolves.

Data analysis

Using location data recorded by the collar, we compared a series of parameters useful to understand the effect of AC on the target animal. We assumed that the presence of the reference shepherd had an influence on the wolf from the first seasonal predation (07/06/2021) up to the last day that it spent in the alpine pastures before beginning transhumance outside the pack's territory (17/10/2021). A total of 132 days were included in the analysis, 73 before the AC and 59 after the AC. To avoid the influence of uncontrolled variables on this large temporal scale, we also performed the analyses on a short temporal scale and considered a period of 20 days before and 20 days after the AC event (from 30/07/2021 to 08/09/2021).

As a first step, we analysed whether the wolf changed spatial behaviour in the period after AC with respect to the period before AC. By using the autocorrelated kernel density estimation method (AKDE; Fleming et al. 2015), we calculated the home range before and after the AC and evaluated their degree of overlap, using the *akde* function of the package "CTMM" in R (Calabrese et al. 2016). Then, we calculated the mean daily distances travelled in the two time periods (before and after AC) by using a continuous-time speed and distance (CTSD) estimation method implemented by means of the *speed* function of the "CTMM" package in R (Calabrese et al. 2016; Noonan et al. 2019). This allowed to overcome some shortcomings of the straight-line displacement estimation, as CTSD method is more accurate and unbiased because considers autocorrelation and tortuosity of location data (Noonan et al. 2019). Next, to investigate the environmental characteristics of wolf locations and whether the monitored wolf selected particular environmental features differently during the two monitored periods, we adopted the resource selection function (RSF) approach with the "use availability design" (Manly et al. 2007). Accordingly, we matched wolf locations (hereafter referred to as "used" locations) to randomly selected locations (hereafter referred to as "available" locations). We sampled available locations generating independent random points inside the 100% minimum convex polygon (MCP), estimated by using the relocations of the wolf collected throughout the monitoring period (07/06/2021 to 17/10/2021). We used a use-available ratio of 1:20 considering it large enough, due to the complexity of the environment in the study area. We then paired available locations to used ones and each pairing (ratio 1:20) was assigned a unique identification code (stratum-ID). Date and time of observation were assigned to its respective used location, as well as to its corresponding available locations. Subsequently, by using GIS software we assigned to all locations (both used and available) the following environmental covariates: altitude (Alt), the environment type (EType), classified in open environment (i.e. meadows, pastures and arable land) and closed environment (i.e. mixed and deciduous forest); the distance to (i) the reference shepherd (dSH), (ii) anthropogenic and residential areas (dAA) and (iii) the nearest road (dR) were calculated as well. Due to the Vaia storm that hit the study area during autumn 2018, 1% of the trees in the forest area had fallen creating optimal shelter sites for several animals, including wolves. Consequently, as a further environmental covariate to be considered in the analysis, we also calculated the distance of each wolf location to the nearest area with fallen trees (dFT). Finally, we assigned to each location (both used and available) a binary variable time related to the AC event date (tACevent): "before" and "after".

We built resource selection functions (RSFs) by using the wolf locations. RSF coefficients were estimated by fitting generalized linear mixed models (GLMMs) with a binary response variable (used = 1, available = 0). We fitted GLMMs by using the glmer function of the lme4 package. Predictor variables such as dSH, dAA, dR, dFT, Alt, EType and tACevent were used in the model. The stratum-ID was included as a random effect in the model. All numerical predictors were scaled [(x-mean)/SD] before running any model to improve glmer convergence (Bates et al. 2015). The variable screening revealed no collinearity (Pearson coefficient $|r_p| < 0.6$) and multicollinearity (Variance Inflation Factor, VIF < 3) among predictors.

We first created a GLMM with a full model structure, based on our expectation on the effect of the predictors in driving wolf resource selection. As we were interested in evaluating whether the selection by the wolf changed after the AC event, all environmental predictors (dSH, dAA, dR, dFT, Alt, EType) were included in the model in interaction with the binary variable tACevent (before/after). We chose the best model by applying a manual step AIC procedure, iteratively removing the worse predictor (that with the highest *P*-value) and re-running the model until achieving a model with the lowest AIC. In so doing, we removed all the predictors that contributed to increase the model AIC from the best model. Finally, the beta coefficients estimated by the most parsimonious model were entered into the resource selection function to obtain RSF scores, which are proportional to the probability of selection. The RSF scores were used to represent the scenarios predicted by the model. We implemented the RSF separately at both temporal scale (large: using data from 07/06/2021 to 17/10/2021, short: from 30/07/2021 to 08/09/2021).

As a final step, we investigated whether the wolf changed its predatory behaviour after the AC event. We analysed the number of wildlife prey and livestock prey found in the cluster sites identified by using the wolf location data before and after the AC event. We used a chi-square contingency test (with Yates correction) to test for a significant association between the two categorical variables prey type (livestock/wildlife) and period (before/after AC).

Results

The AC action using rubber bullets was successfully achieved on August 19, 2021, during the first day that operators attempted the AC approach. The operators fired two rubber bullets hitting the wolf on the thigh of the left hind leg both times, following the protocol. In the following days, the operators did not see the wolf during the survey. During AC, the wolf did not respond aggressively. It quickly fled over 800 m, where it lied down for about 30 min. It subsequently resumed running away at a slower speed until it arrived at the rendezvous site where it remained until the following night. Thanks to videos recorded in the days after the AC event by the camera traps, a vet was able to check the wolf's health condition: no evidence of injury was detected; this was also confirmed by proper movement parameters: it was possible for the wolf to double the distance travelled during the night after AC.

The spatial behaviour of the wolf changed significantly after the AC event with respect to the period before it, as evidenced by an absence of overlapping confidence intervals estimated for the home range size and the mean daily distance. At the large temporal scale, the home range size increased by 170%, from 38.36 km^2 (CI 95% = 32.71–44.95) to 103.58 km^2 (CI 95% = 125.06–84.09; Fig. 1). The overlap between the two home ranges was 98.47%, i.e. the former home range was included in the one after AC. After the AC event, the mean daily distance travelled by the wolf varied significantly, from 31.33 km/day (CI 95% = 30.35–32.31) to 42.88 km/day (CI 95% = 41.64–44.24) and from 26.82 km/day (CI 95% = 25.24–28.42) to 46.56 km/day (CI 95% = 44.27–48.88) when considering the large and the short temporal scale, respectively.

During the monitoring period, the localisations of the wolf were on a mean distance to the reference shepherd of 2464 ± 8 m before the AC and 3715 ± 11 m after it. The mean distance to roads was 109 ± 0.5 m before the AC event and 151 ± 1 m after it. Finally, the wolf before the AC event was at 435 ± 1 m away to anthropogenic structures, while after it the mean distance was 406 ± 1 m.

At the short temporal scale, the most parsimonious RSF model included dSH, dAA, dR, dFT, Alt, EType, tACevent and the interactions tACevent*dSH, tACevent*dR, tACevent*dFT, tACevent*Alt and tACevent*EType (Table 1). Predictions of this model showed that after the AC event the wolf increased the selection for higher altitudes and for areas close to fallen trees (Fig. 2). More in general, the wolf increased selection for open habitats (Table 1). While during the 20 days before the AC event, the wolf strongly selected areas close to reference shepherd, during the following 20 days this pattern of selection disappeared (Fig. 2). On the contrary, before the AC event the distance to roads seemed not to influence spatial selection by wolf, but during the 20 days after the AC event it clearly selected areas farther from roads (Fig. 2). The wolf showed a selection for areas distant from anthropogenic structures, regardless of the AC event (Table 1).

Analysis at the large temporal scale confirmed the results on the short scale, except that the most parsimonious RSF model also included the interactions tACevent*dAA (Online Resource, Table S1), suggesting a modification of the pattern of selection by the wolf also on account of the distance to anthropogenic structures. In general, the predictions of the best model were in accordance with predictions at the short temporal scale: the wolf increased selection for higher altitudes, for open habitats and for areas close to fallen trees (Online Resource, Figure S1). When considering 59 days after the AC event, a stronger selection for areas farther from roads and a weaker selection for areas farther from anthropogenic areas was detected (Online Resource, Figure S1). Moreover, predictions of RSF model at large temporal scale showed a weak selection for areas close to the reference shepherd (Online Resource, Figure S1).

Table 1 Results of the most parsimonious resource selection functions model at the short temporal scale by the monitored wolf before and after the aversive condition event (AC), in the study area located in the north-eastern Italian Pre-Alps (from 30/07/2021 to 08/09/2021)

Coefficients	Estimate	Std. error	z-value	*p*-value
(Intercept)	− 5.37	0.17	− 30.66	< 0.001
Alt	− 0.22	0.11	− 2.04	0.042
dR	− 0.08	0.06	− 1.36	0.173
dAA	0.12	0.04	2.92	0.004
dSH	− 2.43	0.14	− 17.55	< 0.001
dFT	− 1.36	0.11	− 12.91	< 0.001
tACevent (After)	1.90	0.19	10.02	< 0.001
EType (Open)	− 0.68	0.14	− 4.75	< 0.001
tACevent (After) * Alt	0.91	0.13	6.93	< 0.001
tACevent (After) * dR	0.17	0.08	2.16	0.031
tACevent (After) * dSH	2.21	0.15	14.87	< 0.001
tACevent (After) * dFT	0.84	0.12	6.79	< 0.001
tACevent (After) * EType	1.07	0.18	5.85	< 0.001

Alt altitude, *dR* distance from the nearest road, *dAA* distance from anthropogenic and residential areas, *dSH* distance from the reference shepherd, *dFT* distance from the nearest area with fallen trees, *tACevent* binary variable time of the aversive conditioning event (AC), by considering the recording date and time, and the time of the AC event: to all locations recorded before the AC event, we assigned the category "before", while to all locations recorded after the AC we assigned the category "after"; EType Environment type (open = meadows, pastures and arable, land or closed = mixed and deciduous forest)

