Influence of predator and food chemical cues in the behaviour of the house mouse (Mus musculus)

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Abstract

Rodent commensal species produce great damage in agriculture and urban areas. As invasive species they can endanger local species and are carriers and vectors of several important zoonoses. Control methods rely mainly on the use of warfarins, which can be inadvertently be taken up by untargeted species. Warfarins have also lost their efficacy in rodents due to the development of genetic resistance. In addition, these methods are considered inhumane as they cause a slow and painful death due to haemorrhages.

Olfaction is a main source for environmental risk assessment by rodents, and it can be used to modify their use of space. My aim in this thesis was to identify behavioural reactions of the house mouse (*Mus musculus*), using laboratory strains as models of wild animals, to ecologically meaningful chemical messages, including predator and plant chemical olfactory cues. My results showed that mice avoided complex ferret olfactory cues and ethanol which is a ubiquitous chemical related to fruit rotting and ripening. The feline protein Fel d 1, which belongs to the secretoglobin family and is a major cat allergen in humans, did not elicit significant avoidance or alter foraging behaviour in mice. However, Trimethylthiazoline purified from fox faeces, elicited clear avoidance behaviour and stress responses. I carried out a bibliographic review to evaluate and discuss rodent pest control methods from an ethical standpoint. This literature showed that many of the current methods of pest control are considered inhumane, and do not tally with current society concerns and welfare standards in other domains such as farms or laboratory animals.

These results raise new research questions to identify ferret and plant chemical compounds that can induce rodent avoidance, and to carry out next stage of research with wild animals both under laboratory and field conditions.

Key words: Semiochemicals - Rodents - Plant chemical cues - Pest control - Predator-prey interactions - ecological pest management

Résumé

Les rongeurs commensaux sont responsables de grands dommages en agriculture et dans les zones urbaines. En tant qu'espèces invasives, elles peuvent mettre en danger les espèces locales et sont porteurs et vecteurs de plusieurs zoonoses importantes. Les méthodes de contrôle sont basées principalement sur l'utilisation des warfarines, lesquelles produisent un

grand nombre d'intoxications sur des espèces non ciblées et ont perdu une partie de leur efficacité à cause des résistances génétiques constatées chez les espèces cibles. De plus, ces méthodes sont considérées comme inhumaines parce qu'elles causent une mort lente et douloureuse par hémorragies.

L'olfaction est une source principale d'évaluation des risques présents dans l'environnement pour les rongeurs, avec la perception des signaux chimiques des prédateurs ou signaux de toxicité des plants/nourriture. Cette perception olfactive peut être utilisé pour modifier l'utilisation de l'espace des rongeurs. L'objectif de cette thèse était l'identification des réponses comportementales aux messages chimiques importants (par exemple les signaux chimiques émis par les plantes et les prédateurs) dans l'écologie de la souris domestique (*Mus musculus*), avec l'utilisation de souches de laboratoire comme modèle des animaux sauvages.

Nos résultats ont montré que la souris a évité de façon significative les signaux chimiques complexes du furet et un signal chimique ubiquitaire des plantes, lié à la maturation et la pourriture des aliments (l'éthanol). La protéine du chat Fel d 1, laquelle fait partie de la famille des sécrotoglobines et est un allergène majeur du chat, n'a pas modifié le comportement d'exploration de la souris ou son comportement de recherche et de consommation de nourriture. Le composant chimique des fèces de renard, le TMT a induit un évitement clair et des réponses de stress comme cela a été rapporté dans la littérature. De plus, j'ai fait une revue de la littérature pour évaluer et discuter les méthodes de contrôle des rongeurs d'un point de vue éthique, revue qui a démontré que les méthodes actuelles peuvent être considérés inhumaines et ne correspondent pas aux attentes actuelles de la société et aux standards sur le bien-être dans d'autres domaines comme les élevages de production ou les animaux de laboratoire.

Ces résultats ouvrent des nouvelles voies de recherche afin d'identifier les composants chimiques du furet et des plantes liés au comportement d'évitement des rongeurs, les prochaines étapes utilisant des animaux sauvages à la fois en laboratoire et sur le terrain.

Mots-clés : Sémiochimiques - Rongeurs - Messages chimiques des plants - Contrôle nuisibles - Relations predator-proie - Ecological pest management

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ABBREVIATIONS AND ACRONYMS

ABP: androgen binding proteins

AD: Anno domini

Amg: olfactory nuclei of the amygdala

AOB: accessory olfactory bulb

AON: anterior olfactory nucleus

Aps: Alarm pheromones

BAOT: bed nucleus of the accessory olfactory tract

BC: Before Christ

BMR: Basal metabolic rate

BNST: Bed nucleus of the stria terminalis

CFE: cat fur extract

CNS: central nervous system

C57BL6:C57BL/6JRj mice

EC: lateral entorhinal cortex

ESPs: exocrine gland secreting peptides

FPrs: formyl peptide receptors

IPM: integrated pest management

MHC: major histocompatibility complex

MOE: main olfactory epithelium

MUP: major urinary protein

Myr: Million years

OB: olfactory bulb

OBPs: odorant binding proteins

OE: olfactory epithelium

OSN: olfactory sensory neurons

OT: olfactory tubercle

PC: piriform cortex

SO: septal organ

Swiss: RjOrl:Swiss mice

TAARs: trace amine associated receptors

TMT: 2,4,5 Trimethylthiazoline

UV: ultraviolet

VNO: vomeronasal organ

VSNs: vomeronasal sensory neurons

V1r: vomeronasal type 1 receptors

V2r: vomeronasal type 2 receptors

I Introduction

CHAPTER 1: TAXONOMY AND BIOLOGY OF COMMENSAL RODENTS

1.TAXONOMY

The order Rodentia is the largest group of mammals on earth, comprising approximately 40% of mammalian species. Approximately two-thirds of rodent species belong to the superfamily Muroidea (Guénet, Benavides, Panthier, & Montagutelli, 2015). The other third is composed of the suborders Hystricomorpha in Central and South America, which includes capybaras and guinea pigs, and Sciuromorpha, which includes squirrels.

The genus *Mus* (Linnaeus, 1758) includes 38 extant species of mice belonging to the subfamily Murinae in the rodent family Muridae (**Figure 1**). The genus can be distinguished from other murine genera using a combination of morphological features, such as the hind feet with much shorter digits; one and five. Based on morphological characters and diploid chromosome numbers, the genus *Mus* contains four subgenera: *Pyromys*, *Coelomys*, *Nannomys* and *Mus* (Guénet et al., 2015; Marshall, 1977; Veyrunes et al., 2006) and at least forty species.

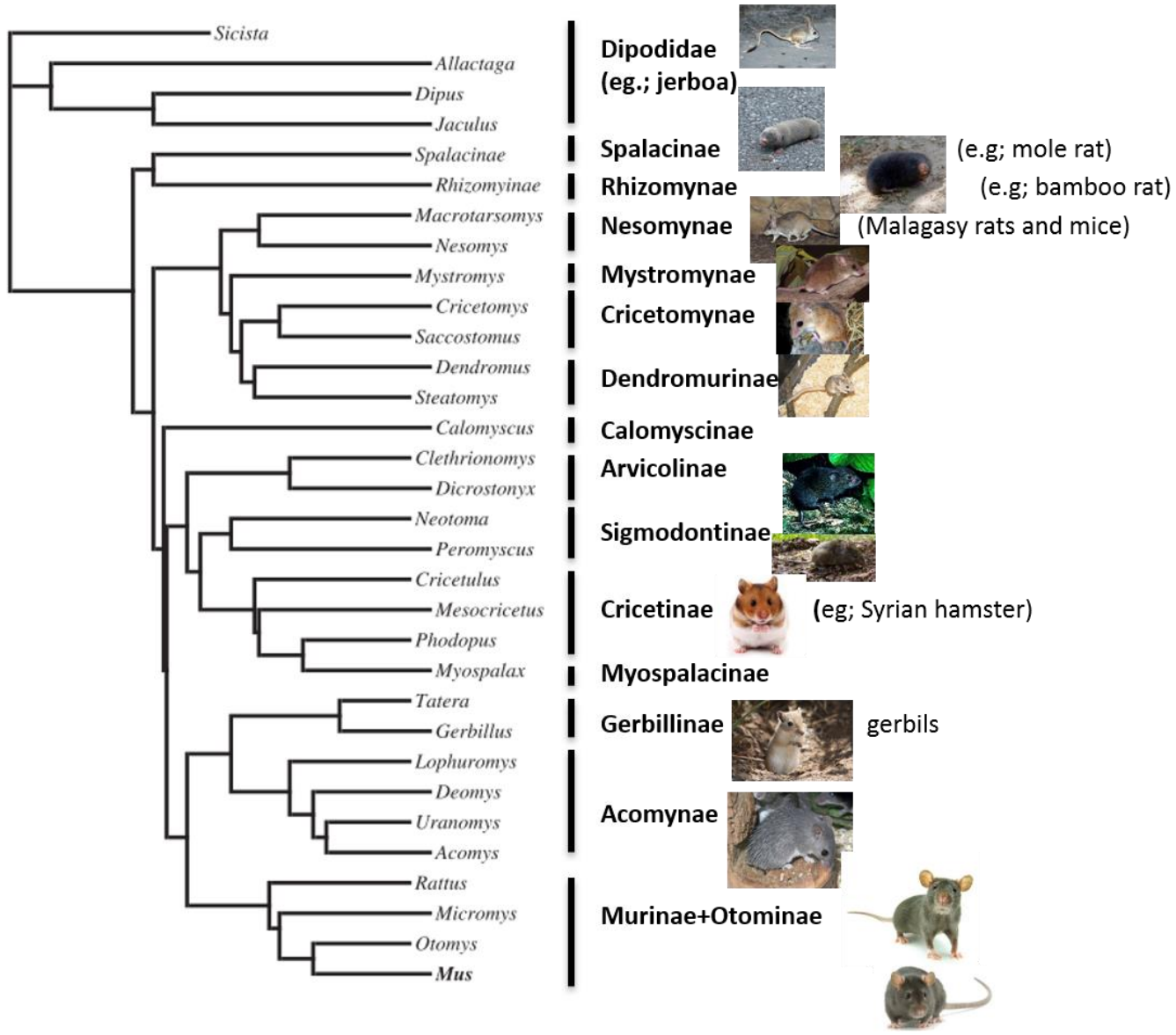


Figure 1 Phylogenetic tree representing the 14 subfamilies of the Muridae family and 32 species of rodents. (Modified from (Guénet et al., 2015) and (Michaux, Reyes, & Catzeflis, 2001)

The divergence between the genera *Mus* and *Rattus* probably occurred approximately 10–12 Myr ago, and the individualization of the subgenus *Mus sensu stricto* occurred approximately 6 Myr ago with the split from three other subgenera (Guénet et al., 2015). All research for this thesis was performed with *Mus musculus*.

2. General anatomy

Dentition and the animal's morphology enable the determination of its diet and the functioning of the animal's dentition and morphology (Ungar, 2015). Masticatory musculature of rodents has evolved to enable gnawing with the incisors and chewing with the molars. The three families of the order Rodentia, Sciuromorpha (squirrels), Hystricomorpha (guinea pigs) and

Myomorpha (rats, mice), exhibit different musculatures that allow for better gnawing in squirrels, molar chewing in guinea pigs and high generalist performance in both myomorph animals such as rats (Cox et al., 2012).

The incisors are the most evident feature of rodents. They have upper and lower pairs of ever-growing, rootless incisors (Britannica, 2017). The structure is formed with hard enamel on the front surface and soft dentine in the back that guarantees a sharp cutting edge. Between the incisors and premolars is a space called the diastema.

Generally, rodent fur is composed of short and thick hairs as well as longer hairs. The fur has varied and complex functions, such as thermoregulation by means of the isolation and position of the hairs, physical protection, sensory input, waterproofing and colouration, which is important for crypsis or camouflage (Dawson, Webster, & Maloney, 2014).

The cranium has a greatly developed masticatory apparatus, and the morphology of the skeleton is characteristic of quadruped mammals that use running for locomotion. Commensal mice and rats have long tails with a thermoregulatory function; the tail has no fur and a large surface to volume ratio that allows heat to be easily dispersed through a great perfusion of blood vessels (Hickman, 1979). The tails are also used for balance as they permit the centre of gravity to be changed and to counterbalance the position of the body (Siegel, 1970). Rodents have five digits each on the front and rear feet. The house mouse has five pairs of nipples over the ventral thorax and the abdomen, and the rat has six pairs, three in the thoracic region and three in the abdominal-inguinal region (Kohn & Boot, 2006). Rodents are capable of digesting cellulose by means of symbiotic bacteria and protozoa (Dehority, 1986); anatomically, this feature is observed with a greatly developed caecum. Many species exhibit caecotrophy.

3. PHYSIOLOGY

3.1 Circadian Rhythm

Most living beings, animals or plants change their behaviour on a daily basis (24 h) with rhythmicity. This daily rhythmicity is mainly controlled by a master clock in the suprachiasmatic nucleus of the hypothalamus (Challet, 2007). The rhythmicity is the result of the combined action of endogenous biological clocks and external time cues. In rodents, the alternation of light and dark is the main synchronizer of circadian rhythms. The synchronizers do not create this rhythmicity but modulate its parameters to help the organism adapt to and anticipate environmental variations (Benstaali, Mailloux, Bogdan, Auzéby, & Touitou, 2001). Even if light is the main synchronizer of this master clock, other stimuli are also capable of shifting this

clock. These factors can be divided into arousal-independent factors such as melatonin and GABA (the main inhibitor neurotransmitter of the CNS) and arousal-dependent factors such as serotonin (Challet, 2007). Given this endocrine plasticity, activity patterns can be adapted to needs, such as access to resources like shelter or food, avoidance of predators or avoidance of dominant individuals during feeding.

The circadian rhythms, such as the locomotor activity, are adapted to photoperiod. In rodents, two oscillators form the basis of these rhythms: “E” for evening and “D” for dusk. Thus, activity patterns are increased between dusk and sunrise. This biological feature is directly connected to sensory processes such as sight or olfaction.

3.2 Thermoregulation

Rodent size is important because it conditions the physiology, metabolic rate, and energetic needs of the animal and consequently its foraging behaviour and environmental requirements. A mouse is 10 times smaller than a rat, 10^3 times smaller than a human and 10^5 times smaller than an elephant (Schmidt-Nielsen, 1984).

Decreasing size exponentially increases the surface/volume ratio of an animal. This trend results in an increase in the surface exposed to the environmental temperature and involved in energetic exchange (Hoyt, Hawkins, St Clair, & Kennett, 2007).

An important parameter for thermoregulation is the basal metabolic rate (BMR), which measures the calories expended per square metre of body surface area or kg of body weight per hour. In mice, the BMR is 13 times higher than in horses, which means that for each gram of body mass, a mouse requires 13 times the calories needed by a horse. There is a specific environmental temperature range in which the metabolic heat generated to maintain the body temperature is optimal. This range varies by species, strain and age. In mice, it is between 29 and 34°C (Hoyt et al., 2007), which is higher than the temperatures used in laboratory animal facilities, but the difference is compensated by nesting material, which allows for better thermic isolation (Gaskill et al., 2012).

4. SENSORY ORGANS

4.1 Olfaction

Olfaction is probably the most developed sensory organ in mice. The olfactory system is composed of the olfactory epithelium, which is connected to the main olfactory bulb and the vomeronasal organ, the septal organ of Masera (SO) and the Grüneberg ganglion (GG), which

are connected to the accessory olfactory bulb. Due to the importance of this sensory organ and its special interest for this thesis, I develop this subject in more details within the section on chemical communication.

4.2 Vision

While vision is developed in rodents, it is poor in comparison to species such as hawks and humans. Absorption of a photon of light by a sensory neuron in the retina generates an amplified neural signal that is transmitted to higher-order visual neurons (Crawley, 2007). These sensory neurons can be rods or cones. The formers are mainly for night vision, and the latter are for day-light vision. The proportion of rods is significantly higher in mice and rats than in diurnal mammals. As in other mammals, rodents typically have two different pigments in the cones. Rodents have UV vision because one of the pigments has its highest absorbance approximately 359 nm, which is within the UV spectrum (**Figure 2**). Twelve percent of the cones have pigments sensitive to UV spectra, and another cone exhibits maximal absorbance approximately 510 nm (Jacobs, Fenwick, & Williams, 2001). The role of UV vision in rodents is not completely understood, but it has been suggested to function in the detection of urinary marks for social communication (Chávez, Bozinovic, Peichl, & Palacios, 2003) as the urine of some rodent species has a high degree of absorbance in the UV spectra (Hurst & Beynon, 2004).

The albino animals commonly used in laboratory animal research have decreased visual acuity. Because the iris is not pigmented, these rodents are not able to regulate the amount of light that enters the pupil.

In rodents, the eyes are positioned laterally, resulting in hemi-panoramic vision that includes a narrow central binocular zone flanked by regions of monocular vision (Priebe & McGee, 2014) (**Figure 3**). The orbit convergence (the difference in orientation between the two eyes) would be similar to that found in herbivores such as goats or cattle, but its lower position according to the size of the animal would confer a minor benefit in terms of visual depth. This wide angle of sight is typical of prey animals. As rodents are mainly nocturnal animals, the proportion of rods is significantly higher, accounting for 1 (rats) to 3% (mice) of the neural receptor cells (Jacobs et al., 2001). Vision has been demonstrated to be useful for avoiding birds of prey or other dangers to rodents; a looming shadow that increases in size triggers freezing or escape behaviours in mice (Yilmaz & Meister, 2013).

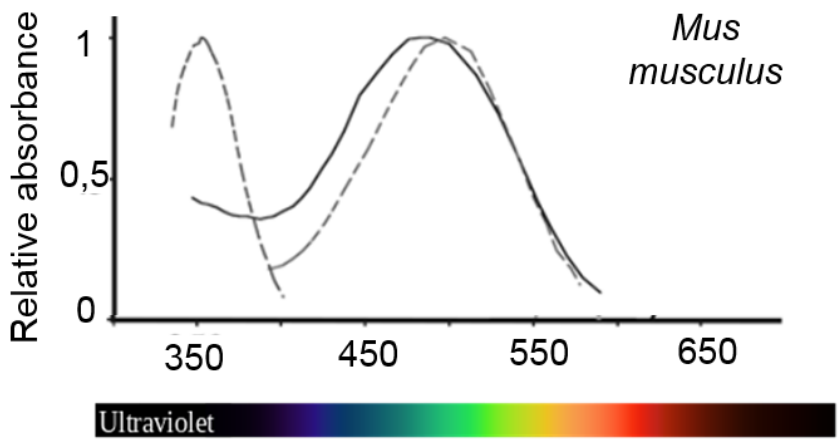


Figure 2 Sensitivity of murine eyes to light of different wavelengths. Modified from (McLennan & Taylor-Jeffs, 2004). The dotted lines are the sensitivity curves of the cones, and the solid line represents the rods

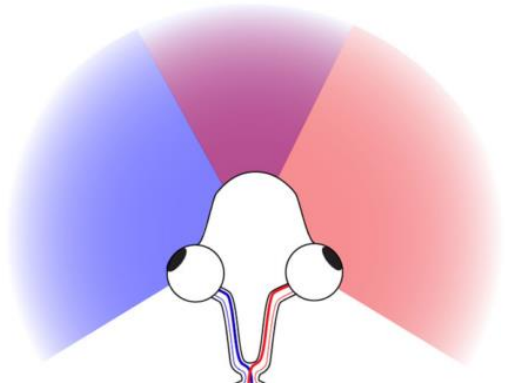


Figure 3 Mouse vision (modified from (Priebe & McGee, 2014))

4.3 Taste

This sense is mediated by a chemical transduction process similar to olfaction. Gustatory receptors are located in the taste papillae and taste buds on the surface of the tongue, and they detect sweet, salty, umami, sour, and bitter flavours to determine the identity and quality of food sources (Yarmolinsky, Zuker, & Ryba, 2009). Buds are composed of clusters of taste cells that express G protein-coupled receptors. They contain 50-120 taste cells and are located in three distinct taste papillae on the tongue, the palate and the pharynx. Two families of receptors are associated with taste: T1Rs for sweet and umami compounds and T2Rs for bitter-tasting substrates (Matsunami & Amrein, 2003). Sour molecules (acids) are detected by a membrane detector named PKD2L1, and salty molecules are detected by the membrane detector ENaC (Briand & Salles, 2016). From a structural perspective, T1Rs are similar to V2Rs in the vomeronasal organ, and T2Rs are similar to V1Rs (Matsunami & Amrein, 2003).

