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Odor cues from predators as a seed protection strategy against foraging rodents

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Abstract

In forest restoration of oak, planting nursery-growth seedlings is related to high operational costs. One cost-efficient alternative is the application of direct seeding. However, acorn removal by rodents is one of the major drawbacks in its implementation. As rodents rely on their sense of smell to allocate their predators and show fear/defense related behaviors, there is a strong potential for the application of predator odor cues as a seed protection strategy. Therefore, the overall goal of this thesis was to investigate to which extent predator odors could be used as a seed protection strategy against foraging rodents. To reach this goal, I established laboratory and field experiments to investigate natural-based treatments and synthetic odor compounds from predators. The first laboratory study (paper I) revealed mink excrement as the most efficient treatment, because it reduced seed consumption and seed touch by rodents, but did not have negative effects on germination. Based on this results, we established a direct seeding field experiment (paper II) were the acorn removal rates were monitored under the application of two mink excrement treatments and other factors such as distance to forest edge and acorn size. Here, in contrast to the laboratory study, the mink excrement did not reduce acorn removal. Factors such as loss of efficiency over time due to excrement desiccation or animal habituation after a long odor exposure were accounted as possible explanations. The results further confirm the importance of reducing suitable habitats for rodents such as post-harvest slash piles, and to select acorn sizes with caution, because although bigger acorns produce better seedlings, they are also removed in higher quantities. Finally, the laboratory and field studies (paper III and IV, respectively) regarding synthetic predator odor compounds showed for the first time that the compound 2-propylthietane was avoided by bank voles in laboratory settings and reduced the acorn removal rates in natural conditions. This result further supports the behavioral relevance of single compounds, which may elucidate fear responses as strong as for complex odor mixtures. In conclusion, this thesis highlights the relevance of synergies between laboratory and field studies with the goal to direct research efforts in finding a better protection strategy during direct seeding.

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Dedication

To Wiebke, Marlene and María del Carmen

The scientific attitude of mind involves a sweeping away
of all other desires in the interests of the desire to know.
   Bertrand A. W. Russell

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Villalobos A.*, Olsson G., Birkedal M. & Löf M. (2019). The effects of four repellents on bank vole consumption and germination of beech nuts and acorns. *New Forests*, 50:241-254.

II. Villalobos A.*, Schlyter F., Olsson G., Witzell J. & Löf M (2020). Direct seeding for restoration of mixed oak forests: Influence of distance to forest edge, predator-derived repellent and acorn size on seed removal by granivorous rodents. *Forest Ecology and Management.* (In review).

III. Villalobos A.*, Schlyter F., Birgersson G., Koteja P. & Löf M (2020). Fear effects on bank voles (Rodentia: Arvicolinae): Testing for repellent candidates from predator volatiles. (Manuscript).

IV. Villalobos A.*, Schlyter F., Dekker T., Larsson-Herrera S., Birgersson G. & Löf M (2020). Predator odor reduces acorn removal by granivorous rodents in mixed oak forest stands. (Manuscript).

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The contribution of Adrian Villalobos to the papers included in this thesis was as follows:

I. Conducted all statistical analysis. Wrote the manuscript in collaboration with co-authors

II. Developed the research idea and planned the experiment together with co-authors. Responsible for data collection together with ML. Conducted all statistical analysis. Wrote the manuscript in collaboration with co-authors

III. Developed research question and design together with co-authors. Conducted data collection and analysis including statistics. Wrote the manuscript in collaboration with co-authors

IV. Planned the experiment together with FS, TD, GB, and ML. Conducted data collection and analysis including statistics. Wrote the manuscript in collaboration with co-authors

1. Introduction

For millennia, a plethora of human activities have altered ecosystems worldwide and especially forests have experienced a continuum of conversion and degradation to fulfil an increasing demand for natural resources. Since the dawn of human civilization (post-Pleistocene), a 45.8% loss of the global number of trees has been estimated (Crowther *et al.*, 2015). One extreme case is Europe, where about 99% of its native forest has been altered (Paillet *et al.*, 2010), and only 0.2% of the temperate broadleaved forests remain in its natural conditions (Hannah *et al.*, 1995). Thus, the European temperate broadleaved forest is one of the most threatened biomes in the world (Venter *et al.*, 2016).

In southern Sweden, human interventions started to have a significant impact on broadleaved forests with the first evidence of extensive agriculture about 2,000 years ago (Lindbladh *et al.*, 2007). During the industrialization, and particularly after the economic boom of the post Second World War era, the forest industry became one of the pillars of the Swedish economy (Törnlund & Östlund, 2006). As a consequence, reforestation and afforestation with fast growing conifers such as Norway spruce (*Picea abies* L.) and Scots pine (*Pinus sylvestris* L.) has been promoted, and are currently dominating what was once a landscape dominated by mixed temperate broadleaved forests (Lindbladh *et al.*, 2014).

The actual *status quo* of the southern Sweden forests can probably not be maintained, not at least because current climate models predict an increase of 2-6 °C in the next 100 years for this area (Löf *et al.*, 2012; Koca *et al.*, 2006) resulting in warmer and more extreme weather. Within this scenario, the Norway spruce and Scots pine are not well adapted as both can be easily affected by increasing heat, disturbances and pests (Hanewinkel *et al.*, 2013). New approaches in forest restoration should therefore include the re-

establishment of more resistant and resilient broadleaved tree species (Bolte *et al.*, 2009). Oaks (*Quercus* spp.), for example, are relatively tolerant to climate-driven disturbances (Götmark & Kiffer, 2014; Epron & Dreyer, 1993) and the regeneration of oak forests or mixed forests with oaks is considered as part of the solution to this challenge (Mette *et al.*, 2013; Löf *et al.*, 2010a).

1.1 Oaks and restoration of oak forests

The genus *Quercus* includes around 600 species of which approximately 80% are distributed in tropical and subtropical ecoregions (Johnson *et al.*, 2019; Mölder *et al.*, 2019). Although only two oak species (pedunculate oak (*Q. robur* L.) and sessile oak (*Q. petraea* L.)) are native to Scandinavia, they are highly valuable as multifunctional forests providing high quality timber for the wood industry, biomass for bio-energy production, areas for recreation and key habitats for biodiversity (Mölder *et al.*, 2019; Löf *et al.*, 2016). Due to their long lifespan and the high durability of the dead wood, more than 50% of all endangered forest animals, plant and fungi are dependent on oak-dominated forests (Felton *et al.*, 2016; Jansson *et al.*, 2009; Lindbladh *et al.*, 2007; Berg *et al.*, 1994). However, currently oaks represent only 2,2% of the total standing tree volume in southern Sweden (Forestry statistics, 2014), and a considerable proportion of these oaks are scattered trees in agricultural lands or consist as small mixtures within Scots pine or Norway spruce forests (Löf *et al.*, 2016; Drößler *et al.*, 2012). The authorities promote the conversion of former Norway spruce stands into more mixed-oak containing stands and subsidies have been made available, but there are still high costs for restoration (Löf *et al.*, 2010b).

Forest restoration can be defined as "the process of recovering destroyed forest or altering existing forest ecosystems towards a predefined historical state by active human interventions or by passive natural regeneration" (Stanturf, 2005; Stanturf & Madsen, 2002). The natural/passive regeneration is a cost-effective method for the restoration of oak (Götmark *et al.*, 2005), but regeneration success depends on the conditions and the level of degradation of the deforested area (Stanturf, 2005). The lack of mature oak trees and seed dispersers, and the magnitude of browsing and seed predation (Leverkus *et al.*, 2016; Annighöfer *et al.*, 2015; Den Ouden *et al.*, 2005;

Kelly, 2002) can hinder passive restoration in certain areas (Bobiec *et al.*, 2018).

The active (or artificial) restoration of oak is the most common practice on former agricultural fields and in forests (Buckley & Ford, 2016; Dey *et al.*, 2008). Usually, this is carried out by planting nursery-grown containerized or bare-root seedlings (Dey *et al.*, 2008). Planting seedlings is often associated with a faster seedling growth (Buckley & Ford, 2016; Löf *et al.*, 2004) and high survival rates (Dey *et al.*, 2008; Valkonen, 2008; but see González-Rodríguez *et al.*, 2011; Löf *et al.*, 2004). However, oak seedlings in nurseries often develop shallow root systems or roots can be easily pruned during transplanting (Castro *et al.*, 2015). Moreover, this technique has the major disadvantage of being expensive and labor intensive (Löf *et al.*, 2004; Bullard *et al.*, 1992). For example, cost estimates of active oak restoration in Sweden revealed between 4,000-7,000 Euros per hectare in 2012 (Löf *et al.*, 2012) and due to the high browsing pressure of wild ungulates (Petersson *et al.*, 2019) additional fencing costs further reduce the cost-efficiency of this type of forest restoration (Bergquist *et al.*, 2009).

