Thorbjörn Sievert

# Indirect and Transgenerational Effects of Predation Risk

## Predator Odour and Alarm Pheromones in the Bank Vole



UNIVERSITY OF JYVÄSKYLÄ

FACULTY OF MATHEMATICS
AND SCIENCE

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## Predator Odour and Alarm Pheromones in the Bank Vole

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# ABSTRACT

Predator-prey interactions are a major evolutionary driver, affecting not only the direct mortality of prey species, but also their behaviours and reproduction. Prey species behavioural adaptations aim to mitigate the effects of predation and to maximise survival and individual fitness. These adaptations include the ability to signal a threat to conspecifics, e.g. via alarm calls or alarm secretions, or to detect predator presence via odours. In this thesis, I studied the effects of predator odours and conspecific alarm secretions on behaviour and reproduction bank voles (*Myodes glareolus*), a small mammal species inhabiting boreal forests. My work focused on three major points in comparing the direct predator cue and indirect conspecific cue: first, how the reproductive behaviour is affected by the predator odour or alarm pheromone, second, whether there are transgenerational effects and how they are exhibited in offspring, and third, what the chemical nature of these alarm secretions is. I conducted four experiments, which included both trials in semi-natural enclosures and under controlled laboratory conditions. I found evidence that exposure to conspecific alarm secretions causes a shift in voles' reproductive behaviour, switching towards terminal investment. This became apparent with an increase in parturitions and an increased growth rate in larger litters, which did not occur when exposed to predator odour. I also found evidence of transgenerational effects, which affect aspects of the offspring's exploratory and foraging behaviour. Additionally, I discovered that these behavioural effects are context-dependent and do not occur in every environment. Lastly, I identified a group of chemicals from voles' alarm secretion, which are likely to be responsible for the observed effects. The results of my thesis fill a knowledge gap concerning chemical communication in mammals, and help to further understand the implications of predator presence on prey behaviour and reproduction.

Keywords: Behaviour; chemical communication; cross-generational effect; fear effect; predator-prey-interaction; terminal investment.

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# TIIVISTELMÄ

Pedon ja saaliin evolutiivinen kilpajuoksu ilmenee kahtaalla, saaliin kuollei-
suutena sekä selvinneen saaliin käyttäytymisen ja lisääntymisen muutoksina, jotka
parantavat sen hengissä säilymistä ja kelpoisuutta. Saalis pystyy välttämään
pedon, jos se havaitsee sen. Saaliseläin voi aistia pedon suoraan sen jättämistä
ärsykkeistä, kuten äänistä tai hajuista, tai epäsuorasti varoituksena muilta oman
lajin yksilöiltä. Väitöstutkimukseni keskittyi petoriskisignaalien, pedon hajun ja
petoriskille altistuneen oman lajin yksilön hajun, vaikutuksiin saaliin käyttäyty-
miseen ja lisääntymiseen. Tutkimuslajini olivat maamme metsien yleinen jyrsijä,
metsämyyrä, ja sen merkittävä spesialistipeto, lumikko. Tutkimukseni neljä osa-
tutkimusta keskittyivät kolmeen pääkysymykseen: miten suora pedon haju tai
epäsuora, lajitoverin kautta tuleva hälytyshaju vaikuttaa metsämyyrän lisään-
tymiseen; onko vanhempien, etenkin äidin, altistumisella korkeaan petoriskiin
vaikutuksia raskausajan tai imetyksen kautta poikasten pedonvälttämiskäyttäy-
tymiseen; sekä mitkä kemialliset yhdisteet olisivat hälytysferomonin välittämän
informaation taustalla. Metsämyyrän lisääntyminen ja käyttäytyminen muuttui-
vat suoran ja epäsuoran petoärsykkeen vaikutuksesta. Hälytysferomoni näytti
muuttavan myyrän lisääntymisstrategiaa siten, että naaras lisääntyi tehokkaam-
min. Tämä näkyi niin lisääntyvien yksilöiden määrässä kuin poikasten kasvu-
nopeudessakin, etenkin isoissa poikueissa. Ilmiö on yhteensopiva niin sanotun
"terminaalivaiheen investointi" -hypoteesin kanssa. Tutkimukseni vahvisti myös
mahdollisten sukupolvien välisten petoriskivaikutusten olemassaolon. Lisäksi
osoitin, että erityisesti kahdella yhdisteellä, 2- ja 1-oktanolilla, saattaisi olla mer-
kittävä rooli petoriskin kemiallisessa signaloinnissa, sekä todensin, että oletus-
temme mukaisesti hälytysferomonin vaikutus luonnossa oli lyhytaikainen.

Avainsanat: Hajuainekommunikaatio; hälytysferomoni; käyttäytyminen; lisäänty-
misstrategiat; peto-saalis –suhde; sukupolvien väliset vaikutukset.

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# CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–IV.

I  Sievert T, Haapakoski M, Palme R, Voipio H, Ylönen H 2019. Secondhand horror: effects of direct and indirect predator cues on behavior and reproduction of the bank vole. *Ecosphere* 10:e02765.
https://doi.org/10.1002/ecs2.2765

II  Sievert T, Kerkhoven A, Haapakoski M, Matson KD, Ylönen O, Ylönen H 2020. In utero behavioral imprinting to predation risk in pups of the bank vole. *Behavioural Ecology and Sociobiology* 74:13.
https://doi.org/10.1007/s00265-019-2791-8

III  Sievert T, Bouma K, Haapakoski M, Matson KD, Ylönen H 2020. Pre- and postnatal fear shape offspring anti-predatory behaviour in the Bank Vole. Submitted manuscript.

IV  Sievert T, Ylönen H, Blande J, Saunier A, van der Hulst D, Ylönen O, Haapakoski, M 2020. Mammalian alarm pheromone chemistry and effects in the field. Manuscript.

The table shows the contributions to the original papers.

| Chapter | I | II | III | IV |
|---|---|---|---|---|
| Original idea | HY, MH, TS | MH, TS, HY | TS, HY, MH | TS, MH, HY, JB |
| Data collection | TS, HV | TS, AK, OY | TS, KB | TS, OY, DH |
| Analyses | TS, HV, RP | TS, AK | TS, KB | TS, AS |
| Writing | TS, HY, MH | TS, HY, MH, KM, AK | TS, HY, MH, KM, KB | TS, MH, HY, AS |

TS = Thorbjörn Sievert, MH = Marko Haapakoski, HY = Hannu Ylönen, HV = Helinä Voipio, RP = Rupert Palme, AK = Arjane Kerkhoven, OY = Olga Ylönen, KM = Kevin Matson, KB = Kerstin Bouma, JB = James Blande, DH = Dave van der Hulst, AS = Amélie Saunier

# 1  INTRODUCTION

## 1.1  The importance of predator-prey interactions

Research on the interplay between predators and their prey has intrigued biologists for decades, leading to a constant refinement of models and theories about population dynamics and evolutionary adaptations throughout the 20th century (Berryman 1992). Earlier work focused on direct mortality, i.e. prey animals being consumed by predators and how this shapes population dynamics and fluctuations (Paine 1966, Taylor 1984b, Krebs *et al.* 1995). A secondary focus has been how and to what extent the evolutionary arms race, i.e. the coevolution of prey to escape and the predators means to increase successful hunts, is a major driver of both predator and prey evolution (Abrams 1986, 2000, Yoshida *et al.* 2003). However, in the last decades, a stronger focus was put on predation-risks effects, as opposed to mere survival, e.g. reduced fitness or reproduction in prey after an increase in perceived predation risk (Taylor 1984a, Lima 1998, Creel and Christianson 2008). The research on these indirect effects became an intensely studied topic in the last decades, leading to a variety of catchy names such as "cost of fear" (Stankowich and Blumstein 2005) or "ecology of fear" (Brown 1999). While direct consumption by a predator seems like the major effect at first glance, experiments have shown that predation-risk effects can have similar effects on prey survival and fitness (Schmitz *et al.* 1997, Nelson *et al.* 2004, Pangle *et al.* 2007).

## 1.2  Detecting predator presence

It is essential for a prey species to reliably detect the presence of a predator, as a wrong choice might be fatal. A failure to recognize a predator can lead to the individual's deaths but interpreting an unrelated cue as an increase in predation risk can affect e.g. foraging and long-term fitness. Predator cues can be categorized into two main groups, "direct" and "indirect". However, depending

on the study, it varies what these terms refer to exactly. As an example, some studies refer to the environment, e.g. availability of cover or moonlight, as an indirect effect (Orrock *et al.* 2004), while others refer to conspecific cues (Barrera *et al.* 2011). In order to avoid confusion, I will use a definition based on the origin of the cue: direct cues originate from the predator, while indirect cues originate from the prey, as a reaction to a direct cue.

Several studies have concentrated on how reliable different cues are. There is no consensus, as the reliability seems to vary between systems and research questions. The main points of disagreement are how reliable chemical cues are (Bourdeau 2010, Barrera *et al.* 2011, Parsons *et al.* 2018), and the reliability of direct compared to indirect cues (Hare and Atkins 2001, Orrock *et al.* 2004, Morrison 2011, Barrera *et al.* 2011). The latter is complicated further by the aforementioned differing definitions.

Prey species can employ all their senses to detect these cues, and for most senses both direct and indirect cues have been studied. A direct and very reliable cue of predation risk is a tactile cue, i.e. a direct attack by a predator. Depending on the predator-prey pairing, the prey's survival chances can increase with prior information about the risk (Mirza and Chivers 2001, Chivers *et al.* 2002), being distasteful (Halpin and Rowe 2016), or by signalling its own fitness, e.g. by loudly singing during a pursuit (Cresswell 1994). Visual cues can either be direct, i.e. seeing the approaching predator, or indirect. Indirect cues include the flight response of conspecifics (Lima 1994, Wong *et al.* 2005) or more aggressive responses such as mobbing (Dominey 1983, Graw and Manser 2007). Auditory cues include predator calls or noise while moving through the environment, both a direct cue (Morrison 2011), but also alarm calls of con- or heterospecifics, an indirect cue (Schmidt *et al.* 2008, Forti *et al.* 2017). Direct chemical cues can consist of the body odour, faeces, urine or marking secretions of predators (Apfelbach *et al.* 2005, Parsons *et al.* 2018). Indirect cues include an array of alarm secretions originating from the prey (von Frisch 1938, Verheggen *et al.* 2010), which will be further explained in the next section.

## 1.3  Adapting to increased risk of predation

### 1.3.1 Adaptations: an overview

Predation can be observed across all taxa. Due to its importance in shaping populations and driving evolutionary adaptations, a variety of antipredator defences can be found in prey (Freeland 1991, Caro 2005). Antipredator adaptations can be morphological, physiological or behavioural. These adaptations can be constitutive, e.g., quills in porcupines (Cho *et al.* 2012, Mori *et al.* 2014) or thorns in plants (Hanley *et al.* 2007), or inducible when predation risk is high, e.g., morphological changes in daphnia (Grant and Bayly 1981), growth and morphology in some tadpoles (Relyea 2004), and a wide range of behavioural adaptations (Sih 1992, Lima 1995, 2009, Bell 2004, Valeix *et al.* 2009). In

environments with consistently elevated risk levels or when the fitness costs are negligible, constitutive defences are favoured. The opposite applies for induced defences (Tollrian and Harvell 1999, Trussell and Nicklin 2002, Heil 2002, Riessen 2012). The cost of these investments can be in the form of negatively affected breeding (Mappes and Ylönen 1997, Fuelling and Halle 2004, Creel *et al.* 2007), chronically elevated stress levels (Sheriff *et al.* 2009), or decreased fitness in the individuals or their offspring (Pangle *et al.* 2007, Sheriff *et al.* 2009, Trebatická *et al.* 2012, Dudeck *et al.* 2018). In extreme conditions, prey can show chronic stress induced disorders, with similar consequences as post-traumatic stress disorders (Clinchy *et al.* 2013). For example, chronically elevated stress levels can negatively affect immunoresponses (Clinchy *et al.* 2013).

## 1.3.2 Signalling conspecifics

Prey can also signal to conspecifics the presence of a predator, often via alarm calls or chemical secretions (Wisenden *et al.* 2004, Collier *et al.* 2017). Alarm calls have been studied in a variety of vertebrates (Schmidt *et al.* 2008, MacLean and Bonter 2013, Collier *et al.* 2017, Barati and McDonald 2017). Their information content can vary from basic indication of predator presence (Blanchard *et al.* 1991), or indicating the level of urgency (Townsend *et al.* 2012) or basic category of the predator (Manser 2001, Slobodchikoff *et al.* 2009), to more detailed predator identification (Ouattara *et al.* 2009). Alarm calls can not only be used by conspecifics but also by eavesdropping heterospecifics (Fichtel 2004, Schmidt *et al.* 2008).

Indirect scent cues excreted by the prey, often called alarm secretion, alarm cue, or alarm pheromone, are a well-studied phenomenon, especially in invertebrates (von Frisch 1938, Bowers *et al.* 1972, Howe and Sheikh 1975, Heath and Landolt 1988). Among the first discovered alarm secretions, called "Schreckstoff" at the time, are those in fish (von Frisch 1938). Fish and other aquatic species differ in the way how alarm secretions are released from most terrestrial species, as they are not released via glands but rather are a result of tissue damage (Smith 2000, Wisenden 2000, Wisenden *et al.* 2001, Ferrari *et al.* 2010). While examples of alarm secretions in insects are abundant and well established (Crewe and Blum 1970, Bowers *et al.* 1972, Heath and Landolt 1988, Collins *et al.* 1989), examples in mammals are from the last few decades and heavily focused on laboratory rodents (Kiyokawa *et al.* 2004, Inagaki *et al.* 2009, 2014, Brechbühl *et al.* 2013), but see Gomes *et al.* (2013) with wild Cabrera's vole (Microtus cabrerae, Thomas 1906).

Behavioural responses to indirect conspecific alarm cues can differ drastically from those to direct predator cues. As an example, cat fur odour commonly elicits avoidance behaviour in rats (Dielenberg and McGregor 2001, McGregor *et al.* 2002), but there are several studies where alarm secretions do not elicits similar behaviour (Kiyokawa *et al.* 2006, 2013). A possible explanation is the difference in information or urgency transferred by each cue. While an alarm secretion does not convey the identity of a predator, a direct predator cue does (Kiyokawa *et al.* 2013). However, an avoidance response has been found in

Cabrera voles (Gomes *et al.* 2013). Other physiological and behavioural changes in response to conspecific alarm cues include changes in analgesic responses (Kavaliers *et al.* 2005), and changes in the reproductive strategy (Haapakoski *et al.* 2018).

Many assumptions arise surrounding the term 'pheromone' . For this work, I will use a very clear definition: Pheromones allow semiochemical communication between individuals of the same species, as opposed to allelochemicals, which facilitate communication between different species (Dicke and Sabelis 1988, Sbarbati and Osculati 2006). To determine whether the pheromones used in this work also possess allelochemical properties goes beyond the scope of the thesis. From here on, the term 'alarm pheromone' solely refers to this definition of intraspecific communication.

### 1.3.3 Behavioural adaptations

Behavioural adaptations in prey include simple mechanisms such as escape, freezing, avoidance or heightened vigilance (Wallace and Rosen 2000, Lung and Childress 2006, Wang and Zou 2017). More complex adaptations are also observable such as mobbing (Dominey 1983, Graw and Manser 2007) or adaptive changes in mating behvaviour (Sih 1994, Creel *et al.* 2007, Adamo and McKee 2017).