Distinct sets of taste-receptor proteins in specific taste cells allow organisms to discriminate between appetitive substances that are generally associated with rich nutrition and bitter-tasting substrates that are typically present in contaminated food sources (Yarmolinsky et al., 2009).

4.4 Touch

Whiskers or vibrissae are prominent sinus hairs found on nearly all mammals that act as specialized sensory organs for touch. In rodents, two kinds of vibrissae can be distinguished, the long facial whiskers (mystacial microvibissae) and the short vibrissae. The short vibrissae have been proposed to function over short distances while the long form a distance detector array that derives distance contours (Brecht, Preilowski, & Merzenich, 1997).

Active touch is used to discern the shape, size and texture of objects. Animals palpate objects during whisking behaviours that last for one second or more, and these forward and backward movements provide sensory information (Mitchinson et al., 2011) and can be repeated several times per second. The importance of whisking as a source of environmental information has been suggested to be higher for nocturnal and climbing animals; in addition, the whiskers of small mammals, such as rodents, have direct contact with the soil in contrast to larger mammals (Mitchinson et al., 2011).

4.5 Hearing

Mice and rats have well-developed hearing and can detect noises from 10 kHz to ultrasounds greater than 100 kHz. The hearing range is determined by cochlear anatomy and the physical characteristics of the head (King et al., 2015). In rats, there is some evidence that ultrasonic calls are used in echolocation and to judge the depth of drops in darkness (Latham & Mason, 2004).

Mice pups emit ultrasonic vocalizations when isolated from the nest that elicit retrieval behaviour in the mother (Portfors & Perkel, 2014).

Adult rats emit two categories of ultrasonic vocalizations, 22-kHz calls and 50-kHz calls. The 22-kHz calls express a negative, aversive state, such as alarm calls in the presence of predators or dangerous situations. The 50-kHz calls serve as affiliative and social-cooperation calls (Willadsen, Seffer, Schwarting, & Wöhr, 2014).

In mice, the role of vocalizations is less clear in adults. Adult males emit vocalizations in the presence of females and female pheromones and vice versa. These vocalizations have also been described as having a territorial function.

The term ultrasound is probably not completely accurate, as mice emit vocalizations that are audible to the human ear (personal observation), while ultrasound means a sound that is inaudible to humans. Rat and mice vocalizations seem to be related to active sniffing and are integrated into the rhythmic orofacial behaviours (Sirotin, Costa, & Laplagne, 2014) and linked to the exhalation phase.

5. ETHOLOGY

5.1 Ontogeny

Mice and rats are altricial species with incomplete development of neural and physical structures at the moment of birth, which makes them especially vulnerable to all predators at this time. They are born blind, deaf and without fur, and they are completely dependent on the mother for nutrition and thermoregulatory control (Weber & Olsson, 2008); however, pups have whiskers and the ability to process tactile as well as olfactory and thermal cues on the first day of life (Brust, Schindler, & Lewejohann, 2015). Mice open their eyes between days 12 and 14, and the first extensive activity outside the nest occurs after this moment (Fuchs, 1981); however, except when exploring, the eyes are often kept tightly closed until day 15 or 16. The ears open around day 3 and can be conditioned to auditory cues from day 4, but the inner auditory structures are not developed until day 13 (Brust et al., 2015). Pups begin to eat solid food at 17 days of age.

Once they reach adulthood, the animals leave the nest to attempt to reproduce in a process called dispersion (**Figure 4**). Male house mice and rats disperse before the females, but dispersion also depends in climate or social behaviours, such as monogamy or polygamy (Gardner-Santana et al., 2009; Pocock, Hauffe, & Searle, 2005).

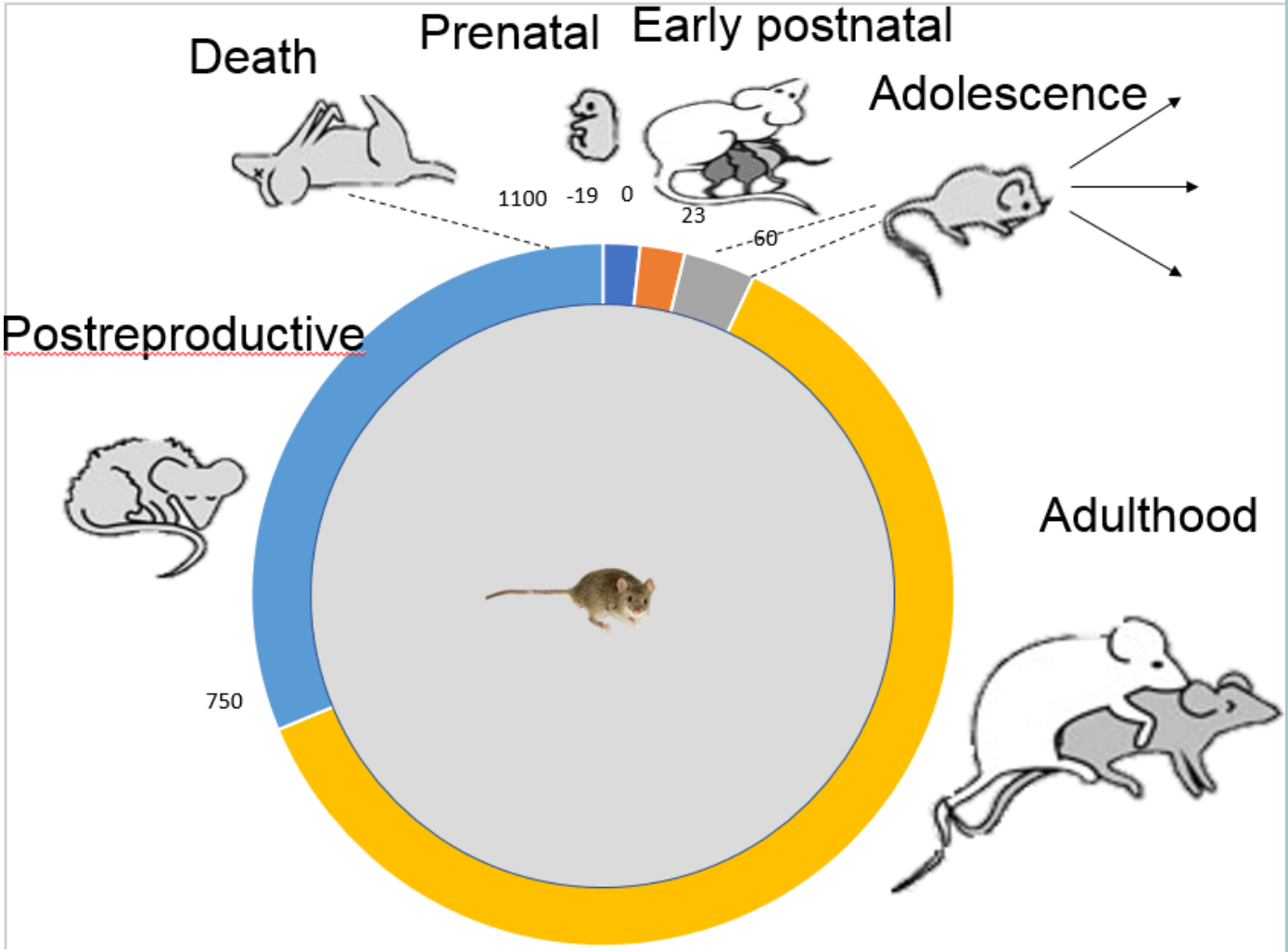


Figure 4 Mouse life cycle. Modified from Brust et al. (2015)

1. Prenatal (-19 d)

Fertilization

SRY gene expression peaks

Intrauterine environmental influences

2. Early postnatal (0 d)

Birth, ultrasonic vocalisation begins

Fur appears, ears open

Ability to be conditioned by auditory stimuli

Standing, self-grooming; full ultrasonic sound spectrum;

Olfactory function efficiently developed

Peak SRY gene expression

Vertical climbing

First agonistic traits

Inner ear structure complete, full hearing, eyes open

3. Adolescence (23 d)

3.1 Prepubescent

Weaning complete

Vagina opens (females)

Competition over food begins

3.2 Pubescent

Elongated spermatozoa present (males)

3.3 Sexually mature

Dispersal (males)

4. Adulthood (60 d)

Full adult physiology and behaviour present

Dispersal (females)

5.2 Social behaviour

In the wild, the commensal house mouse lives in a harem with a dominant male, several females with offspring, sexually immature mice, and subordinate males (Latham & Mason, 2004). Males delimitate territories with urine marks containing major urinary proteins (MUPs), which allow other males and females to identify them as individuals (Hurst et al., 2001). Non-dominant males leave a smaller number of urinary spot marks (Hurst & Beynon, 2004). Population densities vary according to resources and commensal or feral status; commensal populations can live at densities of up to 10 mice per m^2. In contrast, feral populations are less dense, up to 1 mouse/100 m^2 (Pocock et al., 2005), and spatially unstable and found in environments with a seasonally unstable food supply. Females begin to prepare nests before parturition, but nests can be constructed for both the litter and for thermoregulation, which affects both sexes.

House mice use communal nests and also seem to communally nurse their pups. The probability of survival at weaning is higher for communal nests. The male also plays an important role in rearing offspring (Weber & Olsson, 2008).

Under favourable conditions, female house mice reach sexual maturity around the age of 6-8 weeks. Their oestrous cycle varies from 4 to 6 days, and they exhibit spontaneous ovulation and produce large litters of 6-11 pups. The gestation period last up to 19-21 days. The next ovulation period begins 12-18 h after giving birth (Weber & Olsson, 2008).

Introduction of new males will trigger aggressive behaviour in the dominant male to maintain its status in the harem. Pups can be reared by other mother through fostering.

5.3 Vocalizations

Mice pups emit ultrasonic vocalizations when isolated from the nest, and these isolation calls elicit retrieval behaviour in the mother (Portfors & Perkel, 2014). Rat and mice vocalizations seem to be related to active sniffing and are integrated into the rhythmic orofacial behaviours (Sirotin et al., 2014) and linked to the exhalation phase.

In mice, the role of vocalizations in adults is less clear. Adult males emit vocalizations in presence of adult females and female pheromones and vice versa. These vocalizations have also been described has having a territorial function.

5.4 Sexual behaviour and basic reproductive physiology

Sexual maturity occurs between the 4th and 5th week in the house mouse and the brown rat, but animals are not considered adults until approximately the 8th week. In rats and mice, the female reproductive cycle is polyoestrous with cycles of 4-5 days; female rodents do not require induction to ovulate. Female laboratory mice breed until 8-11 months, and males can breed for longer, sometimes up to two years (Guénet et al., 2015). Wild mice have delayed development and are smaller in size, which also delays reproductive activity (Brust et al., 2015; Harper, 2008). The gestation period lasts for 19-21 days in mice and 21-23 in rats; the cycles are influenced by the season with decreasing fecundity during winter (Guénet et al., 2015; Lohmiller & Swing, 2006).

Mating in rats begins with vocalizations. Male mice and rats investigate the anogenital region of the female, as can be observed in other species of mammals, and a male often lifts or pushes the female with his nose. Chemosensory inputs from the main and accessory olfactory systems are the most important stimuli for mating in rodents (Hull & Dominguez, 2007).

A peculiar observation is the presence of a vaginal plug after ejaculation than can remain for 24-48 h in female mice (usually less); the probable purpose is to prevent copulation with another male. Female acceptance is indicated by a lordosis behaviour (Guénet et al., 2015; Madlafousek & Hlinak, 1977).

5.5 Trophic behaviour

Rodents generally avoid open areas and tend to feed close to cover (S. Barnett, 1967). They are generally considered important seed predators (Fedriani & Manzaneda, 2005). *Mus musculus* and *Rattus rattus* are basically herbivorous, but *Rattus norvegicus* can be considered an omnivorous species. Renal functions and food habits demonstrate that *R. norvegicus* is the most prone to thirst, whereas *M. musculus* thrives in dry habitats (Yabe, 2004). Abundance of *M. musculus* during dry periods has been noted in areas such as the Yucatan in Mexico (Panti-May, Hernández-Betancourt, Ruíz-Piña, & Medina-Peralta, 2012). Animals under laboratory conditions eat several small meals, mainly during the dark phase or at night. The three main feeding times include the first in the first few hours after the start of the dark phase, probably to compensate for the energy deficit incurred in the resting phase; the second in the middle of the night; and the final at dawn, when it is necessary to build reserves before the dangerous light phase when predation pressure is highest (Ritskes-Hoitinga & H.Strubbe, 2007).

Stomach content analysis has determined rodent diet preferences; black rats (*R. rattus*) prefer vegetables, house mice prefer arthropods and the brown rat (*R. Norvegicus*) can be classified as a typical opportunistic omnivore that can vary its diet according to the available food resources (Kurle, Croll, & Tershy, 2008; Major, Jones, Charette, & Diamond, 2007). A study in the Hawaiian Islands found that all black rats had fruit in their stomachs, and 90% had seeds. For house mice, 40% had fruit in the stomach contents, and 64% had seeds (Shiels et al., 2013). However, these species can swiftly alter their diets according to the available resources, as demonstrated by their commensal behaviour.

From an anatomical perspective, the rodent digestive tract is more complex in those that are purely carnivorous and less complex than purely herbivorous mammalian species. Rodents have a developed caecum that enables the digestion of plant material such as fibre or starches (Komárek, 2007; Lewis, Ullrey, Barnard, & Knapka, 2006). Water consumption is correlated with food consumption, but this is probably truer under laboratory conditions, in which the percentage of water in the diet is very low.

CHAPTER 2: OF HUMANS AND RODENTS AN ANCIENT HISTORY AND AN ACTUAL PROBLEM

1.HISTORICAL PERSPECTIVE

The close association of rodents with humans first began with the *M. m. domesticus* subspecies approximately 12 000 years ago in the Near East (Pialek, 2012a), when mice first exploited the niche offered by burgeoning human settlements and grain stores (**Figure 5**). Rodents have accompanied humans through trade and transport ever since, reaching a near-global distribution (Pialek, 2012a). In Europe, commercial and demographic expansions of Greeks and Phoenicians acted as vectors throughout the Mediterranean during the last millennium BC (Pialek, 2012a). The westward migrations followed two routes: the continental route (Danubian route) that led to Eastern, Central and Scandinavian Europe and the Mediterranean route that led to the Mediterranean, North Africa and Western Europe (Thomas Cucchi, Vigne, & Auffray, 2005). Before the arrival of the house mouse, this commensal niche in human societies was probably occupied by the wood mouse (*Apodemus sylvaticus* and *A. flavicollis*), which could better adapt to wild environments and already had consolidated populations (Thomas Cucchi et al., 2005). In India, scriptures dating from the 3rd millennium BC describe rodents as pests (Tripathi, 2013).

Figure 5 Profile of a stone pendant discovered in El Kowm (Syria) in the late, pre-pottery Neolithic B (7500-7000 BC.). The pendant, whose base is perforated to allow a chain to pass through, shows the head of a rodent (seen in profile view with the ears on the left and the muzzle on the right) belonging to the subfamily Murinae. ® Picture by B. Bireaud, retrieved from (T. Cucchi & Vigne, 2007)

The origins of black rat (*R. rattus*) commensalism have been proposed in different subpopulations in multiple Asiatic areas including the Himalayan region, Southern Indochina, and Northern Indochina to East Asia. The diversification occurred in the early middle Pleistocene (Aplin et al., 2011). The original natural habitat of the Norway rat is the vast plains of Asia, probably northern China and Mongolia, where rats can be still found in burrows (Hedrich, 2000). Dispersion to Europe probably occurred in the Middle Ages and was associated with trade routes, such as the land-based Silk Road and the maritime Spice Routes (Schmid et al., 2015).

In a second stage, the house mouse and commensal rat species were involuntarily transported from Europe or Asia to the Americas, Australia and other islands by maritime traffic in more recent centuries. Many genetic markers have confirmed these origins (Jones, Eager, Gabriel, Jóhannesdóttir, & Searle, 2013).

Furthermore, human activities promote the dispersal of commensal rodents by eliminating ecological barriers (deforestation and the development of agricultural lands and transportation systems) or by increasing human pressures on natural ecosystems (Cucchi & Vigne, 2007).

2. THE RODENT PARADOX; THE SAME SPECIES DIFFERENT MEANINGS: PESTS, RESEARCH SUBJECTS, PETS, AND FOOD

Commensal rodents and humans have a contradictory relationship. Without doubt, rodents are considered a primary source of knowledge in biomedical and neuroscience research. Of 106 Nobel Prizes in Physiology or Medicine, 96 depended on the use of research animals, 53 of which involved rodents (www.animalresearch.info, 2017). However, within the same laboratory animal facility, if a mouse escapes from its cage and through the door, it is automatically considered vermin, a pest (Herzog, 2010). Similarly, rodents are considered major pests in urban and rural areas, affecting industry, agriculture, networks and dwellings.

As human beings are omnivorous, they can consume rodents as a source of nutrients (Fiedler, 1990); however, with commensal species, this occurs more frequently in times of famine or after war as they are associated with disease and poor hygienic conditions. Rodents are also bred as a source of nutrients for other animal species such as pets: e.g., snakes as well as wild animals in recovery centres for local fauna and in zoos, e.g., birds of prey.

The house mouse and the brown rat are commonly found in pet shops, so humans may desire contact with these species, which is accompanied by empathy and a willingness to be in proximity with them. The common names are “fancy mouse” for *Mus musculus* and “fancy rat” for *Rattus norvegicus*. A long-standing example is the National Mouse Club in the UK

(www.thenationalmouseclub.com), which was inaugurated at the end of the 19th century (1895) to establish breed standards as can be found for other pet species such as dogs. More recently, the National Fancy Rat Society was formed in 1976 (www.nfrs.org).

Domestication and close relations with rodents occurred later than with other species, such as the wolf, as they arrived through agriculture and grain storage. Their reproductive biology characterized by short cycles and large litters allows them to be easily bred in the laboratory.