A more cost-effective alternative for the active restoration of oak forests is the application of direct seeding (Löf *et al.*, 2004; Bullard *et al.*, 1992). This technique has the potential to reduce restoration costs by 30-50% (Ceccon *et al.*, 2015; Löf *et al.*, 2012; Madsen & Löf, 2005) and thus has regained major attention in recent decades (Löf *et al.*, 2019).

1.1.1 Direct seeding of oak

Direct seeding is one of the oldest techniques for artificial regeneration of oaks in which acorns are directly sown into the soil (Abrams & Nowacki, 2008; Dey *et al.*, 2008). Sowing acorns can either be performed using modified agricultural machinery at a recommended row distance of 2.5-4.5 meters, or by manual seeding at sowing rates of 6,000-12,000 acorns per day and person, depending on manual tools used and site conditions (Johnson *et al.*, 2019).

The direct seeding of oak has several advantages in comparison to planting seedlings. First, it can reduce the regeneration costs and easier the transport to sowing sites (Madsen & Löf, 2005; Willoughby *et al.*, 2004). Hence larger areas can be showed in a shorter period (Ceccon *et al.*, 2015), ensuring high stock density for reasonable wood quality at low costs

(Madsen & Löf, 2005). Second, seedlings regenerated after direct seeding, develop a better tap-root architecture and are therefore more suitable to overcome stress conditions in the field such as drought (Zadworny *et al.*, 2014; Tsakaldimi *et al.*, 2009; Pemán *et al.*, 2006). And third, sowing reduces the risk of transporting plant pathogens such as *Phytoptera* spp. from nursery stocks to the field (Sánchez *et al.*, 2005). An overall benefit is that stands established through direct seeding are often mixed with other tree species and result in more complex stand structures (Twedt & Wilson, 2002) which may contribute to increased forest resilience to biotic and abiotic disturbances (Pretzsch, 2020).

1.1.2 Failures in direct seeding of oak: a rodent problem

Failures of direct seeding of oak depend on multiple factors and are not uncommon (Dey *et al.*, 2008). Besides of poor acorn quality due to improper storage or seed selection (Johnson *et al.*, 2019; Dey *et al.*, 2008) and poor soil conditions at the restoration site (Johnson *et al.*, 2019), the consumption and removal of acorns by rodents is the major drawback (Löf *et al.*, 2019; Birkedal *et al.*, 2009; Dey *et al.*, 2008).

The rodent problem has been reported in several studies, where a 80-100% removal of acorns from restoration sites occurred within the first four months after sowing (Martelletti *et al.*, 2018; Van Ginkel *et al.*, 2013; Jinks *et al.*, 2012; Prévosto *et al.*, 2011; Puerta-Piñero *et al.*, 2010; Birkedal *et al.*, 2009). But other factors related to the field preparation or overall habitat/microhabitat conditions can further impact seed predation by rodents. The presence of competing vegetation after sowing improves habitats for rodents resulting in an even higher consumption of acorns (Birkedal *et al.*, 2010; Gómez, 2004). Therefore, a consequent site preparation is one essential prerequisite before direct seeding (Dey et al. 2008; Birkedal et al. 2010). In this context, sowing in small open fields (< 1 ha) surrounded by forest or old field vegetation can increase the rates of acorn predation due to high rodent population sizes (Dey *et al.*, 2008; Stanturf *et al.*, 1998). At last, an improper timing of sowing can lead to high seed predation if restoration operations match the peak of rodent populations (Birkedal *et al.*, 2010).

Another aspect is wild ungulates. Because of their high density in the northern hemisphere (Petersson *et al.*, 2019) and the palatability of oak seedlings to wild ungulates (Van Ginkel *et al.*, 2013), fencing is a common practice of protection (Bergquist *et al.*, 2009). This also keeps away middle-

sized acorn consumers such as wild boars (*Sus scrofa* L.) (Castro *et al.*, 2015). However, there is evidence that rodent activity and abundance increases inside fenced areas, due to the lack of competition form ungulates and also the possible exclusion of rodent mammal predators (Perez-Ramos & Maranon, 2008).

1.1.3   Methods for acorn protection

In the last decades, several techniques were tested to protect sown acorns from consumption and removal by rodents. One extreme was the use of anticoagulant rodenticides as a lethal method (Jacob & Buckle, 2018; Myllymäki, 1975). Today its application has been discouraged as it also represents a threat to non-target species and therefore has unpredictable negative effects on the environment (Gabriel *et al.*, 2018; Joermann, 1998). Simultaneously, several non-lethal acorn protection methods based on ecological, physical, silvicultural or chemical strategies were implemented (Löf *et al.*, 2019).

Ecological methods include the two approaches: attraction of prey species and food satiation. First, by adding perches in forest restoration sites avian predators are to be attracted and consequently reduce rodent presence in the area (Farlee, 2013). This method however, relies on the abundance of birds of prey which is not reliable for many open field areas (Birkedal *et al.*, 2009). Second, the food satiation hypothesis predicts that a high availability of food will increase the probability of seeds escaping predation (Janzen, 1971). It is therefore suggested that sowing during a year of high masting of oak or a high sowed seed density can improve acorn survival (Dey *et al.*, 2008; Perez-Ramos & Maranon, 2008).

Different devices have been developed as a physical protection to minimize rodent access to acorns. Mesh cages and burying wire mesh screens (10-20 cm^2) are successful for restricting access to the acorns but impair seedling development if not removed (Dey *et al.*, 2008; Weitkamp *et al.*, 2001). Another device, consisting of a wire mesh cylinder developed by Reque and Martin (2015), protects the acorn from rodent predation and the germinated seedling from browsing. However, negative effects on seedlings and saplings may occur if not removed in time (Löf *et al.*, 2019). The use of degradable tubes did not show satisfactory results rather they further increase damage to the seedlings due to frost (Madsen & Löf, 2005; Löf *et al.*, 2004). Finally, Castro *et al.* (2015) developed a "seed shelter" with promising

results as it reduces acorn predation by rodents without compromising germination. But the polypropylene plastic seed shelter needs to be removed after seedling emergence, which increases labor intensity.

Today silvicultural methods are predominantly applied to reduce acorn predation or removal during direct seeding of oak (Löf *et al.*, 2019). This techniques include for example, mechanical site operations that remove competing vegetation, which are favored rodent habitats (Johnson *et al.*, 2019; Löf *et al.*, 2019; Dey *et al.*, 2008). Birkedal *et al.* (2010) compared different operations such as disk trenching, patch scarification, top-soil removal, and mounding. Their results indicate that mounding reduces cover vegetation and allows a better seedling establishment. Another silvicultural strategy is to select sowing sites of forest openings bigger than 1-2 ha and far from forest edges (Dey *et al.*, 2008; Stanturf *et al.*, 1998). Moreover, there are indications of lower acorn predation rates if the direct seeding operation is applied in spring rather than autumn which avoids the peak of rodent population (Birkedal *et al.*, 2010; Madsen & Löf, 2005). At last, sowing depths around 5-10 cm can provide a modest acorn protection from rodents (Dey *et al.*, 2008; Nilsson *et al.*, 1996).

Only few studies have addressed the use of non-lethal chemical methods to protect acorns during direct seeding (Löf *et al.*, 2019). As granivorous rodents rely on their olfactory system to allocate food (see section 1.3.1), chemical methods are meant to act mainly as food suppressants or repellents (Hansen *et al.*, 2016). An early study by Bäumler *et al.* (1990) tested the repellent effects of 18 chemicals and plant extracts (e.g., neem oil, L-phenylalanin, grapefruit extract, diesel). Their results show for example repellent effects on diesel and neem oil but with negative effects on acorn germination. Similar results have been observed for the primary compound of chili capsaicin (Leverkus *et al.*, 2013) and diesel (Leverkus *et al.*, 2017).

1.2 Small rodents and acorns

In temperate Scandinavian forest, the yellow-necked mouse (*Apodemus flavicollis* Melchior), the wood mouse (*Apodemus sylvaticus* L.), and the bank vole (*Myodes glareolus* Schreber) are the major mammalian acorn consumers (Birkedal *et al.*, 2009; Hansson, 1971). They are known to play a double role in their interactions with oaks (Gómez *et al.*, 2019). That is, as seed predators (Steele *et al.*, 2005; Sun *et al.*, 2004; Santos & Tellería, 1997;

Crawley & Long, 1995) and as seed dispersers (Steele *et al.*, 2011; Gómez *et al.*, 2008; Pons & Pausas, 2007a). Rodents are scatter hoarders where they store acorns in multiple dispersed catches for later consumption (Lichti *et al.*, 2017; Vander Wall, 1990). Many of these acorns could, however, escape predation (Perea *et al.*, 2016) or end up only partly eaten with the embryo intact (Yang & Yi, 2012; Perea *et al.*, 2011; Steele *et al.*, 1993), allowing germination and seedling establishment if hoarded in suitable habitats (Gómez *et al.*, 2019; Johnson *et al.*, 2019). Thus, scatter hoarder rodents are a key factor in the dispersal of oaks (Jensen & Nielsen, 1986).