While reduced reproduction is often considered a cost, there is an ongoing debate about its adaptive value and effects on population dynamics (Ruxton and Lima 1997, Kokko and Ruxton 2000). Until recently, results concerning reproductive efforts under an increased predation risk have shown a reduced reproductive investment. This can been seen in a decrease of offspring fitness (Feng *et al.* 2015, Owen *et al.* 2018), reduced breeding (Ylönen 1989, Ylönen and Ronkainen 1994, Fuelling and Halle 2004, Haapakoski *et al.* 2012), or both (Sheriff *et al.* 2009). An alternative reproductive strategy is to maximize reproductive efforts when faced with a high-risk situation (henceforth terminal investment). Individuals following this strategy disregard individual fitness cost in order to increase or accelerate reproductive outputs. If any offspring reaches maturity, it compensates the parental loss of fitness or death (Kokko and Ranta 1996, Kokko and Ruxton 2000). Terminal investment has been shown to occur in both vertebrates and invertebrates, such as passerine birds and crickets under high predation risk (Mönkkönen *et al.* 2009, Adamo and McKee 2017). Furthermore, it can also be triggered by infections, for example in ants and sparrows (Bonneaud *et al.* 2004, Giehr *et al.* 2017).

### 1.3.4 Transgenerational effects

Generally, transgenerational effects manifest in offspring after birth, as a result of changes in the parents' environment during gestation and/or lactation. In the case of predator-prey interactions pups whose mothers experienced high predation risks, exhibit different development or antipredator behaviours compared to pups whose mothers experienced low risk. In practice, it could

mean that being chased or even attacked by a predator while gravid can cause differences in offspring development or behaviour. A possible mechanism for such transgenerational effects are maternal hormones, whose production can be altered by stressful events. Maternal hormones can exert an in utero influence on the physiology, behaviour, and life history traits of offspring (Caldji *et al.* 1998, Love and Williams 2008, Sheriff and Love 2013). This has been demonstrated in response to a wide variety of external stimuli or stressors, including foot-shocks (Archer 1973), impoverished environments (Dell and Rose 1987), varied food quality and social environments (Van Cann *et al.* 2019b, a), and scent cues (Champagne and Meaney 2006). Much less is known regarding the role of paternal factors. However, some studies discovered transgenerational effects solely mediated by paternal factors (Rodgers *et al.* 2013, Van Cann *et al.* 2019a).

A growing body of literature has been accumulated in recent years about transgenerational effects caused by direct and indirect predation cues. The transmission of maternal information to in utero offspring has only received more attention in the last decade. Early work in this field showed how direct exposure, for instance via injection into the amniotic sac, to a chemical cue shows later signs of imprinting or conditioning in offspring (Stickrod *et al.* 1982, Smotherman 1982a, b). Recent studies found altered learning behaviour in stickleback offspring (Roche *et al.* 2012, Feng *et al.* 2015), altered stress reaction in the offspring of C57BL/6 mice and Long Evans rats (St-Cyr and McGowan 2015, St-Cyr *et al.* 2017), or changed foraging strategies in Sprague Dawley rat offspring (Chaby *et al.* 2015).

## 1.4 The boreal model-system: Bank voles and weasels

Several decades' worth of research exist on bank voles (*Myodes glareolus*, Schreber 1780) and on the interaction between voles and the least weasel (*Mustela nivalis nivalis*, Linnaeus 1766).

The bank vole is one of the most common small rodents living in a variety of northern temperate and boreal European forest habitats west of the Urals (Stenseth 1985). The species is granivorous-omnivorous, with their diet consisting mainly of seeds and buds, but also of other plant materials or invertebrates (Hansson 1979, Eccard and Ylönen 2006). The gestation period of the bank vole is 19–20 days, and the weaning period is three weeks. Vole pups sexually mature at around 30 days of age. Litter size averages at five to six pups but ranges between 2 and 10. In Central Finland, where this work was conducted, bank voles breed three to five times per season, which lasts from May until September (Mappes *et al.* 1995a, Koivula *et al.* 2003). In the wild, bank voles can live up to two years, but due to high predation rates, estimated survival is one breeding season (Ostfeld 1985, Macdonald 2006). Bank voles in Fennoscandia have typically a three- to four-year population density cycle (Hanski *et al.* 1991, Hansen *et al.* 1999). While the importance of food limitation and maternal effects

has been shown, predation pressure is considered the strongest driver of these cycles (Norrdahl and Korpimäki 1995, Boonstra *et al.* 1998, Huitu *et al.* 2003).

Bank voles are preyed upon by a diverse predator assemblage, including least weasels and stoats (*Mustela ermine*, Linnaeus 1758) (Ylönen 1989, Meri *et al.* 2008). The least weasel is an especially effective vole hunter due to their size and excellent hunting skills, least weasels are likely able to kill bank voles whenever the two species come into direct contact (Tidhar *et al.* 2007, Haapakoski *et al.* 2013). Due to their role as specialist predator, the least weasel is considered the main reason of boreal vole mortality (Korpimäki *et al.* 1991, Norrdahl and Korpimäki 1995, 2000). The capabilities of bank voles to detect their mustelid predators is well established, and voles show a range of behavioural adaptations. Bank voles shift activity patterns and spatial use in the vicinity of weasels (Jędrzejewska and Jędrzejewski 1990, Jędrzejewski and Jędrzejewska 1990, Sundell *et al.* 2008), decrease their movement and foraging efforts (Ylönen 1989, Sundell and Ylönen 2004, Bleicher *et al.* 2018), and use arboreal escape routes when chased by a weasel (Jędrzejewska and Jędrzejewski 1990, Mäkeläinen *et al.* 2014).

## 1.5   Aim of the thesis

The aim of this thesis is to examine the effects of the recently discovered indirect, conspecific alarm pheromones in bank voles compared to direct predator odour cues. With a combination of enclosure and laboratory experiments, I hope to solidify the idea that alarm pheromones in mammals are an important part of the complex interplay that predator-prey interactions represent. As of today, there are only a few studies on this subject. While a meta-analysis in search of common features in vertebrate body odour called for a wider range of study animals (Apps *et al.* 2015), the majority of new studies is focused on traditional laboratory animals. In four chapters, I explore the pheromone's effects on reproduction (I), its effect on growth and behaviour of offspring (II, III and IV), the importance of timing when the parents are exposed to high predation risk (III), and lastly an isolation and identification of the alarm pheromone (IV).

Chapter I lays the foundation of the present work by verifying the effects of alarm pheromones in voles, which have just recently been discovered (Haapakoski *et al.* 2018), and explores how the new results can be incorporated into existing findings. Chapters II and III explore different aspects of transgenerational effects with testing offspring in standardized laboratory environments to assess their exploratory and foraging decisions. After three chapters focusing on the behavioural aspects, chapter IV analyses the body odours of bank voles in order to identify alarm pheromones and provides additional insights on the effect of alarm pheromones compared to predator odour in the field (IV).

# 2 METHODS

## 2.1 Husbandry

All experiments were conducted at the Konnevesi Research Station in Central Finland (62° 37′ N, 26° 20′ E).

In the laboratory, voles were housed in husbandry rooms under a 16L:8D light regime with a constant temperature (22 °C ± 1 °C), males and females were maintained in the same room. All animals were kept individually in 42 cm × 26 cm × 15 cm transparent cages with wire mesh lids and an ad libitum water and food supply. The bedding materials in each cage consisted of wood shavings and hay. The breeding adults used in the study were the F1 generation of wild-caught individuals that were housed in the laboratory during the winter months preceding the study periods. Winter colonies are formed from the last cohort of voles of the previous summer. Thus, their age when paired for the first breeding is around 7 months. The winter population is housed on a short photoperiod (8L:16D) at around 17 °C throughout the winter and male voles' testes are abdominal and female vaginas are closed. Only after adjusting the photoperiod to long day in spring to prepare for breeding, our voles become reproductive again. This is done starting from February when the first voles start to breed also in the field (Haapakoski *et al.* 2012). All animals were individually marked with ear tags (#1005-1L1, National Band & Tag Company, Newport, KY, USA). In experiments where vole pups needed to be individually identified, toe markings were applied.

Weasels for the odour treatment were housed individually in 60 × 160 × 60 cm cages in an outdoor shelter. Each cage had a nest box, and wood shavings and hay as bedding. During the experiments, weasels were fed dead bank voles.

## 2.2 Field enclosures

Large (0.25 ha) outdoor enclosures are situated near Konnevesi municipality. The enclosures are made of galvanised steel sheet (125 cm height), reaching 50 cm underground. This prevents the escape of the experimental animals and intrusion of mammalian predators, while it allows avian predation. Twenty-five live traps (Ugglan special, Grahnab AB, Hillerstorp, Sweden) were distributed evenly in a 5 x 5 grid in each enclosure. All traps were covered with trap chimneys (40 x 40 x 50 cm) made of sheet metal, protecting the traps from the seasons. Sunflower seeds were used as bait. All enclosures were emptied of remaining rodents prior to each experiment.

## 2.3 Odour cues

### 2.3.1 Odour preparation

A range of odour treatments were used for this thesis. The preparation of the three most common ones, alarm pheromone, predator odour and control (Chapters I-III), is detailed in this section.

The predator odour was obtained by collecting used bedding materials, including faeces, urine, and body odour, from least weasels kept at the research station.

The alarm pheromone cue was obtained by collecting bedding materials that were used by male bank voles that were exposed to the weasels on a daily basis. During the exposure, alarm pheromone "donor voles" were placed inside a wire mesh live trap, which was placed inside the weasel cage for 60 s. After the initial exposure, donor voles were placed individually in clean cages with fresh bedding materials to allow their scents to infuse into the bedding materials. The scents produced by the weasel-exposed voles were allowed to accumulate in used bedding materials following successive exposures.

The control odour cue was clean bedding materials without any added cues from voles or weasels.

In order to minimize variation in odour source, the beddings were thoroughly mixed before taking samples of bedding with urine and/or faecal matter. The same was done for the clean bedding. All odour cues were used within 3 h after being taken from its source. No odour cue was stored for later use.

### 2.3.2 Presentation

In in the first experiment, the odour cues were applied directly into a vole's cage, where it accumulated (I). As this was not feasible for the following experiments, which included the odour presentation in the field, we deigned a special

treatment chamber, allowing individual manipulation of each captured vole (Fig. 1). The treatment chamber consisted of two compartments, separated by a perforated wall and could accommodate a live trap. This allowed a more controlled presentation of the odour cue in both a field and laboratory setting (II and III).
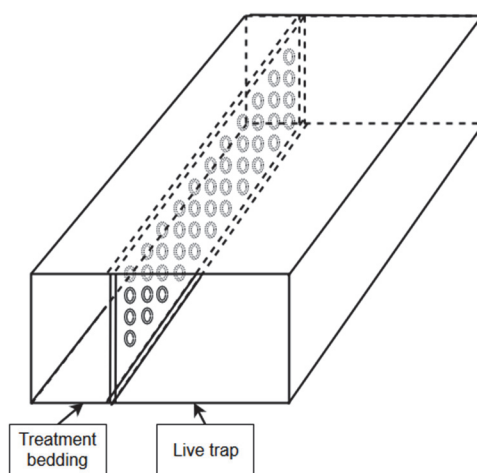


FIGURE 1    Treatment chamber. The odour cue is separated from the trap compartment by a mesh to avoid odour contamination. Closing mechanism not shown. Taken from Chapter II.

## 2.4   Laboratory behaviour trials

Chapters I – III include behavioural tests conducted in the laboratory. Each test was recorded with an overhead camera and later analysed by either a human observer (I and III) or automated software (II) (Noldus *et al.* 2001).

Chapter II used a standard open field arena containing a single odour cue, while chapter I and III use either a four-armed (I) or three-armed (III) maze. In these complex mazes, each arm of the maze was equipped with a different odour cue and means to assess the foraging behaviour.

## 2.5   Experimental procedures

Chapter I focuses on the reproductive behaviour of voles presented with different odour cues. A complementary behavioural experiment served to assess innate reaction and foraging decision in a forced-choice setting.

Chapter II combines natural mating behaviour and controlled odour cue treatments in our enclosures in order to assess their offspring. The resulting vole pups were tested in an open field arena where they encountered the same odour

cues. The emphasis of this chapter is on how the treatment of the parents interacted with the treatment of the offspring.

In chapter III, the focus is on the importance of the timing of the odour treatment. For this, half the animals were treated from mating until parturition and the other half from parturition until weaning. The growth of the pups was monitored, and their exploratory and foraging behaviour was tested.

Chapter IV involves a gas-chromatographical and mass-spectrometric analysis of the volatile odour compounds excreted by voles in order to identify their alarm pheromone. For this, voles were either presented to a weasel (similar with how the alarm pheromone was obtained in the other chapters), handled or left unstressed.

For this, an individual vole was enclosed in a class chamber for 20 minutes, directly after each treatment. Two pumps created an airflow of filtered air around the animals. This airflow was direct through thermal desorption tubes, which collected all volatile components.

A complementary field test was done in order to assess the effect and longevity of the alarm pheromone in a natural environment.

# 3  RESULTS AND DISCUSSION

## 3.1  An overview

Many results and conclusions are summarised in this thesis. For the ease of the reader, I condensed and simplified the main results and conclusions into the table below (Table 1).

TABLE 1    Simplified overview of the experiments (I – IV), their main results and conclusions.

| Chapter | I | II | III | IV |
|---|---|---|---|---|
| Place | Laboratory | Field/laboratory | Laboratory | Field/laboratory |
| Subject | Effect of alarm pheromone | Transgenerational effects | Timing of exposure (pre- or postnatally | Analysis of alarm pheromone Effects in the field |
| Measured | Reproductive output | Behaviour in the open field arena | Foraging behaviour Growth rate | Volatiles Foraging behaviour |
| Main results | Increased parturitions | Transgenerational effects exist | Timing has only minor effect<br><br>Alarm Pheromone favours big litters | Alarm pheromone candidates identified Alarm pheromone short-lived in the field |
| Main Conclusions | Terminal investment | Effects are context dependant | More support for terminal investment | Potential alarm pheromones identified |

## 3.2 Reproductive adaptations to weasel odour and alarm pheromone (chapters I and III)

The ability of prey individuals to inform conspecifics has been well established. A large focus lays on acoustic signals, i.e. alarm calls (Blanchard *et al.* 1991, Forti *et al.* 2017, Barati and McDonald 2017). In some species, these can be elaborate and not only signal threat, but also the predator identity (Manser *et al.* 2002, Ouattara *et al.* 2009, Collier *et al.* 2017, Barati and McDonald 2017). The aspect of added information translates to alarm pheromones, as they are only secreted as a reaction to an acute predator presence, as opposed to predator odours, which may remain in the environment after the acute danger has passed. Prey species can as a result gain more detailed insight of the threat level, e.g. whether heightened vigilance is required as predator is somewhere in the area, or whether more drastic measures need to be taken, as a conspecific barely survived an attack.

In chapter I, I discovered that bank voles, as a reaction to a treatment of conspecific alarm pheromone, showed an increase in parturitions. In fact, while in the control group 36.8% of the females gave birth to a litter, this rate was at 84.5% for those treated with alarm pheromone. No other significant difference from control was observed. In addition, a treatment with predator odour affected the reproductive output. Pups, one day after birth, were significantly heavier when the mother were treated with predator odour.

Chapter II shows an increased mass gain in bigger litters in the alarm pheromone treatment, but increased mass gain in smaller litters in the predator odour treatment. Combined with chapter I, this solidifies the idea that different odour cues carry different information, which in turn allows prey species or individuals to alter their behaviour. Consequently, alarm pheromone exposure causes a change towards terminal investment, with a focus on larger litters, while exposure to predator odours might trigger an investment in a few high-quality offspring.

Contrary to previous studies on bank voles, I did not observe any decrease in reproductive output (Ylönen and Ronkainen 1994, Fuelling and Halle 2004, Haapakoski *et al.* 2012), but rather the opposite, which is in accordance with a more recent study which discovered increased litter sizes in bank voles after an alarm pheromone exposure (Haapakoski *et al.* 2018).

The ambiguity of these results might seem troubling at first glance, but recent work has proposed a new idea for adaptive reproductive strategies (Duffield *et al.* 2017). At low or medium risk levels, a decrease of reproductive output is assumed, as prey individuals focus on their short-term individual fitness. As the perceived risk level passes a threshold, the reproductive strategy may change towards terminal investment, as a single offspring surviving the high risk period and reaching sexual maturity compensates the parental mortality (Kokko and Ranta 1996, Kokko and Ruxton 2000), which is a more long-term fitness investment. This is similar to the insurance hypothesis, in which

reproductive investment increases in expectation of an unfavourable environment (Promislow and Harvey 1990, Forbes 1991, Houston *et al.* 2012).