3. ZOONOSES, EFFECTS ON AGRICULTURE AND FOOD STORAGE, MATERIAL DAMAGE, AND ENDANGERED SPECIES

3.1 Agriculture/food storage

Pest rodents cause losses of 5–10% in various production systems such as agriculture, horticulture, forestry and food grain storage. In India, rice and wheat are the two main staple grains that suffer a similar extent of rodent damage. At a moderate level of 5% pre-harvest damage, the losses amount to approximately 7–8 million tonnes annually (Tripathi, 2013). On a global scale, it was recently estimated that nearly 280 million undernourished people could benefit if greater attention were paid to reducing pre-and post-harvest losses due to rodents (Meerburg, Singleton, & Leirs, 2009).

In addition to direct damage, rodents contaminate stored commodities with their hair, urine and faecal pellets, making them unfit for human consumption (Tripathi, 2013). During their active periods, rodents consume many small meals, thus contaminating a large amount of food. A rodent consumes approximately 10% of its weight per day, but the amount of food lost is much greater due to spillage and wastage that makes it unsuitable for human and livestock consumption. Commensal rat species produce approximately 40 droppings per day (Buckle & Smith, 2015), so a single individual can produce 280 pellets within a week or 14600 pellets within a year.

3.2 Physical damage to property, electrical connections and communications

Rodents must gnaw continuously to maintain the sharpness of their ever-growing incisors. They gnaw through the insulation of electrical wires, causing fires, and occasionally puncture lead pipes and concrete dams (Hegab, Kong, Yang, Mohamaden, & Wei, 2014; Nowak, 1999; Shumake, Sterner, & Gaddis, 1999); communication wires can also be damaged, which can interrupt phone or internet connections (Cogelia, 2000). Furthermore, rodents can destroy building insulation (M.Vantassel, E.Hygnstrom, M.Ferraro, & R.Stowell, 2009), consequently

increasing energy consumption for heating or cooling. Approximately 18% of telephone and 26% of electric manholes inspected in downtown Boston had evidence of rat (*R. norvegicus*) activity (E.tobin & Fall, 2004, from Colvin et al 1998).

Bajomi and Sasvari (1986) claimed that there were an estimated 2 million rats in Budapest, Hungary in the years 1978–1985 that caused US$6.4– 8.5 million worth of damage annually. A survey revealed that approximately 30% of apartment buildings were rat infested, with infestation rates at 17.2% for family houses, 15.2% for non-food-manufacturing plants, 13.3% for food-manufacturing plants and 13.1% for public institutions.

Some burrowing rodent species cause damage, water loss, and the attendant risks of flooding by excavating earthen dams, irrigation canals, or flood control structures (E.tobin & Fall, 2004).

Foraging by rodents can be a major impediment to reforestation efforts. Direct predation on seeds by deer mice (*Peromyscus* sp.) and house mice (*M. musculus*) in the USA (Noltel & Barnett, 2000) can preclude or reduce the success of direct-seeding efforts.

3.3 Endangered species

The introduction of non-native species changes ecosystems functioning, which alters material and energy flows. It is considered the second most important cause of biodiversity loss after habitat destruction and fragmentation (Vitousek 1997, from Courchamp, Chapuis, & Pascal, 2003). One of the commonly reported changes is the extinction of native species due to different ecological processes such as competition, disease, predation and hybridization (Bertolino, di Montezemolo, Preatoni, Wauters, & Martinoli, 2014). House mice have been introduced to more than 200 oceanic islands, impacting flora, invertebrates, seabirds and terrestrial birds (Angel, Wanless, & Cooper, 2009). Invasive predators are drivers of the irreversible loss of global phylogenetic diversity, affecting both mainland and island-endemic species (**Figure 6**).

Islands are delicate ecosystems, to which the introduction of new species can alter and endanger the previous equilibrium for several reasons: the simplicity of the ecosystems and the uniqueness of the species as well as a limited number of species and thus lower redundancy and fewer trophic levels, particularly the virtual absence of terrestrial top predators (Duron, Bourguet, Meringo, Millon, & Vidal, 2017). Some of the most studied islands are Australia and New Zealand, where introductions of new species with human colonization greatly endangered the local fauna and flora.

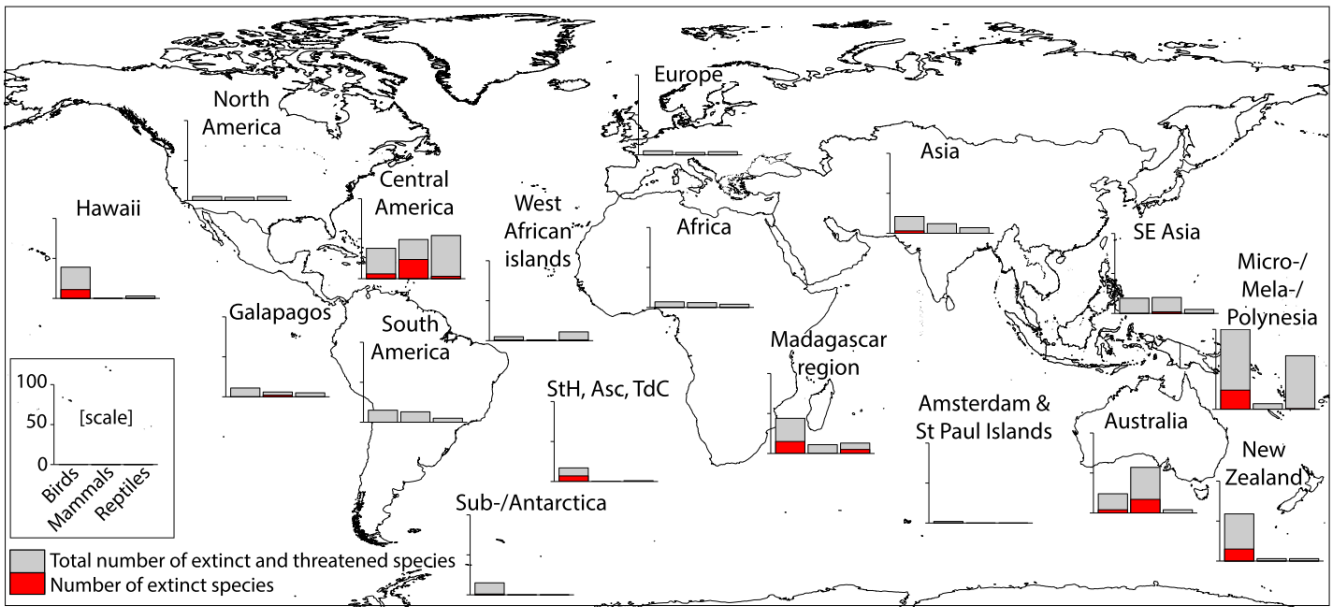


Figure 6 Numbers of threatened and extinct bird, mammal, and reptile species impacted by invasive predators in 17 regions. Grey bars represent the total number of extinct and threatened species, and red bars represent the number of extinct species (including those classified as extinct in the wild). StH, Asc, and TdC indicate the islands of St. Helena, Ascension, and Tristan da Cunha, respectively (Doherty, Glen, Nimmo, Ritchie, & Dickman, 2016)

When introduced outside their natural range, many rodents such as rats, mice, squirrels and coypu may have a detrimental impact on native species and ecosystems (**Figure 7**), requiring the implementation of control or eradication programmes (see Carter & Leonard 2002, Howald et al. 2007, Bertolino et al. 2008, Capizzi et al. 2010). A previous study linked rodents to the extinction of 75 vertebrate species (52 birds, 21 mammals, and 2 reptiles) and the endangerment of 355 species; along with cats, they have been causal factors in 44% of the modern extinctions of bird, mammal and reptile species (after 1500 AD) (Doherty et al., 2016); this study only included 5 rodent species, *M. musculus, R. argentiventer, R. exulans, R. norvegicus,* and *Rattus rattus*. *Rattus rattus* is the rodent species that has been described as affecting the most native species. However, there is increasing evidence of the effects of the house mouse, but knowledge of the effects of this species as an invader remain scarce (Angel et al., 2009; Van Aarde, Ferreira, & Wassenaar, 2004).

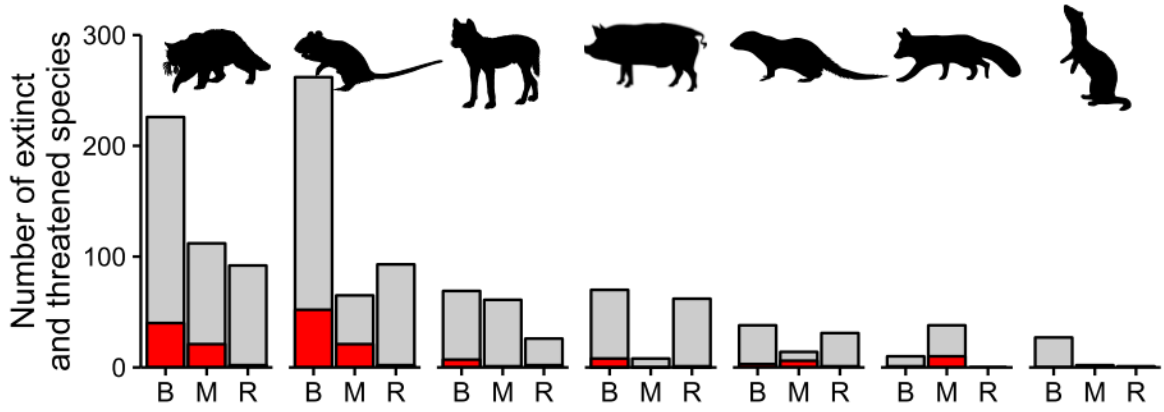


Figure 7 Number of threatened and extinct bird (B), mammal (M), and reptile (R) species negatively affected by invasive mammalian predators. Grey bars are the total number of extinct and threatened species, and red bars are extinct species (including those classified as extinct in the wild). Predators affecting <15 species are not shown. Modified from (Doherty et al., 2016)

Another important concept concerning invasive species is facilitation, as invasion by multiple species can exacerbate their individual impacts on native species. As an example, rodents provide abundant food for cats, which allows high cat densities to be maintained.

As invasive species, rodents can affect the local fauna through the spread of new parasites and competition, either directly or indirectly through interference (Courchamp et al., 2003). The global cost of virulent plant and animal diseases caused by parasites transported to new ranges and presented with susceptible new hosts is currently incalculable (Mack et al., 2000).

Humans allow invader species to colonize new territories through transport, but they also facilitate settlement by providing refuges and food resources until the new population has developed (Mack et al., 2000).

3.4 Health, sanitary issues and zoonoses

Rodents carry and transmit a vast array of diseases to humans and their domesticated animals (Meerburg, Singleton, & Kijlstra, 2009a) (**Table 1**). In the fourteenth century, one of the most famous episodes related rodents and human health occurred, the bubonic plague (the so-called Black Death), that killed between a quarter and a third of the population of Europe within just a few decades (Gage & Kosoy, 2005).

The last pandemic between 1896 and 1911 in India left more than seven million dead (Ginsberg & Faulde, 2008).

Currently, sanitary problems are especially relevant in poor communities, where hygiene and infrastructure (houses, roads with puddles, and sewage and garbage treatment) need improvement, and publics sanitation systems are inexistent or highly deficient (Meerburg, Singleton, & Leirs, 2009).

Rodents can spread diseases by two pathways.

The direct pathway. Rodents can spread pathogens to humans directly by biting, faecal-oral transmission through food or water contaminated with faeces or urine, and respiratory pathways (hantavirus). The faecal viral flora of wild rodents can contain numerous viruses capable of causing human diseases, and analysis of this flora has been described as useful for the prevention and control of outbreaks (Phan et al., 2011).

In terms of epidemiology, rodent bites mainly affect children younger than 15 years as they sleep. One study fixed the median age for children in Philadelphia as 5 years and below (Hirschhorn & Hodge, 1999), while another study in New York found the age to be less than 15 years (Childs et al., 1998). The majority of bites were inflicted on the face and hands and occurred in the bedroom during sleep (Hirschhorn & Hodge, 1999). Both studies highlighted the link between more affected areas and the deterioration of the structures where the bite occurred as well as the adjoining structures.

We can highlight some diseases transmitted by the direct pathway as follows:

Hantavirus pulmonary syndrome (HPS) is caused by several strains of viruses in the *Hantavirus* genus of the Bunyaviridae family. It is considered one of the most dangerous rodent zoonoses as it is transmitted by a wide spectrum of transmission ways including inhalation of aerosolized particles, rodent bites, or direct contact with rodent droppings or urine (Meerburg, Singleton, & Kijlstra, 2009b). It results in a mortality rate of 30-40%, and no treatment currently exists against this pathology.

Haemorrhagic fever with renal syndrome (HPRS) is also caused by the *Hantavirus* genus of the *Bunyaviridae* family, which causes a group of similar illnesses throughout Eurasia and adjacent territories. Approximately 150 000 cases are reported annually (Vapalahti et al., 2003). Transmission is mainly through the inhalation of aerosols of infectious viruses from rodent urine, faeces, and saliva, and the fatality rates range from 5 to 10% (Meerburg, Singleton, & Kijlstra, 2009b).

Lymphocytic choriomeningitis is produced by the lymphocytic choriomeningitis virus (LCMV) from the *Arenovirus* genus, and in humans, the disease is contracted by aerosol dispersion, that is, breathing air contaminated with rodent excrement, especially from *Mus musculus*. The disease can produce intrauterine infection in humans. Infections have been reported in the

Americas, Europe, Australia and Japan. Seroprevalence studies have shown an occurrence between 2-5% in humans (Meerburg, Singleton, & Kijlstra, 2009b).

Lassa fever is an acute viral illness that is endemic to West Africa, and it has been isolated in the multimammate rat (*Mastomys natalensis*). Humans infection can occur through aerosol transmission with air contaminated with rodent excrement or direct contact with rodent droppings or urine. Annual infections in West Africa are estimated between 100.000 and 300.000, with approximately 5000 deaths and a mortality rate between 5-10%. There is no vaccine against this virus (Khan et al., 2008; Yun & Walker, 2012).

Leptospirosis

Rodents are carriers of spirochetes of the genus *Leptospira* and are important infection reservoirs for both humans and domestic animals. Humans acquire infection through the consumption of food or water contaminated by rodent urine or by contact with soil or water contaminated with rodent urine through the skin or mucous membranes. Handling of dead infected rodents can also transmit the disease. Its prevalence is higher in the humid tropics; rice farmers in the Philippines are especially concerned.

The indirect pathway. Rodents can serve as hosts during part of the life cycle of pathogens that can later be transmitted by means of ectoparasitic arthropod vectors (ticks, mites, fleas) (Meerburg, Singleton, & Kijlstra, 2009a). *Yersinia pestis* is probably the most well-known example; the disease produced by this bacterium is commonly known as plague in English (la peste in French and Spanish), a word that also means an epidemic disease that causes high mortality (Oxford, 2010).

Other important examples transmitted via the indirect pathway are as follows:

Lyme disease

Rodents are the main reservoirs of spirochetes from the genus *Borrelia*, which causes Lyme disease. This disease accounts for more than 90% of all vector-borne disease in the United States with 30.000 cases per year compared to 60.000 in Europe (Radolf, Justin D. Caimano, Melissa J. Stevenson & Hu, 2012), and rodents play an important role in spreading spirochetes of the genus *Borrelia*. The cycle of Lyme disease continues with ticks that transmit the spirochetes to humans.

Chaga's disease

Rodents also play a major role in the transmission of Chaga's disease as they are reservoirs of the protozoan parasite *Trypanosoma cruzi* (Bern, Kjos, Yabsley, & Montgomery, 2011).

Eight million people are infected with this parasite, 20-30% of whom can develop potentially life-threatening symptoms.

Table 1 Modified from Meerburg et al. (Meerburg, Singleton, & Kijlstra, 2009a). Overview of pathogens that can be transmitted to humans by rodents

Disease	Agent	Carrier/Reservoir	Population at-risk	Chance	Human Health	Economy
Hantavirus Pulmonary Syndrome	Virus, Bunyaviridae	Carrier	2	1	3	1
Hemorrhagic Fever with renal syndrome (+ other hemorrhagic fevers)	Virus, Bunyaviridae	Carrier	2	2	2	2
Nephropathia epidemica	Virus, Bunyaviridae	Carrier	1	1	1	1
Crimean-Congo hemorrhagic fever	Virus, Bunyaviridae	Reservoir	1	1	3	1
Borna disease	Virus, Bornaviridae	Reservoir	1	1	1	2
Omsk hemorrhagic fever	Virus, Flaviviridae	Reservoir	1	1	1	1
Kyasanur Forest Disease	Virus, Flaviviridae	Reservoir	1	1	1	1
Apoi Virus Disease	Virus, Flaviviridae	Unknown	Unknown	Unknown	Unknown	Unknown
Tick-borne encephalitis	Virus, Flaviviridae	Reservoir	2	1	3	1
Powassan encephalitis	Virus, Flaviviridae	Reservoir	1	1	1	1
Lymphocytic Choriomeningitis virus (LCMV)	Virus, Arenaviridae	Reservoir	1	1	1	1
Lassa Fever	Virus, Arenaviridae	Carrier	2	2	3	2
South American arenaviruses (Junin, Mapucho etc.)	Virus, Arenaviridae	Carrier	2	2	3	1
North American arenaviruses	Virus, Arenaviridae	Carrier	1	1	Unknown	Unknown
Colorado Tick Fever	Virus, Reoviridae	Reservoir	1	1	1	1
Venezuelan quine encephalitis	Virus, Togaviridae	Reservoir	2	2	2	2
Western equine encephalitis	Virus, Togaviridae	Reservoir	1	1	1	1
Hepatitis E	Virus, Caliciviridae	Reservoir	1	1	1	1
Cowpox	Virus, Poxviridae	Reservoir/ carrier	1	1	1	1
Contagious viral animal diseases (Classical Swine Fever, Foot and Mouth Disease)	Virus, Picornaviridae (FMD); Flaviviridae (CSF)	Reservoir?	0	1	0	3
Leptospirosis (Weils' disease)	Bacteria, Spirochaetes	Carrier	2	2	3	2
Lyme disease	Bacteria, Spirochaetes	Reservoir	3	2	1	2
Tick-borne relapsing fever	Bacteria, Spirochaetes	Reservoir	2	1	1	1
Scrub typhus	Bacteria, Alphaproteobacteria	Reservoir	2	1	3	1
Murine typhus	Bacteria, Alphaproteobacteria	Reservoir	3	1	1	1
Sylvatic epidemic typhus	Bacteria, Alphaproteobacteria	Reservoir	1	1	1	1
Queensland tick typhus or spotted fever	Bacteria, Alphaproteobacteria	Reservoir	1	1	1	1
Rocky Mountain spotted fever	Bacteria, Alphaproteobacteria	Reservoir	1	1	3	1
Rickettsialpox	Bacteria, Alphaproteobacteria	Reservoir	2	1	0	1
Bartonella Illnesses	Bacteria, Alphaproteobacteria	Reservoir	2	2	1	1

Disease	Agent	Carrier/Reservoir	Population at-risk	Chance	Human Health	Economy
Human granulocytic anaplasmosis	Bacteria, Alphaproteobacteria	Reservoir	2	1	1	1
Q-fever	Bacteria, Gammaproteobacteria	Reservoir	3	2	3	2
Salmonellosis	Bacteria, Gammaproteobacteria	Carrier	3	1	1	3
Tularemia	Bacteria, Gammaproteobacteria	Carrier	2	1	3	1
E. coli 0157/VTEC	Bacteria, Gammaproteobacteria	Carrier	2	1	3	2
Plague (*Yersina pestis*)	Bacteria, Gammaproteobacteria	Reservoir	2	2	2	2
Campylobacteriosis	Bacteria, Epsilonproteobacteria	Carrier	3	1	1	3
Rat-bite fever and Haverhill fever	Bacteria, Fusobacteria	Reservoir	2	1	3	1
Listeriosis	Bacteria, Bacilli	Carrier	3	1	3	2
Toxoplasmosis	Parasite, Sporozoea	Reservoir	3	2	2	3
Babesiosis	Parasite, Sporozoea	Reservoir	3	2	1	1
Cryptosporidiosis	Parasite, Sporozoea	Reservoir	3	2	1	3
Chagas disease	Parasite, Zoomastigophorea	Reservoir	3	1	3	2
Leishmaniasis	Parasite, Zoomastigophorea	Reservoir	3	2	3	2
Giardiasis	Parasite, Zoomastigophorea	Reservoir	3	2	1	2
Taeniasis	Parasite, Cestoda	Reservoir	1	1	1	1
Rodentolepiasis	Parasite, Cestoda	Reservoir	1	1	1	1
Echinococcosis	Parasite, Cestoda	Reservoir	2	1	3	1
Schistosomiasis	Parasite, Trematoda	Reservoir	3	2	1	3
Human fasciolosis	Parasite, Trematoda	Reservoir	3	1	1	3
Brachylaimiasis	Parasite, Trematoda	Reservoir	1	1	2	1
Alariasis	Parasite, Trematoda	Reservoir	1	1	0	1
Echinostomiasis	Parasite, Trematoda	Reservoir	1	1	0	1
Trichinosis	Parasite, Nematoda	Reservoir	3	2	1	2
Capillariasis	Parasite, Nematoda	Carrier	3	1	1	1
Angiostrongylosis	Parasite, Nematoda	Reservoir	2	1	3	1
Toxascariasis	Parasite, Nematoda	Carrier	1	2	0	2
Baylisascariasis	Parasite, Nematoda	Carrier	1	2	1	2
Aelurostrongylosis	Parasite, Nematoda	Reservoir	0	0	0	0
Amoebic dysentery	Parasite, Lobosea	Reservoir	3	1	3	1
Neosporosis	Parasite, Conoidasida	Reservoir	0	1	0	2

Reservoir: rodents harbor disease-causing organisms and thus serve as potential sources of disease outbreaks, but always via a vector (tick, sand-fly etc.)