As mentioned above (section 1.1.2), acorn removal is especially problematic for restoration of oak forests when direct seeding is applied. Acorns are strongly preferred by scatter-hoarding rodents due to their energy and nutrient content (Jinks *et al.*, 2012) and they even smell acorns that are buried in the soil (Löf *et al.*, 2019; Dey *et al.*, 2008; Vander Wall, 2003). The pattern of acorn removal by rodents can vary in time and space (Lichti *et al.*, 2017; Perez-Ramos & Maranon, 2008) and the distribution of rodents could be influenced by two factors: food quality and quantity (Jensen, 1985). For example, the year after a big acorn crop (mast year), small rodent populations grow with the consequence of higher acorn consumption in late summer and autumn (Ostfeld et al. 1996; Wolff 1996; Schnurr et al. 2002). Furthermore, as bigger acorns have a higher nutrient content than smaller acorns, they are preferred by rodents (Muñoz and Bonal 2008; Pérez-Ramos et al. 2008; Zhang et al. 2008). Acorn removal rates are further influenced by the habitat structure (Jensen & Nielsen, 1986). Rodents increase their foraging in habitats containing dense vegetation (Pérez-Ramos *et al.*, 2008; Pons & Pausas, 2007b; Frost & Rydin, 2000) and close to forest edges (Kollmann & Buschor, 2003; Ostfeld *et al.*, 1997). This change in behavior is well explained by the "predation risk allocation hypothesis" (Lima & Bednekoff, 1999) which is the topic of the next section.

1.3 Rodents and their predators

In the temperate forests of southern Scandinavia, the major predators of granivorous rodents are divided into two groups: specialists and generalist (Erlinge *et al.*, 1983). The more rodent specialists are the least weasel (*Mustela nivalis* L.) and stoat (*M. erminea* L.), both belonging to the family Mustelidae and two birds of prey: the common kestrel (*Falco tinnunculus*

L.) and the long-eared owl (*Asio otus* L.). The more generalist predators are the mink (*Neovison vison* Schreber), the European polecat (*M. putorius* L.), the red fox (*Vulpes vulpes* L.) and two birds of prey: the tawny owl (*Strix aluco* L.) and the common buzzard (*Buteo buteo* L). Both specialist and generalist predators are present all year around and may constitute a persistent predation pressure during all rodent's life cycle (Korpimäki *et al.*, 2005; Hansson & Henttonen, 1985). Therefore, southern Scandinavian rodent populations can fluctuate in densities depending on the presence of predators in the ecosytem (Lambin *et al.*, 2006; Hansson & Henttonen, 1985; Erlinge *et al.*, 1983).

Rodents constantly experience a trade-off between foraging effort and risk of predation (Bedoya-Pérez *et al.*, 2019; Krijger *et al.*, 2017; Apfelbach *et al.*, 2005). This constitutes the basis of the "predation risk allocation hypothesis", which predicts a low foraging effort during short periods of high predation risk (Lima & Bednekoff, 1999). In other words, animals need to fulfill their dietary and body needs without being preyed upon. The assessment of high predation risk is driven by the surrounding habitat structure (Laundré *et al.*, 2010). According to the "landscape of fear" concept, vegetated habitats have a low predation risk, so rodents will forage more intensively than in higher risk open habitats (Laundré et al. 2001; Laundré et al. 2010).

1.3.1 The role of olfaction

As granivorous rodents are mainly nocturnal, their complex olfactory system play a key role in foraging (Vander Wall *et al.*, 2003), identifying conspecifics (Ferkin *et al.*, 2004) and detecting the presence of predators (Apfelbach *et al.*, 2005). The release of chemicals into the environment as a by-products of metabolism is a common pattern in all mammals (Conover, 2007). For example, mammalian predators produce scent marks or odors for intra-specific communication such as individual recognition, breeding, and territory marking (Conover, 2007; MacDonald, 1980). But these odors may also act as kairomones triggering fear/defense responses in prey species (Sbarbati & Osculati, 2006). In general, animals who are prey organisms are stressed to have different mechanism and adaptations to defend themselves and to avoid capture from predators. These defensive responses can be physiological (e.g., changes in the endocrine system), morphological (e.g., presences of spines or amour structure), reproductive (e.g., reproductive

delay) and behavioral (Kats & Dill, 1998). At the presence of predator's odors rodents use detection avoidance, shift their movements to safe habitats, and decrease foraging or feeding as their primary defense mechanisms (Hegab *et al.*, 2015; Ylönen *et al.*, 2003; Hansson, 1971).

1.3.2   Predators scent as repellents

Predator odors have been extensively studied as food suppressants or repellents against rodents (Parsons *et al.*, 2018; Apfelbach *et al.*, 2005), and in recent years there has been an increased interest for their application in an ecological based rodent management framework to replace rodenticides in agriculture and forestry (Sullivan & Sullivan, 2020; Bedoya-Pérez *et al.*, 2019; Krijger *et al.*, 2017). Several volatile compounds have been identified from the odor of feces, urine and anal glands of mustelids (Ferrero *et al.*, 2011; Zhang *et al.*, 2005; Crump & Moors, 1985), and feces and urine from the red fox (Vernet-Maury, 1980). For example mustelid derived compounds such as 2-propylthietane; 2,2-dimethylthietane and indole were tested as synthetic mixtures for rodent repellency in a series of field studies by Thomas Sullivan (Sullivan *et al.*, 1988b; Sullivan *et al.*, 1988a; Sullivan *et al.*, 1988c) or in laboratory experiments as single compounds (Sievert & Laska, 2016; Pérez-Gómez *et al.*, 2015; Brechbühl *et al.*, 2013; Sarrafchi *et al.*, 2013a). Another volatile compound (2-phenylethylamine) identified as a major component of urine in several predators such as bobcats, ferrets, weasels, successfully triggered defensive responses in rodents such as avoidance (Ferrero *et al.*, 2011). Moreover, a compound (2,5-dihydro-2,4,5-trimethylthiazole) derived from the red fox has been widely studied (Rosen *et al.*, 2015; Fendt *et al.*, 2005) showing different fear related behaviors in rodents (Fendt & Endres, 2008; Laska *et al.*, 2005).

However, results of both field and laboratory experiments are rather inconsistent (Apfelbach et al., 2005) and there is still conflicting knowledge regarding of whether single compounds or complex mixtures ("bouquets") of compounds are more optimal to elicit fear responses in rodents (Jackson et al., 2018; Sievert & Laska, 2016; Apfelbach et al., 2015a). Furthermore, only two studies have addressed the use of predators smell to avoid consumption or removal of acorns by rodents (Gallego et al., 2017; Sunyer et al., 2013b). Volatile compounds, however, where not used in these studies. Therefore, there is a great potential in the application of predators smell to

reduce acorn removal during direct seeding operations and to place more research focus to this field.

2. Thesis aims

The overall aim of the present work was to investigate to which extent predator odors can be used as a seed protection strategy against granivorous rodents and to determine their efficiency to protect acorns for the restoration of oak during direct seeding. In this thesis we established laboratory experiments and applied these results to 'real world' conditions in field experiments. While the first two studies investigate natural based treatments, the two last are focusing on synthetic volatile compounds.

In the first part, we investigated in the laboratory the effects of four different 'natural based' seed protection treatments (chili, citronella, mink excrement and sand coating) against bank vole consumption of acorns and beech nuts (Paper I). Based on these results we applied mink excrement as a treatment to protect acorns in two restoration sites during direct seeding of oak (Paper II).

In the second part of this thesis (Paper III and IV), we examined the potential effects of synthetic volatile compounds as repellent candidates from different mammalian predators in order to disrupt foraging or trigger fear related behaviors in granivorous rodents. In paper III, we established a laboratory experiment and determined the most efficient compounds and concentrations to repel bank voles. In paper IV, we implemented the best synthetic repellent candidates from the laboratory study and performed a field experiment to test if the effects of the laboratory setting could also be observed under more natural conditions.

The specific objectives for this thesis were to:

    I. Determine the potential protection effects of four seed protection treatments (chili, citronella, mink excrement and sand coating) on bank vole consumption of beech nuts and acorns.