We identified 49 predations by the monitored wolf: 25 during the period before the AC and 24 after the AC event. The relative number of livestock species (before: $N = 15$, after: $N = 3$) preyed by the wolf varied significantly after the AC event ($\chi^2 = 9.93$; $P = 0.002$), with an increase of predation on wild ungulates with respect to livestock (Online Resource, Figure S2, Table S2). However, it must be noted that the three livestock preyed (two goat and one sheep) were not included in any flock: they were alone in the woods as they had been lost by sheepherders that had moved to the plains. If we consider livestock of the reference shepherd only, the effect of the AC reset out the predations: five

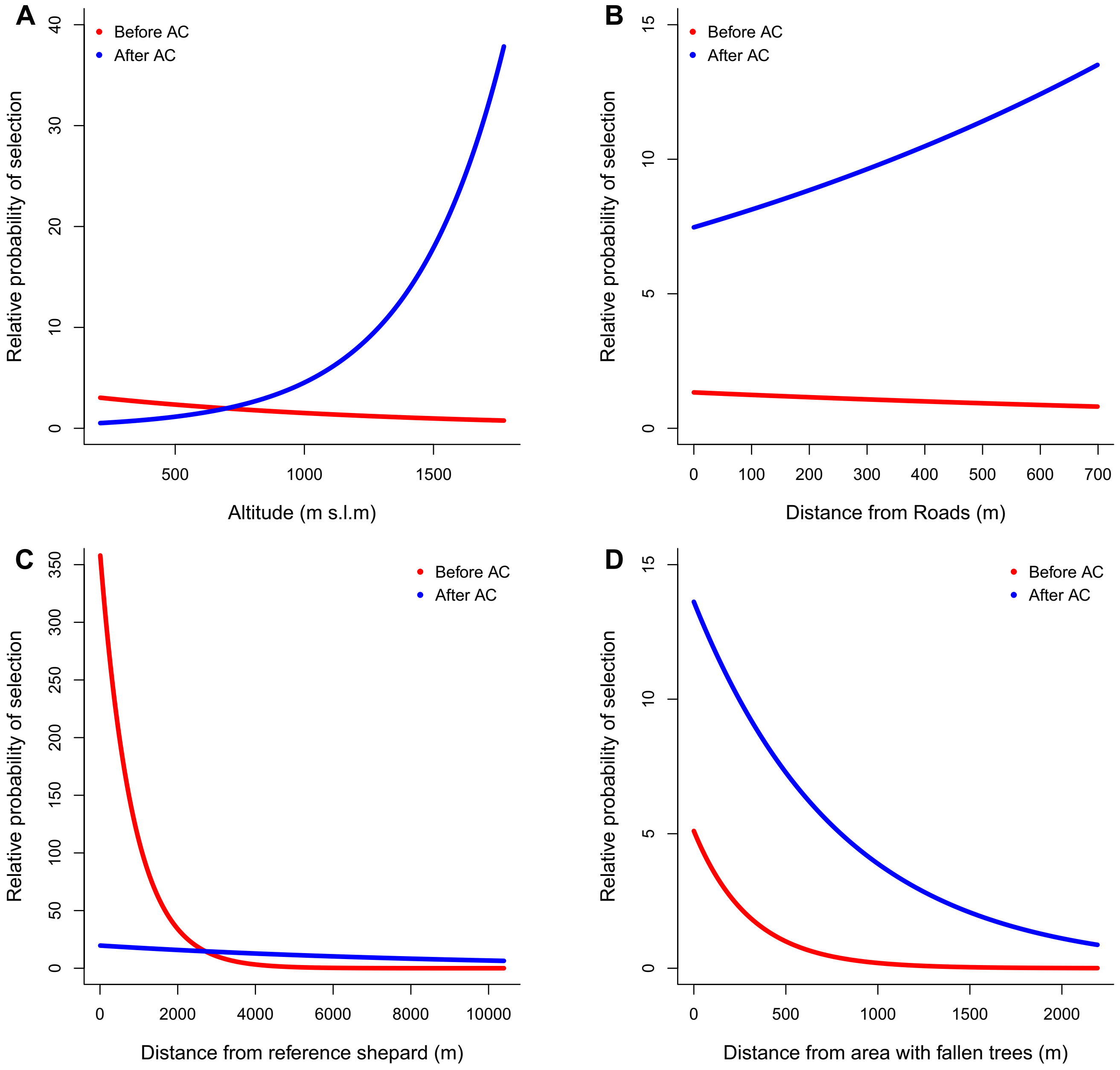

Fig. 2 Relative probability of selection as predicted by the resource selection function, which was built using the wolf observations collected from 30/07/2021 to 08/09/2021 (short temporal scale) in the north-eastern Italian Pre-Alps (Italy). Plots depict the effect of the interaction between time of the aversive conditioning event and A altitude, B distance from the nearest road, C distance from the reference shepherd, and D distance from area with fallen trees. The figure was generated in R

predations were recorded before the AC event and zero predations after it. The experimental wolf stayed with the pack till December 2021 and kept on preying on wild ungulates, then on the 22nd of January 2022, it left the pack and dispersed northward moving 80 km away, reaching an area where it stopped and where on March 14 was filmed with an adult female that became its mate giving birth to 5 pups in May 2022. The monitoring of the wolf ceased on 12 August 2022, when the battery of the collar run out, till then he had preyed upon wild ungulates only.

Discussion

Our study revealed that after just a single AC event, the wolf clearly changed its behaviour during the following 2 months. By taking advantage of unique fine resolution and detailed location data available before and after the AC event, together with a posteriori careful field survey, we showed that the wolf increased its movements, modified its resource selection increasing distances from the shepherd and from roads, and decreased its predation on livestock relying only on stray sheep and goats that were abandoned by shepherds, so ceasing any attack to livestock in farms.

After the AC event, the wolf increased significantly both mean daily distances travelled and the home range size, at both temporal scales (large and short). Daily distance travelled may represent a short-term measure of space requirements that at least in part reflects food resource distribution and foraging strategy (Garland 1983; Carbone et al. 2005). We conjectured that this significant increase in average daily distances travelled may be related to the type of food resource used by the predator. Results on kill rate support this hypothesis: as a matter of fact, the ratio of wildlife and livestock species preyed by the wolf varied substantially after the AC event. Before the AC event, the wolf mainly predated on livestock being typically concentrated in few spots known to the predator, which implied less time and shorter distances travelled to find them. Conversely, wild animals may require more efforts and more time for research since they are generally distributed more uniformly. Moreover, wildlife preys are accustomed to the presence of predators and are able to adopt effective anti-predator behaviours to avoid being predated (Laundré et al. 2001; Caro 2005; Bongi et al. 2008). It has been shown that the domestication process causes inhibition of some behaviours and changes in the quantitative and qualitative nature of responses, such as reactions to predators (Price 1999; Mignon-Grasteau et al. 2005; Brokordt et al. 2006; Ciucci et al. 2020). Thus, when livestock is not adequately protected and managed by farmers, it represents a more advantageous resource than wild prey in terms of cost and benefits trade-off. We hypothesized that the reduction of attacks on livestock by the wolf after the AC event was mainly driven by an increased risk perception around the anthropic areas and activities. However, we cannot exclude that the kill rate modification by the experimental wolf was also influenced by a modification of wild prey availability and behaviour. We cannot also exclude that other members of the pack, which were not collared, may have preyed upon livestock when the experimental wolf was not present, though it is worth noting that the experimental wolf never attacked livestock in farms after AC (but only a stray sheep and two goats) not only during the time he was in the pack area but also when he moved away to create a new pack 80 km north.

Results of the RSF showed that also the spatial selection of the wolf changed significantly during the 2 months after the AC event with respect to the period before it. Most likely, through the AC event the wolf landscape of fear was reshaped (Gaynor et al. 2019). The presence of humans near the sheep's flock, armed with rifles firing rubber bullets, would be perceived by the wolf as a danger, and consequently, also the surroundings would be perceived as a high-risk area (Johnson et al. 2015). Our results support this hypothesis: while before the AC event the wolf strongly selected areas close to the reference shepherd (i.e. the shepherd who suffered more attacks by the wolf and owned the alpine pasture on which we carried out the AC event), during the following 20 days, this pattern of selection disappeared, suggesting that the wolf reduced or zeroed out its visits to the shepherd. The analysis on the large temporal scale showed a weak selection by wolf for areas close to the reference shepherd, suggesting a possible diluted effect of the AC over time. RSF analysis showed that the wolf selected areas distant from anthropogenic structures regardless of the AC event, while selection for areas further away from roads increased after AC, probably because they were perceived by the wolf as higher-risk areas associated to human activity (Eggermann et al. 2011; Muhly et al. 2019).

Our study revealed that a single AC event using rubber bullets succeeded on keeping the wolf away from the shepherd and more in general from preying upon livestock. Our results are consistent with Smith et al. (2020), who showed preliminary results in which in 81% of the cases aversive conditioning by using rubber bullets on wolves was successful in moving predators away from urban areas. This is in contrast with other studies on bears, which showed that the use of rubber bullets did not prevent the predator from returning to urban patches (Rauer et al. 2003; Beckmann et al. 2004; Mazur 2010). Furthermore, in our study case, the effect appears to last beyond a month, specifically for 59 days at least. This is longer than what reported by Beckmann et al. (2004) on black bears, where the AC impact was often not effective beyond a month. Unfortunately, we could not analyse the efficacy of AC in keeping the wolf away from the shepherd for a longer period, since the shepherd left the high-altitude pasture to descend to the valleys 59 days after the AC event. However, the monitoring of the hunting behaviour of the wolf after his dispersal and till the subsequent August allows us to conjecture a long-lasting effect of AC.