Carrier: rodent that shows no or limited symptoms of a disease but harbors the disease-causing agent and is capable of passing it directly onto humans

Population at-risk: focal = 1, regional = 2, more than 2 continents = 3

Chance: chance of contracting the disease (all pathways, not only via rodents): small chance = 1, moderate chance = 2, high chance = 3

Human health: Mortality without treatment <5%=1, 5 to 10% = 2, >10% = 3. No mortality = 0.

Economy: losses in terms of morbidity combined with other losses (e.g. in animal productivity): small losses = 1, moderate losses = 2, huge losses = 3.

CHAPTER 3: RODENT ECOLOGY

1.ECOLOGY OF COSMOPOLITAN RODENT SPECIES

Urbanization creates sets of patches in cities that vary in size and quality. These patches comprise industrial and commercial buildings, residential dwellings, sewers, subways, and natural, semi-natural and sport fields (Gomez, Provensal, & Polop, 2009). Given the unprecedented rates of global urbanization (half of the global population resides in urban areas), commensal rodent infestations and the associated problems will only increase in the future. In 2014, 54% of the global human population will reside in urban areas, and it is estimated that number will increase to 66% by 2050 (United Nations, 2014).

In many places, feral house mice are excluded from field margins due to competition from small mammals such as wood mice (*A. sylvaticus*), so feral house mice are only able to persist in open agricultural and natural habitats with no or few competitors. For this reason, feral house mice are found throughout Australia and New Zealand, but they are generally restricted to isolated islands in Europe and North America (Pocock, Searle, & White, 2004).

In Europe, the black rat (*R. rattus*) has been described as nesting aerially in agricultural crops; as an example in the region of Valencia, in the east of Spain, nests have been encountered in orange trees, olive trees and others (Faus, 1982; Faus & Vericad, 1981, Grau,2016 personal observation).

1.1 Dwellings

Domestic mouse infestations are most likely to accompany poor structural maintenance, poor hygiene and ample internal harbourage.

The density of housing is important, as the higher density of homes in an area, the more likely it is that rodents infesting one home will disperse and colonize the surrounding dwellings. This dispersion is more successful over shorter distances (Ginsberg & Faulde, 2008).

A study in New York found higher levels of mouse infestations in apartment buildings compared to commercial and food establishments. Well-maintained structures and environments had significantly lower rates of mouse infestation (Advani, 1995).

1.2 The underground

Human food and diverse types of solid waste attract rats, mice and other organisms in the underground. These organisms move endlessly upward into human streets and buildings, searching for resources or avoiding dangers such as humans or predators.

For rodents, their use of the urban underground mainly involves sewers, but this environment can be extremely complex, as it is a source of shelter, complex labyrinths and escape routes (**Figure 8**). Rodents also access pedestrian walkways, quarried limestone tunnels (subway, train) and catacomb networks (Forman, 2014). Metro and train stations are sources of food and numerous shelters.

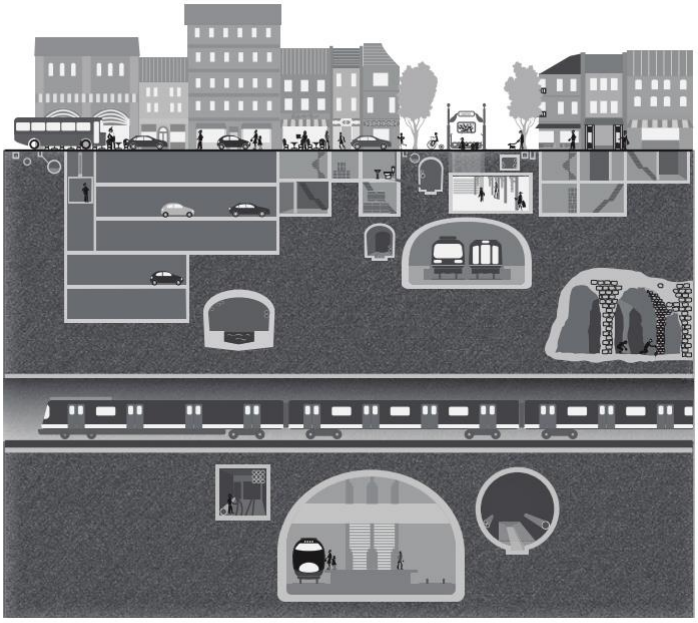


Figure 8 Underground structures at different levels in a city that are mainly based on the extensive, diverse and longstanding underground in Paris. Lower: intercity train, stormwater drain, and electric power system subway. Middle: wastewater system, stormwater system, telephone cable system, clean water supply, heating/cooling pipe system, natural gas supply, and shopping arcade walkway (modified from Clement and Thomas, 2001; in Forman, 2014)

1.3 Sewers

Urban sewers are perfect human-rat habitats because they minimize temperature fluctuations, with cooler conditions in the summer and warmer conditions in the winter and provide a stable flux of food and waste. Additionally, they provide good protection against predators, greatly diminishing or completely negating that risk. These factors contribute to a continual breeding regime without seasonal fluctuations (Ginsberg & Faulde, 2008). The importance of sewer

systems as shelters for rats raises the need for developing control protocols for sewers (CIEH, 2013).

Rats typically do not live in active drains or sewers but instead live in disused pipes in excavations adjacent to cracks or bad joints in pipelines, in dry parts of the network (benching) and in manholes and inspection chambers.

1.4 Movement

House mice normally will not move more than 3-10 m within buildings. The species has been recorded as travelling as far as 2 km, but this is unusual. Blocks of houses can represent individual breeding units as the migration rates between blocks is very low (Ginsberg & Faulde, 2008).

Rats, particularly the brown rat, also do not normally move great distances, especially in urban areas where streets act as barriers. The diameter of the normal home range of the brown rat varies from 25 m to 150 m (Grzimek, 1975). This may not be the case in rural areas, where rats have been reported to move as far as 3.3 km at speeds of 0.5–1.1 km an hour in one night (Taylor & Quy, 1978).

2. CHEMICAL ECOLOGY AND COMMUNICATION IN RODENTS

2.1 Some basic principles

Chemical communication is the most ancient and widespread form of communication in the living world. From intracellular messengers such as mRNA to intercellular connections, neurotransmitters, hormones, individual recognition, transmission of physiological status, all share the same basic components: an emitter of the message, a chemical code (organic or inorganic chemical, protein or mixture of compounds) and a receptor in cellular membranes that will elicit some biological response.

Mice are mainly nocturnal animals that primarily physically keep their noses to the ground. This means two things: the poor information received by sight enhances the value of chemical reception, and the physical positioning facilitates the perception of heavy molecules such as peptides or proteins that can remain attached to the floor and have a lower probability of being transported from the original point.

2.2 Implied anatomical structures

Classically, the anatomical structures implied in chemical communication in rodents include the vomeronasal organ, or Jacobson's organ, as a specialized system that could mediate social, sexual and interspecific interactions.

It was once thought that there is a clear border between the odours associated with learning and the MOE and the innate odours associated with the VNO that could elicit innate responses, however these limits are much more diffuse (Beny & Kimchi, 2014; Griffiths & Brennan, 2015; Turner, Turner, & Lappi, 2006; Xu et al., 2005). Actual evidence indicates that chemical reception in rodents is mediated by four olfactory subsystems consisting of two main structures, the VNO and the MOE, and two smaller structures, the Grüneberg ganglion and the septal organ of Masera. All these structures are physically and physiologically connected with the respiratory system, specifically to the upper respiratory tract beginning with the nostrils and extending to the nasopharynx (Hoyt et al., 2007), as well as indirectly connected to the oral cavity through the incisive or nasopalatine ducts.

2.2.1 Anatomy and histology of the rodent nose

The upper respiratory tract in rodents is comparatively complex when compared, for example, with that of humans. The air enters the vestibule and the nasal valve through the nostrils and reaches the main chamber, which is divided into two symmetrical compartments (**Figure 9**). Once inside, the air travels between the septum and the medial surface of the nasal maxilla and ethmoturbinates. Posterior to the termination of the nasal septum, the air passages emerge as one and travel downward from the nasopharyngeal meatus into the nasopharynx (Reznik, 1990).

The upper respiratory tract has three main kinds of epithelia: squamous, respiratory and olfactory. They transition smoothly from anterior to posterior with the squamous epithelium in the inner part followed by the respiratory epithelium and finally the olfactory epithelium (Gross, Swenberg, Fields, & Popp, 1982). This smooth transition is disrupted by the olfactory epithelium of the septal organ of Masera, the respiratory epithelium of the nasopharynx, and the dual olfactory and respiratory epithelium of the VNO. Olfactory sensory neurons (OSNs) situated in highly stimulated regions have longer cilia and are more sensitive to odorants than those in weakly stimulated regions. Sensory experience and neuronal activity are not required to establish and maintain the cilia length pattern.

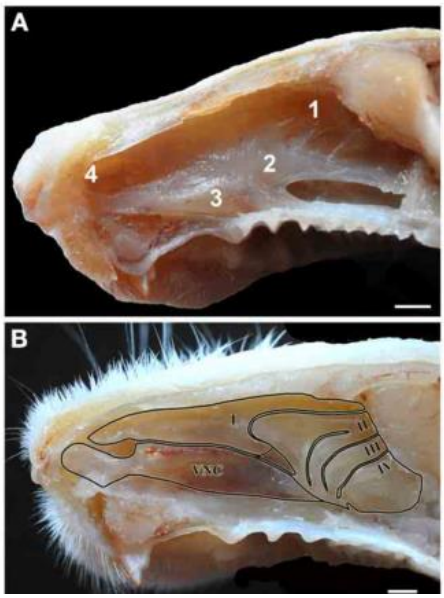


Figure 9 Two views of the nasal cavity: A, the nasal septum and the locations of the main olfactory epithelium (1), the septal organ (2), the vomeronasal organ (3) and Grüneberg's ganglion (4); B, medial view of the nasal conchae and ethmoturbinates following removal of the nasal septum. Modified from (Barrios, Núñez, Sánchez-Quinteiro, & Salazar, 2014)

The upper airway is also the path for chemical communication without the implication of the olfactory receptors and the sensory epithelium. Molecules can pass directly into sanguineous circulation due to the high vascularisation of the respiratory mucosa (Grassin-Delyle et al., 2012), as has been demonstrated by nasal drug administration. Another path is passive diffusion through slow transport to the neural cells or faster transport along the perineural space surrounding the olfactory nerve cells into the cerebrospinal fluid surrounding the olfactory bulbs and the brain. This route has been described as a possible path for lymphatic drainage, and it has been demonstrated by the passage of ink from the cerebrospinal fluid to the nasal mucosa and the cervical ganglions (Kida, Pantazis, & Weller, 1993; Walter, Valera, Takahashi, & Ushiki, 2006). The passage of molecules through this alternative path has been considered negligible in humans due to the small surface proportion of the olfactory epithelium, from 1 to 5% (Grassin-Delyle et al., 2012); in mice or rats, it is almost 50% of the total surface of the nasal epithelium (Gross et al., 1982).

The supply of arterial blood to the nose comes from the external (via the sphenopalatine and facial arteries) and internal carotid systems (via the ophthalmic artery). The arterial blood flow first irrigates a dense bed of capillaries followed by capacitance vessels (i.e., large venous sinusoids) near the turbinate respiratory zone. The venous return involves the sphenopalatine, facial and ophthalmic veins and then the internal jugular vein, which in turn drains (via the subclavian vein and the superior vena cava) into the right heart chambers; this explains the

absence of a hepatic first-pass effect. Nasal blood flow is partly controlled by the autonomic nervous system; stimulation of vascular alpha-adrenergic receptors by the noradrenaline released by sympathetic nerves plays a predominant role in the neuronal control of blood flow and leads to significant vasoconstriction and decreased blood flow.

2.2.2 The Vomeronasal Organ (or Jacobson's organ)

The VNO is an organ that was first described by the Danish anatomist Ludvig Jacobson (Doving & Trotier, 1998; Jacobson, 1813). The VNO is a bilateral, blind-ended tubular structure that occupies a thin cylindrical lamina of bone located on the floor of the nasal cavity adjacent to the vomer and directly above the palate, and it is divided by the nasal septum and laterally surrounded by the nasal mucosa (**Figure 10**). The organ comprises the vomeronasal duct, a blind epithelial tube with a single small rostral orifice of approximately 4 mm of length in mice that connects it with the main nasal cavity(Doving & Trotier, 1998; Ogura, Krosnowski, Zhang, Bekkerman, & Lin, 2010), combined with the surrounding glands, vessels, nerves, and connective tissue. Each half of the organ contains a crescent-shaped sensory epithelium limited to the central levels of the medial wall of the duct (Barrios et al., 2014) that is medial to a fluid-filled lumen and positioned laterally from a non-sensory epithelium and blood vessel, similar to the respiratory epithelium.

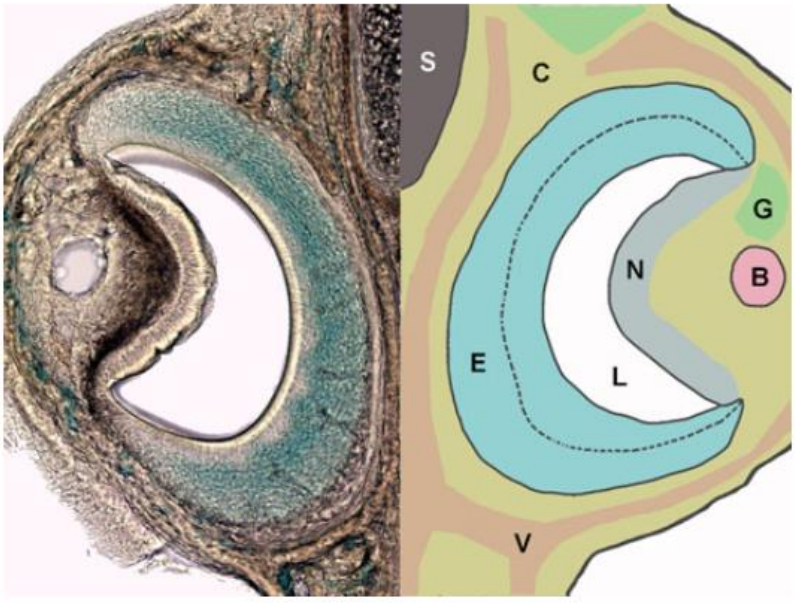


Figure 10 Coronal half-section of the mouse VNO. S: nasal septum, C: cavernous tissue, G: glandular tissue, B: blood vessel, V: vomer, N: non-sensory epithelium, L: lumen, E: sensory epithelium with apical (right) and basal (left) layers on the VNO sensory neurons. Modified from (Ibarra-Soria, Levitin, & Logan, 2014)

In mice, the VNO is indirectly connected with the oral cavity through the nasopalatine canal (**Figures 11, 12**), which opens in the hard palate and connects the end of the VNO with the nasal cavity or nasopharynx caudally. In other species such as carnivores and ungulates, it is connected directly to the VNO (Zancanaro et al 2014). Its blockage has been thought to influence the reception of important semiochemicals (Levy, 2011) and consequent endocrine responses (Booth & Webb, 2010). The vomeronasal canal can pump mucous content into the lumen with the vasodilation of the VNO vessels. Solitary chemosensory cells in its entrance that are connected with trigeminal terminations can prevent irritating or toxic molecules from entering (**Figure 11**) (Ogura et al., 2010). The VNO lumen contains the mucus produced by the VNO glandular cells that can solubilize peptides or proteins.

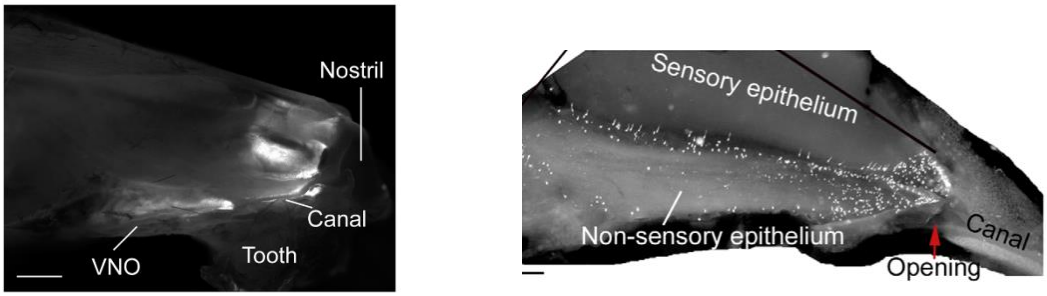


Figure 11 Left Lateral view of the mouse olfactory system showing the entrance canal to the VNO. The figure is a fluorescence image after a dye assay showing rhodamine fluorescence in the VNO and anterior nasal mucosa. Right. Lateral view of the mouse olfactory system showing a high density of solitary chemosensory cells. Modified from Ogura et al 2010

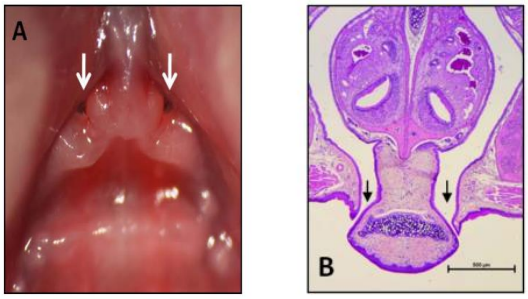


Figure 12 A: Ventral view of the nasopalatine openings (arrows) and the nasopalatine papilla in mice. B: Coronal section of the palate and nasal cavity showing the nasal orifices opening to the nasopalatine ducts. Modified from Levy et al 2011 (Levy, 2011)

The vomeronasal sensory epithelium can be divided into two layers: the apical stratum corresponding to V1R receptors and the basal stratum corresponding to the V2R receptors (Barrios et al., 2014); the axons of the two layers respectively project to the anterior and posterior parts of the posterior accessory olfactory bulb (AOB) through the VN nerve.