II. Elucidate the effects of distance to forest edge, mink excrement, and acorn size on the removal of acorns by rodents.
III. Asses the efficiency and proper concentration of five synthetic volatile compounds from different rodent predators as repellent candidates against bank voles.
IV. Determine if synthetic volatile compounds can reduce acorn removal rates by rodents and if the removal rates are depending on the distance to the odor source.

3. Methods

To address the specific objectives of this thesis, laboratory and field experiments were conducted including the study of natural based treatments (Paper I and II) and synthetic volatile compounds (Paper III and IV). In this section, the applied methods are briefly summarized. More detailed information can be found in each individual paper.

3.1 Natural-based seed protection treatments

Within this work we considered secondary plant metabolites and animal by-products as our natural-based seed protection treatments. Here, we selected: chili (*Capsicum chinense* Jacq) with coconut fat as a carrier, citronella (*Cymbopogon winterianus* Jowitt) with rapeseed oil as a carrier, mink excrement diluted in water, and sand coating (mixture of potato starch and sand). Our rationals for this choice were based on accessibility of materials, feasible application on seeds, and previous knowledge on their potential repellent and seed protection effects (Sunyer *et al.*, 2013a; Nordlander *et al.*, 2009; Biswas & Biswas, 2006; Nolte & Barnett, 2000).

3.1.1 No-choice laboratory study (Paper I)

No-choice feeding experiments were performed at the Astrid Fagraeus Laboratory, Solna, Sweden to test if the natural-based seed protection treatments can hinder bank voles to consume acorns (*Q. robur*) and beech nuts (*Fagus sylvatica* L.). Both seed types were prepared with chili/ coconut fat, citronella/ rapeseed oil, mink excrement or sand coating. As control treatments, seeds were only submerged in water.

The no-choice experiment was performed in the standard cages, which are similar to the home cages of the bank voles (dimensions: 60 x 30 x 40

cm^3). Bedding materials and water was provided *ad libitum* to the animals tested. Either 10 treated beech nuts or 5 treated acorns were placed at the opposite side of the cage entrance. Each bioassay lasted 7 hours and at the end of each bioassay the amount of consumed seeds was measured in order to compare the five treatments. Bioassays were conducted in two sessions on five consecutive days. Thus, each animal experienced one treatment per day arranged in a series of 5 × 5 Latin Squares. We used 20 different bank voles for each combination of treatment and seed species resulting in a total of 40 animals.

Both beech nuts and acorns were weighed before and after each trial. To avoid bias due to water loss or uptake by the seeds, seed samples were placed in open jars in the same room as the trials and weighed before and after. Furthermore, we recorded at the end of experiments if seeds were handled or let untouched in each cage.

For each treatment and seed species combination a sample of 200 seeds (total: 2,000 seeds) were used to test if the treatment affects the germination capacity. These germination tests were carried out at the Statsskovenes Planteavlsstation nursery, in Humlebæk, Denmark and lasted 14 weeks.

3.1.2   Direct seeding field study (Paper II)

To investigate if mink excrement has an effect on acorn removal we conducted a field experiment at two forest clear-cuts (Skrylle and Klåveröd) in Scania (southern Sweden) between May and September 2019. Both study sites were a stand dominated by Norway spruce before felling in December 2017 for Skrylle and June 2018 for Klåveröd. These two clear-cuts were patch scarified (ca. 80 cm wide and 3 m long in tracks at ca. 2 m spacing) before spring in 2019 and were direct seeded with pedunculate oak by the Scania Landscape Foundation in late April, whereas no direct seeding was applied in our experimental areas.

At both clear-cuts we randomly selected four blocks with split-split plots (Figure 1a). This consisted of two main treatments (habitats), which were: open area inside the clear-cut and an area near the forest edge. The distance between the two main treatments was between 20-24 m. At each habitat, three sub-plots (1 x 2 m) contained respectively: (i) mink excrement directly applied on top of the sown acorn (ME), (ii) seed coating with mink excrement (MEC), and (iii) control acorns without mink excrement (C) as sub-treatments. The distance between sub-plots was 5 m. Each sub-plot was
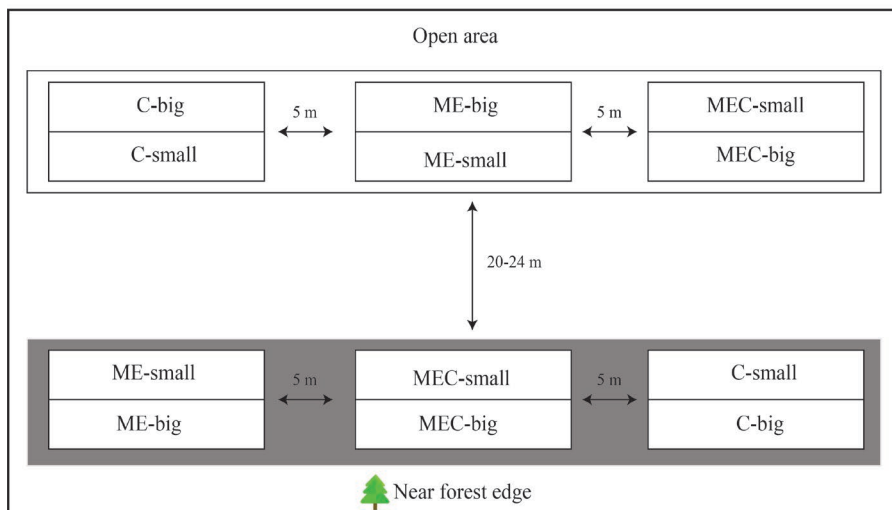
further divided in two rows (split-split plot) for direct seeding of two different sized acorns (small: < 4.8 g, and big: ≥ 4.8 g fresh weight).

Ten acorns were sown manually at 5 cm depth for each split-split plot (960 acorns in total). To detect the presence of acorns after sowing a small hole was drilled (ø 0.8 mm) at the basal end opposite the embryo side of each acorn, and a flat metal washer (ø 15 mm) was attached using 5 cm of wire thread (Figure 1b). Thereafter, with the use of a pin-point metal detector we monitored presence or absence of acorns after 15, 30, 60, 90 and 120 days of sowing. We also recorded emergence of seedlings (Figure 1c) during each monitoring visit. At the end of the experiment, seedling height was measured, and the percentage of ground vegetation was estimated visually for each main plot.

3.2   Synthetic volatile compounds

We selected the following synthetic volatile compounds: (i) 2-phenylethylamine (2-PEA, ≥ 99% purity, Sigma-Aldrich, Darmstadt, Germany) a general compound of carnivore's smell (Ferrero *et al.*, 2011), (ii) 2-propylthietane (2-PT, ≥ 95% purity, Chemspace, Riga, Latvia) from anal gland secretions of mustelids (Crump, 1980), (iii) indole (≥ 99% purity, Sigma Aldrich, Darmstadt, Germany) from anal gland secretion of mustelids (Brinck *et al.*, 1983), and (iv) 2,5-dihydro-2,4,5-trimethylthiazoline (2,5-TMT, ≥ 97% purity, Bio SRQ, Sarasota, Florida, USA) form fox urine and feces (Vernet-Maury, 1980). In addition, we used heptanal (≥ 95% purity, Sigma Aldrich, Darmstadt, Germany), which was identified has the most pronounced compound form mink fur (for more details please see supplementary material in Paper III). This selection was based on previous studies (Ferrero *et al.*, 2011; Apfelbach *et al.*, 2005; Lindgren *et al.*, 1995; Sullivan *et al.*, 1988a) and compound stock availability by different chemical vendors. In the field study (Paper IV), only 2-PEA, 2-PT and indole were used.

a)

b)

c)

Figure 1. a) Experimental design for one block with split-split plots. Main plot treatments were the open area of the clear cut and the area near the forest edge (gray rectangle). Subplots consisted of the sub treatments ME (mink excrement), MEC (Mink excrement coating) and C (control). Each split-split plot contained one row of 10 *small* acorns and another row with 10 *big* acorns. b) Acorns of pedunculated oak with attached metal washer used during direct seeding. c) Seedlings of oak three months after sowing (photos: Adrian Villalobos).