The majority of mammalian studies focusing on terminal investment cover senescence (Ericsson *et al.* 2001, Hoffman *et al.* 2010, Weladji *et al.* 2010), therefore it is challenging to assess the adaptive and evolutionary aspects of terminal investment in mammals as a response to predator cues. However, a very recent study found similar results in Brandt's voles (*Lasiopodomys brandtii*, Radde 1861) (Gu *et al.* 2020), so this could be a more widespread phenomenon than assumed at the moment.

Signalling conspecifics immediate threat is most often seen in social species such as suricates (Manser *et al.* 2002) or bees (Johnson *et al.* 1985). However, bank voles are commonly not considered a social species. Nevertheless, despite the fact that females are very territorial during breeding season (Bujalska 1973), during winter, bank voles tend to form communal nests for better thermoregulation (Ylönen and Viitala 1985, 1991). Additionally, young female offspring are tolerated in their mothers territory for a prolonged time (Mappes *et al.* 1995b). These indications of temporarily close social groups could be the basis of alarm pheromone communication in bank voles.

## 3.3  Transgenerational effects (chapters II and III)

In the current literature on transgenerational effects, the consensus is that predator exposure (or predator odour exposure) causes increased anxiety-like behaviour in offspring (Abe *et al.* 2007, Brunton and Russell 2010, Brunton 2013, St-Cyr *et al.* 2017). The majority of the studies in this area use an open-field arena (or similar neutral environments), which is a neutral, blank environment without any kind of stimuli. As the range of behavioural adaptations to predation are vast, an open field arena certainly is a simplification of the natural environment.

In an attempt to bridge this gap, the voles in chapter II were put in such an arena, but one of the odour cues was present. While this is still a simplified environment, it presents the animal with an ecologically relevant stimulus, unlike a completely empty environment. However, the assumption of an open-field arena is that spending time next to its outer limits, i.e. walls, can be interpreted as a proxy for anxiety (Treit and Fundytus 1988).

In chapter I, the focus was on proportion of time spent in the centre zone. I was able to replicate previous experiments, i.e. increased anxiety in pups, whose parents encountered a predator odour, in a neutral environment. Interestingly, I also found interactions between the parents' treatment and the odour encountered in the arena, meaning that the combination of prenatal predator odour with either predator odour or alarm pheromone in the testing environment resulted in offspring reacting less fearfully. Thus, transgenerational effects are highly context-dependent.

Chapter III used a more complex testing environment. Vole offspring were observed in a three-armed maze. Each arm contained a foraging patch along with

an odour cue. In this set-up, no transgenerational effects were found when it came to foraging or investigating the predator odour arm. Voles still showed an innate avoidance of areas treated with a predator, but independent of any parental treatment. In contrast to this, pups with a parental treatment of alarm pheromone also explored the area of the maze with alarm pheromone first.

An increase in boldness, resulting from in utero imprinting, can be adaptive when triggered in a high-risk environment. During the short life span of a vole, a significant change in predation pressure might not occur. As predator densities follow prey densities with a time lag, a high predator-to-prey ration outlasts a prey generation (Hanski *et al.* 2001, Sundell *et al.* 2013). In that scenario, bold individuals are more likely explore, forage or mate (Ylönen *et al.* 2002, Korpela *et al.* 2011, Mella *et al.* 2015) instead of waiting for an safer environment.

These results also show the importance of the test environment, as some transgenerational effects become only apparent under specific conditions.

## 3.4 Timing of the exposure (study III)

Several studies have shown how predation risk affects prey offspring's fitness and behaviour (Bestion *et al.* 2014, Sheriff *et al.* 2015). To date, an overarching consensus on when the cue has to be encountered to trigger an effect is still lacking. Nevertheless, explanations for the different forms of information transmission exist. In utero transmission of the information is likely to occur via hormone transmission in the placenta (Sheriff *et al.* 2017, Kuijper and Johnstone 2018). Postnatally the difference in e.g. behaviour can be explained by different hormone contents during lactation (Brummelte *et al.* 2010, Sullivan *et al.* 2011), or with differences in maternal care (Bauer *et al.* 2015).

In contrast to my expectations, only negligible differences in the offspring behaviour were explained by the timing of parental treatment. The only affected aspect was the foraging. There, vole pups from mothers treated with alarm pheromone after parturition foraged 0.1 g more compared to animals from the control group. The difference of 0.1 g needs to be put into context, as in this chapter millet seeds were used. Thus, the difference in foraging is about 19 millet seeds with diminishing returns, which require a significant time investment to find from the substrate.

Similar to the conclusion drawn in the previous section, this is possibly an adaptation to a longer lasting high-risk environment, in which individual are forced to forage in order to survive.

## 3.5 Identification of vole alarm pheromone (study IV)

The work on invertebrate alarm pheromones is already extensive (e.g. Bowers *et al.* 1972, Howe and Sheikh 1975, Heath and Landolt 1988), however literature on

terrestrial vertebrate alarm pheromones are lacking. To be precise, "extensive" solely refers to the number of studies on invertebrates compared to vertebrates, since in relation to the number of invertebrate species, the numbers become less impressive. In terrestrial vertebrates, there is a clear overrepresentation of studies on laboratory rodents (Kiyokawa *et al.* 2005, Inagaki *et al.* 2009, 2014, Brechbühl *et al.* 2013). On the other hand, there are only a few studies on body odours of any kind in wild animals (Charpentier *et al.* 2008, Apps *et al.* 2015), and very few on alarm pheromones in wild terrestrial vertebrates (Gomes *et al.* 2013). This is partly due to a historical lack of non-invasive sampling techniques, which have just been developed in recent years (Birkemeyer *et al.* 2016, Weiß *et al.* 2018).

Chapter IV aimed at identifying possible alarm pheromones in bank voles. I was able to narrow down the possible candidates to three potential chemicals. Of these three, one is an unknown compound, while the other two, 2-octanone and 1-octnol, have previously been found in alarm secretions of other species. However, 1-octanol has solely been found in insects (Johnson *et al.* 1985, Collins *et al.* 1989, Hunt *et al.* 2003, Yamashita *et al.* 2016) and 2-octanone mostly in ants (Crewe and Blum 1970, Dumpert 1972, Brand *et al.* 1989), with the exception of two loris species (Hagey *et al.* 2007).

In the aquatic environment, examples of chemical cross-phyla communication exist (Kaliszewicz and Uchmański 2009), however for terrestrial vertebrates, a communication across taxonomic classes has only been shown with alarm calls (Vitousek *et al.* 2007, Lea *et al.* 2008). Despite the evidence that our identified compounds are secreted in invertebrates, my results do not allow drawing conclusions about a common underlying structure or chemical features of alarm pheromones. A previous meta-analysis looking for a common denominator in chemical communication of terrestrial vertebrates concluded that the current studies are merely scratching the surface of the topic and data on more species is needed to draw meaningful conclusions (Apps *et al.* 2015).

I supplemented this analysis with a field experiment on the effect and longevity of alarm pheromones in the field compared to direct predator odour. The experiment showed a drastic increase in foraging in the foraging patches treated with the alarm pheromone after just one day. This can be explained in two ways. Either, as I expected, the volatile alarm pheromone only efficiently carries the information on immediate risk short-term. The remaining odours, after the vanishing of the fear signal, merely indicates the presence of a conspecific, in turn sending safety signal, as only odours of conspecifics remain, which increases investigation and foraging. Alternatively, the alarm pheromone is still present on the second day, but in such low concentrations that that triggers increased investigation and intensified use of the foraging patch (Parsons *et al.* 2018), which in turn results in the discovery of the foraging patch. Both options indicate a minimal information value concerning predator presence on the second day. It also supports the role of alarm pheromones to indicate an acute and immediate risk, as argued in chapter I.

# 4  CONCLUSIONS

Chemical communication in predator-prey relations, between conspecifics sharing the same risky environment, is a slowly growing field that only has gained more traction in the last decade. Especially pheromones, which allow chemical communication between conspecifics, have been mostly ignored in terrestrial mammals until recently. Even though there has been a call for more extensive work on semiochemicals, there has only been little progress made in the last decade on identifying the nature and effects of body odours in terrestrial vertebrates.

In this thesis, I investigated the predator-prey interaction between bank voles and least weasels, with a focus on direct and indirect chemical cues of predation. I also studied how these cues affect the reproductive behaviour, offspring growth and behaviour, and finally the chemical nature of vole alarm pheromones. I approached the research questions with a combination of enclosure and laboratory studies.

Firstly, I showed how voles associated different odour cues with different risk levels. While the direct odour of a predator represents a medium threat level, as it is constantly in the vole's environment, alarm pheromones represent an acute and high risk (chapter I – III). This leads to my second point, alarm pheromone triggers terminal investment on several levels in bank voles (chapters I and III). Specifically, alarm pheromone triggers an increase in parturitions (I), an increase in litter sizes (Haapakoski *et al.* 2018), and an accelerated growth in big litters (III). Thirdly, I showed that both predator odour as well as alarm pheromones trigger transgenerational effects in bank voles. However, these effects are highly context-dependent, e.g. they depend on the test environment and stimuli provided. This in turn has potential consequences on existing literature, as often-used neutral and simplified environments trigger different behaviours, compared to enriched environments with odour stimuli. However, I also showed that the timing of when parents encounter these cues only has minor effects. Fourthly and lastly, I identified a group of chemicals that are likely to act as alarm pheromones in bank voles (IV). In the process, using enclosure experiments, I also confirmed the only short-lasting effect of alarm pheromone

as a foraging deterrent in the field, which vanished after just one day. Thus, the concept of different threat levels or different threat immediacy associated with different odours is verified.

In this thesis, I have gathered new information on the importance of alarm pheromones for the assessment of predation risk in small mammals, as well as how it shapes a new range of behavioural adaptations. Terminal investment, as a result of high predation risk, is currently a rarely researched topic but pheromone communication in prey is also often overlooked. I hope that this thesis helps to develop further the field of predator-prey research by providing a fresh aspect to it. I am confident that pheromone communication occurs in a wider range of terrestrial mammals than is currently known. Further, I recommend that the next generation of research on chemical cues in antipredator adaptations should strongly focus on conspecific odour cues.

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# YHTEENVETO (RÉSUMÉ IN FINNISH)

## Petoriskin epäsuorat ja sukupolvien väliset vaikutukset: pedon haju ja hälytysferomonit metsämyyrä–lumikko –suhteessa

Peto–saalis-suhteen evoluutio ja ilmentymät populaatioissa ja yhteisöissä ovat kiinnostaneet tutkijoita jo vuosisadan ajan. Evolutiivinen kilpajuoksu pedon ja saaliin välillä on johtanut moniin morfologisiin ja fysiologisiin sopeumiin sekä käyttäytymisen muutoksiin. Varhaisempi peto–saalis-suhteen tutkimus keskittyi petojen aiheuttamaan kuolleisuuteen ja sen populaatiovaikutuksiin. Viime aikoina tutkimuskohteina ovat olleet yhä useammin pedon epäsuorat vaikutukset saaliin käyttäytymiseen ja tämän adaptiiviset seuraukset saaliin hengissä säilymiselle ja lisääntymiselle ja näin ollen lajin populaatioille. Pedon välttämisen edellytys on se, että saalis havaitsee pedon. Saaliseläin voi aistia pedon läsnäolon suoraan pedon jättämistä ärsykkeistä, kuten äänistä tai hajuista, tai se voi saada epäsuorasti viestin muilta oman lajin yksilöiltä. Havaitessaan pedon saaliseläin muuttaa käyttäytymistään siten, että sen selviytyminen paranee ja kelpoisuus lisääntyy. Saalis saattaa puolustautua morfologisesti, kuten ahven, joka nostaa selkäevänsä piikit kohtisuoraan ylös petokalan, esimerkiksi hauen havaitessaan. Saaliin yleisimpiä vasteita kohonneeseen petoriskiin ovat käyttäytymisen muutokset, kuten jähmettyminen, jotta peto ei havaitse saalista, tai pakeneminen. Pedon läsnäolo voi myös muuttaa saaliin lisääntymiskäyttäytymistä.

Saalis voi myös viestittää petoriskistä lajitovereilleen pelkoreaktion kautta erittyvillä hajuilla, niin sanotuilla hälytysferomoneilla. Nämä kemialliset viestit saattavat sisältää tarkempaa, akuutimpaa informaatiota petoriskistä kuin pedon haju. Suorien käyttäytymisvasteiden lisäksi kohonneella petoriskillä saattaa olla sukupolvienvälisiä vaikutuksia. Vanhempien, varsinkin äidin, kokema riski saattaa välittyä kehittyviin poikasiin niin raskauden kuin imetyksenkin aikana ja vaikuttaa poikasten kykyyn reagoida petoärsykkeisiin pesästä lähdön jälkeen.

Selkärankaisilla on tehty vain vähän tutkimuksia hälytysferomoneista ja niiden merkityksestä saaliseläinten keskinäisessä kommunikaatiossa. Väitöstutkimukseni keskittyi petoriskiä viestittävien signaalien, pedon hajun ja oman lajin toisen, petoriskille altistuneen yksilön erittämän hajun, vaikutuksiin saaliin käyttäytymiseen ja lisääntymiseen. Tutkimuslajini olivat maamme metsissä yleinen jyrsijä, metsämyyrä, ja sen merkittävä spesialistipeto, lumikko. Lumikko pystyy pienenä ja pitkulaisena saalistamaan myyriä niiden käytävissä, pesissä ja myös lumen alla.

Tutkimukseni keskittyi kolmeen pääkysymykseen: miten suora pedon haju tai epäsuora, lajitoverin erittämä hälytyshaju vaikuttaa metsämyyrän lisääntymiseen; onko vanhempien, etenkin äidin, altistumisella korkeaan petoriskiin vaikutuksia raskausajan tai imetyksen kautta poikasten pedonvälttämiskäyttäytymiseen; sekä mitkä kemialliset yhdisteet olisivat hälytysferomonin

välittämän informaation taustalla. Tutkin näitä kysymyksiä neljässä osatutkimuksessa, joista osa tehtiin laboratoriossa ja osa luonnonmukaisissa ulkotarhoissa.

Tutkimukseni todensi sekä saaliseläimen lisääntymisen että käyttäytymisen muuttuvan suoran ja epäsuoran petoärsykkeen vaikutuksesta. Hälytysferomoni näytti muuttavan myyrän lisääntymisstrategiaa siten, että naaras lisääntyi tehokkaammin petoriskistä huolimatta. Tämä näkyi niin lisääntyvien yksilöiden määrässä kuin poikasten kasvunopeudessakin, etenkin isoissa poikueissa hälytysferomonille altistuttaessa. Ilmiö on yhteensopiva niin sanotun "terminaalivaiheen investointi" -hypoteesin kanssa, jossa yksilö pistää kaiken yhden kortin varaan vaikka henki menisi. Yksikin lisääntymisikään selviävä poikanen kompensoi mahdollisen oman kuoleman.

Laajemmassa mittakaavassa tulokseni näyttävät olevan ristiriidassa aiempien tutkimusten kanssa, joissa on osoitettu, että korkea petoriski saa aikaan lisääntymisen siirtymisen tai estymisen. Oletan kuitenkin, että toisen yksilön erittämä hälytyssignaali on luonteeltaan erilainen ja tuo viestin akuutista, korkeasta vaarasta, johon reagointi voi olla erilaista kuin elinympäristöön jäänyt pedon haju. Molemmilla lisääntymisstrategioilla saattaa olla vaikutusta yksilön tai yksilön jälkeläisten selviämiseen, ja reaktio korkean petoriskin signaaleihin saattaa olla joustava elinkierron eri tilanteissa.