2.2.3 The main olfactory epithelium

The main olfactory epithelium is the major area of the four olfactory subsystems. It is a sensory epithelium situated in the dorsal area of the nasal cavity, approximately starting over the entrance of the VNO and occupying the mucosal lining of most of the nasal cavity except the ventral concha (turbinates). From a histological perspective, we mainly find three types of cells: the basal cells that are undifferentiated and could play a role in olfactory plasticity (Griffiths & Brennan, 2015), the mature neurons and the supporting cells (Buck, 2000).There is a continuous turnover of mature sensory cells. This continuous turnover permits changes in the expressed olfactory receptors depending on external factors and internal factors such as the endocrine state (Brennan & Keverne, 2015; Griffiths & Brennan, 2015). External and internal factors can alter the expression of receptors in new sensory cells coming from the undifferentiated basal cells that replace the old cells.

The olfactory glands are tubuloalveolar serous-secreting glands lying in the lamina propia of the mucosa; these glands deliver a proteinaceous secretion via ducts onto the surface of the mucosa. The role of this secretion is to trap and dissolve odour molecules, and the constant mucous flow permits old stimuli to be washed out. The MOE olfactory sensory neurons project to the main olfactory bulb. The MOE only receives the stimuli via the airstream flowing through the turbinates.

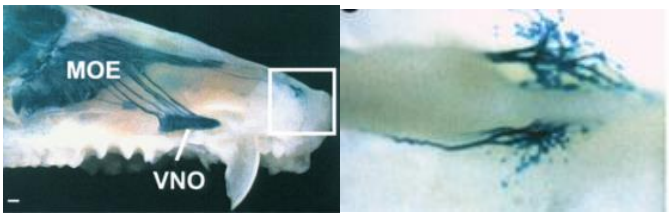
2.2.4 The Grüneberg ganglion

The Grüneberg ganglion is the smallest of the olfactory subsystems. The sensory epithelium is located under the nasal mucosa, and it is a small, bilateral cluster of neurons situated in the rostral nasal vestibule (**Figure 13**); the ganglions are surrounded by blood vessels, which are rich in this area (Roppolo, Ribaud, Jungo, Lüscher, & Rodriguez, 2006). This anatomic region was accidentally discovered in 1971 by Hans Grüneberg (Grüneberg, 1973), who had already hypothesized that it could have a role as a chemoreceptor or thermoreceptor due to its apical location. Its histological structure is analogous to the main olfactory epithelium, as can be seen in **Figure 13** (Barrios et al., 2014). There has been renewed interest in this organ during the last ten years, and it has been proved to play a role in the detection of alarm pheromones (Brechbühl, Klaey, & Broillet, 2008), predator molecules and derivatives such as pyridine analogues(Brechbühl, Moine, Tosato, Sporkert, & Broillet, 2015) and other molecules (Mamasuew, Hofmann, Breer, & Fleischer, 2011). It has also been suggested to have a role in thermoreception, especially in neonates, as it exhibits c-fos expression when pups are separated from the mothers and exposed to lower temperatures (Mamasuew, Breer, & Fleischer, 2008; A. Schmid, Pyrski, Biel, Leinders-Zufall, & Zufall, 2010). This neural activation

in cold temperatures has been suggested to play a role in social stress (Mamasuew et al., 2008), but a simpler explanation is probably the dual function of the nasal cavity as a chemoreceptor and the first part of the respiratory tract, which functions in warming the airstream (Hoyt et al., 2007). Thermoreceptors could stimulate vasodilation to increase the temperature of the air (Charkoudian, 2003). Contrary to the other olfactory subsystems, the GG appears to be complete and functional at birth {Formatting Citation}, ensuring immediate alarm pheromone sensing and increasing the chances of survival in the wild.

The axons of its neurons project to the glomeruli necklace in the olfactory bulb (Koos & Fraser, 2005; Roppolo et al., 2006).

a.



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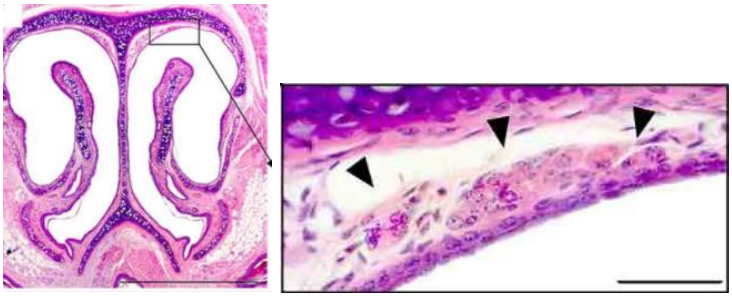


Figure 13 Location of Grüneberg's ganglion **a.** General view from the olfactory subsystems (left) and macroscopic view of the Grüneberg ganglion showing its bilateral structure (right). Modified from Roppolo et al (Roppolo et al., 2006). **b**. Transverse section of the nasal cavity in mice showing the location of the Grüneberg's ganglion. Scale bar 500 µm. Modified from Barrios et al 2014 (Barrios et al., 2014)

2.2.5 The septal organ of Masera

The septal organ of Masera is the third olfactory subsystem according to the surface of the sensory epithelium, and as with the others, it is also bilateral. It is an isolated patch (Weiler & Farbman, 2003) located in the basal part of the septum (**Figure 14**). It was first described by Broman (Broman, 1921) in new-born mice, and Rodolfo-Masera (1943) later described it in different species. Histologically, it is different from the main olfactory epithelium; Bowman's glands frequently intrude in contrast to MOE, where only the ducts are found. Respiratory

glands are found beneath the lamina propia in contrast to MOE, where they are not found (Weiler & Farbman, 2003).

The opening of the nasopalatine duct near the septal organ has been proposed as a chemosensory information path to this organ through licking in species without a direct connection between the nasopalatine ducts and the VNO (Weiler & Farbman, 2003).

Grosmaitre et al (2007) demonstrated a dual function of the septal organ neurons in chemosensation and mechanoreception, so the organ could have a role in synchronising the activity of the olfactory bulb with respiration.

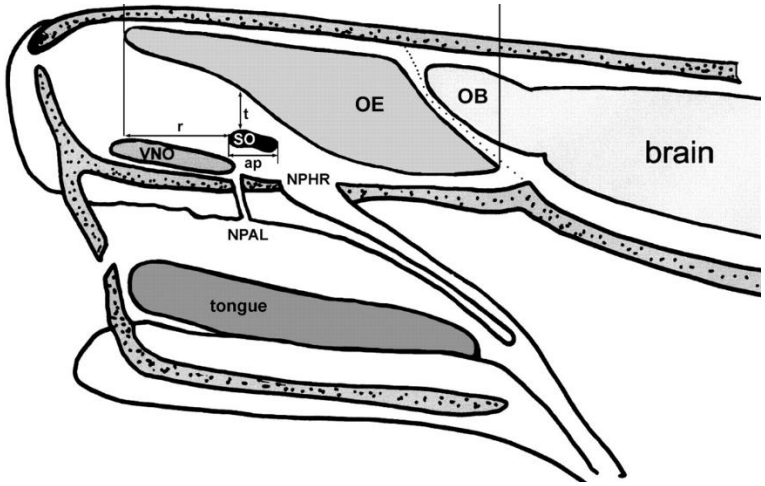


Figure 14 Position of the septal organ (SO) and nasopalatine duct in the rat. From Weiler and Farbman 2003 (Weiler & Farbman, 2003). OE: olfactory epithelium, OB: olfactory bulb, VNO: vomeronasal organ, NPAL: nasopalatine duct, and NPHR: nasopharyngeal duct. r, distance of rostral SO to rostral end of OE; t, length of ZE (epithelium between OE and SO)

2.3 Respiratory and olfactory physiology

Mice are obligate nasal inspirators due to close apposition of the epiglottis to the soft palate (Reznik, 1990), which has been suggested as an anatomical feature related to the primary function of the mouse nasal cavity, olfaction, that inspire between 106-230 times per minute (Hoyt et al., 2007) with a tidal volume between 0.15-0.29 ml and ventilation from 23-47.5 ml/min. This activity is related to a high metabolism, but it indirectly permits a large amount of chemical information that is attached to the airstream. The regulation of breathing patterns has been suggested to be related to the chemosensory systems involved in olfaction (Mori, Manabe, & Narikiyo, 2014).

The nasal cavity has been shown to metabolize molecules based on the enzymes found in this area (Bogdanffy, 1990; Dahl & Hadley, 1991), such as carboxylesterases, aldehyde dehydrogenase, cytochrome P-450, epoxide hydrolase, and glutathione S-transferases. The distribution of these enzymes appears to be cell type-specific, and the presence of an enzyme may predispose particular cell types towards enhanced susceptibility or resistance to chemical-induced injury, sustains the role of the nasal cavity in processing xenobiotic chemicals. These enzymes can also affect odorant perception and olfactory bulb activation; for example, functional groups such as aldehydes and esters can be converted in the corresponding acids and alcohols in the mouse mucus (Nagashima & Touhara, 2010). More specifically, mammal and insect (Kaissling, 2009) pheromones, such as the rabbit mammary pheromone, can be transformed by enzymes in the nasal cavity (Legendre et al., 2014) This active catabolism in the OE can therefore contribute to terminating the sensory impact of the pheromone by clearing it from the peri-receptor space and keeping the receptors free to encounter new stimuli (**Figure 15**). The nasal microbiota plays an active role in modulating the physiology of the olfactory epithelium and participates in the transduction of nasal enzymes, and its absence in germ-free animals increases the response to odorants (François et al., 2016).

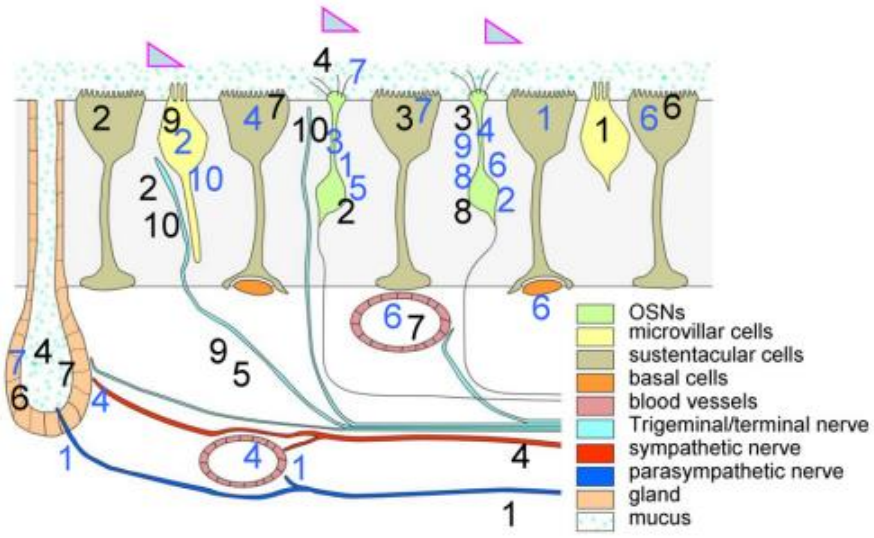


Figure 15 Peripheral modulation of olfaction (Lucero, 2013)**.** Diagram showing complex modulation of odorant responses in OSNs. Numbers in black represent the source, and numbers in blue are the target of each neuromodulator. (1) Acetylcholine (Ach), (2) ATP, (3) endocannabinoids, (4) dopamine or catecholamines, (5) GnRH or LHRH, (6) insulin, (7) leptin, (8) nitric oxide, (9) NPY, (10) substance P, and odorants (triangles). The trigeminal and terminal nerves are combined for simplicity. Gland refers to Bowman's glands and deeper nasal glands

Before reaching the neuronal membrane, the odorant molecules must cross a thick layer of mucus containing high concentrations of several classes of proteins that may interact with the volatile compounds. The olfactory mucus, similar to other types of protective mucus, is very complex in composition, and several aspects remain to be investigated. The mucins are large proteins (250 to 1000 kDa) that are highly glycosylated, which gives consistency and thickness to the mucus. Apart from these structural proteins, the mucus is rich in antibodies, antibacterial proteins such as lysozyme, carrier proteins, detoxifying enzymes, and other proteins (Pelosi, 1994). The odorant-binding proteins (OBPs) are small, abundant extracellular proteins belonging to the lipocalin superfamily (Briand et al., 2002). The terminology is confusing as the word odorant is very unspecific and could refer to any molecule with a molecular weight lower than 300-400 that is sufficiently volatile to reach the nose (Tegoni et al., 2000); nevertheless, the molecular weights of OBPs fall within a narrow range (approximately 18 kDa) (Briand, 2009). Three hypotheses have been proposed about the function of these proteins such as a buffer, where OBPs could more efficiently trap molecules at high concentrations, narrowing the wide range of stimuli intensities. As carriers, OBPs could bind the hydrophobic molecules and carry them to the receptors of the olfactory epithelium or remove them from the olfactory receptors. Finally, as transducers, they could bind odorants and interact as a complex, a mechanism that could be involved in the discrimination of odours (Pelosi, 1994). The best ligands bind with dissociation constants within a micromolar range of 0.1-1 µM and include heterocyclic derivatives such as pyrazines and tyazoles, terpenoids and derivatives such as menthol and thymol and medium-sized aliphatic alcohols and aldehydes. Molecules with poor affinity include terpenoids or those with a rounded structure, such as camphor, and polar compounds such as short-chain fatty acids (Tegoni et al., 2000). These ligands have been observed in several vertebrate species such as cow, pig, rabbit, mouse, rat, elephant, and human. Two lipocalins were found expressed in the vomeronasal organ of mice and in glands opening in the lumen of the VNO (Miyakawi, Matsushita, Rio, & Mikoshiba, 1994).

2.4 Neural pathways: learned versus innate

Neural chemical communication pathways are based in different areas. The discovery of the basis of the mechanisms involved in olfactory sensation was rewarded in 2004 with the Nobel Prize in Physiology or Medicine to Linda Bucks and Richard Axel.

The interaction between taste and olfaction as chemical senses and with anatomical proximity has also been demonstrated for example, by decreasing the thresholds for flavour perception (Dalton, Doolittle, Nagata, & Breslin, 2000).

2.4.1 Sensory pathways from the main olfactory epithelium

The olfactory epithelium contains millions of olfactory sensory neurons as well as supporting cells and a basal layer of stem cells that continuously replace the sensory neurons as they have a short life. At the surface of this sensory epithelium, each neuron extends cilia into the nasal lumen, which allows contact with the odorants dissolved in the nasal mucus.

These cilia are the last ramification of the neural dendrites, where the chemical message will be transformed into an electric signal through transduction that will finally converge in the body of the olfactory sensory neurons, and depolarization will continue to conduct the message through the axons until the glomeruli of the main olfactory bulb (**Figure 16**) (Mori et al 1999). These axons enter the cranial cavity through the cribriform plate of the ethmoid bone (**Figure 17**), where the synapses between the olfactory sensory neurons and the dendrites of the mitral cells occur (Bird, Amirkhanian, Pang, & Van Valkenburgh, 2014). The OSNs of the main olfactory epithelium situated in highly stimulated regions have longer cilia and are more sensitive; this pattern is innate and thus does not require experience or sensory stimulation (Challis et al., 2015).

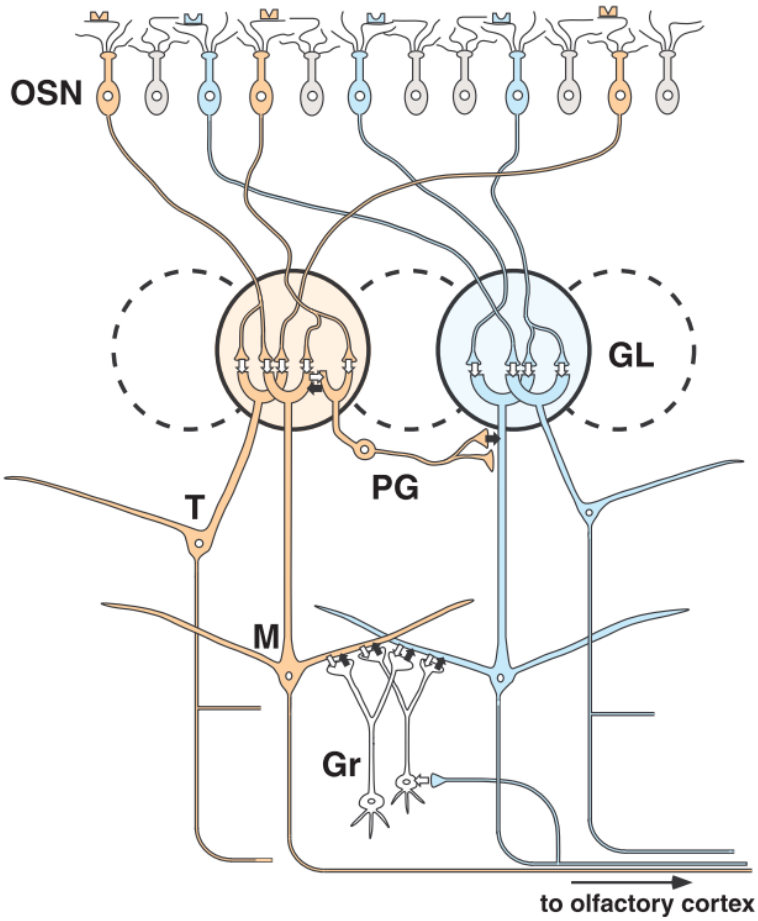


Figure 16 Basic circuit diagram summarizing the synaptic organization of the mammalian MOB. Two glomerular modules (brown and blue) represent two different types of odorant receptors. Mitral cells (M) and tufted cells (T)

are output neurons, and granule cells (Gr) and periglomerular cells (PG) are local interneurons. Each glomerulus receives afferences of only one receptor. GL: glomerulus Modifed from Mori et al (1999).

The input from these synapses will arrive in the body of the mitral cells, and the output will continue through the axons to different areas of the olfactory cortex: the anterior olfactory nucleus, piriform cortex, olfactory tubercle, olfactory nuclei of the amygdala and the lateral entorhinal cortex (Buck, 2004). The amygdaloid neurons also project to the hypothalamus.

The olfactory tract runs inferiorly to the frontal lobe. As the tract reaches the anterior perforated substance, it divides into medial and lateral stria.