Management implications

With the return of large carnivores, there is an increasing need for prevention systems to solve conflicts with humans while preserving these species (Woodroffe 2000; McManus et al. 2015). The use of rubber bullets may be an effective aversive conditioning method to limit the damages caused by large carnivores, to prevent them from approaching urban areas and to avoid illegal solutions to manage the problems. It is, however, imperative that aversive conditioning is implemented as part of a comprehensive wildlife coexistence program (Sampson and Van Patter 2020). In this sense, the use of alternative less invasive preventive techniques (e.g. guard dogs, electrified fencing: Shivik et al. 2003; Shivik 2006; conditioned food aversion: Tobajas et al. 2020), together with the education and engagement of the community and stakeholders should also be promoted to obtain a more compassionate and ethically correct coexistence with wildlife (Bonnell and Breck 2017; Breck et al. 2017; Sampson and Van Patter 2020). A limitation of the present case study is that we were not able to verify the effectiveness of AC on a wider sample size; however, this study represents a first step in gaining knowledge that can be useful for comparison with other studies in the future. Further research evaluating other less risky and harmful aversive conditioning methods (e.g. conditioned food aversion: Tobajas et al. 2020, community-level hazing; Bonnell and Breck 2017) should be conducted to improve conservation of wild carnivores. We encourage other researcher to implement and publish similar studies in order to provide a useful and widely tested tool for administrations and operators involved in wildlife conservation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10344-023-01693-z.

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Author contribution Conceived and designed the experiment: M. A.; performed the experiment: M. Z., D. B., S. L., L. C., D. F.; analysed the data: M. Z., F. B.; wrote the paper: M. Z., F. B. All authors read and approved the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics statement This research complies with all national and regional laws dealing with ethics and animal welfare. Capture and manipulation protocols were approved by The Italian Ministry of Environment on 5.7.2018 n 0014897 for wolves captures, on 16.8.2022 n 0089653 for the dissuasive action. The research adhered to the ASAB/ABS Guidelines for the Use of Animals in Research.

Conflicts of interest The authors declare no competing interests.

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DISCUSSION AND CONCLUSIONS

My results shed light on various aspects of the spatial and trophic ecology of the wolf with an appropriate consideration of its temporal dimension, allowing an interesting consideration of a more coherent conservation of this species and greater management of conflicts with humans and their activities.

Chapter 1 highlighted how wolves tend to keep as far away from humans as possible but can also permanently occupy human populated contexts to cope with the reduced availability of space. Basically, if there is space, wolves tend to occupy natural environments, when the free space runs out as the territory is close to saturation, they smoothly occupy more and more human dominated areas where they find refuges and trophic resources. The anthropic environment is also used by wolves to select advantageous environmental features to prey upon, as confirmed in Chapter 2 where human related environmental characteristics like roads and human infrastructures as shown to favor predation, particularly in cover habitats.

Chapter 3 once again marks the ecological role of wolves, but also highlights how wolves exploit human related food sources to meet their daily energy needs in human dominated landscapes at certain times of the year. These results shedding light on the wolf's ecological role in these contexts question on inappropriate waste management practices. Finally, in Chapter 4 we show the first fine-scale results of a deterrence event using rubber bullets that was effective on a bold wolf.

Even though wolves tend to occupy natural environments and avoid more anthropized environments staying far from human settlements, we have shown that when wolves increased in number and saturated the most suitable environments, the presence of stable packs also occurs in the vicinity of large cities. This phenomenon was probably mediated by a density-dependent dispersal, with young wolves forced to move from territories at high elevation a low human presence to plains where humans are densely settled in order to establish new territories. This phenomenon is supported by our results showing that as the wolf's presence increases, the possibility of choosing suitable locations gradually decreases. Furthermore, the colonization of more anthropized environments by the wolf occurred during the historical period in which human density was more concentrated in urban contexts, decreasing in more rural areas (Reynaud et al., 2020). On the one hand, if this phenomenon increased the rapidity with which the wolf reached saturation in mountain and hill areas, on the other hand, it supports the hypothesis that this colonization did not depend on the variation in human density. Otherwise, since habitat selection is dependent on consumer density and/or resource availability (Avgar et al., 2020), we have reason to believe that urbanization of prey species may favour wolf expansion into human dominated areas.

Studies conducted so far had not predicted such a significant return of the wolf in such anthropized contexts. In fact, our study highlights how the wolf's tendency to avoid humans may have delayed the recolonization of human-dominated landscapes, leading many researchers to define as suitable habitats for the wolf only forest areas with low anthropic impact (Ahmadi et al., 2014; Gehring and Potter, 2005; Jędrzejewski et al., 2008, 2005; Massolo and Meriggi, 1998; Mladenoff et al., 1995; Mladenoff and Sickley, 1998; Potvin et al., 2005; Salvatori et al., 2002), where they have been effectively confined as ecological refugees (Kerley et al., 2012).

The process of urbanisation of the wolf, which chapter 1 suggests, has already been recorded for other carnivore species, particularly for other species that are phylogenetically very close, such as the coyote, which due to its plasticity in behaviour not only exploits, but thrives in many environments modified by humans (Gese and Bekoff, 2004). As demonstrated in Chapter 2, in certain contexts, human-modified environments can also be a resource for the wolf. Chapter 2 contributed substantially to highlighting the plasticity of this predator in killing different prey (both wild and domestic) in different environmental contexts, even in particularly anthropised settings. This led the wolves studied to periodically select the same (prey) species specific killing sites that presented different environmental characteristics depending on the species preyed upon, as hypothesised by McPhee et al. (2012). In fact, our results suggest that in a multi-prey environment the wolf adapted to prey by increasing the probability of encountering prey, preying on them in environments where the prey species was likely to be most present (Hebblewhite et al., 2005) but killing them by exploiting the presence of natural and/or artificial barriers whenever possible. Indeed, wolves did not only use anthropogenic elements to increase prey killing success, as they also exploited the presence of anthropogenic elements, selecting roads and anthropogenic structures, probably to increase encounter rates (Newton et al., 2017). Anthropic structures were selected more in the closed environment. In contrast, the open environment conditioned wolves to select kill sites distant from anthropogenic features such as roads and houses. This suggests that kill site selection by wolves in anthropised environments is often driven by the trade-off between predation success and the probability of being detected by humans, who are perceived as a threat. It is an interesting scenario if we consider the evolution of the environment in western countries. On the one hand, we have a nature that is reclaiming its space by rapidly chasing the abandonment of the rural environment (Navarro and Pereira, 2015). As a result, we have more and more wildlife and potential wolf prey near heavily anthropised environments that use the anthropogenic environment to their advantage (Podgórski et al., 2013; Stillfried et al., 2017), both as a concentration of trophic resources and protection from predators by making greater use of the 'human shield effect' (Rogala et al., 2011). On the other hand, we have an opportunistic predator with a high trophic plasticity. If we consider that wolves generally select hunting sites where the species is most abundant (McPhee et al., 2012), we can better understand why wolves, having reached environmental saturation in the most suitable environments, can use increasingly anthropized environments (Chapter 1). These results agree with the hypothesis that Wolves indeed exploit anthropogenic features only when the risk of mortality due to human presence becomes a back- ground noise and the human environment becomes favorable from a feeding opportunity perspective (Muhly et al., 2019). The behavioural plasticity of the wolf is also suggested in Chapter 3 where I highlight differences in kill rates for different packs and in different seasons with different prey species abundance. I have shown how in a human dominated landscape a wolf pack can maintain its ecological role by preying mainly on wild animals and by selecting animals with a low body condition. On the other hand a wolf pack in these environments can also adapt to resources that come from humans and that provide a better cost-benefit trade-off such as carrion and waste that is not disposed of properly, compromising its ecological role (Ciucci et al., 2020). Indeed, the fluctuations in kill rate are influenced not only by the number of wolves per pack but also by the scavenging rate, that is related mainly on kitchen scraps and on carcasses not properly disposed of by livestock farms. This is another aspect that favors the urbanization of the wolf, influencing not only the number of individuals per pack but probably also the survival of the more debilitated individuals. Effective management of conflicts between wolves and humans must involve educating the human populations in how to manage waste and trophic resources in the most correct way. If this is not enough, there are also other prevention tools that can be integrated in these situations to prevent habituation by the predator (Shivik, 2006). In Chapter 4, we demonstrated how an aversive conditioning event with rubber bullets worked in deterring and driving away a particularly bold wolf from preying on domestic livestock. After a single aversive conditioning intervention, the bold wolf resumed killing wild prey again by increasing its home range and increasing the average daily distances travelled to find alternative preys to domestic livestock, thus ceasing any attacks on livestock on farms. Most likely, through aversive conditioning the wolf landscape of fear was reshaped (Gaynor et al., 2019). Our study suggests that aversive conditioning with rubber bullets applied properly on the wolf, unlike what has been seen on the bear (Beckmann et al., 2004; Mazur, 2010; Rauer et al., 2003), could be used more successfully as a prevention tool (Smith et al., 2020) by restoring the wolf to its ecological role within natural ecosystems.

The presence of the wolf, although conditioned by the presence of man, is spreading in the most anthropised areas without a saturation point. Increasing knowledge of landscape and spatial use, even if in a small way, may be fundamental for the management and conservation of wolf populations soon. The importance of woods and thick vegetation cover in general, as a key element that can facilitate wolves in exploiting human dominated habitat, may in the future increase the presence of this species closer to large cities, as more and more rural areas are being lost in Europe and forests are becoming closer to urban settlement. Indeed, Chapters 1 and 2 highlighted how the urbanisation process for the wolf in some Italian contexts may already be a reality. In these contexts, it is probably no more advantageous for the wolf to avoid humans in order to reduce the risk of mortality caused by them than it is to take advantage of feeding opportunities. Instead, they exploit the characteristics of the environment, such as roads and infrastructure, to their advantage to find and kill wild and domestic prey. Chapter 3 showed how the human dominated habitatis not only favorable for the wolf to kill but also to find food sources. This chapter once again shown the great plasticity of wolves, general opportunistic predators, which in a human dominated context have achieved their daily needs by also taking advantage of kitchen scraps and incorrectly disposed of carrion, achieving the best trade-off between costs and benefits. It will therefore be important to raise the awareness of the population in large urban settlement, and not only in rural and remote areas, to prevent inappropriate actions.
With the return of large carnivores, it will also be important to increase knowledge of prevention systems such as aversive conditioning to re-educate the behaviour of bold wolves. Chapter 4 showed how, with aversive conditioning, the wolf regains its ecological role. It is, however, imperative that aversive conditioning is implemented as part of a comprehensive wildlife coexistence program. In this sense, the use of alternative less invasive preventive techniques (e.g., guard dogs, electrified fences, conditioned food aversion), together with education and engagement of the community and stakeholders should also be promoted in order to achieve a more compassionate and ethical coexistence with wildlife.