3.2.1 Two-choice laboratory study (Paper III)

In a two-choice design we evaluated three fear/defense related behaviors in bank voles at the presence of five synthetic volatile compounds (2-PEA, 2-PT, indole, heptanal, and TMT) at two concentrations (1% and 5% (w/w) diluted with pentane (*puriss p.a.* $\geq$ 99% purity, Sigma Aldrich, , Darmstadt, Germany). Experiments were carried out at the Institute of Environmetal Sciences (Jagiellonian University, Krakow, Poland). In total 33 female and 33 male laboratory-bred bank voles were randomly selected from a control line colony (Sadowska *et al.*, 2008). For the two-choice design we used three Y-mazes (Figure 2a) placed simultaneously in the experimental room. Each Y-maze consisted of three arms: One arm was selected as a "shelter" zone. The two remaining arms were randomly selected as treatment arm where clean air (control arm) or air mixed with the volatile compound (compound arm) was puffed inside the Y-maze. At the distal end of the treatment arms, rodent food was placed *ad libidum.* An air inlet connected with polytetraflouroethylene (PTFE teflon®) tubing distributed the odors inside the arms (Figure 2a). To avoid the odor plumes entering the shelter zone, two air outlets at 25 cm from the arm ends were connected with PTFE tubing to a reversed air pump (Figure 2a: red arrows). During 1 hour, odors were air puffed at every 5 min for 1 min (total 10 air puffs/h). Before the experiment, each bank vole was acclimatized to the experimental set-up for 10 hours. Furthermore, animals experienced just one single compound at one concentration. In total, 6 behavioral trials per diluted compound were performed. All experiments were carried out during the beginning of the artificial night cycle of the animals and were video recorded.

At each air puff, we examine the following fear related behaviors: (i) *Food contact* was scored as a binary response. If the animals poked their noses into the food cage (Figure 2a) for more than 5 consecutive seconds it was noted as 1, otherwise as 0. (ii) To estimate the *Area avoidance*, we defined a 10 cm virtual detection zone from the food cage to the center of the Y-maze at each treatment arm (Figure 2a) and measured the cumulative time spend in each detection zone. (iii) *Move to shelter*, was recorded binary and if the bank voles returned to the shelter and stayed inside for more than 10 consecutive seconds it was noted as 1, otherwise as 0.

3.2.2   Effects of synthetic compounds on acorn removal (Paper IV)

The experiment was carried out at two broadleaved forest stands in southern Sweden (Alnarp and Skrylle). At each forest site we examined the efficiency of three synthetic compounds (2-PEA 5%, 2-PT 1%, and indole 5% (w/w)) in reducing acorn removal by granivorous rodents. In both sites we conducted a randomized block design with four blocks (ca 45 x 45 m each) and five plot treatments in each block. The plot treatments consisted of a circular metal cage with a diameter of 60 cm, a height of 30 cm, and a mesh size of 2.5 cm. At the center of the cage, a plastic pole holding a cartoon delta house was placed at ca. 5 cm above ground (Figure 2b). Inside the delta house 1 g of SPLAT (Specialized Pheromone Lure Application Technology, ISCA Technologies, Riverside, CA, USA) pellet was positioned. Each pellet of SPLAT functioned as a chemical dispenser, releasing the mixed in active ingredient. The five treatments were: 2-propylthietane + SPLAT (2-PT-S), 2-phenylethilamine + SPLAT (2-PEA-S), indole + SPLAT (I-S), control without active volatile compound + SPLAT (C-S) and control without SPLAT pellet and delta house (C). Inside the mesh cages 12 acorns of sessile oak were positioned in an arrangement of one acorn at 5, 10 and 15 cm from the odor source in four different directions (Figure 2b). In addition, three pieces of rodent food were placed aside of each acorn.

Following the start of each experimental period (4 pm), we visually determined for each plot treatment, and then after 4, 8, 12, 16, 24, 28, 32, 36, 40 and 48 hours, whether acorns and rodent food were removed. Experiments were replicated in two sessions at each site. For the second experimental session, we randomly changed the plot treatment position within the blocks, and new acorns and rodent food were placed inside the mesh cages.
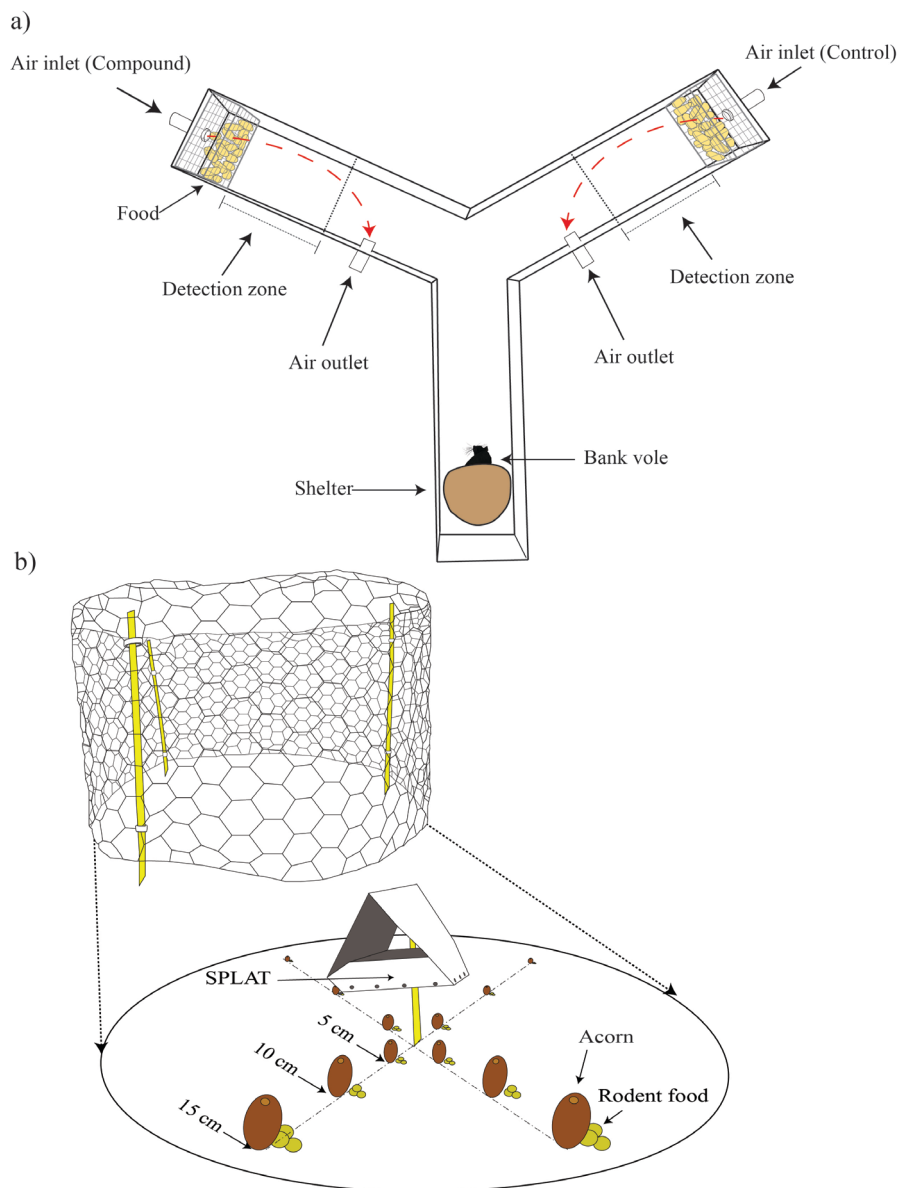
Figure 2. a) Design of the Y-maze (Paper III) with the following arm dimensions: L×W×H: 37.5 × 15 × 11 cm. At each treatment arm a mesh cage with food was provided at all times during the experiment. Doted lines at 10 cm from the food cages indicate the detection zone. Dashed red arrows show the odor plumes direction. A coconut half-shell was used as a shelter. b) Design of the wire mesh treatment cage and the acorn-rodent food arrangement from Paper IV. A treated pellet SPLAT was placed inside the delta house at the center of the mesh cage.

4. Main results and discussion

4.1 The seed protection effects of four natural-based products

The results from paper I show that the application of mink excrement on both beech nuts and acorns was the most successful treatment as seed protection because it reduced the consumption of seeds by male and female bank voles (Figure 3a), reduced the handling of seeds by rodents about 50% (Figure 3b) and did not have major negative effects on seed germination (germinated beech nuts, mink excrement = 61%, control = 79%; germinated acorns, mink excrement = 70.5%, control = 73.5%). For the protection of seeds during direct seeding operations, it is highly important that the method applied does not only reduce consumption but also reduces catching and removal of seeds from restoration sites (Takahashi *et al.*, 2006). Moreover, treatments should not have negative effects on seed germination (Johnson *et al.*, 2019; Dey *et al.*, 2008). Hence, this combined result highlights the potential applicability of mink excrement as a seed protection method during direct seeding.