- The lateral stria sends the axons to the olfactory area of the cerebral cortex (also known as the primary olfactory cortex).
- The medial stria carries the axons across the medial plane of the anterior commissure where they meet the olfactory bulb on the opposite side.

Figure 17 The cribriform plate: the skull of a grizzly bear (*Ursus arctos*) viewed from a caudal aspect, from Bird et al 2014

Tufted cells and granular cells also form part of this glomeruli, and they can modulate the output from this synapsis, inhibiting the depolarization. Receptors found in the dendrite villi from the olfactory sensory neurons from the main olfactory epithelium converge in the glomeruli of the main olfactory bulb (**Figure 18**). The glomeruli are formed by the axons of the sensory neurons and the dendrites and bodies of the mitral cells (Buck, 2004). Each of these mitral cell projects to different areas of the olfactory cortex.

Figure 18 Axons of the olfactory epithelium neurons converging in a glomerulus of the main olfactory bulb. R. Axel 1995 ® b. Sensory neuron of the olfactory epithelium and cilia where the receptors can be found. R. M Costanzo and E. E. Morrison ®

From the VNO, neurons are relayed through the accessory bulb to the medial amygdala and then to the hypothalamus.

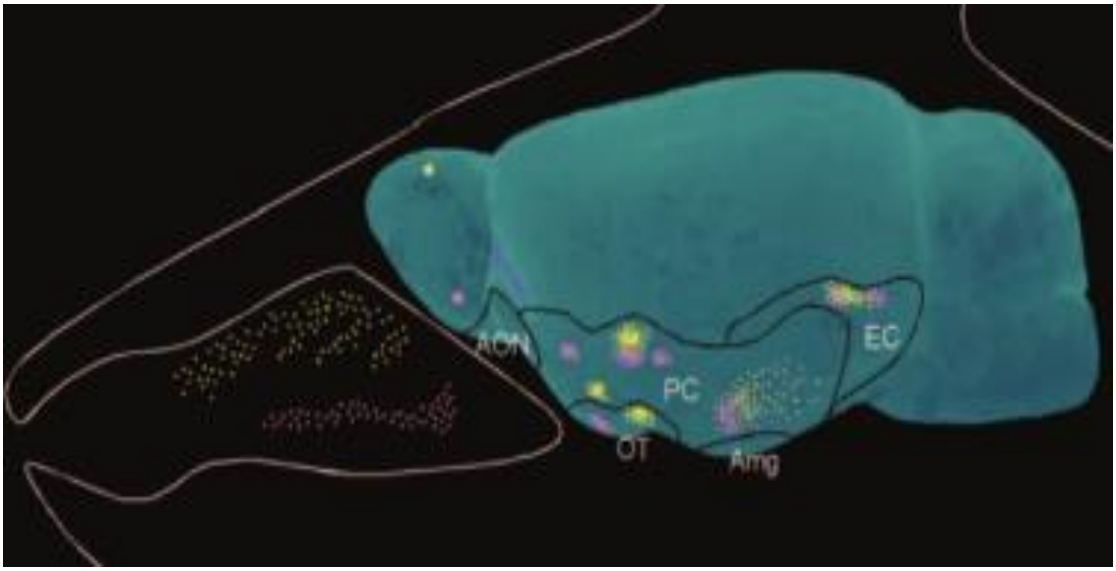


Figure 19: Areas of the olfactory cortex related to reception in the olfactory epithelium. Black lines and abbreviations indicate different areas of the olfactory cortex. AON: anterior olfactory nucleus, PC: piriform cortex, OT: olfactory tubercle, Amg: olfactory nuclei of the amygdala, EC: lateral entorhinal cortex. Modified from Buck 2004 (Buck, 2004).

2.4.2 Sensory pathways from the VNO

Unlike olfactory sensory neurons (OSNs), the dendritic knob of VSNs lacks cilia and instead contains up to 100 microvilli (**Figure 18**). In the rat, VSN microvilli are 2-4µm in length and

approximately 100 nm in diameter (Vaccarezza, et al., 1981). The primary chemotransduction events are thought to take place in these microvilli.

The axons of the VSNs form the vomeronasal nerves pass through the cribriform plate and project to the accessory olfactory bulb and into glomeruli, where they form the synapsis with the mitral cells and the tuft cells (Buck, 2000). The major output neuron of the AOB, the mitral cell, has a strikingly different structure from the mitral cells of the MOB. It has been known for nearly a century that AOB mitral cells possess multiple apical dendrites, up to five, that each ramify within a different glomerulus (Cajal, 1911).

Axons of the vomeronasal sensory neurons (VSNs) project to the accessory olfactory bulb (AOB), which in turn transmits sensory information to the bed nucleus of the stria terminalis (BNST), the bed nucleus of the accessory olfactory tract (BAOT), and the amygdala, that is, the medial amygdaloid nucleus (Me) as well as the posteromedial cortical amygdaloid nucleus (PMCo). The information is then transmitted from the amygdala to specific nuclei of the hypothalamus. These tertiary projections to the hypothalamic region are also known as the neuroendocrine hypothalamus. This area controls the release of hormones by the pituitary, and it therefore modulates the endocrine status of the animal (Zufall, Leinders-Zufall, & Puche, 2008).

2.4.3 Sensory pathways from the Grüneberg ganglion

This compact cluster of neurons projects its axons from the nasal vestibule along the septum and passes through the cribiform plate; the axons course along the dorsal part of the OB until the olfactory necklace of the olfactory bulb, in the rostral part of the AOB and the dorso-caudal region of the OB (Joerg Fleischer & Breer, 2010; Koos & Fraser, 2005). It is still not clear how the molecules could be detected as there is no direct contact with the nasal lumen.

Initially, it was thought that the Grüneberg ganglion does not express olfactory receptors (Roppolo et al., 2006). However, it was later discovered that it expresses trace amine-associated receptors (TAARs), mainly in the embryonic and early stages (Fleischer, Schwarzenbacher, & Breer, 2007), and a V2R receptor, VRr83 (Fleischer, Schwarzenbacher, Besser, Hass, & Breer, 2006).

2.4.4 Sensory pathways from the septal organ of Masera

In adult rats, the septal organ projects two nerve bundles to ≈ 1% of the glomerular population in the main olfactory bulb (Ma et al., 2003), mainly to 30 “septal glomeruli” although some fibres innervate glomeruli shared with the main olfactory epithelium axons. The SO area decreases in adults, which could diminish the number of septal glomeruli. In contrast to the VNO and the MOE, the SO has shown limited capacity for regenerating neurons (Weiler & Farbman, 2003).

2.4.5 Sensory pathways in the perception of predator cues

The medial amygdala plays an important role in modulating predator chemical sensory information, either conditioned or unconditioned (**Figure 20**).

The bed nucleus of the stria terminalis has been shown to play an important role in detecting TMT (Kobayakawa et al., 2007) and predator urine (Dewan, Pacifico, Zhan, Rinberg, & Bozza, 2013; Ferrero et al., 2011). It is a key structure of the network of the amygdala that is involved in behaviours related to natural reward, drug addiction and stress (Puente et al., 2010).

The ventral hippocampus (**Figure 21**) has dense reciprocal connections with the medial amygdala and other nuclei. Lesion in this structure has been shown to impair avoidance and risk assessment of coyote urine (Wang et al., 2013).

The medial amygdala and the bed nucleus of the stria terminalis also project to *medial hypothalamic nuclei*, which regulate reproductive, ingestive and defensive behaviour. Three hypothalamic nuclei, the anterior hypothalamic nucleus, the dorsomedial part of the ventromedial nucleus, and the dorsal premammillary nucleus, are hypothesized to underlay a medial hypothalamic defensive system (Canteras, 2002), where the premammillary nucleus can play a highlighted role.

Little is known about the sensory pathways involved in predator reception. A recent study showed that multiple areas of the amygdala were capable of stimulating corticotrophin-releasing factor neurons in the hypothalamus, but only a specific area of the olfactory cortex was discovered as capable of inducing stress hormone responses to volatile predator odours such as TMT or bobcat urine, the amygdalo-piriform transition area (Kondoh et al., 2016).

The medial prefrontal cortex modulates the innate fear elicited by predator odours (Takahashi, 2014), and it is connected to the amygdala, hypothalamus, and periaqueductal grey, structures that are involved in the fear of predator odours. Some studies have shown c-fos expression in response to cat odour that was not found in response to TMT (Chan et al., 2011; Staples, Hunt, van Nieuwenhuijzen, & McGregor, 2008). Interestingly, the c-fos response to cat stimuli was found to increase with age from young rats to adults (Chan et al., 2011), which is comparable to the increased perception of risk in human adults compared to adolescents due to the slower maturation of this area of the brain (Steinberg, 2008).

The periaqueductal grey receives inputs from the hippocampus, the amygdala and the prefrontal cortex, and its role in threatening situations and related behaviours, such as flight and freezing, has been described in rodents (Comoli, Ribeiro-Barbosa, & Canteras, 2003; Tovote et al., 2016); however, no predator olfactory stimulus has been tested for these reactions.

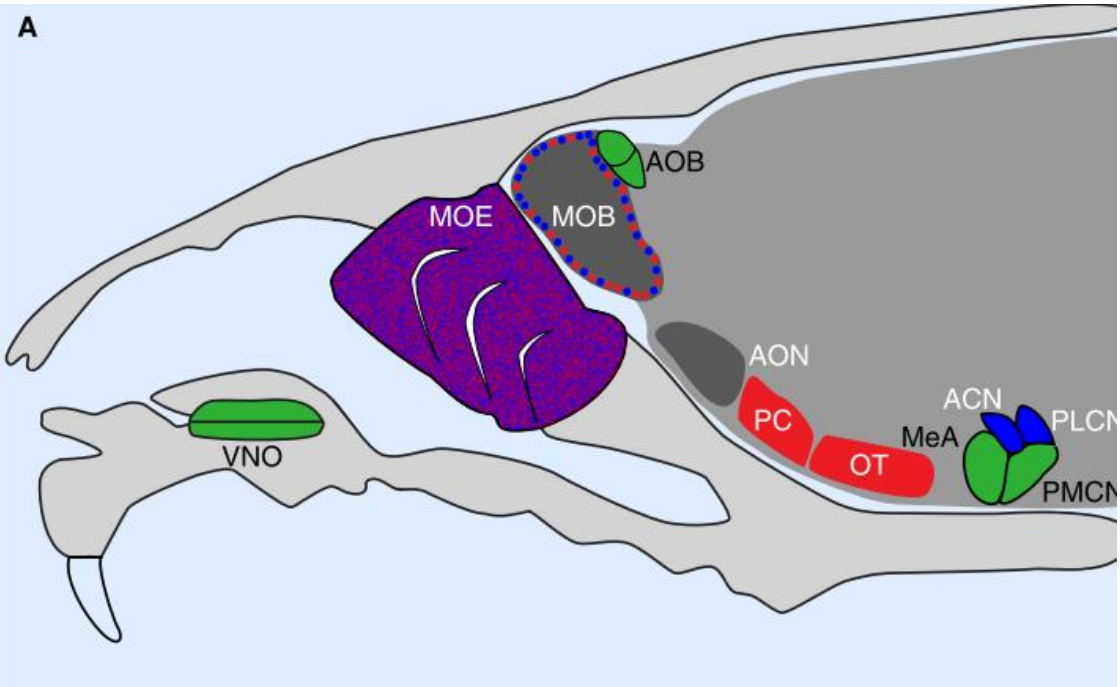
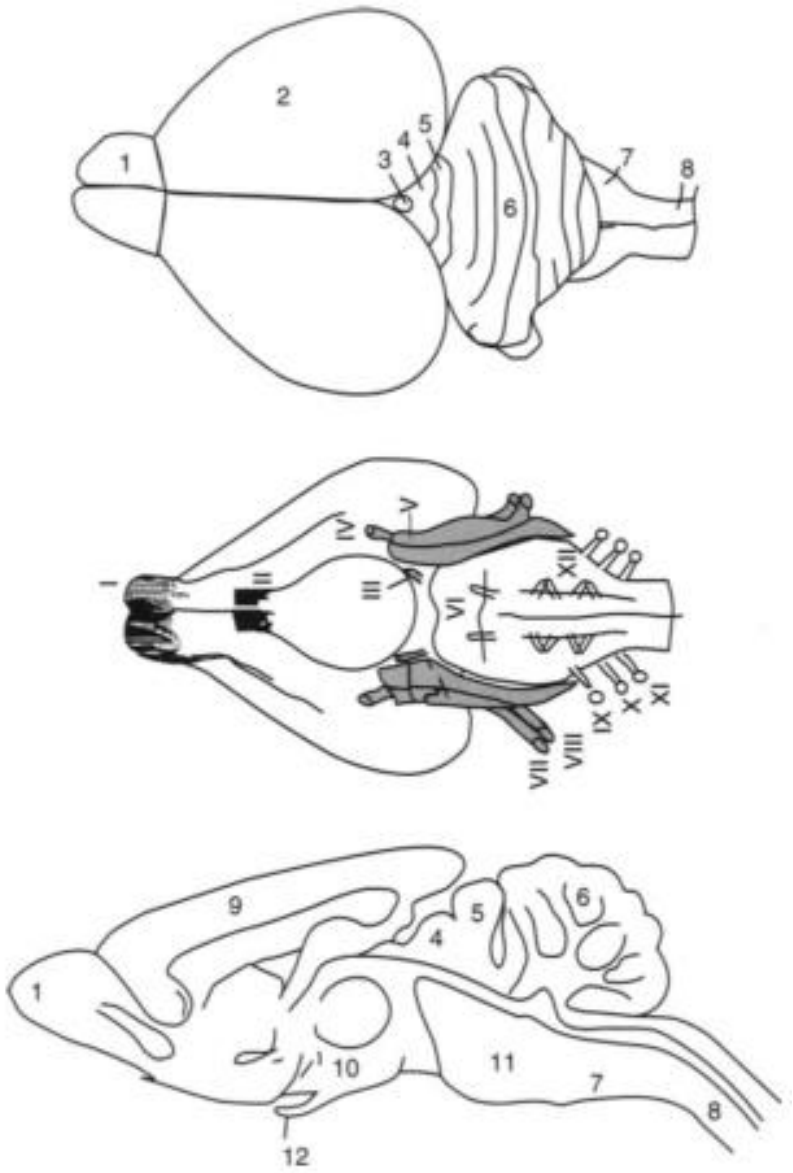


Figure 20 Olfactory pathways in the mouse, modified from Li & Liberles 2015 (Li & Liberles, 2015). Main olfactory systems: blue (innate responses) and red (learned responses). Signals from the MOE are transmitted to the MOB and then to several brain regions including the anterior olfactory nucleus (AON), piriform cortex (PC), olfactory tubercle (OT), posterocolateral cortical amygdaloid nucleus (PLCN) and anterior cortical nucleus (ACN). Accessory olfactory pathway (green): signals from the VNO are sent to the AOB and then to the medial amygdala (MeA) and the posteromedial cortical amygdaloid nucleus (PMCN)



1. bulbus olfactorius
2. hemispherium cerebri
3. glandula pinealis
4. colliculi rostralis
5. colliculi caudales
6. cerebellum
7. medulla oblongata
8. medulla spinalis
9. cortex telencephalic
10. hypothalamus

I nervus olfactorius
II n. opticus
III n. oculomotorius
IV n. trochlearis
V n. trigeminus
VI n. abducens
VII n. facialis
VIII n. vestibulocochlearis
IX n. glossopharyngeus
X n. vagus
XI n. accesorius
XII n. hypoglossus

Figure 21 Gross anatomy of the mouse brain: dorsal view, ventral view and midline section. Modified from Komarek (2007) (Komárek, 2007)

2.5 Receptors

In mammals, three endogen systems use chemical communication: the nervous system with neurotransmitters, the endocrine system with hormones and the immune system with cytokines. Two senses are used to receive the chemical information from the environment: taste and olfaction.

Olfactory system receptors can be considered chemical neuronal receptors that are specialized to communicate with the surrounding world. They receive information about conspecifics, predators, food and noxious or poisonous stimuli and send it to the main and accessory olfactory bulbs (**Figure 24**). In general terms, olfactory receptors share many basic features with the rest of the chemical neuronal receptors in mammals that are used for communication between cells, tissues or regions and in some cases, with the immune or endocrine systems.

G-protein coupled receptors is a big superfamily of receptors, including odorant receptors, VNO receptors, trace amine receptors and formyl peptide receptors. This kind of receptor is formed by 7 transmembrane hydrophobic sequences, when the ligand binds the receptor there is a conformational change which allows its interaction with the G-protein (**Figure 22**). G-protein is constituted of 3 subunits: α, β, and γ. Through this activation, the G protein provides the signal transduction by being dissociated and interacting with an effector which is an enzyme or ionic channel, this leads to an increase in intracellular calcium, the cellular response and the start of the electric signal (Klein, 2005).

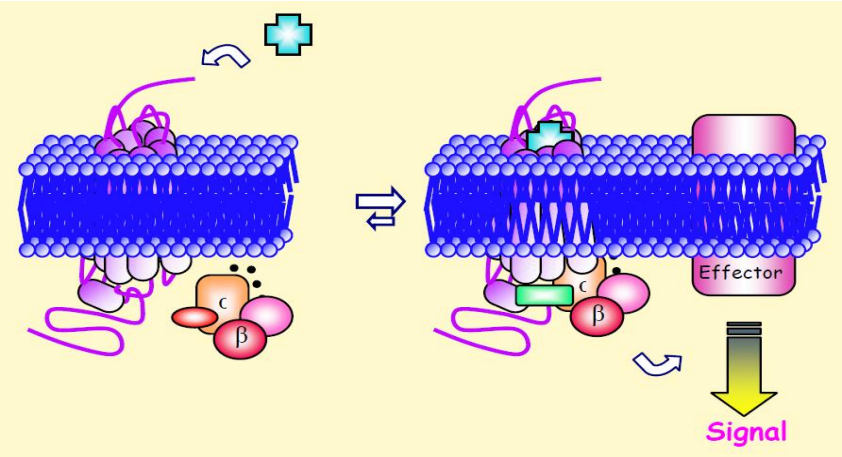


Figure 22. G-protein coupled receptor with the G-protein and the effector. Modified from Klein 2005.

2.5.1 Odorant receptors (ORs)

These are the main group of olfactory system receptors and the first to be identified (Buck & Axel, 1991), which was rewarded with a Nobel Prize (Axel, 2004; Buck, 2004). These receptors are coded for more than 1000 genes in mice (Godfrey, Malnic, & Buck, 2004) and are related to the main olfactory epithelium, but they are also found in the SO and the VNO to a lesser extent. This family of genes is by far the largest in vertebrate genomes (Joerg Fleischer, 2009), and the genes can be divided into classes I and II, of which the majority of mammal receptors belong to class II; class I genes belong to fishes, probably due to the solubility of the molecules. The vast majority of neurons express only one receptor, but they can respond to more than one odorant molecule and an odorant can activate neurons with different degrees of specificity (Tirindelli, Dibattista, Pifferi, & Menini, 2009). This rebounds in a combinatorial code that allows an almost unlimited number of ligands to be perceived (Malnic, Hirono, Sato, & Buck, 1999).

2.5.2 Vomeronasal receptors

These particular G-protein receptors account for almost 250 putative pheromone receptors identified in the mouse VNO.