In conclusion, the proper consideration of the temporal dimension in the study of wolf ecology has greatly expanded the information available on this species in a human dominated landscape. This approach has advanced our knowledge of various aspects of the wolf's trophic and spatial ecology, while at the same time achieving an overview of possible future scenarios. The results of this study could have important implications for the future management of this species on both a small and large scale, as its densities are increasing throughout Europe.

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Appendix

Estimation of spatial and temporal overlap in three ungulate species in a Mediterranean environment

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ORIGINAL PAPER

Estimation of spatial and temporal overlap in three ungulate species in a Mediterranean environment

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Abstract
Interspecific interactions are key drivers in structuring animal communities. Sympatric animals may show such behavioural patterns as the differential use of space and/or time to avoid competitive encounters. We took advantage of the ecological conditions of our study area, inhabited by different ungulate species, to investigate the spatial and temporal distribution of *Capreolus capreolus*, *Dama dama* and *Sus scrofa*. We estimated intraspecific interaction arising from the concomitant use of resources by using camera trapping. We collected 2741 videos with the three ungulates, which showed peculiar activity patterns. The three species were observed in all the habitat types of the study area over the four seasons, thus highlighting an evident spatial overlap. Moreover, our analysis demonstrated that the three species did not avoid each other through temporal segregation of their activities, rather showing a high overlap of daily activity rhythms, though with differences among the species and the seasons. Despite the high spatial and temporal overlap, the three species seemed to adopt segregation through fine-scale spatial avoidance: at an hourly level, the proportion of sites where the species were observed together was relatively low. This spatio-temporal segregation revealed complex and alternative behavioural strategies, which likely facilitated intra-guild sympatry among the studied species. Both temporal and spatio-temporal overlap reached the highest values in summer, when environmental conditions were more demanding. Given these results, we may presume that different drivers (e.g. temperature, human disturbance), which are likely stronger than interspecific interactions, affected activity rhythms and fine-scale spatial use of the studied species.

Keywords Activity rhythms · Fallow deer · Interspecific interaction · Roe deer · Spatio-temporal overlap · Wild boar

Introduction

Interspecific interactions are key drivers in structuring animal communities (e.g. Gause 1934; Hutchinson 1959) and may affect distribution, resource use, behaviour and population dynamics of interacting species (Sinclair and Norton-Griffiths 1982; Putman and Putman 1996; Forsyth and Hickling 1998; Latham 1999; Murray and Illius 2000). Among animal species, at least four types of interactions were described (Krebs 1985): two positive (mutualism and commensalism) and two negative (predation and competition) ones. Interspecific competition occurs when species of the same trophic level share the same resources with limited availability (De Boer and Prins 1990), which may result in a species negatively affecting the fitness of the other. Competition was described across several taxa (insecta: Human and Gordon 1996; reptiles: Polo-Cavia et al. 2009; fish: Bergstrom and Mensinger 2009; birds: Maron et al. 2011), and it is the most frequent interspecific interaction in ungulates (Latham 1999). Researchers described two main patterns of competition in ungulates: resource and interference competition. The former refers to direct interactions between two or more species which use and compete for shared resources (food and space, Latham 1999). The latter includes adverse social interactions as well as the negative impact of a species on the environment, thus reducing its quality for other species (Latham 1999).

Competitive interactions may occur at both spatial and temporal levels. However, spatial and temporal interactions are not always estimated properly and simultaneously

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(Lewis et al. 2015; Swanson et al. 2016; Karanth et al. 2017; Cusack et al. 2017). Sympatric animals may show such behavioural patterns as the differential use of space and/or time to avoid competitive encounters (Karanth and Sunquist 1995; Durant 1998). Subordinate competitors may avoid locations in which activity levels and/or population density of dominant species are high (Sherry 1979). Likewise, species may adapt their circadian activity patterns to reduce temporal activity overlaps (Carothers et al. 1984).

Wild animals showed a vast array of daily and seasonal activity patterns, which are the result of a complex compromise between best time for feeding, social activity and environmental constraints (Aschoff 1963). Theoretically, time budgeting is usually considered a process of optimisation. The time spent for an activity may increase until costs do not exceed benefits (MacArthur and Pianka 1966). Consequently, we may expect changes in activity patterns as the quality and quantity of environment resources change. Moreover, activity rhythms are likely influenced by predation risk as well as by interspecific competition. For these reasons, the study of spatial and temporal distribution of activity in sympatric species may contribute to understand interspecific competition.

Camera traps are cost-effective, non-invasive and highly efficient tools to collect data and are increasingly used to determine the potential relationship among sympatric species (Di Bitetti et al. 2009; Foster et al. 2013; Tambling et al. 2015; Cusack et al. 2017; Mori et al. 2020). Camera trapping has been widely used in ecology and conservation to investigate the distribution of species, estimate population density and assess biodiversity (O'Connell et al. 2011; Burton et al. 2015; Steenweg et al. 2017). This methodology was also applied to the study of activity rhythms with encouraging results (Tobler et al. 2008; Centore et al. 2018; Caruso et al. 2018; Lashley et al. 2018). More specifically, camera trapping offers the possibility to consider the activity patterns and space use of different species at the same time in the same recording area (Monterroso et al. 2014; Centore et al. 2018; Caruso et al. 2018; Mori et al. 2020) to estimate intraspecific competition arising from the concomitant use of resources.

We investigate the spatial and temporal distribution of activity of roe deer *Capreolus capreolus*, fallow deer *Dama dama* and wild boar *Sus scrofa*. We took advantage of the ecological conditions of our study area, a large fenced area inhabited by these species, and ascertained whether they adopted behavioural strategies to avoid potential competitive encounters among each other. Given the similar feeding habits of the two deer species, their different size and social habits and the competition that may arise between them (Ferretti et al. 2011), we predicted (1) a limited overlap of their activity rhythms. Moreover, given the wild boar's predatory habit on deer fawns, we also predicted that (2) the activities of these species seldom overlapped during deer fawning period, i.e. from late April to the end of June.

Materials and methods

Study area

The Presidential Estate of Castelporziano is a protected area located at about 20 km south-west of Rome. It represents one of the most important Mediterranean forests still existing in Italy.

The area is part of the Mediterranean climatic region, in particular, the mesothermal Mediterranean region (Blasi 1996), characterised by hot and dry summers and cold and rainy winters. During the year of data collection, monthly mean temperature ranged from 6.4 °C in January to 26.6 °C in August (Fig. S1 in Online Resource). The estate represents a biologically interesting environmental system thanks to the presence of a wide variety of natural environments, such as newly formed and old dunes, wetlands, Mediterranean scrubland, evergreen (*Quercus ilex*, *Quercus suber*, *Pinus pinea*, *Eucalyptus spp*) and deciduous forests (*Quercus robur*, *Quercus frainetto*, *Quercus cerris*, *Carpinus orientalis*) Grignetti et al. 1997; Pignatti et al. 2001).

The estate is a 6000-ha wide, fenced, rather flat area. In the past, it was mainly used for farming, forestry, livestock breeding and hunting activities. Nowadays, about 600 ha are devoted to cereal crops and livestock breeding (horses and cows). Wild ungulates in this area are wild boar, fallow deer, roe deer and red deer (*Cervus elaphus*), with an estimated population size of 2600 wild boars, 695 fallow deer, 150 roe deer and 128 red deer (ISPRA 2017). Wild boar and fallow deer are culled each year (mean ± standard deviation, 410.67 ± 251.44 and 218.67 ± 86.29 heads during last 3 years, respectively) during autumn/winter in order to keep their number stable.

Data collection

We monitored the ungulate species by using camera trapping during four 30-day sessions, one for each season: autumn (from 12 November to 16 December 2016), winter (from 11 February to 12 March 2017), spring (from 8 May to 6 June 2017) and summer (from 31 July to 28 August 2017).

In order to select camera stations, we overlaid a 1 × 1 km grid onto the study area. From this grid, we randomly selected 40 cells and put the camera waypoint in their centroids. The randomisation of the 40 stations was stratified on the area size of each habitat, meaning that the proportion of stations inside each habitat mirrored the proportion of that habitat inside the study area (Table S1 in Online Resource). Camera waypoints were digitised in Quantum GIS (3.4.4) and located in the field by means of a handheld GPS. Within each camera waypoint, we searched for the spot with the best light condition in close proximity and placed the camera station. On average, we placed camera stations 946.32 ± 196.63 m apart. During each season, the survey was conducted by using 20 camera traps (UOVision UV595-HD and IR PLUS BF 110°), which were placed for 2 weeks in 20 stations randomly selected out of the 40 available ones and then relocated in the other 20 stations for 2 more weeks.

Camera traps were secured to trees and wooden poles at an average height of 60–70 cm from the ground and adequately hidden from the animals' sight. To prevent too many animals being attracted and avoid any modification of their behaviour, no lure or bait was used at the camera stations. The position of camera traps and the range of vision were the same during the four sampling seasons. At all camera stations, we set the cameras to operate 24/7. Cameras were triggered by motion and programmed to take a 30-second video, with a 5-second delay between consecutive triggers. We checked camera stations at least weekly to replace camera batteries and memory card when needed.

We extracted the habitat type surrounding each camera station from a 10-m resolution digital map of vegetation (Grignetti et al. 1997). We pooled the habitats recognised by Grignetti et al. (1997) in 5 main classes: deciduous oak forest, evergreen oak forest, pine forest, mixed forest and grassland (Fig. 1).

All applicable international, national and institutional guidelines for animal care and use were strictly followed.