Tutkimukseni vahvisti myös mahdollisten sukupolvien välisten petoriskivaikutusten olemassaolon. Vaikutukset näkyivät yksilön liikkumisessa, riskinotossa ja ruokailukäyttäytymisessä, mutta tulokset eivät olleet yksiselitteisiä. Tutkimukseni toi uutta tietoa eläinten käyttäytymistutkimuksen metodiikkaan ja suosittaa luonnonmukaisempia testausympäristöjä yksilön säilymiseen liittyvien käyttäytymispiirteiden tutkimukseen laboratoriossa.

Viimeinen tutkimus selvitti yhdisteitä, jotka saattaisivat toimia hälytyssignaaleina pelästyneen yksilön erittämissä hajuissa. Tulokseni osoittivat, että erityisesti kahdella yhdisteellä, 2-oktanonilla ja 1-oktanolilla saattaisi olla merkittävä asema petoriskin kemiallisessa signaloinnissa, koska nämä yhdisteet on löydetty myös muiden lajien pelkovälitteisissä hajuaineissa. Havaitsin myös, että oletustemme mukaisesti hälytysferomonin vaikutus luonnossa oli lyhytaikainen.

Yhteenvetona totean, että tutkimukseni tuotti uutta tietoa nisäkkäiden hälytysferomonien koostumuksesta ja niiden vaikutuksesta saaliseläimen käyttäytymiseen ja lisääntymiseen. Tutkimukseni vertasi näitä vaikutuksia pedon hajun suoriin vaikutuksiin. Tulokset osoittivat yksilöiden lisääntymisstrategioiden mahdollisen joustavuuden. Varsinkin tulokseni lisääntymispanostuksen kasvusta korkean petoriskin aikana on harvinainen nisäkäsekologiassa, vaikka se onkin havaittu useilla muilla lajeilla selkärangattomista lintuihin.

# ZUSAMMENFASSUNG (RÉSUMÉ IN GERMAN)

**Indirekte und Transgenerationale Effekte des Prädationsrisikos: Raubtiergeruch, Alarmpheromone und deren Auswirkungen auf Rötelmäuse**

Das Zusammenspiel von Beutegreifern und ihrer Beute fasziniert Forscher bereits seit Jahrzehnten. Einer der vielen Gründe für diese Faszination ist, dass diese Interaktionen einer der größten evolutionären Treiber ist und dadurch zu einer Art „Wettrüsten" mittels physiologischen Adaptionen und Verhaltensänderungen führt. Frühe Forschung war stark auf die direkte Mortalität der Beutetiere. Dies führte zu einer Fokussierung auf die resultierenden Veränderungen in der Populationsdynamik. Heutzutage liegt der Fokus vermehrt auf nicht konsumptiven Effekten, wie verändertes Verhalten und dessen Konsequenzen.

Beutetiere können ihre Fressfeinde anhand von direkten, ausgehend von dem Beutegreifer, oder indirekten, ausgehend von Artgenossen, Signalen erkennen. Die bereits erwähnten Veränderungen in Verhalten oder Physiologie der Beutetiere ermöglichen eine maximale Überlebenschance und eine erhöhte individuelle Fitness. Die Anpassungen können permanent sein, wie zum Beispiel Stacheln bei Stachelschweinen, oder auch induziert, das heißt nur auftretend wenn benötigt. Letzteres kann sowohl morphologische wie auch Verhaltensänderungen umfassen. Das Spektrum der Verhaltensänderungen bei Beutetieren reicht von einfachen Anpassungen wie Flucht, erstarren, oder vermeiden von Orten, bis hin zu komplexeren Veränderungen, zum Beispiel in der Fortpflanzung. Eine weitere komplexe Anpassung sind Effekte, die erst bei den Nachkommen auftreten, sogenannte transgenerationale Effekte. Diese werden entweder in utero oder während des Säugens ausgelöst, und führen zu einer Reihe von Veränderungen wie zum Beispiel verändertem Lernverhalten, veränderten Stressreaktionen oder Veränderungen bei der Futtersuche. Weiterhing haben Beutetiere die Möglichkeit Artgenossen vor Gefahr zu warnen, entweder mittels Alarmrufen oder Alarmsekretion, zum Beispiel Alarmpheromone. Diese konspezifischen Signale können einen höheren Informationsgehalt haben, als Signale von Fressfeinden. Sie können Auskunft geben über die Identität des Prädators oder über das aktuelle Risikoniveau informieren.

Bislang hat nur eine Handvoll von wissenschaftlichen Studien die Alarmsekretionen und die durch sie ausgelösten Adaptionen in Wirbeltieren untersucht. In meiner Dissertation untersuchte ich die Auswirkungen von Raubtiergeruch und konspezifischen Alarmpheromonen auf das Verhalten und die Fortpflanzung von Rötelmäusen (*Myodes glareolus*), eine Wühlmaus der borealen Wälder. Als Prädator für meine Experimente diente das Mauswiesel (*Mustela nivalis nivalis*), einer der kleinsten Vertreter der Marder und sehr effektiver Nagetierjäger. Bedingt durch seinen schlanken Körperbau ist das Mauswiesel fähig den Wühlmäusen in ihren Tunneln zu folgen und ist dadurch einer der Hauptursachen der borealen Wühlmaussterblichkeit.

Meine Arbeit fokussiert sich auf drei Hauptpunkte um die Auswirkungen von Prädatorgeruch und konspezifischen Alarmpheromonen zu vergleichen:

erstens, wie wird das Fortpflanzungsverhalten verändert; zweitens, gibt es transgenerationale Effekte und wie äußern sie sich; und drittens, was genau sind die Alarmpheromone in Rötelmäusen.

In meinen Studien habe ich Hinweise gefunden, dass die Konfrontation mit konspezifischen Alarmpheromon einen Wechsel der Fortpflanzungsstrategie zu „terminal investment" verursacht. Dies wurde durch einen Geburtenanstieg nach einer längeren Alarmpheromonbehandlung deutlich. Zusätzlich war auch ein beschleunigtes Wachstum der Jungtiere bei größeren Würfen zu beobachten. „Terminal investment" beschreibt die Idee, dass im Falle eines extremen Risikos Individuen ihre Fitness steigern, indem alle Ressourcen für die Fortpflanzung verwendet werden, unabhängig von den eigenen Nachteilen. Sobald ein einziger Nachkomme geschlechtsreif wird, kompensiert dies jegliche Investition der Eltern.

Ein zusätzlicher Aspekt ist, dass Rötelmäuse ihre Fortpflanzungsstrategie an die implizierten Informationen eines Geruchs anpassen können. Frühere Studien mit Raubtiergeruch beschreiben meist eine Verminderung der Fortpflanzung, was in Kontrast zu meinen Ergebnissen steht. Allerdings, unter der Annahme, dass Alarmpheromone, im Gegensatz zu Raubtiergeruch, eine sehr akute und zeitlich begrenzte Gefahr signalisieren, zeigt der Wechsel der Fortpflanzungsstrategie zu „terminal investment" eine Plastizität des Verhaltens der Rötelmäuse.

Weiterhin habe ich Hinweise auf transgenerationale Effekte gefunden, die sich auf das Erkundungs- und Futtersuchverhalten auswirken. Während frühere Studien die Nachkommen in neutralen Umgebungen, zum Beispiel „open field arena", testen, untersuchte ich das Verhalten des Nachwuchses unter dem Einfluss von Gerüchen. Dies zeigte, dass das veränderte Verhalten abhängig von der Umgebung ist und nicht unter jeden Umständen auftritt. Im Gegensatz zu früheren Studien, die Anzeichen für erhöhte Ängstlichkeit gefunden haben, zeigen meine Ergebnisse, dass dies nur in neutralen Umgebungen zutrifft, und dass Nachwuchs weniger ängstlich agiert sobald Raubtiergeruch zugegen ist.

Schlussendlich habe ich eine Gruppe von chemische Verbindungen aus den Alarmsekretionen der Rötelmäuse identifiziert, die mit hoher Wahrscheinlichkeit für die beschriebenen Ergebnisse verantwortlich sind. Besonders zwei chemische Verbindungen, 2-Octanon und 1-Octanol, sind wahrscheinliche Kandidaten, da sie bereits in den Alarmpheromonen anderer Arten nachgewiesen wurden. Ein Vergleich mit anderen Säugetieren gestaltet sich schwierig, da bisher nur einige wenige Studien die chemische Natur der Alarmpheromone in Wirbeltieren untersucht haben. Weiterhin hat sich gezeigt, dass die vergrämende Wirkung von Alarmpheromonen lediglich einen Tag unter natürlichen Bedingungen anhält. Dies untermauert die Relevanz von Alarmpheromonen für zeitlich begrenzte Informationen.

In meiner Dissertation habe ich neue Informationen zu der Relevanz von Alarmpheromonen für die Beurteilung des Risikos durch Prädatoren für Kleinsäuger gesammelt. Sowohl „terminal investment", ausgelöst durch Raubtiere, wie auch Alarmpheromone bei Säugetieren, sind bisher wenig erforschte Phänomene. Meine Ergebnisse füllen eine Wissenslücke zur chemischen Kommunikation bei Säugetieren und helfen das Verständnis von dem Zusammenspiel zwischen Beutegreifern und deren Auswirkungen auf die

Fortpflanzung und das Verhalten der Beutetiere zu vertiefen. Zukünftige Studien werden intraspezifische Kommunikation in ihre Ergebnisse einbeziehen müssen, um die Gesamtheit der Räuber-Beute-Interaktionen zu verstehen.

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# ORIGINAL PAPERS

# I

## SECONDHAND HORROR: EFFECTS OF DIRECT AND INDIRECT PREDATOR CUES ON BEHAVIOR AND REPRODUCTION OF THE BANK VOLE

by

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# Secondhand horror: effects of direct and indirect predator cues on behavior and reproduction of the bank vole

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**Abstract.** Risk recognition by prey is of paramount importance within the evolutionary arms race between predator and prey. Prey species are able to detect direct predator cues like odors and adjust their behavior appropriately. The question arises whether an indirect predation cue, such as the odor of scared individuals, can be detected by conspecifics and subsequently affects recipient behavior. Parents may also transfer their experience with predators to their offspring. In two experiments, we assessed how direct and indirect predation cues affect bank vole (*Myodes glareolus*) foraging behavior, reproduction, and pup fitness. Weasel (*Mustela nivalis*) odor served as the direct cue, whereas the odor of weasel-scared conspecifics, alarm pheromones, was used as an indirect cue and both of those were compared to a control odor, clean wood shavings. Alarm pheromones attracted female voles, measured as time in proximity to the treatment and foraging. Both predator odor and alarm pheromones enhanced reproduction compared to the control odor. Females treated with alarm pheromone had significantly higher pregnancy rates, and pups from predator-treated mothers were significantly heavier at birth. Our study provides two novel ideas. First, the impact of a predator can be socially transmitted. Second, predation risk likely triggers terminal investment in reproduction.

**Key words:** alarm pheromone; ecology of fear; *Mustela nivalis; Myodes glareolus*; odor; stress response; terminal investment.

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## Introduction

Predators decrease an individual's survival probability (Sih et al. 1985, Murdoch et al. 2003). Predation, and the indirect effects of predator presence, has been recognized as strong life-history determinants across different taxa (Sih 1994, Ylönen and Ronkainen 1994, Werner and Peacor 2003, Nelson et al. 2004, Ylönen and Brown 2007, Sheriff et al. 2009). Historically, ecological research has focused on the aforementioned direct predation effects (Paine 1966, Taylor 1984, Krebs et al. 1995). However, in the last decades, the focus has shifted more and more toward the indirect effects of predation (see reviews by Lima 1998, Creel and Christianson 2008), and it has been recognized that perceived predation risk alone can have large fitness or survival effects on the population level as direct mortality by predators (Schmitz et al. 1997, Nelson et al. 2004, Preisser et al. 2005, Pangle et al. 2007).

Co-evolution of predator and prey species suggests prey evolved a number of sensory and behavioral adaptations in order to recognize and avoid predators. In many mammalian prey species, this includes behavioral changes such as

freezing, avoidance, and heightened vigilance, but also the ability to detect and correctly recognize the odors emitted by predators, from here on predator odor (PO), which serve as triggers for the adaptive behaviors previously mentioned (Kats and Dill 1998, Dielenberg and McGregor 2001, Sundell and Ylönen 2004, Conover 2007, Osada et al. 2014, Apfelbach et al. 2015, Sievert and Laska 2016). Indirect effects of predation, for example, decreased reproduction (Ylönen and Ronkainen 1994, Sheriff et al. 2009, 2015), as well as the interaction of risk and competition, are drawing increasing attention in current literature (e.g., Apfelbach et al. 2005, Parsons et al. 2017).

In a natural environment, the odor of a predator might be abundant in the form of excrement or markings. It is therefore not surprising that several studies have reported rapid habituation to predator-born odor in a natural environment (Cox et al. 2010, Elmeros et al. 2011, Bytheway et al. 2013). This leads to the assumption that prey, while detecting PO, considers it as an ambient risk (Brown et al. 2015). A study by Bleicher et al. (2018) showed that vole's reaction to predator odor returns to baseline levels after being confronted with a live predator. This indicates that actual predator presence outweighs the information content of an olfactory cue alone and that there is no increase in perceived risk toward a predator odor cue. Prey species then need different means to convey actual threatening or acute predation risk, allowing them to dynamically adjust their behavior to different threat levels (Duffield et al. 2017). This role is most likely covered by intra-species communication.

Intra-species communication and signaling about increased risk, for instance, through Schreckstoff (Frisch 1938) or alarm pheromones (henceforth AP), are evolutionarily widespread in many taxa (Bowers et al. 1972, Howe and Sheikh 1975, Stowe et al. 1995, Boissy et al. 1998, Beale et al. 2006, Gutiérrez-García et al. 2007). In several social species of fish, insects, and mammals, AP secretions are recognized as a signal to protect their colony, group, or family when in danger (Breed et al. 2004, Kiyokawa et al. 2004*a*, Gomes et al. 2013). Despite some papers raising concern about the categorization of APs, arguing that these chemicals cannot be classified as real pheromones (Magurran et al. 1996, Viney and

Franks 2004), the behavioral response is the same, given the correct context (Magurran et al. 1996). While for most mammals, the chemical structure of APs is still unknown, it has been identified in, for example, aphids (Bowers et al. 1972, Beale et al. 2006), sea anemones (Howe and Sheikh 1975), and several insects (Crewe and Blum 1970, Heath and Landolt 1988, Kuwahara et al. 1989). To fulfill their sensory warning role, APs should be volatile or hydrophilic (Kiyokawa et al. 2005, Inagaki et al. 2009). Given the majority of experiments on mammalian APs have been done on lab animals, their chemical structure has been described only for mice (C57BL/6J and OMP-GFP strains; Brechbühl et al. 2013) and Wistar rats (Inagaki et al. 2014). Brechbühl et al. (2013) state that both mouse APs and mammalian predator olfactory cues share structural similarities, specifically sulfur-containing molecules. In this paper, we utilize the concept of APs similarly as in previous studies, although we acknowledge that in most studies the chemical or biological nature of the different odors of stress is not yet properly determined.

High predation risk affects mating behaviors and reproductive success (Sih 1994, Ruxton and Lima 1997, Kokko and Ruxton 2000). There is strong support for the notion that predation risk negatively affects breeding success (Sih 1994). This is manifested as delayed breeding in bank voles and gray-sided voles (*Myodes rufocanus*; Mappes and Ylönen 1997, Fuelling and Halle 2004), hindering copulations in bank voles (*Myodes glareolus*; Ronkainen and Ylönen 1994), elevating stress levels in snowshoe hares (*Lepus americanus*; Sheriff et al. 2009), or decreasing weights of breeding individuals or their offspring in snowshoe hares and bank voles (Sheriff et al. 2009, Trebatická et al. 2012). However, the mechanisms and adaptive value of delayed or suppressed breeding under risk are not clear and continue to be debated (Ruxton and Lima 1997, Kokko and Ruxton 2000). Several publications have already explored the effects of increased risk of predation in parents' environment on offspring behavior and fitness, finding altered learning behavior in three-spine sticklebacks (*Gasterosteus aculeatus*; Roche et al. 2012, Feng et al. 2015), altered stress reaction in C57BL/6 mice and Long Evans rats (St-Cyr and McGowan 2015, St-Cyr et al. 2017), or changed foraging

strategies in Sprague Dawley rats (Chaby et al. 2015).