Vomeronasal type 1 receptors (V1r)

This family was discovered in 1995, again by the Axel laboratory (Dulac & Axel, 1995). These receptors can distinguish structural classes of steroids such as androgens, oestrogens and glucocorticoids, and they could serve as detectors of the physiological status of an animal (Isogai Yoh et al, 2012). They are expressed in the apical layer of the VNO and are differentiated by the $G_{\alpha i}$ membrane proteins. They account for more than 100 receptors in mice.

Vomeronasal type 2 receptors (V2r)

In this case, the research race was even more competitive with three laboratories publishing the discovery of a new receptor expressed in the basal layer of VNO neurons that expressed G_0 proteins, in contrast to the cells from the apical layer, at the same time in three major journals (Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). In mice, they represent more than 150 receptors.

These receptors seem to encode information about the identity of the emitters and can detect large peptides and protein families (Isogai Yoh et al, 2012). Peptide ligands from the major histocompatibility complex (MHC) (Leinders-Zufall et al., 2004), MUPs and ESP tear proteins have been proved to be detected by these receptors in mice. Specifically, the α fraction of the g-protein complex is implied in the detection of this peptidic molecule, as was demonstrated

by the creation of conditioned transgenic mice in which this protein was absent (Chamero et al., 2011).

2.5.3 Formyl peptide receptors (FPrs)

These receptors were first discovered in the 1990s as part of the immune system (Boulay, Tardif, Brouchon, & Vignais, 1990), and their ligands were associated with leukocyte chemotaxis in the 1970s (Schiffmann, Corcoran, & Wahl, 1975), but was not until 2009 that they were found in the VNO (Riviere et al., 2009). In mice, these VNO FPrs are coded by 7 genes.

These receptors are expressed by approximately 1% of the VNO neurons (Joerg Fleischer, 2009). They are activated by disease-related proteins that have been associated with the detection of infected conspecifics or contaminated food (Riviere et al., 2009), which can be related to their role in the immune system in host defence against bacterial infections and the clearance of damaged cells (Le, Murphy, & Wang, 2002).

2.5.4 Trace amine-associated receptors (TAARs)

The name of these receptors comes from the original discovery of a group of G protein-coupled receptors in the central nervous system. Instead of being stimulated by the main amine neurotransmitters such as serotonin or noradrenaline, the TAARs were activated by amines found in trace amounts in mammals such as tyramine, octopamine or B-phenylethylamine (Borowsky et al., 2001). TAARs are found in the amygdala in humans and rodents and could be related to affective disorders. Later, Liberles and Buck (Liberles & Buck, 2006) found these receptors in the main olfactory epithelium in mice as well as in fishes and humans, contradicting previous results that failed to find these receptors in the central nervous system and thus assumed that they only existed in the olfactory system. TAARs were found to be able to detect volatile amines and were suggested to participate in the detection of social cues. In fishes, detection and avoidance of the diamines cadaverine and putrescine were related to these TAARs (Hussain et al., 2013). The importance of TAARs importance in the aquatic environment is easily indicated by the number of coding genes, reaching up to 112 in zebrafish but only 15 in mice (Li et al., 2015). 2-phenylethylamine, which is present in high amounts in carnivorous mammals such as the bobcat, activates TAAR4 (Ferrero et al., 2011).

They receptors were found also in the Grüneberg ganglion, mainly in late embryonic and neonatal stages (Joerg Fleischer et al., 2007).

2.5.5 MS4A protein receptors

The last family of olfactory receptors was discovered very recently in 2016 by Paul Greer and collaborators (Dey & Stowers, 2016; Greer et al., 2016). These new receptors from the 4-pass transmembrane protein family (**Figure 23**) are found in the olfactory sensory neurons of the recesses of the olfactory epithelium whose axons project to the necklace zone from the olfactory bulb. Different from the previously known olfactory sensory neurons, these neurons can simultaneously express multiple receptors and are able to detect ethologically relevant ligands such as pheromones. As formyl peptide receptors, they were first identified in the immune system (Eon Kuek, Leffler, Mackay, & Hulett, 2016).

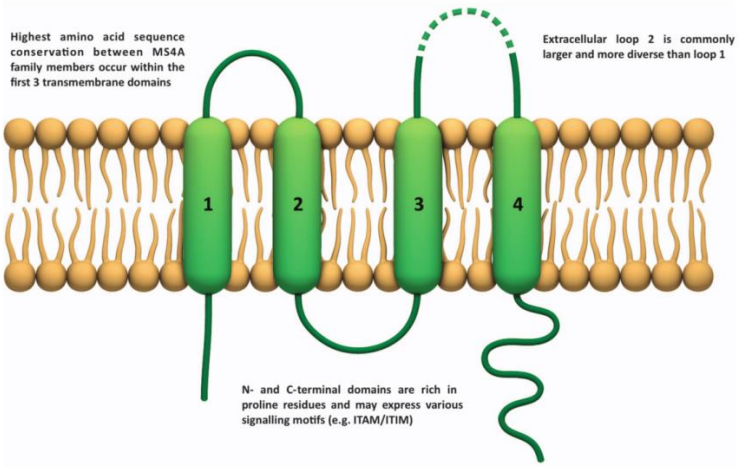


Figure 23 Topology of an MS4A protein

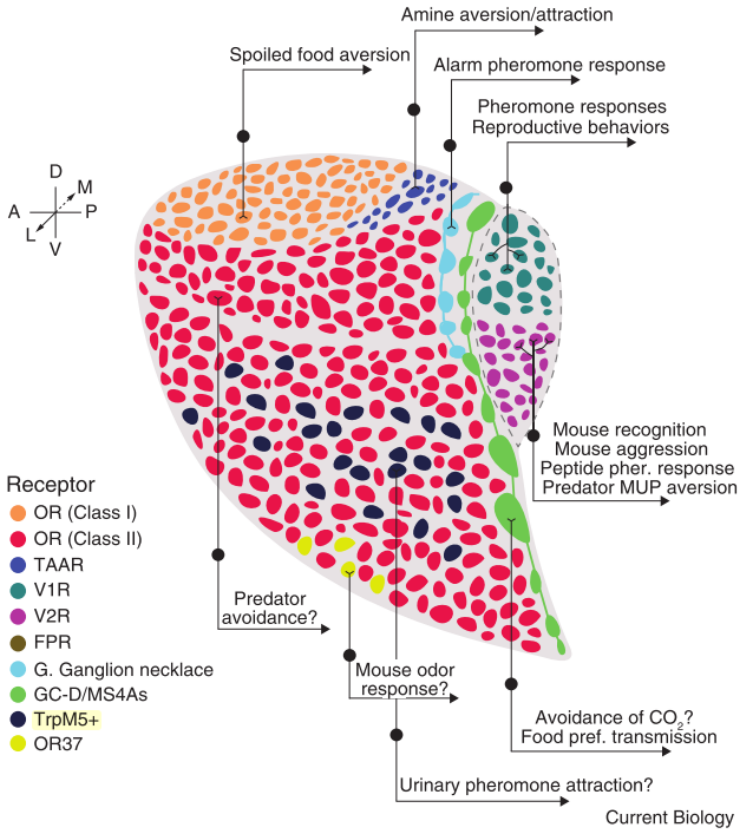


Figure 24 Spatial and molecular organization of projection targets and behavioural responses of mouse olfactory glomeruli in the main and accessory olfactory bulbs. Modified from (Bear, Lassance, Hoekstra, & Datta, 2016)

2.6 Genetics and transgenic technology in chemical communication

The diverse chemical structures of odours do not exhibit continuous variation in a single parameter such as vision or hearing, so they cannot be accommodated by a small number of receptors (Axel, 2004), as is the case for other senses.

Laboratory mice and rats are the result of 100 years of controlled breeding and selection for determined features, such as behaviour, longevity or pathologic phenotypes, as human disease models (Guénet et al., 2015). Two main groups of genetically different models are today bred for laboratory facilities, inbred and outbred strains.

Inbred strains are the result of inbreeding over at least 20 generations; animals are crossed with kin which results in very homogenous strains, which has been useful for increasing reproducibility and reliability between laboratories around the world while decreasing variability, thus enabling the use of fewer animals to detect differences. These strains are phenotypically and genetically defined, permitting the development of transgenic technology by eliminating genes from the genetic repertoire (knock outs). From a research perspective, this has been described as "genetic ablation" (Ben-Shaul, Katz, Mooney, & Dulac, 2010; Harkema, Carey, & Wagner, 2006; Zufall et al., 2008), which means that animals without concrete genes, due to their being "ablated", will not express the phenotype or features linked to these genes.

Genetic dissection of the VNO began with the detection of TRcpc2 in 2002 (Leypold et al., 2002; Stowers, Holy, Meister, Dulac, & Koentges, 2002), when two laboratories discovered that this cation channel could be implied in the cascade activation of the VNO neurons, thereby inhibiting the depolarization of these neurons. Trp2 knock-out animals exhibit impaired social behaviour such as aggressiveness (Keverne, 2002; Leypold et al., 2002; Stowers et al., 2002) and a lack of response to predator proteins (Papes, Logan, & Stowers, 2010). Recently, Chung and collaborators demonstrated impaired sexual behaviours in mice lacking genes related to the expression of androgen-binding proteins. (Chung, Belone, Vošlajerová Bímová, Karn, & Laukaitis, 2017), and in the last few years, a newly developed technology in genetics called the CRISPR-cas system, which is based in the immune systems of bacteria and archaea (Horvath & Barrangou, 2010; Mali Prashant, 2014), will allow the faster and easier development of new models of transgenic mice to study and regulate genes related to olfaction.

Outbred strains, in contrast, are phenotypically defined strains with a higher degree of variability between animals. Crosses between close relatives are avoided to control inbreeding,

but even with these measures, these strains show some degree of inbreeding. The most widely used outbred mouse strain is commonly called CD-1.

2.7 Different Classifications in chemical communication

2.7.1 According to its chemical nature

In animals, cells can communicate via different kinds of chemicals within and among vertebrate organisms, but the main division is between non-peptidic ligands and peptidic ligands.

Working at the Max Planck institute, Karlson and Luscher originated the term pheromone, in agreement with other experts (Karlson & Luscher, 1959). As most neologisms in science come from Greek, the term was derived from the words *pherein* (to transfer) and *hormon* (to excite), which followed an even more ancient term, ectohormone (Bethe, 1932). Both terms adopted the word hormone from the father of endocrinology, Ernest Starling (Starling 1905 from Tata, 2005).

Peptidic ligands

These molecules are used as pheromones by many terrestrial and aquatic species of invertebrates and vertebrates (Wyatt, 2014b), and they are the main group of signal molecules (e.g., hormones) in the animal kingdom (Nicolau, 2012); we can classify them according to size, which conditions their structure and soluble properties.

Small peptides: these have fewer than 15 amino acids, and they have amphiphilic properties, exhibiting hydrophilic and hydrophobic components, and they do not present a tertiary structure as in larger peptides.

Peptides with secondary structure: in this group, we find peptides with a size between 15-50 amino acids that include those secreted by the extraorbital lacrimal gland that are called exocrine gland-secreting peptides (ESPs) such as ESP1 (Kimoto, Haga, Sato, & Touhara, 2005), which is implied in sexual behaviours in mice including the stimulation of lordosis and sexual acceptance in females (Haga et al., 2010), and ESP22, which is produced by juvenile males and inhibits mating behaviour in adults. The family has 38 genes in mice and 10 in rats (Kimoto et al., 2007).

Polypeptides

In this group, we can find peptides with a large number of amino acids. Due to this feature, they usually present a globular structure with hydrophobic residues in the inner part and hydrophilic residues in the outer part.

The protein superfamily of lipocalins includes the most well-known mouse proteins implied in communication, the major urinary proteins. They are produced in the liver in large amounts (Finlayson, Asofsky, Potter, & Runner, 1965) and excreted in the urine, where they have a role in individual recognition (Hurst et al., 2001) and territory marking; they are also related to dominance between males. MUPs are associated with a gradual release of volatile ligands, extending the life of these signals (Hurst, Robertson, Tolladay, & Beynon, 1998). Investment in MUPs is costly but varies with environmental and social conditions. Mice increase urinary scent marking and MUP production to defend their territory when other males are present, and production is decreased, for example, when mice are housed individually in laboratory facilities (Michael Garratt et al., 2012). The MUP profile is constant within individuals but varies between individuals; these variations are due to amino acid-coding sites and differential transcription (Sheehan et al., 2016).

Darcin is a male MUP implied in female attraction and aggressiveness as well as competition between males. The production of MUP declines with age in males, and the ability to release volatile organic compounds (VOCs) also decreases with age (Garratt, Stockley, Armstrong, Beynon, & Hurst, 2011).

Non-peptidic ligands

Like other organic molecules, non-peptidic ligands are based on a carbon chain and attached to hydrogen atoms (Howse, Stevens, & Jones, 1998). The hydrogen atoms attached to the carbon backbone lie in two planes, and the structure is often simplified to only the carbon backbone (**Figure 24**). The systematic nomenclature for hydrocarbon structures depends on the number of carbon atoms in the straight portion of the carbon chain; it uses the Greek-based numbering system devised by the International Union of Pure and Applied Chemistry (IUPAC).

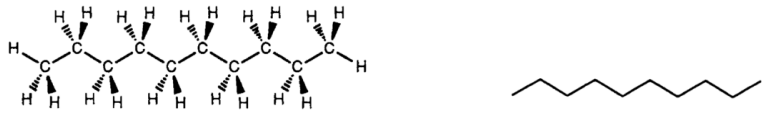


Figure 25. Schematized hydrocarbon structure: left, classic, and right, simplified. Replacing one hydrogen with atoms of other chemical elements results in functional groups.

Table 2. Prefixes and suffixes for common functional groups

Functional group	Formula	Prefix	Suffix
Alcohol	-OH	Hydroxy-	-ol
Aldehyde	-CH=O	Formyl-	-al
Amine	$-NH_2$	Amino-	-amine
Carboxylic acid	-COOH	Carboxy-	-oic acid
Ester	-COOR	*R*-oxycarbonyl-	-*R*-oate
Ketone	>C=O	Oxo-	-one

Modified from Howse et al.(1998).

Compounds composed of atoms bonded to form a ring are commonly divided into carbocyclic compounds, in which the ring consists entirely of carbon atoms, and heterocyclic compounds, in which one (or more) atom(s) of a different element (O, N and S are the most common) is incorporated into the ring (Howse et al., 1998). One example of a heterocyclic compound is the fox faeces molecule 2,4,5-trimethylthiazoline, with a heterocyclic ring of thiazoline (**Figure 25**) containing nitrogen and sulphur, which could be derived from peptides (Gaumont, Gulea, & Levillain, 2009) or amino acids such as cysteine (Charpentier, Barthes, Proffit, Bessière, & Grison, 2012; Walsh & Nolan, 2008).

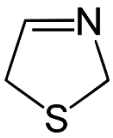


Figure 26: Thiazol ring

The use of secondary metabolites as chemical mediators in intra- and interspecific interactions is at the root of chemical communication. Secondary metabolites are produced by a relatively small number of essential intermediates derived from five biosynthetic pathways (Charpentier et al., 2012):

1. The Shikimate pathway enables the biosynthesis of aromatic hydrocarbons and is exhibited by microorganisms, algae and plants.
2. The acetate pathway is the source of the production of phenolic derivatives and fatty acids.

3. The amino acid pathway is the precursor of most nitrogenous heterocyclic compounds.
4. The mevalonate pathway, which is derived from the acetate pathway, leads to the production of isoprenoids: terpenoids, steroids and carotenoids.
5. The methylerythritol 4-phosphate pathway enables plants and bacteria to produce isoprenoids through a pathway other than 4.

2.7.2 According to its role in chemical communication

Intraspecific: Pheromones

The original definition of pheromones proposed by Karlson and Lüscher was “substances secreted to the outside of an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or developmental process” (Karlson & Luscher, 1959).

More recently, Wyatt slightly modified the definition of pheromones as molecules that are evolved signals, which occur in defined ratios in the case of multiple component pheromones, that are emitted by an individual and received by a second individual of the same species, in which they cause a specific reaction, for example, a stereotyped behaviour or developmental process (Wyatt, 2014a). The same pheromone (or components of it) can have a variety of effects depending on the context or the receiver (Wyatt, 2010), such as the endocrine state (Griffiths & Brennan, 2015). These innate responses can be influenced by past experience and associative learning, especially in reproductive responses, or the by the gender of the receiver (Beny & Kimchi, 2014; Stowers & Liberles, 2016).

Wyatt proposed to separate pheromones and signature mixtures, defining signature mixtures as the subsets of variable molecules from the entire chemical profile that are learnt by other conspecifics and used to recognize an organism as an individual or as a member of a social group (Wyatt, 2010). One example could be major urinary proteins that indeed have been described as bar code to identify individuals (Kaur et al., 2014).

Sexual pheromones in mice

Sexual behaviour is paramount in the life of mammals. The search for and evaluation of adequate partners to exchange genes and ensure viable progeny is probably the primary motivation in adults’ life. Such important social behaviour, which requires detailed information about individuals of the other sex and rivals of the same sex, is largely covered by chemical communication in rodents because olfaction is the primary sensory process in these species.

Most likely, for these reasons, the largest number of pheromones has been identified in rodents. In mice specifically, we find urine pheromones such as pyrazines, acetates, thiazole amines and lipocalin and secretoglobin proteins.

The response to sexual pheromones can vary depending on the gender, reproductive physiology and experience of the receiver. This response variability is due to modulation of the pheromone processing circuitry at different levels from periphery receptors to the amygdala (Stowers & Liberles, 2016).

Androgen-binding proteins are secretoglobins produced in the lacrimal and submandibular glands. A role in sexual communication has been proposed as animals in a Y maze spend more time exploring areas with saliva from the other gender (Chung et al., 2017).

The peptide ESP22, which is secreted in the lachrymal gland, is released in the tears of 2-3-week-old mice, and it inhibits male sexual behaviour towards juveniles (Ferrero et al., 2013). Transgenic males lacking the VNO gene Trcp2 did not reduce attempts to mount juveniles. The ESP1 peptide from the same family, which is also secreted in the lachrymal gland, enhances female sexual receptivity behaviour upon mounting by males, exhibiting increased lordosis. This peptide is recognized by a specific vomeronasal receptor, V2Rp5, and its absence in transgenic mice induces neither neural activation nor sexual behaviour (Haga et al., 2010). Major urinary proteins also have an important role in sexual behaviour that was described in a previous section and is summarized in the next table (**Table 3**).

Table 3 Sexually dimorphic odours and pheromones in mice

Sexually dimorphic odors and pheromones of mice

Chemical	Biosynthesis	Behavior	Detection pathway
Trimethylamine	Male urine	Scent attraction	MOE; response lost in TAAR5 knockout mice
(Methylthio)-methanethiol	Male urine	Scent attraction	MOE
(*Z*)-5-Tetradecen-1-ol	Male urine	Scent attraction	MOE, includes Olfr288
2-*sec*-Butyl-4,5-dihydrothiazole	Male urine	Aggression	Activates OE and VNO
2,3-Dehydro-*exo*-brevicomin	Male urine	Aggression	Activates OE and VNO
Major urinary proteins	Male urine	Aggression, scent countermarking, attraction	VNO neurons containing V2Rs
Exocrine-gland secreting peptides	Male tears (ESP1, ESP24)	Lordosis behavior (ESP1)	VNO neurons containing V2Rs; ESP1 response lost in V2Rp5 knockout mice
Modified steroids	Female urine (estrogen derivatives) Male urine (androgen derivatives)	Courtship behavior	VNO neurons containing V1Rs

Modified from Stowers & Liberles, 2016

Alarm pheromones

Alarm pheromones (APs) are semiochemicals secreted by threatened or injured conspecifics. Intraspecies communication through APs is an evolutionarily widespread phenomenon that presumably occurs in all animal phyla. Social species of fish, insects and mammals use APS secretion as an altruistic signal to protect their colony/group or family in dangerous situations

(Brechbühl.J, 2013). These alert cues may derive from compounds that evolved to make the flesh unpalatable or toxic to predators, and their primary function could have been the control of skin pathogens. In insects and fish, APs of variable chemical structures have been identified such as terpenes, hydrocarbons, ketones or nitrogen/sulphated heterocyclic compounds.