Data analysis

For each camera trap record, we identified ungulate species, date, time and habitat type. We defined distinguished records of the same species at the same camera station as independent when pictures were taken at least 30 min apart (Linkie and Ridout 2011b), thus reducing pseudoreplication biases (Meredith and Ridout 2014). Only independent records were used in the subsequent analyses. We estimated activity levels of ungulate species for which we had a reasonable number of records, defined by inspecting the distribution of sample sizes. Red deer were detected only 39 times, while roe deer, fallow deer and wild boar were recorded 267, 737 and 1737 times, respectively. As a limited sample size may negatively affect the accuracy and precision of activity curve estimates (Lashley et al. 2018), we excluded red deer from the analyses.

Spatial overlap analysis

For each camera station and species, we calculated capture frequency as the number of independent sightings per camera-day, by dividing the total number of recorded individuals of that species by the number of days in which the camera trap was active. To prevent biased analyses owing to the different population sizes of each species, detection probability for each season was obtained by dividing the number of daily detections (capture frequency) at each station by the total number of detections for the corresponding species (wild boar, fallow deer and roe deer):

$$DP = \left(\frac{CF_{station\ i}}{\sum_{station\ 1-i} CF} \right)$$

in which DP stands for detection probability and CF for capture frequency.

In order to verify whether the three ungulates showed a differential use of space over the four seasons, we modelled DP by using generalised linear mixed models (GLMMs) with a Gaussian distribution of errors by using the *nlme* package in R (Pinheiro et al. 2018). DP was arcsin-root-transformed in order to improve normality of residuals and to reduce skewness. Species, season, habitat type and their interactions were included in the model as fixed factors. Camera station ID was fitted as a random intercept to control for the influence of camera-related factors (e.g. vegetation cover, distance to water). Based on the model predictions, we derived the estimated marginal means (EMMs) for each factor and interaction included in the model. We tested pairwise comparisons of EMMs by using the *emmeans* package in R (Lenth 2020).

Temporal overlap analysis

To carry out the temporal overlap analysis, each individual captured by a camera trap record was treated as a single observation in the dataset. The temporal distribution of observations of each species was used to represent its daily activity budgets. Firstly, we converted the time of each capture event into radians to account for the circular distribution of the time of day (Meredith and Ridout 2014; Rowcliffe et al. 2014). For each species, we estimated seasonal activity patterns by fitting a circular kernel density distribution to radian time-of-day data by means of the *fitact* function in the *activity* package in R (Rowcliffe 2016), which provided the percentage of activity time (activity level) during the day (24 h). Then, we calculated the percentage of activity time during daylight hours by means of the *densityPlot* function, by taking into consideration sunrise and sunset times for the study area (obtained from the website https://www.usno.navy.mil/). We calculated the percentage of activity time during nocturnal hours by subtracting the percentage of daylight activity from the total (24 h). Furthermore, we used the *compareAct* function, which uses a Wald test, to compare the activity levels of each species during the different seasons.

To determine the activity overlaps among the three species, we calculated the coefficients of overlapping (Δ) in a pairwise

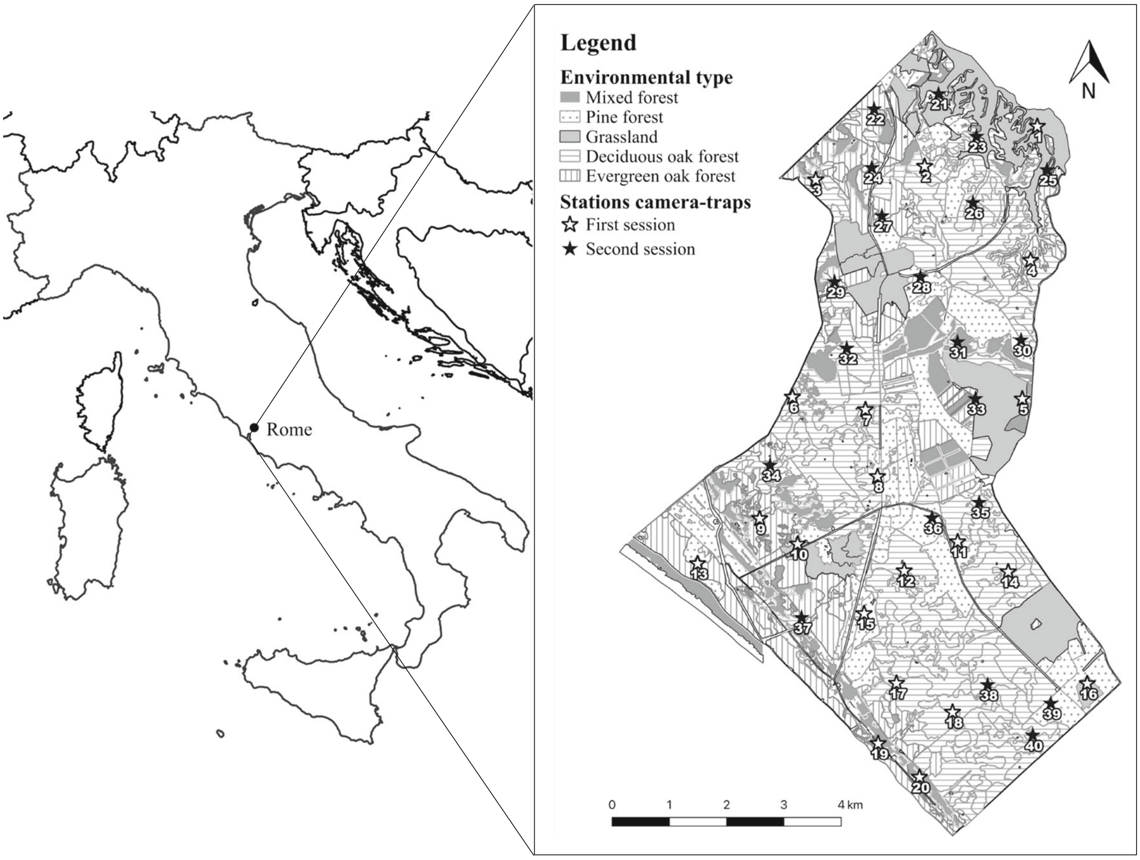


Fig. 1 Map of Italy (left) showing the localisation of in the Estate of Castelporziano (Rome, Italy) and an enlargement of the map (right) with the distribution of camera traps inside the study area.
We used Quantum GIS (3.4.4) Medeira graphics program to create this figure.

manner for each season by using the *overlap* package in R (Ridout and Linkie 2009). The Δ coefficient measures the extension of the overlap between two kernel density estimates by taking the minimum density function from two sets of samples compared at each point in time. The area under both density curves was considered an overlap. The coefficient of overlapping ranged from 0 (no overlap) to 1 (complete overlap; Ridout and Linkie 2009; Linkie and Ridout 2011a). We used Δ_4 estimator, which was recommended for large sample sizes (>75 camera records Meredith and Ridout 2014). We calculated the 95% confidence intervals of each overlap index by using smoothed bootstrap with 1,000 resamples (Meredith and Ridout 2014).

Spatio-temporal overlap analysis

We evaluated the spatio-temporal overlap following the methodology proposed by Karanth et al. (2017). For each season and species, we created a matrix in which we verified the hourly presence of the species at all camera stations: the rows of the matrix represented the camera stations and the columns the hourly intervals of the diel cycle. Each cell of the matrix contained the total number of detections of the species at a particular site during a specific hourly interval, aggregated throughout the entire season. We then calculated the proportion of camera stations, at each hourly interval, when (i) each species was detected alone, in the absence of the other species, (ii) detection of activity of any two species overlapped and (iii) all three species were active. The proportions were calculated for each hourly interval by dividing the number of camera stations where the species were recorded (alone, in pairs, all together) by the total number of stations where the species were actually detected. Finally, for each species and season, we calculated the hourly average and its relative standard error.

Results

During the four sampling sessions, we recorded 2741 videos: 1737 videos with wild boars, 737 with fallow deer and 267 with roe deer. Throughout the year, all the species were recorded in the five types of environment considered (Table S2 in Online Resource).

Table 1 Effect of predictor variables (habitat type, species, season and their interactions) on the detection probability of wild boar, fallow deer and roe deer over the four seasons of data collection in the Estate of Castelporziano (Rome, Italy)

	numDF	denDF	*F* value	*P* value
(Intercept)	1	385	270.59	< 0.001
Habitat type	4	35	0.59	0.672
Species	2	385	4.18	0.016
Season	3	385	0.34	0.793
Habitat*Species	8	385	5.50	< 0.001
Habitat*Season	12	385	1.85	0.038
Species*Season	6	385	0.42	0.868
Habitat*Species*Season	24	385	0.86	0.658

The results of the GLMM (Table 1) showed that *DP* significantly varied according to the species, with a higher probability to detect wild boar. However, the pairwise comparison of EMMs showed that this difference was not statistically significant (Table S3 in Online Resource). The two-way interaction between species and habitat type showed that throughout the monitoring period, the patterns of *DP* were significantly different among the species. Specifically, wild boar was detected more frequently in grassland (*DP* = 0.22 ± 0.02, mean ± standard error) with respect to both fallow deer (*DP*, 0.06 ± 0.02) and roe deer (*DP*, 0.07 ± 0.02). Moreover, it was detected more frequently in deciduous oak forest (*DP*, 0.14 ± 0.02) with respect to roe deer (*DP*, 0.10 ± 0.02, Table S4 in Online Resource). However, when comparing *DP* of the three species in the different habitat types during each season, no significant difference was found (Table S5 in Online Resource). This result highlighted that, when considering the seasonal patterns, the three species showed a clear seasonal spatial overlap (Fig. S2 in Online Resource).

During summer and autumn, wild boar showed higher activity level with respect to winter and spring (Tables 2 and 3), with an equal distribution during daylight and nocturnal hours (Table 2). During winter, wild boar showed mainly nocturnal activity patterns, while during spring its activity was mainly concentrated during daylight hours. Throughout the year, wild boar reached the maximum peak of activity at dusk, with the exception of summer, when it showed two distinct peaks of activity, at dawn and dusk (Fig. 2).