The repellent effects of several predator odors on different rodent species have been previously described (Apfelbach et al. 2005; Apfelbach et al. 2015 and references therein). The more natural-based application of this strategy is normally performed by placing predator excrement (Koivisto & Pusenius, 2003; Nolte *et al.*, 1994), spraying urine (Borowski, 1998; Epple *et al.*, 1993), placing pieces of bedding materials from the predators burrow (Sullivan *et al.*, 1985), or placing a fabric cloth which was previously in contact with the predator close to food (Masini *et al.*, 2005). Other studies showed a reduction of acorn removal by rodents when excrements of the common genet (*Genetta genetta* L.) were sprayed on acorns (Gallego *et al.*,

2017; Sunyer *et al.*, 2013a). However, the effect on the consumption of mink excrement treated seeds has – to the best of our knowledge – not formerly been documented; neither has the influence of any predator excrements on germination capacity been reported in the literature.
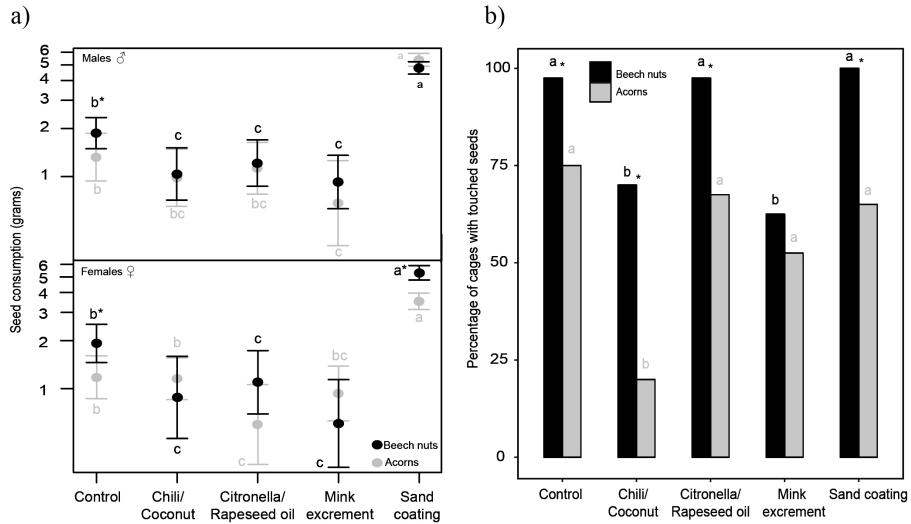

Figure 3. a) Consumption of beech nuts and acorns by male and female bank voles. Different letters represent significant differences between the treatments for beechnuts (black) and acorns (grey). Asterisks indicate significant differences between beech nuts and acorns. b) Percentage of cages with touched beech nuts and acorns. Letters indicate significant differences between the treatments. Asterisks indicate significant differences between seed species (Villalobos *et al.*, 2019).

Contrary to our results with mink excrement, the treatments with chili/coconut fat and citronella/rapeseed showed a clear trade-off between reducing bank vole consumption of seeds (Figure 3a) and also the germination capacity (germinated beech nuts, chili = 18%, citronella = 5.5%; germinated acorns = 51.5% for chili and citronella). In addition, the citronella treatment did not decrease the handling of seeds as showed by the chili/coconut treatment, thus depleting its repellent effect (Figure 3b). This results are in line with studies from Nolte and Barnett (2000), Jensen *et al.* (2003), and Willoughby *et al.* (2011) where repellent effects of the chili compound capsaicin were observed. Besides, Leverkus *et al.* (2013) found negative effects in germination of capsaicin treated acorns. Similarly, Biswas and Biswas (2006) described a reduced consumption of rice grains by

weevils when citronella was applied but with negative effects on germination rates.

Finally, our results show that sand coating as a physical barrier failed to protect seeds rather increased the consumption. One reason for this could be the material used for creating the coat, potato starch is palatable and nutritious to rodents. In summary, the treatments chili/coconut fat and citronella/rapeseed, and the sand coating method cannot be recommended for future applications.

4.2 Direct seeding of oak: The effects of distance to forest edge, acorn size and mink excrement

In our direct seeding experiment (paper II), we applied mink excrement as an acorn protection method based on our results from the previous laboratory experiment (paper I). We further observed the effects of sowing near or at far distance from the forest edge as well as of sowed small or big acorns. Our results revealed no seed protection effects for the two treatments with mink excrement (treatment MEC and ME), rather in most of the cases, removal of acorns increased under these treatments (Figure 4). This is in contrast to our laboratory results from paper I.

Different attempts have been made to use predator odors as natural repellents but dissimilar results between laboratory and field studies are common (Apfelbach et al., 2005). Particularly in our study, there may be several reasons for this contradiction. First, it is possible that the olfactory cues from the mink excrement dissipated quickly due to desiccation, despite having been buried in the soil (Bytheway et al., 2013). Second, we cannot discard that habituation to the predator smell by rodents has occurred. There are strong possibilities that after a long-lasting exposure to predator odor cues without real attacks from a predator, the prey animals habituate and do not alter their foraging behavior in response (Apfelbach et al. 2005). Above all, our results are in line with some other work where feces of the least weasel did not reduce foraging behavior in bank voles (Sundell et al., 2004), and neither did rabbits (*Oryctolagus cuniculus* L.) reduced foraging at the presence of mink excrement (Bakker et al., 2005).
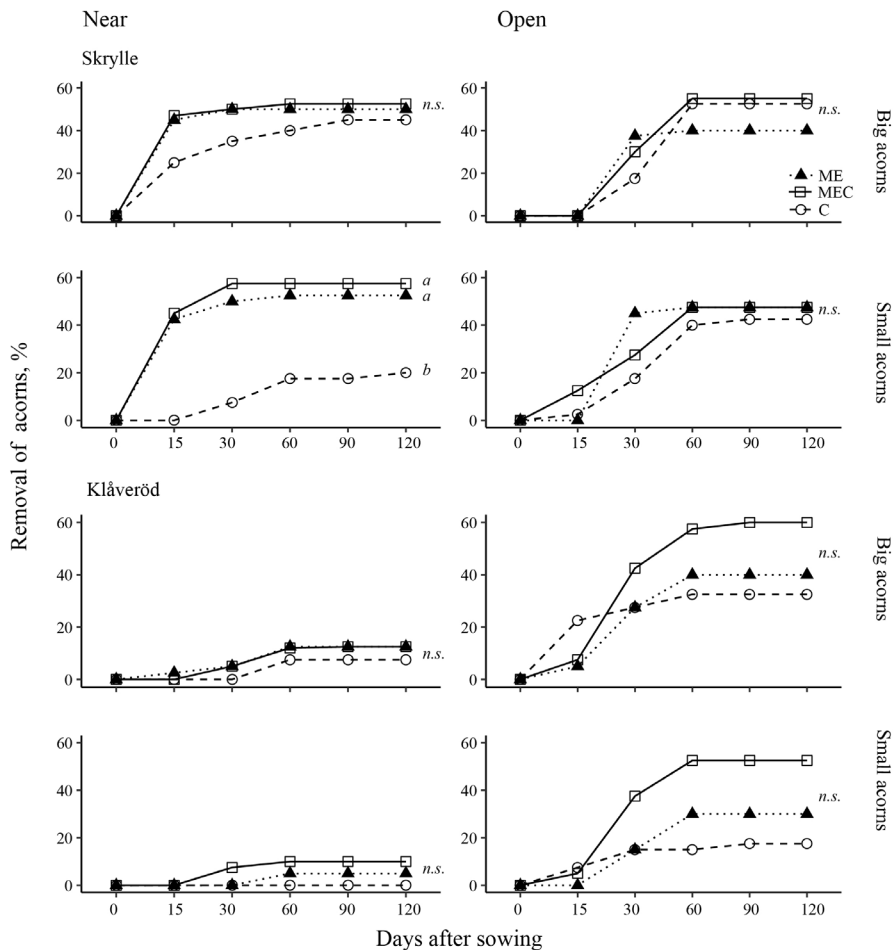
Figure 4. Removal of acorns from two acorn sizes (big and small) in two habitats (*Near* forest edge and *Open* cleat-cut), and at two clear-cut sites: Skrylle (top) and Klåveröd (bottom). The treatments are: ME = Mink excrement, MEC = Mink excrement coating and C = Control. Different letters show significant differences (*p* < 0.05) between the repellent treatments (paper II).

In general, at the end of the experiment 120 days after sowing no more than 60% of sown acorns were removed at both field sites (Figure 4). This is another contrasting result to several previous studies where nearly 100% of acorn removal has been reported during direct seeding (Martelletti *et al.*, 2018; Van Ginkel *et al.*, 2013; Birkedal *et al.*, 2009; Den Ouden *et al.*, 2005; Madsen & Löf, 2005; Schnurr *et al.*, 2002). However, our experiments were laid out simultaneously as a direct seeding operation by the Scania

Landscape Foundation outside our plots but at the same sites. Thus, more direct-seeded acorns were available. This result supports the predator-satiation hypothesis (Janzen, 1971), where it is expected that a higher availability of food resources leads to an increase of the probability of seeds escaping consumption.