An alternative explanation suggests that parents will maximize reproductive efforts at all costs in risky conditions (henceforth terminal investment). In this scenario, individuals breeding in a risky environment will enhance, or speed up, reproduction in order to maximize fitness by producing a number of strong offspring despite the high costs for the parents' or mother's survival. If offspring survive and reach a fertile age, this then compensates for parental disappearance from the reproductive pool (Kokko and Ranta 1996, Kokko and Ruxton 2000). This strategy of bet-hedging or terminal investment has been shown in experimental studies in passerine birds breeding under increased predation risk (Mönkkönen et al. 2009) as well as in crickets (Adamo and McKee 2017). Additionally, it has been shown as a reaction to infections in ants and sparrows (Bonneaud et al. 2004, Giehr et al. 2017).

The relationship between weasels and voles has been intensively studied as the weasel is a specialist predator of rodents and is the major cause of mortality in boreal voles, especially during a population's decline (Korpimäki et al. 1991, Norrdahl and Korpimäki 1995, 2000). As an adaptation against the dramatic predation pressure by weasels, voles are able to detect the odor of mustelids as an antipredator measure and change their behavior accordingly. Bank voles decrease their movement and foraging when exposed to weasel odor (Ylönen 1989, Sundell and Ylönen 2004, Bleicher et al. 2018). They shift their activity times and spatial use to avoid weasels (Jędrzejewska and Jędrzejewski 1990, Jędrzejewski and Jędrzejewska 1990, Sundell et al. 2008) and use more arboreal escape under predation risk (Jędrzejewska and Jędrzejewski 1990, Mäkeläinen et al. 2014). In the study by Mäkeläinen et al. (2014), weasels rarely followed bank voles into a tree, if the bank vole climbed one, showing the efficiency of bank voles' antipredator responses.

Here, we studied in two experiments how increased predation risk, either direct risk in the form of least weasels (*Mustela nivalis nivalis*) odor, or indirect risk in the form of odor emitted by weasel-scared conspecifics, influenced behavior and reproductive investment in bank voles.

The effect was assessed in both behavioral trials and a breeding experiment with cue exposure of parents and monitoring subsequent offspring performance. Social cues, such as pheromones, have previously been shown to be sufficient to trigger cross-generational changes (Koyama et al. 2015). In order to differentiate between the effects of PO, AP, and mere social odor, we also used non-stressed conspecific bedding as a second control in addition to clean wood shavings.

In the behavioral experiment, we predicted that:

1. Voles would feel safer in control and social odor treatments and spend more time in boxes containing those treatments. This would lead to increased foraging in those treatments and foraging to be lowest in PO treatment and second lowest in AP treatment. This would be in accordance with previous studies (Osada et al. 2014, Sánchez-González et al. 2017).

In the fitness experiment, we predicted, based on the existing body of research, that predation cues have a detrimental effect on reproduction. Specifically, we predicted that:

2. The direct predation cue, PO, would decrease the breeding success of parent voles (measured as number of breeding females and litter size) more than AP.
3. Both predation cues, PO and AP, will decrease the number of breeding females and cause the production of smaller litters (Kokko and Ruxton 2000, Fuelling and Halle 2004).
4. Both PO and AP treatments will cause pups to be smaller (Sheriff et al. 2009, 2015).
5. There would be no effect of social odor or control odor on condition, breeding of parent voles, or size of offspring.

## Materials and Methods

### Study species

Bank voles are common rodents in boreal forest areas. Vole populations cycle in Scandinavia and specialist predators have a large role in causing this cyclicity (Hanski et al. 2001). Regular high predation pressure in the wild maintains

bank vole antipredator behavior at a high level. The breeding season of the bank vole in central Finland usually begins at the end of April and lasts until September. During the breeding season, breeding female bank voles are strictly territorial and male territories overlap with several female territories (Bujalska 1973). The gestation period is about 20 d, after which 3–6 pups are born. These pups mature after 30 d.

The least weasel is a specialist predator of small mammals and lives in the same habitat as its prey. Due to its small size, the weasel is able to hunt in tunnels and burrows of voles during both summer and winter, leaving only a few safe places for the voles (Norrdahl and Korpimäki 1995, 2000). Weasels are adapted to the harsh winter conditions by a coat change in late autumn. The weasel, like all small mustelids, uses strong odors in its intraspecific communication, giving the prey a means to evaluate the current predation risk.

The studies were conducted in the laboratory at Konnevesi Research Station in Central Finland (62°37′ N, 26°20′ E). In the laboratory, the voles are kept in light and climate-controlled husbandry rooms with a 12-L:12-D daily cycle. The animals were kept individually in 42 × 26 × 15 cm transparent cages with wire mesh lids with ad libitum water and food supply. Each cage had wood shavings and hay as bedding. Males and females were kept in the same room. Study animals were the F1 generation of individuals housed in the lab during the winter months. The average initial weight of the voles was 16.3 g ± 2.8 g (mean ± SD). All animals were individually marked with ear tags (#1005-1L1, National Band & Tag Company, Newport, Kentucky, USA).

Weasels for the odor treatment were housed individually in 60 × 160 × 60 cm cages in an outdoor shelter. Each cage had a nest box and wood shavings and hay as bedding. During the experiment, weasels were fed dead bank voles.

### Odor cues

For this experiment, the following odor cues were used:

Predator odor (PO): 1 mL of odor solution. The PO was obtained by collecting 6 dL of weasel bedding (wood shavings soiled with urine and fecal matter) and mixing it with 6 dL of diethyl phthalate (CAS 84-66-2), a solvent for a broad variety of chemical substances and often used for fragrances (Api 2001). The mix was left overnight in a refrigerator, and the liquid phase was extracted after 24 h (±2 h). The odor solution was renewed every 7 d and stored in a stable temperature of +4°C (±0.2°C) in a refrigerator in-between application. The use of extracted olfactory cues allowed for even exposure to all animals and reduced the stress to our captive weasels. Alarm pheromone (AP): 1 dL of vole beddings from individuals directly exposed to a predator. To obtain AP, two male voles were individually exposed to a weasel for 1 min every other day. Each individual was placed in a wire mesh cage, which was then put directly into the weasel cage. The animal was immediately returned to its cage afterward. When the treatments were applied, all the bedding of both animals was thoroughly mixed together. If the voles were scared on the same day the treatments were applied, the bedding was collected at the earliest 1 h after the animal returned to its cage. Social odor (SO): 1 dL of vole beddings collected from two male voles that were not handled before collection nor exposed to weasel. The bedding of both animals was carefully mixed before application. The control (C) odor consisted of clean vole bedding, that is, fresh wood shavings changed between each trial. The odor cues were renewed for each trial.

### Experimental design—behavioral assays

For the first experiment, we used 50 bank voles (28 males, 22 females). We applied two behavioral measures to study the response to olfactory cues in the voles: The first was a test measuring the individual's perceived risk using optimal patch use (Brown 1988, Lima and Dill 1990) and the other investigating spatial avoidance or preference.

Brown (1988) framed the harvest rate an animal makes at a given patch as a balance of the energetic gains and costs attributed to foraging effort, predation, and missed opportunity costs. The density of food remaining in a patch after the forager stops foraging is called a giving-up density (GUD; Brown 1999) and reflects the point where the energy remaining in the patch is equal to or outweighed by the combined costs to the forager. The GUD, as a method, has been

adapted to test a large variety of elements affecting the strategic decisions animals take (Bedoya-Perez et al. 2013) and has been widely applied as a measure for habitat use (Ylönen et al. 2002, Orrock et al. 2004, Bleicher 2017, Bleicher et al. 2018).

Each individual was placed in a 190 × 190 cm cross-shaped system (Appendix S1: Fig. S1) for three hours. At the center of the cross is a release cage (20 × 20 cm). Going outward in the four horizontal directions, the odor chamber is connected via an opaque tube (10 cm long, 4 cm diameter) to an antechamber (30 × 20 cm) with a metal grid lid. This prevented the odors entering and mixing in the central area of the maze and minimized the chances of an odor contamination. From there, going outward, an opaque tube (5 cm long, 4 cm diameter) led to a closed and opaque odor chamber (40 × 25 cm). This tube was considered as part of the odor chamber for the analysis. Each odor chamber contained one of four odor treatments together with a box acting as a foraging patch (henceforth patch). The odor cues were attached to the lid of the chamber to avoid contaminations by the vole and renewed for each trial. PO was applied to filter paper (article no. 120002, grade 1001; Munktell Filter AB, Falun, Sweden). The spatial orientation of the odors was randomly changed for each trial to avoid a spatial bias. Two mazes were used simultaneously, both were located in a dimly lit room 2 m apart. The ventilated experimental room was 7.5 × 7.5 m with the height of 4 m, allowing a large overhead space to dilute escaping odors from the systems. The experiment was performed during day time. Two trials were run simultaneously for a total of four to six trials per day. After each trial, every segment of the maze was cleaned with denatured ethanol (70%) and dried, to avoid odor contamination between trials.

The design of the patches was a lidless box (19 × 19 × 6 cm) containing 8 dL of sand into which 20 husked sunflower seeds were mixed. Each animal was allowed to forage in the system for three hours (henceforth trial). The optimal trial length was determined beforehand with pilot trials. After each trial, the sand was sieved and the remaining untouched seeds were counted to obtain the GUD. To avoid cross-contamination of olfactory treatments, the sand was

left to air out for 3 d between trials. Bytheway et al. (2013) showed that even though predator odor still elicited increased investigative behavior after 24 h, it no longer elicited a change in foraging behavior. Based on this, it seems reasonable to assume that if the voles were still able to detect the odor after 72 h, the information conveyed drastically changed. To encourage foraging in the novel systems, the animals were starved for three hours prior to each trial.

Each trial was recorded using a GoPro4 for later analysis. During the video analysis, the following parameters were measured for each of the four arms: choice of the first odor box entered, time spent in the connection tubes, and time spent in the odor box. The first hour of each trial was analyzed separately from the whole duration to account for a possible habituation effect.

*Experimental design—trans-generational effects*

The 240 bank voles (120 males, 120 females) were divided equally into four treatment groups for the second experiment. Prior to grouping the animals, every individual was weighed and the dominance of the male individuals was assessed following the urine marking of males as described by Horne and Ylönen (1996) and Klemme et al. (2006). The males were placed in the urine marking arena for 4 h and had access to a small amount of food and water. The urine markings were analyzed twice by two observers independently and the average score was recorded. Each individual received a dominance score from 1 (no marking, a subordinate male) to 6 (markings all over the arena, a dominant male).

During the group assignment, we made sure that all treatment groups consisted of an equal number of males and females, the weight distribution for each sex was similar and that the dominance distribution for each treatment was similar. Within these constraints, the animals were assigned randomly into four different husbandry rooms.

The voles were kept in the rooms for seven days to acclimate to their new husbandry rooms.

The treatments consisted of the following four odor cues (measurements per cage). Predator odor (PO): 1 mL of odor solution on filter paper (article no. 120002, grade 1001; Munktell Filter AB, Falun, Sweden), Alarm pheromone (AP):

1 dL of male vole beddings from scared individuals, social odor (SO): 1 dL of male vole beddings and control (C): 1 dL of dry, aired wood shavings. Each treatment was directly applied through the lid of the cage, without handling the animal or the cage itself. Treatments were applied three times per week for a total of seven weeks. The treatments were collected and prepared identically to what was outlined before. All animals were moved to clean cages after the mating phase. This is the standard procedure in our laboratory. It allows the pregnant female to build a nest in a cage free of the odor of a male conspecific. Furthermore, it reduces the need to disturb the female to clean its cages during pregnancy/lactation.

After the first week, the animals within the treatment were randomly paired for mating, avoiding pairing of first-degree siblings. For pairing, the animals were housed in a joined cage for seven days. From 18 d on after the beginning of the pairing, female cages were checked for pups twice per day. When litters were found each pup was weighed one day after birth and the size of the litter was recorded. The treatments were stopped as soon as all pregnant individuals had given birth. All individuals were weighed again and the dominance of the males was reassessed. The females were weighed again 5 d after giving birth. The experiment and all measurements ended at this point.

At the end of the habituation, prior to the odor treatment, fecal samples were collected from all voles for stress analysis. The voles were put individually in smaller cages without bedding for a maximum of three hours, after which all fecal pellets not contaminated with urine were collected into Eppendorf tubes then stored at −20°C. This procedure was repeated for all individuals, including nursing females, after the treatment was stopped. Corticosterone metabolites in the samples were analyzed following the method outlined by Sipari et al. (2017) at the University of Veterinary Medicine in Vienna.

*Statistical analyses*

All statistical analyses were performed in R (R Core Team 2018). Plots were generated with ggplot2 (Wickham 2009) and ggsignif (Ahlmann-Eltze 2017). To analyze the directional choice of voles as they entered the behavioral assays, a multinomial log-linear regression (MLM), package nnet (Venables and Ripley 2002), was run. This was combined with a Wald z-test to determine $P$-values, package AER (Kleiber and Zeileis 2008). In order to analyze not only the distribution of litter sizes between treatments but also the differences in successful pregnancies, and GUDs, zero augmented generalized linear models, from the package pscl (Zeileis et al. 2008) were used. The time spent in each compartment, the differences in weight, the weight of the pups, and the difference in stress metabolites were analyzed with a linear model (LM) or linear mixed model (LMM) for repeated measurements, packages lme4 (Bates et al. 2014) and lmerTest (Kuznetsova et al. 2017). Other measurements were analyzed with linear or generalized (mixed) models, depending on the measurement in question. Data points with missing observation were excluded from the data set, resulting in an effective sample size for the statistical tests of 93 breeding pairs for the breeding success part of the experiment.

For each analysis, the most complex model included an interaction between Treatment and Sex. Other factors, such as litter size and weight, were added to the most complex model if appropriate, but never in interaction with other factors. To achieve the best model fit, first the interaction was removed, then other factors, only leaving Treatment for the simplest model. The individual animal was always included as a random factor in analyses with repeated measurements. Each treatment was compared to the C (control) treatment. For each analysis, the most fitting distribution and model were chosen based on AICc, package MuMIn (Barton 2018). A model was considered the best if the difference in AICc from the next model was greater than 2.5. In the cases where there was no clear best model, all models within a ΔAICc of 2.5 were weighed based on their differences to the best fitting model and weighed averages of the parameter estimates were reported. The tables with all fitted models for each statistical test can be found in Appendix S1: Tables S1–S14.

## RESULTS

*Foraging behavior and giving-up densities*

The first choice of animals did not show a significant preference for or avoidance of entering

any specific odor compartment. However, we found a tendency (MLM, $P = 0.056$, df = 3, $n = 46$) that voles were 2.25 times more likely to enter the SO compartment first. Otherwise, it seems likely that the sex of the individual had a negligible role in the decision to enter either odor compartment of the maze, as it was not included in the best model.

In contrast to the first choice for odors, we found that over the full experimental duration of three hours there was a significant interaction between the sex of the vole and the time spent in the AP tube (LMM, $P = 0.031$, df = 10, $n = 50$, Fig. 1). When both sexes were analyzed together, female voles spent on average 2.5 min (147.5 s) longer in the AP tubes compared to the males. When the two sexes were analyzed separately, male voles did not show a preference or avoidance for the tubes (LMM, $P > 0.05$, df = 6, $n = 28$), but females spent two minutes (121.4 s) longer in the tubes connecting the AP compartment (LMM, $P = 0.027$, df = 6, $n = 22$) compared to the tubes leading to C compartment (close to four minutes, 225.2 s). For the time spent in the odor compartment, there are no significant differences for the whole trial (LMM, $P > 0.05$, df = 10, $n = 50$).

The analysis of the GUD showed that about 1.1 seeds more (weighted average) were harvested from the AP compartment compared to control independent of the animal's sex (GLMM, Poisson, $P = 0.019$, df = 5, 6, $n = 50$, Fig. 2).