The activity levels of fallow deer were not significantly different during the four seasons (Tables 2 and 3) and diurnal activity was prevalent during spring only. Surprisingly, during autumn and winter, fallow deer showed mainly nocturnal activity patterns (Table 2). Fallow deer did not show well-defined activity peaks during autumn and winter, while its activity seemed to peak at dawn and dusk during spring and summer (Fig. 2).

Roe deer showed similar activity levels over the four seasons (Tables 2 and 3). No clear distribution of activity patterns was observed either during the day or at night: they were mainly diurnal during spring (Table 2) and almost equal during daylight and nocturnal hours in autumn and winter (Table 2). During summer, roe deer shifted to a mainly nocturnal activity (Table 2). During winter and summer, it showed two clear peaks of activity at dawn and dusk. The peak at dawn was delayed during spring and autumn, while the peak at dusk disappeared in spring (Fig. 3).

In general, the activity overlap among the three species was high in all seasons with a Δ_4 never lower than 0.63 (Figs. 2, 3

Table 2 Estimates of the proportion of active time during daylight hours and the percentage of active time during the 24-hour cycle over the four seasons of data collection in the Estate of Castelporziano (Rome, Italy)

Species	Seasons	*N*	Prop. diurnal	Activity level	*SE*	*CI*
Wild boar	Autumn	859	49%	0.48	± 0.03	0.42–0.54
	Winter	214	30%	0.34	± 0.03	0.28–0.40
	Spring	262	64%	0.36	± 0.04	0.30–0.46
	Summer	402	47%	0.50	± 0.04	0.42–0.58
Fallow deer	Autumn	184	40%	0.38	± 0.05	0.30–0.47
	Winter	93	37%	0.55	± 0.08	0.40–0.71
	Spring	199	53%	0.49	± 0.05	0.38–0.59
	Summer	261	45%	0.46	± 0.05	0.38–0.57
Roe deer	Autumn	47	50%	0.52	± 0.08	0.33–0.64
	Winter	87	52%	0.36	± 0.05	0.25–0.46
	Spring	62	65%	0.48	± 0.07	0.33–0.59
	Summer	71	36%	0.35	± 0.07	0.23–0.50

n = number of individual records (prop. diurnal = proportion of active time during daylight hours; activity level = estimated percentage of active time during the 24-hour cycle (see Rowcliffe et al. 2014 and the Methods section for more details); *SE* = standard error for the estimated activity level; *CI* = 95% confidence intervals for the estimated activity level) and 4). Overall, the season with the highest coefficients of overlapping was summer (range of mean Δ_4, 0.82–0.88) showing that the drivers of activity forced all the species to be active simultaneously. During autumn, the general overlap estimates provided a range of Δ_4 from 0.76 to 0.84, whereas in winter (Δ_4, 0.64–0.71) and spring (Δ_4, 0.63–0.77) results showed a decrease of overlap.

Considering the paired coefficients of overlapping, the highest activity overlap was found between fallow deer and roe deer (Fig. 4), particularly during summer ($\Delta_4 = 0.84$, $CI =$ 0.75–0.88). Conversely, the overlap between wild boar and either roe deer or fallow deer was not homogeneous throughout the year, reaching the minimum values in spring (wild boar-roe deer, $\Delta_4 = 0.63$, $CI =$ 0.53–0.73, Fig. 3) and winter (wild boar-fallow deer, $\Delta_4 = 0.64$, $CI =$ 0.56–0.72, Fig. 2).

Patterns of combined spatio-temporal overlap showed that the species pair overlap was much lower when compared to the exclusively temporal overlap. Generally, the three species were detected together (same station and hourly interval) in less than 12% of cases, with the only exception of roe deer, which in summer was observed with the other two species at 27% of the stations where it was detected (Table 4). By comparing pairs, the species that showed the highest level of overlap were fallow deer-wild boar and roe deer-wild boar, particularly during autumn and summer (Table 4). Roe deer was generally the species with the highest level of spatio-temporal overlap (Table 4), reaching the highest values in summer when it was detected alone only in 26% of the stations. On the contrary, spring was the season when its degree of overlap reached the lowest values. Unlike the temporal overlap, the level of spatio-temporal overlap between roe deer and fallow deer was quite low and never higher than 11% during all seasons. From a seasonal point of view, the season that showed the lowest percentages of overlap was winter, while summer showed the highest levels of spatio-temporal overlap (Table 4).

Table 3 Results of Wald test used to compare activity levels of each species during four different seasons

Species	Seasons	Difference	*SE*	*W*	*P*
Wild boar	Autumn vs winter	0.14	0.05	8.88	0.003
	Autumn vs spring	0.12	0.05	5.48	0.019
	Autumn vs summer	−0.02	0.05	0.23	0.632
	Winter vs spring	−0.02	0.05	0.14	0.711
	Winter vs summer	−0.16	0.05	9.58	0.002
	Spring vs summer	−0.14	0.06	6.44	0.011
Fallow deer	Autumn vs winter	−0.17	0.09	3.43	0.064
	Autumn vs spring	−0.11	0.07	2.48	0.116
	Autumn vs summer	−0.09	0.07	1.55	0.213
	Winter vs spring	0.06	0.10	0.44	0.507
	Winter vs summer	0.09	0.10	0.80	0.371
	Spring vs summer	0.02	0.07	0.09	0.760
Roe deer	Autumn vs winter	0.15	0.10	2.52	0.112
	Autumn vs spring	0.04	0.11	0.11	0.736
	Autumn vs summer	0.16	0.11	2.33	0.127
	Winter vs spring	−0.12	0.09	1.61	0.204
	Winter vs summer	0.01	0.09	0.01	0.915
	Spring vs summer	0.13	0.10	1.51	0.219

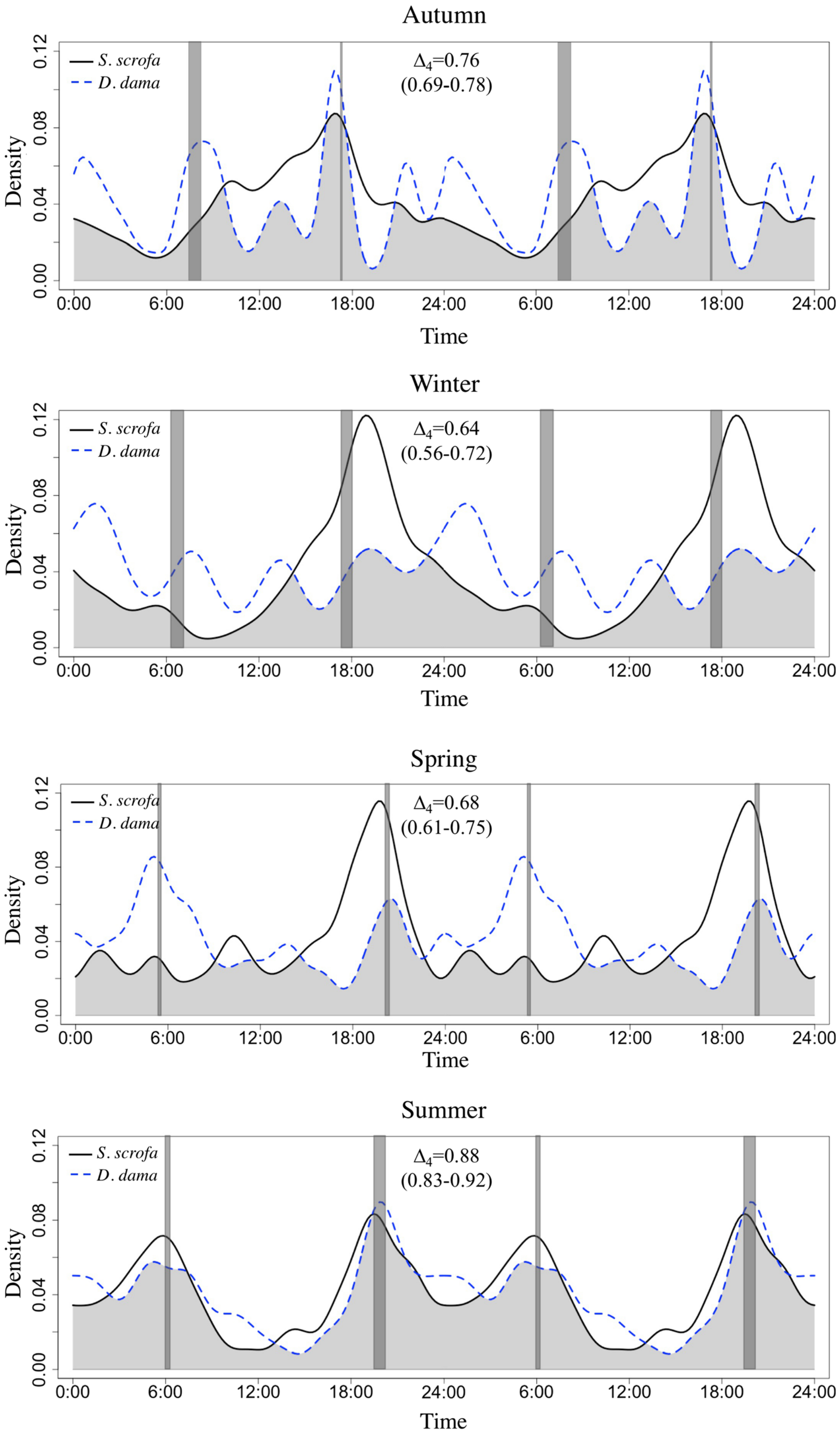
Difference = differences between activity estimates during the different seasons; *SE* = Standard errors of the differences; *W* = Wald statistics; *P* = *p* values (H_0 is no difference between estimates)

Fig. 2 Seasonal activity overlap between wild boars and fallow deer. The grey lines show dawn and dusk. Δ = index of overlap, value in brackets for confidence interval. Records are double plotted on a 48-h time scale to help the interpretation. ▶
We used R (3.6.1) software graphics program to create this figure.