The acorn removal rates were significantly higher in the open area compared to near the forest edge at one of our sites (Klåveröd, Figure 4). Although previous research showed higher seed removal by rodents at the edge of a deciduous forest (Kollmann & Buschor, 2003), our result can be explained by the presence of large post-harvest slash piles (Figure 5) close to our open area sub-plots. Indeed, findings of Birkedal *et al.* (2010) reported higher rodent captures at sowing sites closer to slash piles in a clear-cut. Thus, it is possible that slash piles are suitable rodent shelters, providing a safe zone without risk of predation. This is in line with the "landscape of fear" concept, where habitats with a low predation risk will be used more intensively than higher-risk habitats (Laundré *et al.*, 2010).

Figure 5. Presence of post-harvest slash piles at the clear-cut in Klåveröd. Acorn removal was higher near the slash piles as they may represent a suitable shelter for rodents (photo: Adrian Villalobos).

Overall, bigger acorns produced larger seedlings regardless of any treatment combination (habitat or mink excrement). But a larger acorn size did not lead to higher germination rate. Similar results were obtained by Xiao et al. (2004) on the jolcham oak (*Q. serrata* Murray). However, for northern red oaks (*Q. rubra* L.), there is evidence that bigger acorns increase germination rates and overall seedling performance during direct seeding (St-Denis et al., 2013; Kormanik et al., 1998). If a bigger acorn size is recommended for direct seeding, it shall be taken with caution as we observed higher removal rates for big acorns. Thus, a conflict between small and big acorns emerges because sowing big acorns may improve the early growth of the seedling but they are also preferred by rodents (Muñoz & Bonal, 2008; Kormanik et al., 1998).

4.3 Synthetic predator odors as potential seed protection strategies: from the lab to the field

To determine if synthetic predator odor compounds have the potential to be applied as a seed protection strategy we first developed a laboratory study (paper III) using previously known predator compounds which have had repellent effects against rodents from previous studies (see sections 1.3.2 and 3.2). Our first results show such effects on bank voles with the compounds 2-phenylethylamine (2-PEA), 2-propylthietane (2-PT) and more modest in indole. Thereafter, we brought these three compounds to the field (paper IV) and observed if similar results could be detected in a more natural environment. In the following sections, I will discuss our results in more detail.

4.3.1 Testing synthetic predator odors on bank voles

Strong avoidance effects of the bank voles were observed when the compound 2-PEA was applied in the Y-maze setting (paper III). Here bank voles spent more time in the control arm compared to the compound arm at both tested concentrations (Figure 6). The 2-PEA compound is a biogenic amine as a product from decarboxylation of the amino acid phenylalanine - a general component of urine from different carnivore mammals (Ferrero *et al.*, 2011). Ferrero *et al.* (2011) found that 2-PEA in a 10% concentration elicits innate avoidance behavior, increases the plasma corticosterone levels indicating stress, and activates multiple olfactory sensory neurons in rodents,

which support our results. Furthermore, we detected fewer contact to the food cages at the presence of 2-PEA, which may suggest feeding suppression effects, and more moves to shelter were also observed. However, the few studies which implemented 2-PEA to their trials do not have information regarding feeding suppression or escape behavior in rodents (Wernecke, 2016; Ferrero *et al.*, 2011). Thus, our results seem to be new for rodents.

The compound 2-PT at a 1% concentration (w/w) significantly reduced the time spend in the compound arm (Figure 6) and reduced the food contact by bank voles. This results are consistent with previous laboratory experiments highlighting that 2-PT treated areas were avoided by mice (Sievert & Laska, 2016; Pérez-Gómez et al., 2015; Brechbühl et al., 2013; Sarrafchi et al., 2013b), and consumption of food pellets by Long Evans rats (Rattus rattus domestica L.) was reduced (Heale & Vanderwolf, 1994). Moreover, a study from Sarrafchi et al. (2013b) found that mice can recognize and avoid 2-PT in concentrations as low as 1.3×10^{-9} mol/L. Hence, from our experiments we could expect that a 1% concentration of 2-PT is sufficient to elicit avoidance response in bank voles.

A clear avoidance effect was observed for indole at a 5% concentration (Figure 6). However, we did not find significant effects on food contact or moves to shelter. Previously, there are no studies regarding the implementation of indole as a single compound in rodents. Previous field studies have used indole in mixtures with sulfurous compounds (Sullivan et al., 1988a) or other nitrogenous compounds (Swihart et al., 1995). These mixtures showed different results. For example Sullivan et al. (1988a) found feeding suppression in meadow voles (Microtus pennsylvanicus Ord) and montane voles (Microtus montanus Peale), whereas Swihart et al. (1995) did not observe feeding reduction in meadow voles.

The fox related compound TMT and the mink fur compound heptanal did not show avoidance effects (Figure 6). Unexpectedly, voles were attracted to the compound TMT. This is in contrast to previous results where a clear avoidance behavior and feeding suppression have been observed in rats during two-choice laboratory experiments (Endres & Fendt, 2007; Burwash et al., 1998). Although, we do not have a clear explanation for the observed attraction to TMT by our bank voles, previous studies described different avoidance responses depending on the rat strain (Rosen et al., 2006; Staples & McGregor, 2006; McGregor et al., 2002). Thus, our results could further suggest that responses to TMT may differ depending on the rodent strain or

species. We found the compound heptanal as the most pronounced volatile compound from mink fur. Other studies have also described this compound in urine from ferrets (Mustela furo L.) (Zhang et al., 2005) and otter (Lutra lutra L.) spraint (Kean et al., 2015). However, heptanal was also found in products of non-predator mammals such as cattle fur (Isberg et al., 2016) and different organic materials such as plant flowers (Deisig et al., 2012). Thus, this compound seems to be a more general compound in origin and alone does may not denote the presence of a predator to rodents.
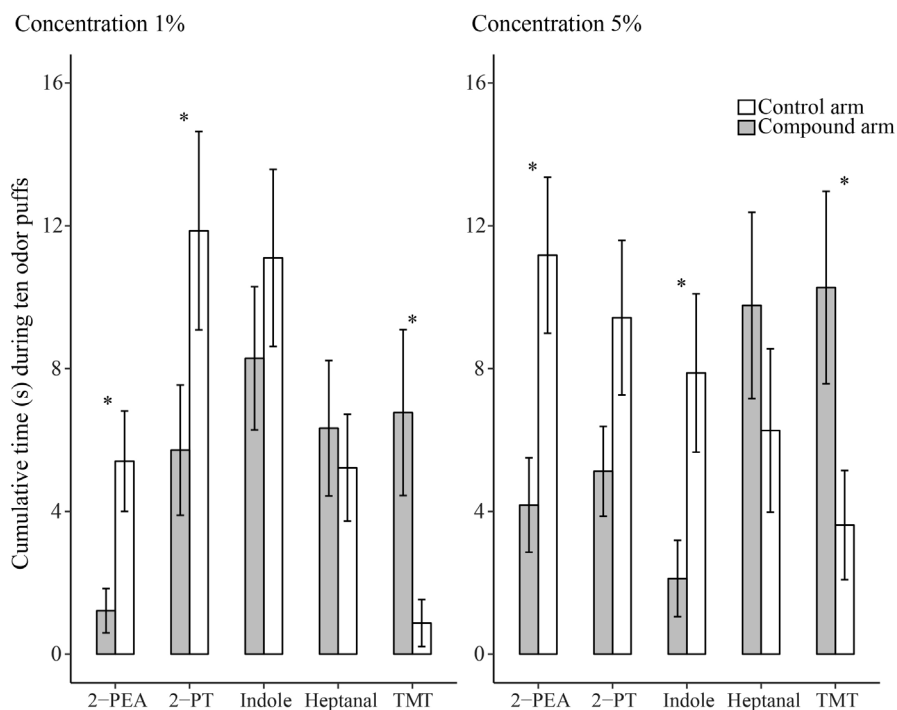

Figure 6. Cumulative time spend by bank voles in the Y-maze detection zone at the control arm (white) and the compound arm (gray) during 10 air puffs. Asterisks indicate significant difference between the arms of the y-maze. Error bars represent the standard error of the cumulative time spend by bank voles (paper III).