### Effect on parents and offspring

*Weight change in parental generation.*—On average, female voles gained 0.92 g in weight during the experiment. However, the weight gain of females was solely dependent on the number of pups born, as for every additional pup the females gained 1.2 g of weight (LM, $P < 0.001$, $n = 93$, df = 3) and was not affected by the treatments (Appendix S1: Fig. S2).

The two best models show a significant (LM, $P = 0.023$, $n = 120$, df = 5, 6, $n = 93$) weight increase for the males in the SO treatment. The weighted average of those models indicates that the male voles in the SO treatment gained about 1.49 g more weight than males in the control group (Fig. 3). Change in male dominance may also play a role in the weight change since it was included in the second best model. Our treatments, however, did not affect male dominance (LM, $P > 0.05$, df = 5, 6, $n = 93$).

*Breeding success and offspring weight.*—During the experiment, a total of 74 litters were born from 120 breeding pairs. The analysis of the litter
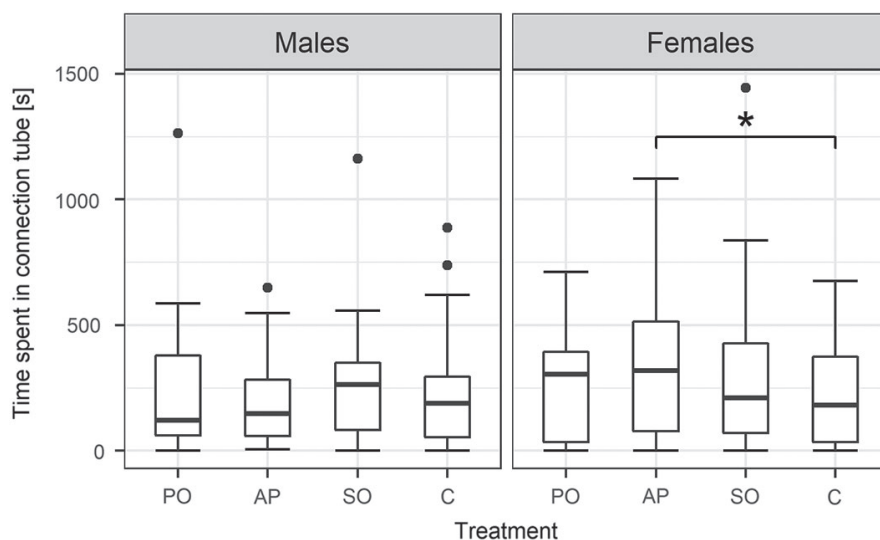


Fig. 1. Time spent in the connective tube by sex and treatment. Females reacted significantly different from males to AP ($P < 0.05$). Asterisks indicate a significant difference from C at $P < 0.05$.
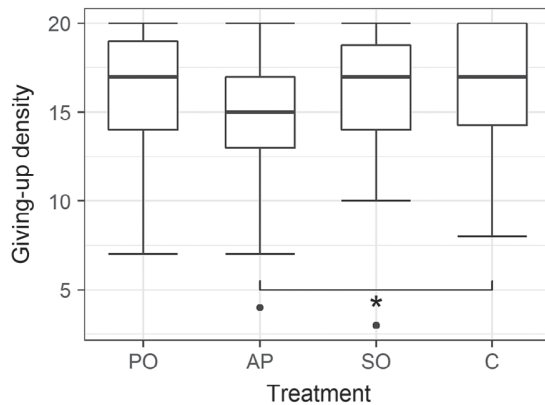
Fig. 2. Giving-up density by treatment. Asterisk indicates a significant difference from control at $P > 0.05$.



Fig. 4. Female average pregnancy rates for each treatment weighted by AICc. Whiskers show the 95% confidence intervals. Asterisks symbolize a significant difference from the control group at $P > 0.01$.

rate per treatment showed a clear best model. Significantly, more female voles gave birth under the AP treatment compared to those in the control treatment (Hurdle GLM, Poisson & Binomial, $P = 0.0095$, df = 8, 10, $n = 93$). About 36.8% of the females in the control treatment successfully gave birth, whereas about 84.5% of those under AP treatment gave birth (weighted averages, Fig. 4).

Seventy-four litters resulted in a total of 290 pups across all treatments. There were no significant differences in litter sizes between the treatment groups (Hurdle GLM, Poisson & Binomial, $P > 0.05$, df = 8, 10, $n = 93$). However, pups

from the PO group weighed about 2.81 g and were significantly heavier one day after delivery than the control pups which weighed about 2.44 g (LMM, $P = 0.026$, df = 6, 7, $n = 262$ pups, 64 mothers, Fig. 5). This was independent of litter size.

*Fecal corticosterone metabolite levels.*—From the 240 experimental animals, we non-invasively collected pre- and post-exposure fecal samples from 230 individuals. The difference of stress metabolites per 50 mg of fecal matter between the two measurements was assessed with a LM. No clear
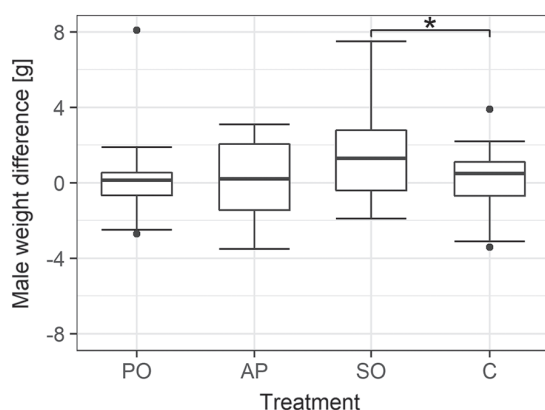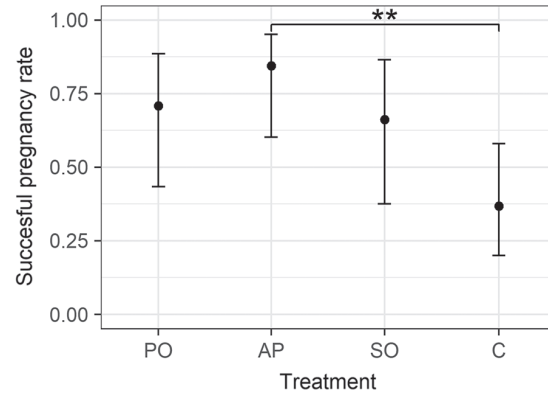


Fig. 3. Boxplots of the weight change of male voles separated by treatment. Asterisks symbolize a significant difference from the control group at $P > 0.05$.
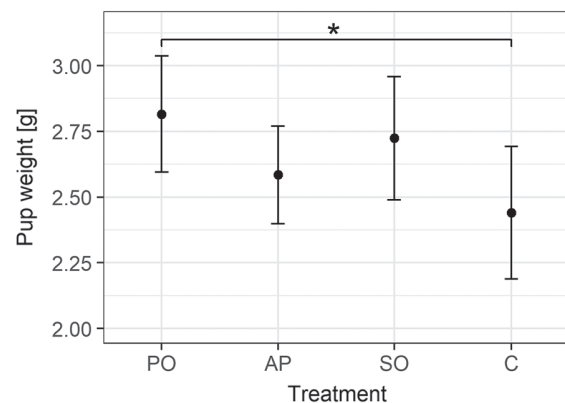


Fig. 5. Weighted (by AICc) model averages of the individual pup weight at day 1. Whiskers show 95% confidence intervals. Asterisk indicates a significant difference at $P < 0.05$.

best model was found; however, both models within the AICc range show a significant difference between the C and SO treatments (LM, $P = 0.037$, $n = 225$). In the control treatment stress metabolites rose by 82.7 ng/50 mg but in the SO treatment the metabolites only rose by 32.5 ng/50 mg (weighted averages by AICc, Fig. 6).

## DISCUSSION

Our study brings new insights into the complex system of predator–prey interactions. We propose a novel way how prey can determine predator presence and how prey change their behavior in response to olfactory cues. First, we show that the voles were able to distinguish between an ambient or conservative level risk, that is, PO (Duffield et al. 2017) and a cue of an acute or reliable risk signaling life-threatening imminent possibility of predator attack, that is, AP. Second, we also show how these different
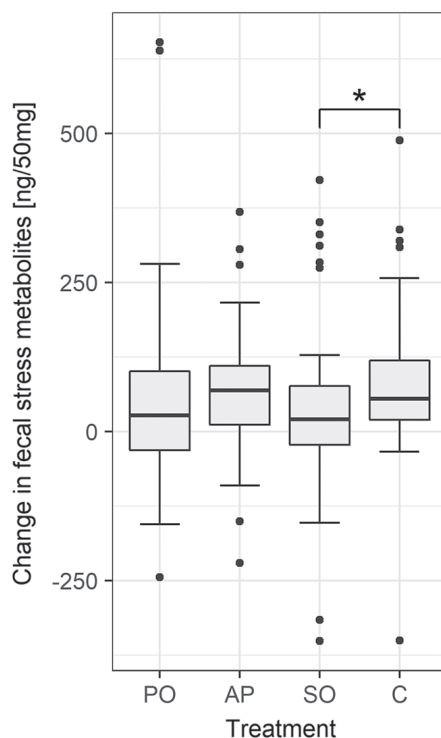


Fig. 6. Difference in fecal stress metabolites pre- and post-treatment per 50 mg fecal matter. Asterisk indicates a significant difference at $P < 0.05$.

perceived threat levels differently affected reproductive investment and success. In the AP treatment, most of the females were breeding, and in the PO treatment, voles were investing in larger pup size.

Compared to the information that a direct predator odor presents a more long-lasting habitat level risk, the acute risk information in the form of odor of a recently scared conspecific vole, seemed to outweigh in importance the weasel odor. Actually, the conspecific carried cues may include both predation risk levels, as the predator must have been close enough to scare a prey individual, who carries then the immediate threat cue to other conspecifics. Non-olfactory conspecific cues, for example, vocalizations, have already been known to convey information about predator presence (Blanchard et al. 1991, Barati and McDonald 2017, Forti et al. 2017), the perceived risk, or even the identity of the predator (Manser et al. 2002, Ouattara et al. 2009, Barati and McDonald 2017, Collier et al. 2017). Thus, in the total assessment of predation risk both cues may well be complimentary: Predator odor increases vigilance from the base level and a scared conspecific vole having survived a close encounter with a predator may signal more accurately and more rapidly for a group of conspecifics that the real danger is acute and near.

We found females treated with both PO and AP showed a positive response in their reproductive states compared with control females. This manifested itself in two major ways: (1) AP-treated females had a higher successful insemination rate, and (2) PO-treated parents had heavier pups shortly after birth. This further indicates that both predator presence cues and alarm cues from conspecifics can work at the same time, both to increase vigilance but also to trigger enhancement of reproduction in the form of terminal investment. Contradictory to our expectations derived from previous experiments (Ylönen and Ronkainen 1994, Fuelling and Halle 2004, Haapakoski et al. 2012), voles' breeding effort increased under elevated predation risk. However, our results are in accordance with Haapakoski et al. (2018) where female bank voles had larger litters in the AP-treated field enclosures compared to social odor treated females.

We cannot rule out that AP produced by our voles exposed to predator might communicate

more than just alarm. We do not know yet what kind of physiological processes are involved in the odor production of a scared individual; that is, the odor may be a combination of being scared but also relief due to being able to escape predation. This issue needs further studying. In fact, at least two studies show that male mice exposed to competitors or predators are more attractive to females. In the first one, chronic exposure of cat odor enhanced aggression, urinary attractiveness, and sex pheromones in mice (Zhang et al. 2008). In the second one, chronic co-housing with rats increased the competitiveness of male mice and their urines were more attractive to females (Liu et al. 2017). Liu et al. (2017) also found that the levels of major urinary proteins (MUP) and some volatile pheromones were increased in the co-species-housed mouse urine, along with their serum testosterone levels. It is known that MUP functions as a pheromone and stimulates sexual attraction (Roberts et al. 2010) and estrus in female mice (Marchlewska-Koj et al. 2000). We have an ongoing bioassay study for clarifying and analyzing the body odor compounds of AP voles compared to non-disturbed voles. After this information, we hope to find more answers to the role of MUP and AP on the vole reproduction.

The behavioral experiment suggested that the voles were more likely to inspect the maze arm containing the AP cue than the arm containing the control cue. In Haapakoski et al. (2018), vole females also preferred the AP odor compared to SO while males preferred SO over AP odor. This is also partly reflected in our result that female voles spent significantly more time in the maze arm leading to the AP compartment compared to males and significantly more time compared to the control. We attribute this to exploring the arms to gain information from the signal (Barocas et al. 2016, Parsons et al. 2017). In contrast, AP cue enhanced foraging compared to C, causing lower GUDs in the experimental patches containing the AP, which is suggestive of lower vigilance (Embar et al. 2011). Further, this could be a result of a heightened energetic need (Arenz and Leger 2000) and the first indicator of terminal investment.

Increasing reproductive investment despite severe negative changes in the breeding environment seems maladaptive at first glance.

However, Duffield et al. (2017) propose a new dynamic model for adaptive reproductive strategies. At low-to-medium perceived risk levels, reproduction is affected negatively, as parents invest in own survival. Above a certain threshold a coping mechanism, that is, terminal investment, would be triggered to compensate for the loss in an individual's own reproductive value. A similar idea is described with the insurance hypothesis, where individuals increase their reproductive investment in anticipation of an unfavorable environment (Promislow and Harvey 1990, Forbes 1991, Houston et al. 2012). The increased number of offspring or increase in fertility is designed to counteract expected low survival chances of offspring. While this has been mainly shown in birds (Anderson 1990a, Forbes 1990), there is also evidence in humans (Anderson 1990b, Strassmann and Gillespie 2002). As the majority of studies about terminal investment in mammals focuses on the aspect of senescence (e.g., Ericsson et al. 2001, Hoffman et al. 2010, Weladji et al. 2010), it is difficult to assess the benefits of this strategy on an evolutionary scale considering high predation pressure. We invite others to investigate this phenomenon further and to incorporate it into existing evolutionary frameworks.

For our study species, the bank vole, Eccard et al. (2011) describe a similar pattern for a dramatic increase in breeding effort after a critical threshold vole density was surpassed. Breeding bank vole females require a breeding territory and if the breeding habitat is occupied, the surplus females cannot breed. In the Eccard et al. (2011) study, the number of females was gradually increased from normal to four times the sustainable number of territory owners. As the density of females became far too high and no opportunities for an individual's own breeding territory existed anymore, all females started to breed regardless of costs (Eccard et al. 2011). This was explained by incomplete control of a social behavior (Reeve et al. 1998). Similarly, Ylönen et al. (2002) found in their study with Australian house mouse during a plague, that despite an extremely high predation risk, mice were taking high risks in exploiting food sources in open habitats with a diverse guild of predators including mammals, birds, and snakes. As the number of competitors becomes intolerably high

and food becomes scarce, risk-taking is the only solution. The authors described the desperate behavior of the mice as Stalingrad effect, after the behavior of desperate soldiers during the siege of Stalingrad in World War 2. While those two studies do not investigate the effect of predator odor exposure, they investigate the results of extreme stress situations due to crowding and social cues or crowding and direct predation risk. It is conceivable that the underlying mechanism is similar to what we found in our study in giving up a conservative strategy for risky strategies under high risk.

Levels of stress hormone metabolites rose significantly less in the SO, compared to C, while no change was observed for the two predation cues. This was unexpected as the SO, signaling competition environment, was affecting male weights as discussed below. It also contradicts our hypothesis as we expected to see a strong increase in those metabolites under both predator cues. However, as the treatment period lasted a total of seven weeks, it is possible that the treatments elicited an initial spike in corticosterone metabolites, but then adapted to the new perceived stress level, causing only mildly elevated levels due to new environment and handling. With this in mind, it was interesting to see that the SO treatment, the odor of an unstressed conspecific male, reduced the increase in stress hormone metabolites. This could be caused by social buffering, the decreased impact of stress by a social interaction. It has been shown before in rodents that the presence of an unfamiliar conspecific is sufficient to cause social buffering (Terranova et al. 1999, Kiyokawa et al. 2004*b*, Klein et al. 2015, Kiyokawa and Hennessy 2018). A potential explanation is that voles are potentially able to adapt to a prolonged period of stress, while the benefits of social interactions do not vanish with prolonged exposure.