Discussion

The three species were observed in all the habitat types of the study area over the four seasons of our data collection, thus highlighting an evident spatial overlap. Our results showed that the three species did not avoid each other by means of temporal segregation of their activities as their daily activity rhythms highly overlapped. Nevertheless, by using a finer scale analysis of the spatio-temporal dimension, we highlighted the three species' ability to reduce interspecific interactions either by being active at the same hours but in different areas or by using the same areas at different times of the day. This spatio-temporal segregation indicated that, under the ecological conditions of our study area, the three species developed the skills to implement complex and alternative behavioural strategies, which likely facilitated intra-guild sympatry.

According to the limiting similarity theory by Macarthur and Levins (1967), competing species should differ at least for one dimension of their ecological niche: space, time or resource exploitation. In our study, we did not find spatial segregation among the three species. In contrast to other studies (e.g. Mori et al. 2020), spatial partitioning did not seem to play a major role in structuring interspecific coexistence in our study system. It is worth noting that the study area is fenced and surrounded by a territory strongly affected by human presence as it is located in the suburbs of the largest city in Italy (i.e. Rome). Consequently, the spatial overlap we found may be the result of the limitations in dispersal opportunity. Given the high spatial overlap, a considerable potential for overlap in resource exploitation might be expected, particularly between roe deer and fallow deer (Ferretti et al. 2011). On the other hand, interference competition with wild boar might be expected primarily on account of its destructive feeding


Autumn
S. scrofa
D. dama
Δ_4=0.76
(0.69-0.78)
Density
0.00
0.04
0.08
0.12
0:00
6:00
12:00
18:00
24:00
6:00
12:00
18:00
24:00
Time
Winter
S. scrofa
D. dama
Δ_4=0.64
(0.56-0.72)
Density
0.00
0.04
0.08
0.12
0:00
6:00
12:00
18:00
24:00
6:00
12:00
18:00
24:00
Time
Spring
S. scrofa
D. dama
Δ_4=0.68
(0.61-0.75)
Density
0.00
0.04
0.08
0.12
0:00
6:00
12:00
18:00
24:00
6:00
12:00
18:00
24:00
Time
Summer
S. scrofa
D. dama
Δ_4=0.88
(0.83-0.92)
Density
0.00
0.04
0.08
0.12
0:00
6:00
12:00
18:00
24:00
6:00
12:00
18:00
24:00
Time

habits (i.e. rooting). Consequently, we expected a differential exploitation of the temporal niche by these sympatric species to avoid resource and interference competition, as shown in other studies (Di Bitetti et al. 2009; Monterroso et al. 2014). For instance, Di Bitetti et al. (2010) showed that the ability of pumas (*Puma concolor*) and oncillas (*Leopardus tigrinus*) to adjust their activity patterns to local conditions resulted in a temporal segregation which may facilitate their coexistence and explain the lack of spatial segregation in this assemblage. Contrary to our expectation (1), the three sympatric ungulates apparently did not develop a strategy to avoid being active at the same time. Indeed, our results showed a high temporal overlap among the three species during all seasons. This is consistent with the findings of Mori et al. (2020), which pointed at a high temporal overlap among ungulates species, particularly between wild boar and roe deer. It is interesting to note that, despite the high temporal overlap of activity among the studied species, our results showed that during autumn, winter and spring, roe deer and fallow deer had a peak of activity at dawn, when wild boar was less active. This may be a strategy adopted by the two deer species to avoid being active at dusk, when wild boar reached its peak of activity. Moreover, the three species adopted segregation through fine-scale spatial avoidance as the proportion of sites where the species were observed together was relatively low. In this framework, the species which showed the higher spatio-temporal overlap with other ungulates was roe deer, which was less frequently observed alone, likely on account of its lower density inside the study area. Wild boar, being numerically prevalent, was detected more frequently and this affected the probability of spatio-temporal overlap with fallow and roe deer.

Interestingly, the season when both temporal and spatio-temporal overlap reached the highest values was summer, i.e. the most limiting season in the Mediterranean environment, when food resources were scarce due to drought. On account of these results, we may presume that different drivers, which are likely stronger than interspecific interactions, affected the activity rhythms and fine-scale spatial use. It is now well established that animal activity patterns rely on endogenously fixed rhythms, which are regulated by biological clocks but are also regulated by environmental stimuli, the so-called "zeitgebers" (Aschoff et al. 1982). As a result, activity patterns are strongly affected by different external factors, which may be either environmental (e.g. photoperiod, moon phases, weather conditions, food and water availability) or biotic (e.g. social signals, the presence of predators and human activities; Maloney et al. 2005; Paul et al. 2008). Ambient temperature was repeatedly shown to be one of the most important factors affecting the activity rhythms (Maloney et al. 2005; Pagon et al. 2013; Brivio et al. 2016, 2017; Grignolio et al. 2018) and spatial behaviour of ungulates (Mysterud and Østbye 1999; Marchand et al. 2015; Mason et al. 2014, 2017). By reducing activity when it is warmer and selecting cooler microclimates in their environment (Mysterud and Østbye 1999; Marchand et al. 2015; Brivio et al. 2019), animals may be able to avoid heat stress, while reducing the costs for autonomic thermoregulation (Terrien et al. 2011). Our results are consistent with these findings and suggest that temperature strongly affected the behavioural strategies of the monitored individuals. Indeed, both the temporal and spatio-temporal overlaps were particularly high exactly during the season in which ambient temperature reached the highest levels, i.e. summer (Fig. S1 in Online Resource). On the one hand, the high proportion of nocturnal activity (higher than 50%, Table 2) suggested that high temperatures in summer likely forced the populations involved in this study to be active during the coolest time of the day (i.e. nocturnal hours; Beier and McCullough 1990; Berger et al. 2002; Scheibe et al. 2009; Pita et al. 2011). On the other hand, the high hourly overlap found in the spatio-temporal analysis suggested that the three species in our study area simultaneously used the same habitats. We may suppose that a common driver constrained spatio-temporal choices of the three species: indeed, the high summer temperatures likely pushed the animals towards the coolest parts of the study area.

The activity overlap between wild boar and roe deer, both at temporal and spatio-temporal levels, reached the lowest value in spring, thus confirming our prediction (2). This result was likely affected by the birth of the roe deer fawns and the territorial activity of roe deer males—both occurring in spring. In white-tailed deer (*Odocoileus virginianus*), females with vulnerable fawns were reported to alter their temporal activity patterns arguably to reduce the risk of encounters with potential predators (Higdon et al. 2019). Wild boar can prey upon small mammals and fawns during their early weeks of life (Loggins et al. 2002; Wilcox and Van Vuren 2009). Consequently, the lower activity and fine-scale spatial overlap in this season might suggest a strategy adopted by roe deer to avoid encounters with wild boar to reduce risks for their fawns.

Our results confirmed the great behavioural plasticity of wild boar (e.g. Cousse et al. 1995; Caley 1997; Russo et al. 1997; Keuling et al. 2008; Barrios-Garcia and Ballari 2012; Podgórski 2013), with considerable variations of its activity rhythms. In our study area, wild boar activity showed a single peak around dusk in autumn, winter and spring, as found in other populations (e.g. Mori et al. 2020). In summer, on the other hand, wild boar showed two distinct peaks at dawn and dusk. Throughout the year, activity patterns switched from predominantly diurnal to predominantly nocturnal to a quite equal distribution between day and night. This is in contrast with the results regarding other Italian populations which were found to be nocturnal throughout the year (Russo et al. 1997; Brivio et al. 2017; Mori et al. 2020). It was suggested that the switch from diurnal to nocturnal activity may be a response to anthropic disturbance in wild boar as well as in other animal species (Keuling et al. 2008; Ohashi et al. 2013; Gaynor et al.