4.3.2   Reducing removal of acorns with synthetic predators' odor

Our field experiment from paper IV revealed a significant reduction of acorn removal by rodents when 2-PT was used at field sites (Figure 7). This result further support our observations in laboratory bioassays from paper III. To our knowledge, this is the first time 2-PT has been applied as a single

compound to control the removal of any type of seeds. Moreover, only few experiments have tested the effect of 2-PT to reduce foraging in rodents during field settings. For example, a reduction of bark and vascular tissue feeding on apple trees by meadow voles has been observed (Sullivan *et al.*, 1988a), and stem damage to Scots pine by the red-backed vole (*Myodes gapperi* Vigors) were significantly reduced in China (Sullivan *et al.*, 1991). However and contrary to our study, 2-PT has then only been implemented as a 1:1 mixture with 3-propyl-1,2-dithiolane in these studies. Furthermore, we observed the lowest rate of acorn removal (between 25% and 45%) at the close distance from the odor source (5 cm) in the 2-PT treatments. For the longer distance (15 cm), between 65% and 80% of the acorns were removed. In contrast, a semi-field study by Sundell *et al.* (2004) showed that when mustelid excrement was placed at two different distances (1 m and 3 m) from food, the closest distance did not reduced foraging on rodents. Though, a distance of 1 m might be too far and therefore our results may suggest that distances farther than 5 cm could reduce the intensity of the odor signal. Indeed, Gire *et al.* (2016) states that under natural conditions the encounter rate of strands of an odor often decreases with distance from the odor source due to forces of turbulent diffusion and shifting winds (Cardé & Willis, 2008) and this might be particularly relevant in forests.

Contrary, to our laboratory study from paper III, we did not find the expected repellent effects for the compound 2-PEA as acorns were greatly removed at one of our field sites (Figure 7). This is also in contrast to the strong avoidance behavior observed in rats and mice in a previous study (Ferrero *et al.*, 2011). One possible explanation for our result could be that the concentration of 5% (w/w) 2-PEA used into the dispenser matrix (SPLAT) was not appropriate for field studies. In a laboratory experiment by Wernecke (2016) rats were avoided by the compound 2-PEA only at lower concentrations (0.04, 0.4; and 4 µmol/mL). Though, their results were not verified under field conditions.

The compound indole did not reduce the removal of acorns at our two field sites (Figure 7). During semi-field conditions, findings from Arnould *et al.* (1998) found that domestic sheep (*Ovis aries* L.) did not avoid indole as a single compound. Though, no studies from rodents can be found in the literature when indole is applied as single compound. Moreover, our field results on indole are also in contrast to the avoidance effect observed from paper III. One possibility for this dissimilar results can be that for certain

compounds, laboratory-bred and wild type rodents could differ in their modes of action. Animals under more natural conditions have had previous experiences with a wider variate of odorants cues released by different sources (e.g. predators, conspecifics, plants) (c.f. Conover, 2007). Compounds such as indole are found in different organisms from mustelid (Crump & Moors, 1985; Brinck *et al.*, 1983), invertebrates (Tomberlin *et al.*, 2017) to different plants (Bischoff *et al.*, 2015). It is therefore possible that during field conditions rodents cannot assess this compound as predator scent.

The experimental plots in the forested areas of Alnarp were surrounded by a mixture of open areas, orchards and crop fields, which might show a richer habitat with more rodents compared to the site of Skrylle (formed by a more ordinary mixed forest dominated by Norway spruce managed for timber production). This may explain the higher trend on acorn removal in Alnarp compared to Skrylle (Figure 7).
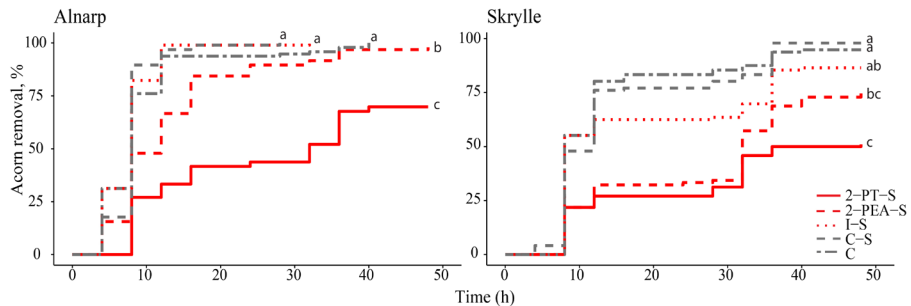

Figure 7. Percentage of acorn removal over time between the three compound treatments (red lines) and the two controls (gray lines). Treatments are: 2-PT-S = 2-propylthietane in SPLAT, 2-PEA-S = 2-phenylethylamine in SPLAT, I-S = indole in SPLAT, C-S = SPLAT dollop without compound, C = control treatment without SPLAT nor delta house. Figure on the left shows results from Alnarp and to the right results from Skrylle. Data from the three distances (sub-treatments) and from two sessions are polled in the figure. All curves have censored data. Different letters represent significant differences (paper IV).

5. Conclusions and future perspectives

In this thesis I provide insights for the potential use of predator odors as a tool for future improved forest restoration using direct seeding. I could also demonstrate the importance of determining potential synergies between controlled laboratory settings and close to the real world field studies. This is particularly important in areas such as animal behavior or when strategies are to be recommended for future applications in forestry and agriculture. However, the present work also shows the complexities of how prey animals react to predator odors under different environments.

The main results from paper I (laboratory study) and paper II (field study) show indeed different results. In paper I we concluded that mink excrement could be used as a potential seed protection strategy. Contrary, our results from paper II did not show the expected results and instead a higher acorn removal was observed under the treatment with mink excrements. Although, inconsistent results between laboratory and field studies regarding the behavioral responses of pray animals to predator odors are difficult to explain (Apfelbach et al., 2005), one explanation derived from our results could be that the mink excrements applied in the field desiccated fast and the olfactory cues volatized quickly (Bytheway et al., 2013). Furthermore, if the olfactory signal was lost but previously identified by rodents, it could have had a counter-productive effect, so that rodents learned where the acorns were buried. Therefore, future research efforts should focus on different application modes of excrements to ensure its efficiency over longer periods. Here one possibility could be the combination of different seed protection strategies. For example, physical barriers such as the seed shelter developed by Castro et al. (2015) in combination with mink excrement. Placing mink excrement inside the shelter could reduce the possibilities of excrement desiccation and may facilitate the use of biodegradable materials for the

construction of the seed shelter. Additionally, further research on the application of excrements from more prey specific predators such as the least weasel and the stoat may give significant results in the field. There is evidence of stronger aversive effects in rodents if odors are from sympatric predators with longer shared evolutionary history (Apfelbach et al., 2005).

Results from paper II further highlight the relevance of removing post-harvest slash piles from clear-cuts and controlling the vegetation in order to reduce suitable habitats for rodents. Moreover, selecting big acorns may improve the early seedling growth. But rodents prefer big acorns, implying a conflict between acorn size and removal rates. In addition, our overall results from paper II suggest a food satiation effect (Janzen, 1971) when an additional supply of acorns is available in a clear cut. We, therefore, recommend future studies on the application of higher acorn densities or to satiate rodents with alternative food during direct seeding of oak.

The application of synthetic chemical strategies for seed protection is unexplored (Löf et al., 2019) and the few studies following the non-lethal chemical approach have been rather inconsistent in reducing acorn removal by rodents (Leverkus et al., 2017; Leverkus et al., 2013; Bäumler et al., 1990). Moreover, there are no studies regarding the application of single synthetic compounds to protect acorns. Therefore, the results from this thesis are new and show a potential for the application of the synthetic odor compound 2-propylthietane (2-PT) as a seed protection strategy. We found similar effects for this compound in both, the laboratory (paper III) and the field (paper IV) study. 2-PT triggered avoidance behavior in bank voles in the laboratory and reduced acorn removal by rodents in the field. This further supports the relevance of single compounds which may elucidate fear responses as strong as for complex odor mixtures (Jackson et al., 2018; Saraiva et al., 2016). Although, these results are encouraging, we need to address several limitations. First, both field and laboratory experiments were carried out under short periods compared to the time needed for an acorn to germinate and produce a seedling (circa. two months). Second, our field experiments only revealed protection of acorns at a close distance to the predator odor source (5 cm). Therefore, further studies should focus on the application of 2-PT for longer periods. Moreover, more research is needed to optimize the release rates of compounds together with the use of chemical dispensers such as SPLAT. Lastly, different methods to increase the effective distance from the odor source should be determined. In this way, we may in

the future develop cost-efficient strategies for restoration and direct seeding of oak. Finally, my results further contribute to the understanding of the complex behavioral patterns involving the role of olfaction in the prey-predator dynamics and revived questions to be addressed in future studies.

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Acorn removal by small rodents is one of the major causes of restoration failures using direct seeding of oak. New unexplored strategies for acorn protection are based on prey-predator interactions and rodent's sense of smell. In this thesis, such new potential methods are investigated based on the application of natural-based- and synthetic predator odors.

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