The weight of the voles varied significantly throughout the experiment. The changes we observed suggest that there is a difference in how the sexes respond to different cues. In females, the strongest effect was offspring care— the larger the litter, the greater the weight increase. However, we also found an effect of conspecific cues. Only the odor cue of conspecific rival males caused an increase of weight for the males. The SO treatment possibly simulated a high-density environment with increasing intra-sexual competition in males. So it might be favorable for the males to invest in growth in order to outcompete competitors, as shown in Kalahari meerkats (*Suricata suricatta*; Huchard et al. 2016). It is therefore interesting that the alarm pheromone treatment, which is the odor of a stressed/anxious male vole, failed to elicit a similar response. Thus, it is possible that the addition of the external risk pheromone cancels out the effect of the social cue, as alarm pheromones are only excreted in extreme situations.

Our study contributes to the picture of how mammals with highly developed olfactory senses can interpret the information carried in olfactory signals correctly and are able to differentiate between ambient level predation risk and socially validated risk. We collected evidence that voles are able to gather information about acute risk levels from conspecific odor cues, which in turn triggered a higher successful insemination rate and heavier offspring. This is in accordance with terminal investment ideas. If a female takes the risk of investing in producing offspring (Ylönen and Ronkainen 1994 vs. Kokko and Ranta 1996), then it pays to put all efforts into breeding (Mönkkönen et al. 2009). As predator odor and alarm pheromones yield such different results, we propose that both odors provide the voles with different information, that is, the predator odor simulates a low-to-medium threat environment, but the alarm pheromone clearly represents a high and immediate threat.

## Acknowledgments

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## DATA AVAILABILITY

## SUPPORTING INFORMATION

**II**


**IN UTERO BEHAVIORAL IMPRINTING TO PREDATION
RISK IN PUPS OF THE BANK VOLE**



by

Thorbjörn Sievert, Arjane Kerkhoven, Marko Haapakoski, Kevin D. Matson, Olga
Ylönen, Hannu Ylönen 2020

**FEATURED STUDENT RESEARCH PAPER**

# In utero behavioral imprinting to predation risk in pups of the bank vole

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## Abstract

In the predator–prey arms race, survival-enhancing adaptive behaviors are essential. Prey can perceive predator presence directly from visual, auditory, or chemical cues. Non-lethal encounters with a predator may trigger prey to produce special body odors, alarm pheromones, informing conspecifics about predation risks. Recent studies suggest that parental exposure to predation risk during reproduction affects offspring behavior cross-generationally. We compared behaviors of bank vole (*Myodes glareolus*) pups produced by parents exposed to one of three treatments: predator scent from the least weasel (*Mustela nivalis nivalis*); scent from weasel-exposed voles, i.e., alarm pheromones; or a control treatment without added scents. Parents were treated in semi-natural field enclosures, but pups were born in the lab and assayed in an open-field arena. Before each behavioral test, one of the three scent treatments was spread throughout the test arena. The tests followed a full factorial design (3 parental treatments × 3 area treatments). Regardless of the parents' treatment, pups exposed to predator odor in the arena moved more. Additionally, pups spend more time in the center of the arena when presented with predator odor or alarm pheromone compared with the control. Pups from predator odor–exposed parents avoided the center of the arena under control conditions, but they spent more time in the center when either predator odor or alarm pheromone was present. Our experiment shows that cross-generational effects are context-sensitive, depending on the perceived risk. Future studies should examine cross-generational behavioral effects in ecologically meaningful environments instead of only neutral ones.

## Significance statement

We exposed bank voles to odors signaling predation risk to assess the effects parental predation exposure on the behavior of their offspring. Besides predator odor, we also assessed the role of a conspecific alarm cue as a novel way of spreading the predation risk information. Pup behaviors were assessed in the open-field arena, a standard way of assessing animal behavior in a wide range of contexts. We found that also alarm pheromone increased the time pups spend in the center of the arena similarly to predator odor. While previous studies suggested that offspring would be more fearful, our results indicate that the cross-generational effects are very context-dependent; i.e., they differ significantly depending on which scent cue is presented in the open-field arena. This shows the need for better tools or measurements to translate laboratory results into ecologically meaningful frameworks.

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Thorbjörn Sievert and Arjane Kerkhoven contributed equally to this work.

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## Introduction

Predator–prey interactions are among the strongest drivers of evolution (Abrams 1986, 2000; Yoshida et al. 2003). These effects work via multiple mechanisms, e.g., from simple acute behavioral modifications to intergenerational and long-term physiological adaptations by prey animals (Abrams 2000). Thus, in the context of an evolutionary arms race, early recognition of predation risk by prey is of paramount importance for their survival. For instance, when small mammals and raptors interact, antipredation tactics used by the prey include reliance on visual predator cues and avoidance of open areas where aerial predators can hunt most effectively (Lima and Dill 1990; Kotler et al. 1991). In predator–prey interactions between mammals, olfactory cues (and the associated sensory abilities) are more important. These cues and senses are critical to predators and prey in locating and avoiding each other in the covered or enclosed habitats that they share, including tunnels and cavities where the animals live or nest (Haapakoski and Ylönen 2013; Haapakoski et al. 2013; Ylönen et al. 2019).

The different types of cues that prey use to monitor the presence of predators can be transmitted directly or indirectly. Direct cues originate from predators. An attack is probably the most unambiguous direct cue, but predator scents also qualify. Indirect cues originate from other prey and include warning calls (Townsend et al. 2012; Collier et al. 2017), other behavioral changes (Graw and Manser 2007; Cornell et al. 2012), and alarm secretions (Verheggen et al. 2010). Indirect cues are typically the result of direct ones. For example, if a prey animal survives a predator encounter or attack, then the fear response can result in the prey producing olfactory signals, or alarm pheromones, which can alert conspecifics about predator presence (Breed et al. 2004; Kiyokawa et al. 2004). The antipredatory responses of the recipients of these indirect scent cues are expected to resemble the responses of conspecifics receiving olfactory cues directly from predators. This hypothesis is supported by recent results of behavioral and physiological studies: for example, alarm cue–induced avoidance responses (Gomes et al. 2013), analgesic responses (Kavaliers et al. 2005), and changes in reproduction (Haapakoski et al. 2018). The similar effects of indirect and direct scent cues may result from biochemical similarities between the alarm pheromones produced by prey and scents produced by predators, as reported by Brechbühl et al. (2013).

An area of research on predator–prey interactions that remains relatively unexplored in recent years relates to the transmission of information about predation risk from prey parent to offspring. Specifically, the extent to which information on predation risk is transferred by predation cue recipient mothers to their in utero offspring, i.e., cross-generational effects via in utero imprinting did not receive much attention over the last decade, but is again gaining traction with researchers. The earliest work in this area demonstrated that direct exposure (i.e., via injection into the amniotic sac) to a chemical cue in utero can be used for imprinting/conditioning of the offspring (Smotherman 1982a, b; Stickrod et al. 1982). In general, cross-generational effects could mean that pups whose mothers experienced higher than normal predation risks exhibit different development or antipredator behaviors compared with pups whose mothers experienced low risk. Mothers that were chased or even attacked by a predator while gravid might also produce pups that develop or behave differently.

A possible mechanism for such cross-generational effects is maternal hormones, whose production can be altered by stressful events. Maternal hormones can exert an in utero influence on the physiology and possibly the behavior and life history traits of pups later in life (Caldji et al. 1998; Love and Williams 2008; Sheriff and Love 2013). If unborn pups are exposed to hormones related to stress caused by high predation risk, then the pups' behavioral responses to that stressor can be programmed differently in utero compared with unexposed pups. Such effects have been demonstrated in response to a wide variety of external stimuli, including foot-shocks (Archer 1973), impoverished environments (Dell and Rose 1987), and scent cues (Champagne and Meaney 2006). Much less is known regarding the role of paternal factors, but Rodgers et al. (2013) found that lifelong paternal experience can induce germ cell epigenetic reprogramming and impact offspring HPA stress axis regulation in mice.

In this study, we compared the cross-generational effects of predation risk on pup behavior using three different scent cues: predator odor (PO), scent of frightened conspecifics (alarm pheromone, AP), and an "unscented" control with no added scent (C). Parents living in semi-natural field enclosures were exposed to the scent cues individually. Mothers were brought to the lab to give birth, and at about 5 weeks of age, pups were behaviorally assayed in an open-field arena containing the PO, AP, or C cue. We expected that if parental exposure to cues of predation risk has cross-generational effects, then we would see differences between the parental treatments regardless of the pup treatment in the open-field arena. More specifically, we expected the combined parental–pup exposure to predator odor (i.e., PO-PO) would decrease pup exploration behavior, as measured by movement in the open-field arena, more than either PO-C or C-PO (Norrdahl and Korpimäki 1998). Decreased exploration behavior would indicate a greater antipredator response (Norrdahl and Korpimäki 1998). Should there be a difference

in the effect sizes between the two experimental scent cues, then we expected PO would exert stronger effects than AP.

## Material and methods

### Study animals

The bank vole (*Myodes glareolus*) is one of the most common small rodents living in a variety of northern temperate and boreal European forest habitats west of the Urals (Stenseth 1985). The species is granivorous-omnivorous, with their diet consisting mainly of seeds and buds, and also of other plant materials or invertebrates (Hansson 1979; Eccard and Ylönen 2006). The gestation period of the bank vole is 19–20 days, and the weaning period is 3 weeks. Litter size averages 5–6 pups but ranges between 2 and 10. In Central Finland, where this work was conducted, bank voles breed three to five times per season, which lasts from May until September (Mappes et al. 1995; Koivula et al. 2003). Bank voles are preyed upon by a diverse predator assemblage, including least weasels (*Mustela nivalis*) and stoats (*Mustela erminea*) (Ylönen 1989; Meri et al. 2008). The least weasel is an especially effective hunter of voles due to their size and excellent hunting skills; least weasels are likely able to kill bank voles whenever the two species come into direct contact (Tidhar et al. 2007; Haapakoski et al. 2012).

We conducted our study at the Konnevesi Research Station in Central Finland (62° 37′ N, 26° 20′ E). In the laboratory, voles were housed in husbandry rooms under a 16L:8D light regime with a constant temperature (22 °C ± 1 °C); males and females were maintained in the same room. All animals were kept individually in 42 cm × 26 cm × 15 cm transparent cages with wire mesh lids and supplied with *ad libitum* water and food. The bedding materials in each cage consisted of wood shavings and hay. The breeding adults used in the study were the F1 generation of wild-caught individuals that were housed in the lab during the winter months preceding the study period. Winter colonies are formed from the last cohort of voles of the previous summer. Thus, their age when paired for the first breeding is around 7 months. The winter population is housed on a short photoperiod (8L:16D) at around 17 °C throughout the winter and male voles' testes are abdominal and female vaginas are closed. Only after adjusting the photoperiod to long day in spring to prepare for breeding, our voles become reproductive again. This is done starting from February when the first voles start to breed also in the field (Haapakoski et al. 2012). All animals were individually marked with ear tags (#1005-1L1, National Band & Tag Company, Newport, KY, USA).

Weasels for the scent treatment were housed individually in 60 cm × 160 cm × 60 cm cages in an outdoor shelter. Each cage had a nest box and wood shavings and hay as bedding. Throughout the experiment (and during the 2-week period before its initiation), weasels were exclusively fed dead bank voles.

### Scent cues

The predator odor (PO) was obtained by collecting used bedding materials, which included feces, urine, and body odor, from the three captive least weasels. The odor cues were always used within 1 h after being taken from the weasel cage. No odor cue was stored for later use. The alarm pheromone cue (AP) was obtained by collecting bedding materials that were used by male bank voles that were exposed to the weasels on a daily basis. During the exposure, AP "donor voles" were placed inside a wire mesh live trap, which was placed inside the weasel cage for 60 s. After the initial exposure, donor voles were placed individually in clean cages with fresh bedding materials to allow their scents to infuse into the bedding materials. The scents produced by the weasel-exposed voles were allowed to accumulate in used bedding materials following successive exposures. Bedding materials were only used as an AP cue after at least three consecutive exposures. The control odor cue (C) was clean bedding materials without any added cues from voles or weasels. In order to minimize variation in odor source, the beddings were thoroughly mixed before taking samples of bedding with urine and/or fecal matter. The same procedure was used for the clean bedding.

### Field phase

Fieldwork was conducted using nine 0.25-ha outdoor enclosures that are part of the Konnevesi Research Station in Central Finland (Haapakoski et al. 2012). The enclosures, which were emptied of other rodents by live trapping before the experiment was started, were split evenly among the three treatment groups. Each enclosure contained 25 multiple-capture live traps (Ugglan Special, Grahnab AB, Gnosjö, Sweden) arranged in a 5 × 5 grid with 10-m intervals between traps. The traps were placed inside lidded metal chimneys to protect captured individuals from exposure to the elements.

In mid-June, six females were released in each enclosure, allowing them to establish territories. Two days later, four males were added to each enclosure. After a 1-day acclimation period following the addition of the males, the traps were activated and checked two times per day. Thus, a total of 54 female and 36 male voles, all of which were 11–13 months old, were used in the field.

For every capture event throughout the full 14-day experimental period, the ID of each captured vole was recorded, and captured individuals of both sexes were exposed to scent cues according to the treatment randomly assigned to their enclosure at the start of the experiment. The voles were presented with scent cues in a treatment chamber, a tightly closing box

made of plywood with a perforated divider (i.e., a plastic wall with holes; see Suppl. material Fig. A). Two dl of PO, AP, or C bedding materials was placed on one side of the divider, an individual vole in a live trap was placed on the other side, and the chamber was closed for 3 min. Immediately after the treatment, voles were released at the site of capture. The odor cues, each in their own dedicated treatment chamber, were renewed twice per day. Recapture rate was similar in each treatment (see "Results" for more details).

From day 15 onwards, captured voles were removed from the enclosure and brought back to the laboratory where the pregnant females gave birth.

## Laboratory phase

In the laboratory, all females were checked daily for parturition from day 17 onwards. The pups were weaned, separated, sexed, and when 23 days old, ear-tagged. Pups were then caged individually in separate husbandry rooms, as described above, according to their parents' experimental treatment.

Pups from each parental treatment group (PO, AP, C) were assigned to one of three pup treatment groups (also PO, AP, or C; Fig. 1) to study cross-generational effects on behavior. Pups from the same mother were distributed among treatment groups; the sex ratios in each parental-pup treatment combination were kept similar. Once over 30 days of age ($37.7 \pm 5.5$ days, mean $\pm$ standard deviation), pups were behaviorally assayed on an individual basis in an open-field arena, which allowed for easy measurement for fearfulness and vigilance in exploratory behavior. Immediately prior to assaying a pup, 1 dl of bedding materials corresponding to the pup's treatment was spread equally throughout the arena. The arena was separated into a border zone (a total of 20 $10 \times 10$ cm squares) and a center zone (a total of sixteen $10 \times 10$ cm squares; Suppl. material Fig. B). After a pup was released in the central circle of the arena, its behavior was filmed from above for 10 min with a GoPro 4 camera. After each trial,

the bedding was removed, and the arena was thoroughly cleaned with 70% alcohol and allowed to dry.

Video analysis was done automatically with the EthoVision XT (Noldus et al. 2001) and overseen by a single researcher (AK). In the program, the different zones were marked, and the settings were calibrated separately per video. Movement frequency between the center zone and the border zone, total distance moved, time spent in the border zone, and movement bouts were measured automatically. A movement bout was defined as constant movement with less than 5 s of no movement. If an individual remained still for $\geq 5$ s, a new bout was recorded.

It was not possible to record data blind because our study involved scent cues, which are easily visually or by scent distinguishable.