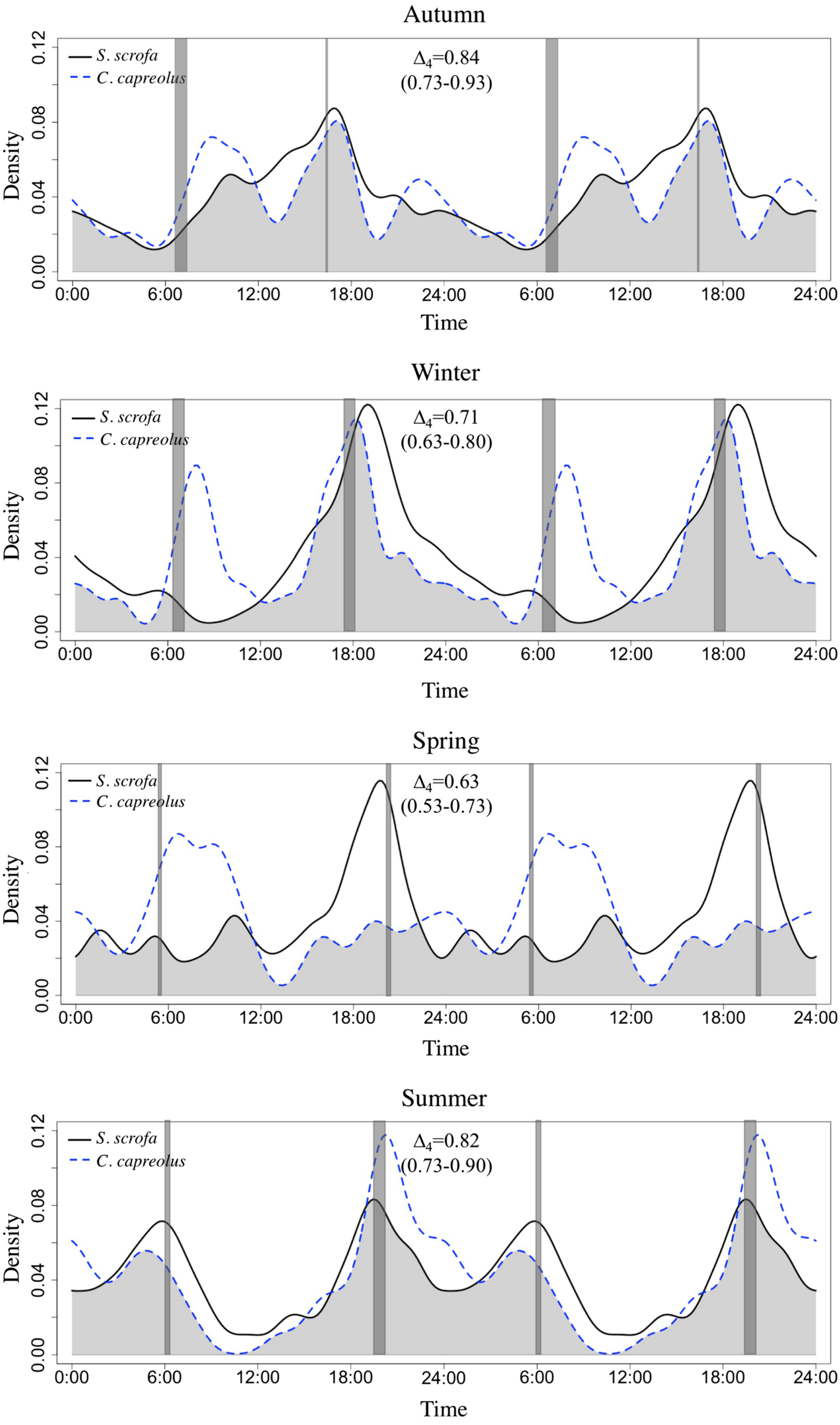


Fig. 3 Seasonal activity overlap between wild boars and roe deer. The grey lines show dawn and dusk. Δ = index of overlap, value in brackets for confidence interval. Records are double plotted on a 48-h time scale to help the interpretation.
We used R (3.6.1) software graphics program to create this figure.

Fig. 4 Seasonal activity overlap between fallow deer and roe deer. The grey lines show dawn and dusk. Δ = index of overlap, value in brackets for confidence interval. Records are double plotted on a 48-h time scale to help the interpretation. We used R (3.6.1) software graphics program to create this figure.

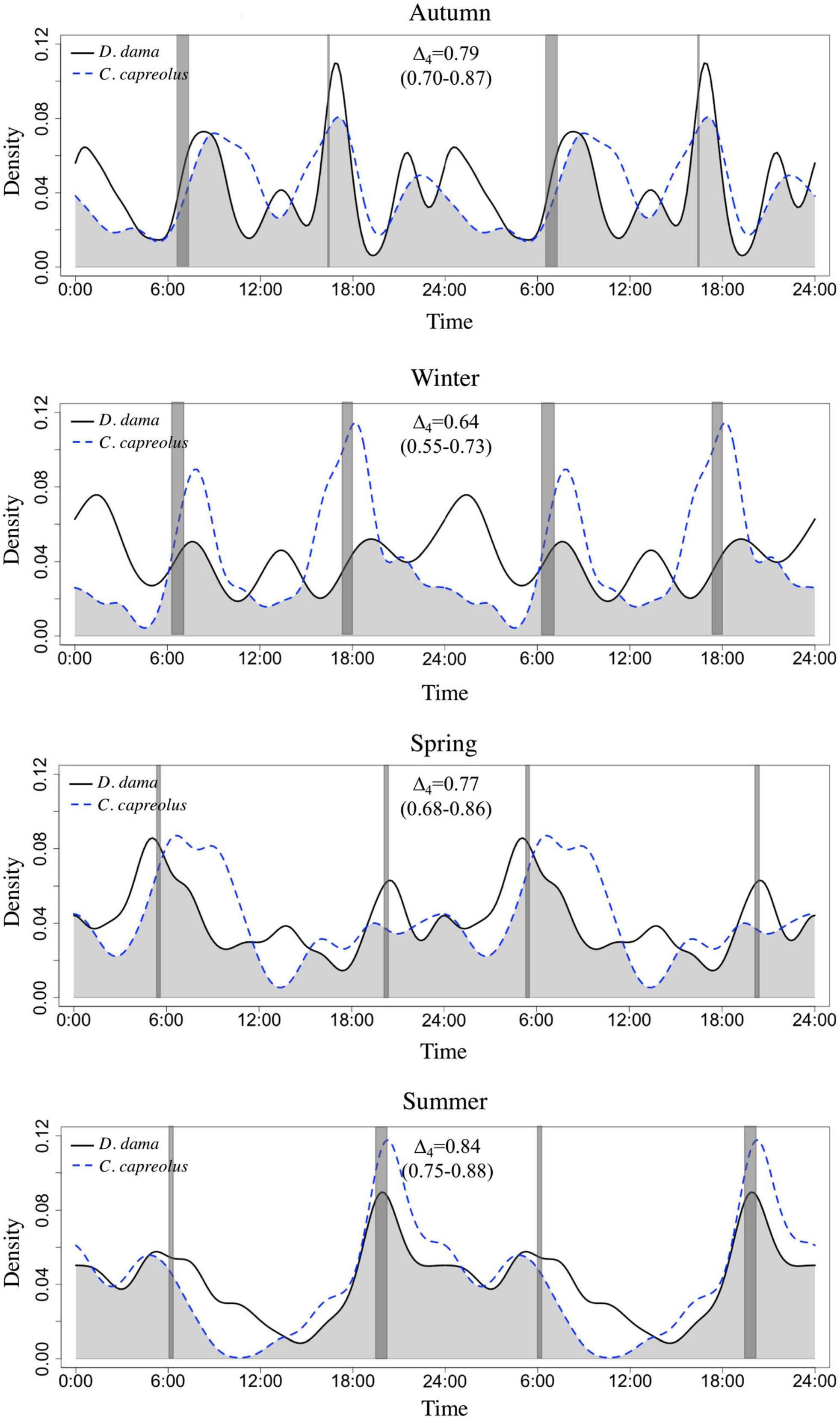
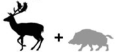
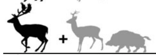
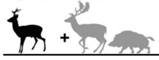


Table 4 Proportion of camera stations, averaged over hourly intervals, when (i) each species was detected alone, in the absence of the other species, (ii) detection of activity of any two species overlapped and (iii) all three species were active

Type of interaction	Autumn	Winter	Spring	Summer
	0.86 (0.09)	0.86 (0.14)	0.83 (0.15)	0.67 (0.23)
	0.11 (0.01)	0.06 (0.02)	0.11 (0.02)	0.23 (0.04)
	0.03 (0.01)	0.07 (0.02)	0.03 (0.01)	0.05 (0.02)
	0.004 (0.003)	0.02 (0.01)	0.03 (0.02)	0.04 (0.01)
	0.48 (0.06)	0.76 (0.04)	0.76 (0.05)	0.56 (0.05)
	0.47 (0.06)	0.09 (0.03)	0.17 (0.05)	0.31 (0.04)
	0.04 (0.02)	0.11 (0.03)	0.05 (0.02)	0.02 (0.02)
	0.01 (0.01)	0.04 (0.03)	0.02 (0.01)	0.09 (0.04)
	0.53 (0.07)	0.65 (0.07)	0.67 (0.07)	0.26 (0.05)
	0.33 (0.08)	0.19 (0.06)	0.13 (0.04)	0.38 (0.08)
	0.11 (0.04)	0.09 (0.03)	0.10 (0.04)	0.09 (0.03)
	0.04 (0.03)	0.07 (0.05)	0.11 (0.05)	0.27 (0.06)

The values in brackets are the relative standard errors.

The values in brackets are the relative standard errors

2018). The high nocturnal activity that we found during winter, when culling occurred, supports this theory and is in contrast with the results found by Brivio et al. (2017), which showed that hunting activities did not influence wild boar activity patterns. These differences may be the consequence of the very low levels of human disturbance which characterised our study area throughout the year, with the only exception of the culling period. Consistently, the other culled ungulate, i.e. fallow deer, showed predominantly nocturnal activity during winter in our study area.

Our results on the activity patterns of fallow deer were among the few available data on this species in the Mediterranean environment. According to Caravaggi et al. (2018), whose data referred to Northern Ireland, fallow deer showed prevalently diurnal activity patterns. On the contrary, we found a prevalently nocturnal activity, with the only exception of spring, when it was quite equal during day and night. During winter, the activity pattern was characterised by the presence of several peaks, with a reduced magnitude. However, it is important to stress that we examined the behavioural patterns of a small population for a single year and, therefore, these results have to be taken with caution and further studies are necessary to fully describe the activity patterns of this species. Generally, fallow deer and roe deer are thought to be crepuscular species, showing the highest activity levels at dawn and dusk (Náhlik et al. 2009; Sandor et al. 2011; Krop-Benesch et al. 2013; Pagon et al. 2013; Mori et al. 2020). Several management activities, such as census, rely on this feature. However, our findings did not completely support this statement: only during two seasons (winter and spring for roe deer and fallow deer, respectively), the studied individuals were clearly crepuscular. During summer, they were mostly nocturnal, while during the other seasons fallow deer showed several peaks during diurnal and nocturnal hours, while roe deer seemed to postpone activity after crepuscular hours, particularly in the morning. These results suggest the need to improve knowledge in order to better define management activities.

In conclusion, our study indicated a high degree of spatial and temporal overlap, though a lower overlap was found when data were analysed at a finer scale (i.e. spatio-temporal overlap). This suggests that, even though the species used the same habitats and had similar activity rhythms, they may be able to avoid interspecific interaction by using space during different time periods. On the other hand, by definition, competition can only exist when resources are actually or potentially lacking (Putman and Putman 1996; Tokeshi 2009). The three sympatric ungulates under scrutiny may be able to avoid interspecific competition by using different resources. Diet analysis of each species will likely improve our understanding of the actual interspecific competition among them.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13364-020-00548-1.

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Author contributions FB and MA originally formulated the idea. MZ and FB conducted fieldwork. MZ, FB and SG performed analyses. FB and MZ wrote the manuscript and other authors provided editorial advice.

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Compliance with ethical standards

Ethical approval All applicable international, national and institutional guidelines for animal care and use were strictly followed.

Competing interests The authors declare that they have no competing interests.

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