## Data analysis

All statistical analyses were performed in R (R Core Team 2019), and figures were produced using "ggplot2" (Wickham 2016) and "ggsignif" (Ahlmann-Eltze 2019). Litter sizes were analyzed using a zero-augmented model in "pscl" (Zeileis et al. 2008). To analyze the potential effect of the treatments on the recapture rate per day in the field, we used the package "marked" (Laake et al. 2013). A recapture rate exceeding 100% was possible because we trapped twice per day. Two variables from the laboratory-based behavioral assay, total distance moved (cm) and time spent (s) in the border zone, were analyzed with a linear mixed model (LMM) using "lme4" (Bates et al. 2015) and "lmertest" (Kuznetsova et al. 2017). The third behavioral variable, proportion of time in center (time in center: total time), was analyzed with a generalized linear mixed model (GLMM) with a binomial distribution using "lme4" (Bates et al. 2015). The independent variables included in models examining cross-generational effects were parental treatment, pup treatment, pup sex, litter size, and pup age during the test. The initial model contained the three-way interaction of parental treatment × pup treatment × pup sex. Additionally, the ID of the
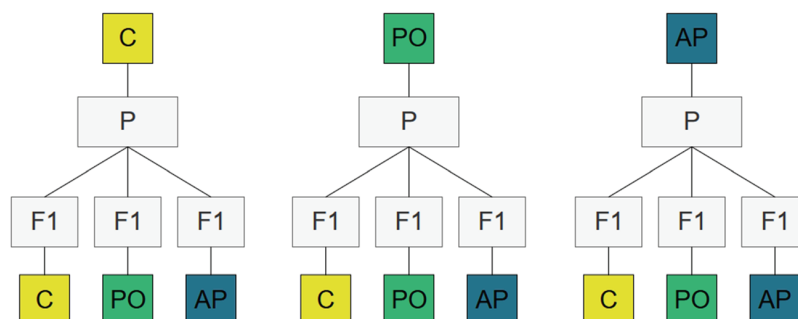


Fig. 1 Experimental design showing parental and pup treatments. All assayed pups experienced a parental treatment via their mothers (in utero) exposure and a pup treatment directly in the open-field arena: C,

control bedding; AP, alarm pheromone treatment with weasel-exposed bank vole bedding; and PO, predation odor treatment with weasel bedding

mother was included as a random factor. Non-significant interactions were discarded one by one, always starting with the three-way interaction of parental treatment × pup treatment × pup sex. The two-way interaction of parental treatment × pup treatment was always the last to be removed because this interaction was central to our research question. The full model and all models that resulted from the backwards selection procedure were ranked by AICc. If a single model was identified as best (i.e., > 2 AICc units from second best), then this model was used when reporting the significance of explanatory variables. If more than one model were identified as best (i.e., within 2 AICc units of the best model), the average $p$-values and model estimates, weighted by AICc, are reported. AICc assessment and model averages are obtained with "MuMIn" (Barton 2019) (more detailed information about the model selection procedure is provided in the supplemental material (Tables S1-S5 and R code)).

## Results

### Parents

The recapture rate of adults in the field enclosures did not differ among odor treatments but did differ between sexes. Males had a recapture probability of $116\% \pm 15\%$ per day; females had a recapture probability of $54\% \pm 16\%$ per day (weighted average ± weighted standard error by AICc). For four of 24 models, $\Delta AIC \leq 2$ (Suppl. material, Table S1). All four models included sex as a factor, and only one included treatment as a factor. The combined additive weight by AICc of the models not containing the treatment is 0.55, whereas the model containing the treatment has a weight of 0.17.

Thirty-one of 54 females were found to be pregnant: 11 C (sired $4.72 \pm 1.27$ pups, mean ± standard deviation), 10 AP ($5.2 \pm 1.14$), and 10 PO ($6.1 \pm 1.1$). Litter sizes did not differ significantly by treatment ($n = 31$, df = 6, PO vs. C $p = 0.579$, AP vs. C $p = 0.142$).

### Pups

In total, 135 pups were tested in the open-field arena. The total distance moved differed among pup treatments (LMM; $n = 135$; Table S2, $p < 0.001$, weighted average by AICc); PO pups moved significantly more than C pups (about 998 cm; weighted model average by AICc; Fig. 2). Parental treatment and litter size were not included in the best models based on AICc.

The proportion of time pups spent in the central zone of the open-field arena differed significantly depending on both parental and pup treatments separately (GLMM, $n = 135$, df = 12, Table S3; Fig. 3). Pups from control parents exposed to control bedding materials in the arena (i.e., C/C, parental/F1)
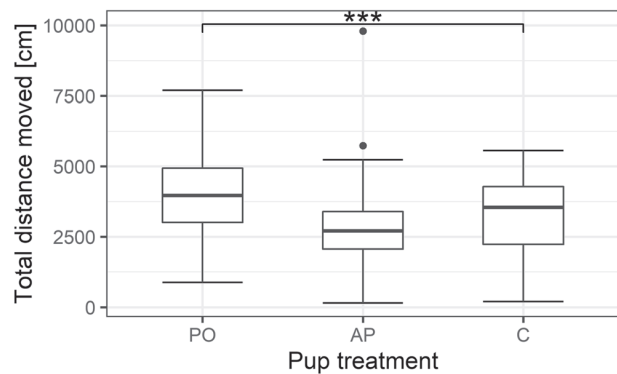


**Fig. 2** Total distance moved in the open-field arena by treatment during a 10-min period. Three asterisks indicate a significant difference from C ($p < 0.001$). Each boxplot consists of the following elements: median value (bold horizontal line), 0.75 and 0.25 percentiles (upper and lower box limits, respectively), and the highest and lowest values (upper and lower whisker ends, respectively). The boxplot whiskers are limited to 1.5 times the interquartile range. Outliers are marked as dots

spent about 18% of the time in the center zone. Some pups spent significantly less time in the center zone (C/PO decreased by 12%; $p = 0.012$); some spent significantly more (PO/C increased by 25%; $p < 0.0001$ and AP/C increased by 21%; $p = 0.0017$; all results from GLMM above). Aside from the single effects of the treatments, the interaction resulted for PO/PO in 22% of time spent in the center zone ($p < 0.0001$, the same GLMM as above). Similarly, AP/PO spent 26% of the time in the center ($p < 0.0001$, the same GLMM as above). Additionally, regardless of treatment, females spent 6% less time in the center zone than males ($p = 0.0099$, the same GLMM as above), and older pups spent less time in the center than younger ones (by about 3% per day; $p < 0.0001$, the same GLMM as above).

Lastly, the time spent in the border zone correlated negatively and significantly with the number of movement bouts in that zone (LMM, $n = 135$, Table S4; all models $p < 0.0001$; Fig. 4), while the time spent in the center zone correlated positively and significantly with the number of movement bouts in that zone (LMM, $n = 135$, Table S5; all models $p < 0.0001$; Fig. 4). For each movement bout in the border, the time spent in the border zone was 1.4 s less; for each movement bout in the center zone, the time spent in the center zone increased by 2.5 s (both by weighted average).

## Discussion

We examined the extent to which the predation experiences of parents are transmitted cross-generationally, via in utero imprinting, to their pups. Further, we examined how the environment of the pups influences the manifestation of any behavioral effects of imprinting. We did this by first exposing parents and then subsequently their offspring to one of three scent
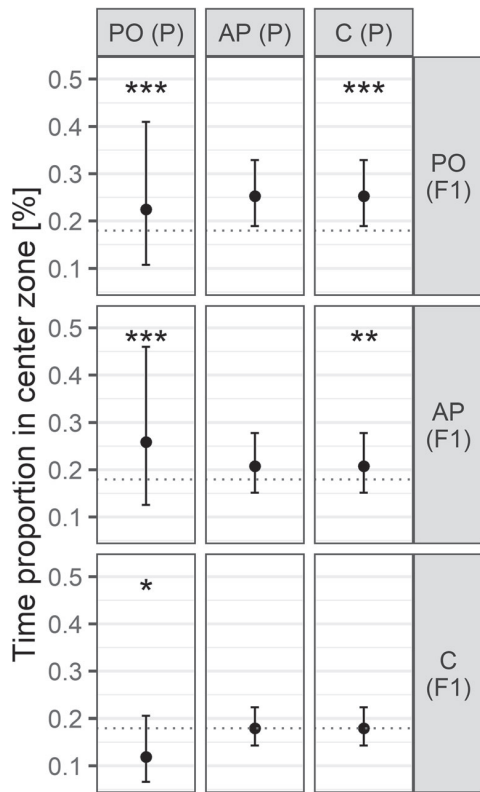
**Fig. 3** Proportion of time in the center zone of the open-field arena during a 10-min period. Columns show the parental treatment; rows show the offspring (F1) treatment in the open-field arena. One asterisk indicates significance at $p < 0.05$, two asterisks at $p < 0.01$, and three asterisks at $p < 0.001$. All comparisons are with C/C. The dotted line indicates the C/C value and serves to aide in comparisons. All model averages (dots), weighted by AICc, are shown with their respective 95% confidence intervals (whiskers)

treatments (direct predator odor, PO; indirect alarm pheromone, AP; or control, C) in a full factorial design and by testing the behavior of pups in an open-field arena. We found cross-generational effects: pups from predator odor–exposed
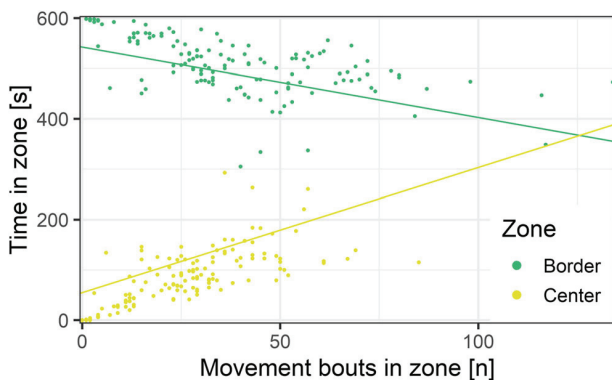


**Fig. 4** Time spent in the border (green) and center (yellow) zones in relation to the number of movement bouts per zone during a 10-min period. Each dot represents a single individual and each line represents the model estimate (weighted by AICc)

parents sought shelter along the edges of the arena when exposed to the control cue, but pups from that same parental exposure group took more risks by spending more time in the center of the arena when exposed to predator odor or alarm pheromone.

Contrary to our hypothesis based on previous research (Jędrzejewski et al. 1993; Norrdahl and Korpimäki 1998), pups exposed to predator odor during behavioral testing, regardless of their in utero treatment, did not decrease their total distance moved. In general, prey animals like voles are expected to make use of safe places within their broader environment to reduce their chances of encountering and being attacked by a predator. For example, prey can flee to a safe place to avoid a predator (Mäkeläinen et al. 2014), or they can decrease movement or even freeze in place if they are already in a safe place and predators are in the vicinity (Sundell and Ylönen 2004; Apfelbach et al. 2005). In our open-field arena, retreat to a safe place was not possible since the risk-cue was evenly distributed throughout and no hiding places or refuges were offered, which both could help explain our results.

The pups exposed to the experimental scent cues during testing may have kept moving to avoid encountering a predator (Jędrzejewski et al. 1993), but they also could have been gathering information about the odor landscape in an attempt to identify a relatively safe place (Parsons et al. 2018). In other studies (Jędrzejewski et al. 1993; Norrdahl and Korpimäki 1998), safety was offered in the form of hiding places. However, because exposure to alarm pheromone treatment during behavioral testing did not elicit the same effects as the predator odor treatment, behavioral responses to predator cues can depend solely on scent traits and olfaction and do require the activation of other senses. This might raise the question whether the method to collect AP is suitable. However, studies using a similar method (Haapakoski et al. 2018; Sievert et al. 2019) clearly showed significant changes in voles when presented with the AP cue.

Antipredator behavior is extremely complex, and split-second responses of animals may make the difference between life and death. For these reasons, an open-field arena test is certainly a simplification of the natural situation. Nevertheless, spending more time away from the center of an open-field arena is commonly interpreted as a consequence (and therefore a proxy) of anxiety (Treit and Fundytus 1988). In our study, pups exhibited significant cross-generational differences in the proportion of time spent in the center zone. While treating parents with predator odor resulted in their offspring exhibiting increased anxiety-like behavior in the control testing environment, the combination of prenatal predator odor with either predator odor or alarm pheromone in the testing environment resulted in offspring reacting less fearfully. Thus, our results indicate that cross-generational changes in behavior are highly context-dependent, while simultaneously supporting earlier cross-generational studies that

documented higher levels of anxiety-like behavior when off-spring of stressed parents are tested in neutral environments (Abe et al. 2007; Brunton and Russell 2010; Brunton 2013; St-Cyr et al. 2017). Cross-generational effects have been proposed to act as an adaptive bridge between the maternal and offspring environment (Love et al. 2013). Our study supports this view.

Increased boldness in a high-risk environment, as a result of in utero imprinting, might be adaptive. Being bold might equate to a greater willingness to explore, forage, and mate instead of waiting for the risk to abate (Ylönen et al. 2002; Korpela et al. 2011; Mella et al. 2015). In fact, over the lifespan of a short-lived rodent, predation pressure is unlikely to decline rapidly. Predator density is known to track prey density with a time lag, and this lag can mean a high predator-to-prey ratio lasts longer than the prey generation time (Hanski et al. 2001; Sundell et al. 2013).

Our experiment was designed so that the prey voles can encounter fresh predator cues daily. This design represents a situation with a high predator-to-prey ratio, such as the case when vole populations are declining. Small mammal populations fluctuate cyclically in boreal landscapes (e.g., Hanski et al. 2001), and weasel populations follow the vole density with an approximately half-year time lag (Korpimäki et al. 1991). Independently of population cycles, weasel densities during summer in deciduous forest can approach 4.5 individuals/km$^2$ (Jędrzejewski et al. 1995). Under such densities, voles likely frequently encounter weasels or signs of weasel presence, and this encounter rate is further elevated by female weasels nesting in the vicinity of voles. Finally, in addition to weasels, voles may encounter other small mustelid predators such as stoats (Erlinge et al. 1982) and pine martens (*Martes martes*), both of which produce distinctive mustelid odors (Brinck et al. 1983).

Weasel movement ecology and hunting efficiency have proven hard to study experimentally, but Norrdahl and Korpimäki (1995) estimated that weasels and stoats account for almost 80% of the total mortality for three vole species during the period of vole population decline. Though weasels are effective lethal predators, voles are well adapted to weasel presence and evasion. In a radio-tracking study in the same enclosures as used for the current experiment, we observed a clear relationship between weasel inactivity and vole activity. When weasels were resting, voles were active, regardless of the time of the day or night (Sundell et al. 2008). In addition to temporal avoidance of predation risk, bank voles also use arboreal escape routes when weasels are giving chase (Mäkeläinen et al. 2014). These different survival strategies represent scenarios in which prey voles are expected to produce AP.

Our study focused on cross-generational effects of different scent-based predation cues on offspring behavior. Using behavior as a starting point to understand possible fitness effects,

our results highlight the need for future studies to test this type of cross-generational behavioral effect in pups living in or exposed to different environmental conditions. Ultimately, the goal should be to investigate the interaction of pre- and post-natal cues about predator pressure on reproduction and survival in ecologically meaningful instead of neutral environments.

**Data availability** The datasets analyzed during the current study are available in the figshare repository, www.doi.org/10.6084/m9.figshare.8057303

The R code to analyze the data is available in the figshare repository. www.doi.org/10.6084/m9.figshare.8057276

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All experiments were performed according to applicable European, national and institutional guidelines. Procedures were conducted according to national and institutional ethical standards (permit ESAVI/6370/04.10.07/2014). Keeping weasels in captivity for experimental use was done under the permission KESELY/2022/2015.

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**III**

**PRE- AND POSTNATAL FEAR SHAPE OFFSPRING ANTI-PREDATORY BEHAVIOUR IN THE BANK VOLE**

by

Thorbjörn Sievert, Kerstin Bouma, Marko Haapakoski, Kevin D. Matson, Hannu Ylönen 2020

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# IV

# MAMMALIAN ALARM PHEROMONE CHEMISTRY AND EFFECTS IN THE FIELD

by

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