Brechbül and collaborators (Brechbühl.J, 2013) reported the first mammal compound identified as an alarm pheromone, 2-sec-butyl-4,5- dihydrothiazole (SBT), which shares the thiazole ring with fox kairomone 2,4,5- trimethylthiazoline (TMT). This molecule was sampled from a CO_2 euthanasia chamber for mice.

Kiyokawa and collaborators (Kiyokawa, Kodama, Kubota, Takeuchi, & Mori, 2013) found a putative native alarm secretion for rats following electrocution of anaesthetised animals that increased freezing and risk assessment behaviours. However, the pheromone per se remains unidentified. This laboratory also criticized the alarm pheromone identified by Brechbül et al as the molecules were sampled both during and after the euthanasia and consequently could be associated with the decay process and the carcasses of the animals.

Interspecific: Allomones, synomones, kairomones, and apneumones

Interspecies chemical communication has a key role in the ecology and behaviour of species (**Table 4**). It can be related to species of the same animal class, such as mammals, but such communication can also cross larger taxonomic borders such as orders and kingdoms.

Allelochemicals are defined as semiochemicals that mediate interactions between two individuals who belong to different species (Dicke and Sabelis, 1988) from Sbarbati & Osculati, 2006)).

Allomones are chemical substances produced or acquired by an organism that, when they come in contact with an individual of another species in a natural context, evokes a behavioural or physiological response in the receiver that is adaptively favourable to the emitter but not the receiver (Lewis 1977, from (Sbarbati & Osculati, 2006)). Allomones produced by predators are mainly prey attractants (Blum, 1996) and those produced by preys are predator repellents (Apfelbach, Blanchard, Blanchard, Hayes, & McGregor, 2005).

Kairomones are allelochemicals that are pertinent to the biology of an organism (organism 1) that, when they come in contact with an individual of another species (organism 2), evokes in the receiver a behavioural or physiological response that is adaptively favourable to organism 2 but not organism 1 (Dicke & Sabelis, 1988).

Synomones can be defined as allelochemicals that are pertinent to the biology of an organism (organism 1) that, when they come in contact with an individual of another species (organism

2), evokes in the receiver a behavioural or physiological response that is adaptively favourable to both organism 1 and 2 (Dicke & Sabelis, 1988).

*Apneumone*s are substances emitted by non-living material that evokes a behavioural or physiological reaction that is adaptively favourable to a receiving organism but detrimental to an organism of another species that may be found in or on the non-living material (Nordlund & Lewis, 1976).

Rollo and collaborators (Rollo, Czvzewska, & Borden, 1994) proposed the term necromone for chemicals that play a role in the recognition of dead co- or heterospecific individuals and that may be adaptive to organisms of more than one species, e.g., the avoidance of disease or corpse management in social insects (Sun & Zhou, 2013).

Table 4 Signalling molecules with behavioural activities in mice

No.	Species	Chemosignal	Source	Receptor	Organ (Neuron)	Behavior
1	*M. musculus*	Hexanal	Decaying food	MOR1-1, etc	MOE	Unpleasant odor
2		Hexanoic acid	Decaying food	MOR31-2, etc	MOE	Unpleasant odor
3		Isoamylamine	Decaying food	TAAR3	MOE	Unpleasant odor
4		Carbon disulfide	Rodent breath	GC-D	MOE	Food preference
5		Uroguanylin	Rodent feces	GC-D	MOE	Food preference
6		2-*sec*-Butyl dihydrothiazole	Male mouse urine	?	MOE or VNO	Female attractiveness
7		Dehydro-*exo*-brevicomin	Male mouse urine	?	MOE or VNO	Female attractiveness
8		α- and β-farnesene	Male mouse urine	?	MOE or VNO	Female attractiveness puberty acceleration
9		(Methylthio)methanethiol	Male mouse urine	MOR244-3	MOE	Female attractiveness
10		(Z)-5-Tetradecen-1-ol	Male mouse urine	Olfr288	MOE	Female attractiveness
11		2-Heptanone	Female mouse urine	MOR139-1, etc V1Rb2	MOE or VNO	Estrus extension
12		Trimethylamine	Male mouse urine	TAAR5	MOE	Attractiveness
13		ESP1	Male mouse tear	V2Rp5	VNO	Female lordosis
14		ESP22	Juvenile mouse tear	?	VNO	Inhibition of male sexual behavior
15		ESP36	Female ELG	?	VNO	?
16		MHC class I peptide	Mouse urine	V2r1b	MOE or VNO	Pregnancy block Social preference
17		MUP20	Male mouse urine (C57BL/6)	?	VNO	Female attractiveness Learned spatial preference
18		Sulfated steroids	Stressed female mouse urine	V1rj2, etc	VNO	?
19		N-formylmethionine-leucine–phenylalanine	Mouse urine	mFpr1, mFpr2	VNO	?
20		2-Phenylethylamine	Carnivore urine	TAAR4	MOE	Avoidance
21		2,5-Dihydro-2,4,5-trimethylthiazole	Fox feces	?	MOE	Avoidance
22		MUP13	Rat urine	?	VNO	Defensive behavior
23		Feld4	Cat saliva	?	VNO	Defensive behavior

Modified from (Ihara, Yoshikawa, & Touhara, 2013)

CHAPTER 4: METHODS FOR CONTROLLING PEST RODENTS

Four processes can be manipulated to manage a pest population: birth, death, immigration and emigration (Buckle & Smith, 2015). Modern ecology stresses the role of spatial heterogeneity in population dynamics (Shorrocks & Swingland, 1990). Animals can be distributed between patches where resources are found, and some migration occurs between these patches (**Figure 26**). This is termed a metapopulation.

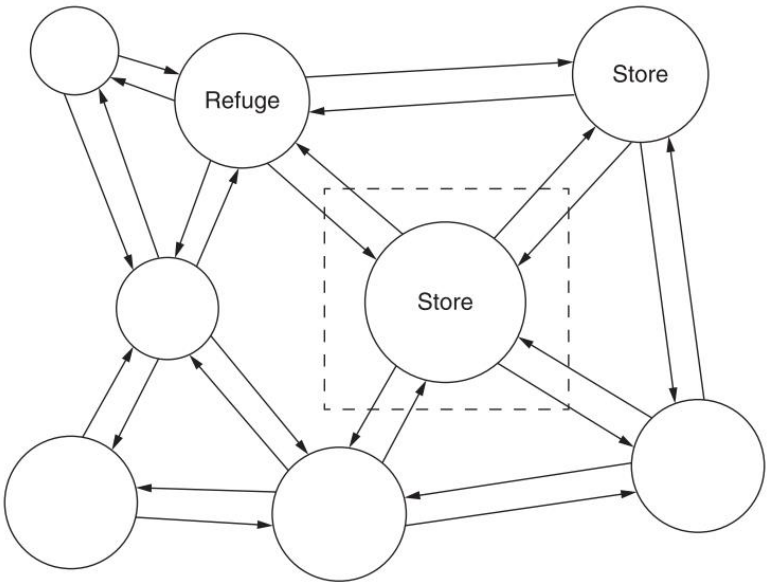


Figure 27 Population dynamics. The dynamics of pest populations may depend on migration between local populations in patches of suitable habitat as much as on within-patch dynamics. Exclusion isolates a patch from the metapopulation. Modified from (Singleton & Krebs, 2007)

Control of rodent pests mainly relies on increasing deaths through lethal methods without special interest in their ecology. Within lethal methods, we found two large groups: chemical and physical.

1. CHEMICAL METHODS

Lethal chemical agents (rodenticides) are presently the mainstay of all rodent-control programmes that involve the removal of extant infestations. This occurs in urban and agricultural environments and in species conservation efforts, and this situation is not expected to change in the near future (Buckle & Smith, 2015). Classically, two features are sought in rodenticides. The first, the efficacy, means that it must be toxic to target rodents and preferably

be lethal in small amounts with a mild speed of action to avoid learning and a mode of action that does not induce resistance.

The second feature is safety. A wide spectrum of activity is an important feature, but specificity to rodents is highly desirable in terms of safety and to avoid poisoning non-target species. However, it has been virtually impossible to develop a rodent-specific poison. Within the chemical methods, we distinguish acute and chronic methods depending on the time between application and the death of the animal.

1.1 Acute

Acute compounds are defined as those that kill the animal in 24 h or less after the administration of a lethal dose. These molecules are generally used at high concentrations and are mostly unsophisticated and therefore cheap to produce. In terms of security, they do not have a specific antidote, but the fast mode of action would mean a short time for administration. In terms of security, these features highly limit the use of acute compounds in urban environments to isolated locations by professional teams and specialized equipment. There are no valid patents for these chemicals, so they are not technically supported by major international companies.

The use of acute chemical control in rodent pest management has been proposed for large-scale agriculture programmes and invasive species removal (Buckle & Smith, 2015).

As many rodent species, especially rats, are suspicious of new objects (neophobia), they are highly reluctant to immediately feed on a novel food and may take only small quantities during initial feeding bouts (S. A. Barnett, 1988; Kronenberger & Médioni, 1985). To avoid this possible neophobic response, it is common to use a pre-baiting strategy, i.e., no rodenticide the first time, to increase the chances of success as rodents would be less suspicious of baits that they already know. This neophobic behaviour to food has recently been questioned (Modlinska, Stryjek, & Pisula, 2015).

Up to 1950, all rodenticides were non-anticoagulants, and most were acute or fast acting. This changed after the introduction of warfarin.

Zinc phosphide

This is the most commonly used acute rodenticide and the only one widely available for use. The mode of action of zinc phosphide is by the evolution of phosphine gas in the acid environment of the stomach; the gas enters the bloodstream and causes heart failure and damage to internal organs. It is generally available as a grey or black powder at a concentration of 80-95% with a strong garlic odour.

Strychnine

It is an alkaloid extracted from the seeds of the tree *Strychnos nux-vomica*, and it has been used worldwide for rodent control since the mid-18th century. In the US, it can only be used underground, and in Europe, it was removed from the market after the last Biocidal Directive (EU, 1998). The signs of poisoning are restlessness and muscular twitching progressing to convulsive seizures and muscular spasms before death.

Sodium fluoroacetate

Commonly known as 1080, this chemical acts by blocking the tricarboxylic acid cycle, causing the accumulation of citric acid and leading to convulsions and either respiratory or circulatory failure. As this cycle is fundamental to vertebrate physiology, the poison is non-specific. It is only used in a few countries such as Australia and New Zealand.

Alphachloralose

This narcotic with a rapid effect was previously used as a hypnotic, sedative and general anaesthetic in human and animal medicine. It slows several essential metabolic processes, such as brain activity, the heart rate and respiration, inducing hypothermia and eventual death. Its reliance on hypothermia restricts its use to ambient temperatures below 15-16°C, and it is unsuitable for rats due to its lower area: volume vertebrates. It can generate good results without the need for pre-baiting.

Cyanide

Cyanide disrupts energy metabolism by preventing the use of oxygen in energy production, causing cytotoxic hypoxia in the presence of normal haemoglobin oxygenation. When the dose is optimized, the cytotoxic hypoxia depresses the central nervous system, the site most sensitive to anoxia, resulting in rapid respiratory arrest and death.

Calciferol and cholecalciferol

A form of vitamin D, these substances interfere with calcium homeostasis, causing mobilization of calcium from the bone matrix and increased uptake in the gut. In the UK, it is used at a concentration of 3-4% (Mason & Littin, 2003). The resulting hypercalcaemia and other symptoms are often difficult to reverse, and victims usually die from hypercalcaemia, kidney failure, and/or the side effects of soft-tissue calcification, particularly metastatic calcification of the blood vessels and nephrocalcinosis. It can be formulated as one feed bait, requiring no pre-baiting.

These chemicals are also called subacute rodenticides because even if the lethal dose cannot be consumed in the first 24 h, repeated feeding can occur, and death is normally delayed for

several days. There is a characteristic period of anorexia that can be problematic if the animals have ingested sub-lethal doses (Buckle & Smith, 2015).

Bromethalin

This rodenticide is used at 0.005 or 0.01%. It is a pale-yellow, crystalline solid that is effective against rodents, including strains resistant to anticoagulants. The mode of action is by uncoupling oxidative phosphorylation in cells of the central nervous system (CNS), and it produces anorexia after the consumption of an effective dose. Symptoms of poisoning include tremors, convulsions, prostration and hind-limb paralysis. No specific antidote is available, and it is not authorized in the EU.

Para-aminopropiophenone (PAPP)

This is a new acute toxin registered in New Zealand in 2011 (Buckle & Smith, 2015), and its toxic effects are based on its ability to reduce the oxygen-carrying capacity of red blood cells through the formation of methaemoglobin. Symptoms are clearly identifiable; animals receiving a lethal dose are unconscious within 30-45 m, and death occurs in 2h. Methylene blue is considered an antidote. At the moment, PAPP is considered insufficiently potent against rodents.

Powdered corn cob

This is formulated into bait pellets containing approximately 90% powdered corn cob for use as a rodenticide. The chemical compounds are complex as they are natural products, and the mode of action it is not completely understood. Animals lose weight severely, primarily due to dehydration linked to reduced drinking.

1.2 Chronic

Anticoagulants

These compounds originated from research conducted in the 1930s in the USA to determine the origin of a haemorrhagic disease of cattle (Buckle & Smith, 2015). The causative agent of the disease was found to be a chemical contaminant of spoiled sweet clover hay; naturally occurring coumarin in sweet-clover hay is converted to dicoumarol by fungi (Murphy, 2007). Afterwards, a range of molecules were synthesized. Warfarin takes its name, in part, from the Wisconsin Alumni Research Foundation; it was the first anticoagulant rodenticide introduced into the market shortly after World War II and became widely used in many countries.

All anticoagulant rodenticides are either hydroxycoumarins or members of a related group, the indane-diones (Buckle, 1993); they differ little in their chemical properties (**Figure 27**), but their

toxicity to target rodents varies. The first hydroxycoumarin and rodenticide was warfarin, and other compounds in the same family include coumachlor, coumafuryl or coumatetralyl. Some compounds of the related family, indane-diones, are pindone, diphacinone and chlorophacinone.

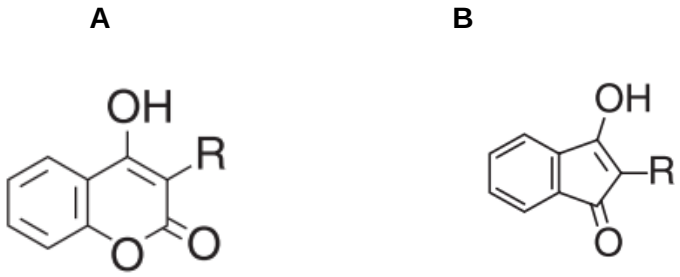


Figure 28 A. Base structure of warfarins B. Base structure of indane-diones

They have a chronic mode of action, interrupting the vitamin K cycle in liver microsomes.

First-generation anticoagulants

Generally, these chemicals have moderate toxicity with acute LD50 values ranging from 10 to 50 mg/kg body weight (Murphy, 2007). The active form of the vitamin, vitamin K hydroquinone, is required as a cofactor in the blood-clotting cascade. Anticoagulants block the recycling of the active hydroquinone form of the vitamin, and as vitamin K recycling is blocked, only dietary vitamin K is available, which is insufficient to maintain clotting factor synthesis.

These anticoagulants are not sufficiently toxic to rodents to cause death after a single exposure. In fact, they are only effective at blocking the vitamin K cycle for relatively short periods and must be taken over several days to have a sufficiently prolonged effect to cause death. Therefore, success depends on the animals having continuous access to baits during a period of several days to several weeks. For this reason, surplus baiting developed, in which relatively large quantities of baits are used at the bait points.

Resistance

The occurrence of spontaneous mutations in mammalian genomes results from errors occurring during either meiosis or in the process of DNA replication that are not mended by cellular (DNA) repair mechanisms (Guénet et al., 2015). These spontaneous mutations can be transmitted to the next generations if they occur in germinal cells, and the rate in mice is 0.54 × 10−6 per locus per gamete. Thus for each gene, there is approximately one possibility out of 2 million (Schlager & Dickie, 1967). Accounting for rodent population numbers and the fast

reproductive cycle, it is not difficult to find mutations, which arise rapidly in populations if they confer an evolutionary advantage.

Mice and rats developed genetic resistance to warfarins, which was discovered in the beginning of the 21st century with improvements in genetics technology. The gene expressing the vitamin K epoxide reductase multiprotein complex 1(VKORC1), which contributes to renewing disposable vitamin K in the organism, was found to be the basis of genetic resistance to anticoagulants (Pelz et al., 2005; Rost et al., 2004). Its overexpression inhibited the action of warfarins. Since then, multiple mutations concerning this gene have been discovered in mice, rats (Oldenburg, Müller, Rost, Watzka, & Bevans, 2014; Rost et al., 2009), and humans (Watzka et al., 2011) that have important clinical relevance for the resistance to antithrombotic drugs.

Sensitivity to warfarin-based rodenticides may also be pharmacokinetically based, arising from increased warfarin biotransformation, and this mechanism may be responsible for resistance to some of the superwarfarins, such as difenacoum. A third form of resistance may arise from an enhanced capacity to synthesize vitamin K from menadione, a commonly used additive in animal foods on farms (Thijssen, 1995). In addition, natural tolerance is observed in xerophilous rodents, those adapted to arid regions, in North Africa and the Middle East.

Second-generation anticoagulants

Superwarfarins, or second-generation warfarins, were developed following the emergence of resistance in rodents. They have been commercially available as rodenticides since 1979, and they have much longer half-lives and a stronger affinity to vitamin K epoxide reductase, therefore causing death in warfarin-resistant rodents. The increase in use was accompanied by an increase in accidental poisonings that reached >16 000 per year in the United States (Feinstein et al., 2016). Treatment of superwarfarin poisoning with vitamin K is limited by extremely high cost; it can require daily treatment for long durations (one year or more). Risk of exposure has become a concern since up to hundreds of kilograms of rodent bait are applied by aerial dispersion over infested regions. Superwarfarins are normally provided in baits at 0.005%, and difenacoum and brodifacoum have exhibited the highest toxicity to warfarin-sensitive and resistant rats.

Rodenticide intoxication

Rodenticides have been described as the most common toxicants found in domestic carnivores such as dogs and cats (Murphy, 2002) and ferrets (Overman, 2015). Exposure in

domestic pets occurs through ingestion of the product from the bait container or from the environment to which the rodents have carried the bait.

Most cases of rodenticide exposure occur in younger animals. In the US, the most commonly reported cases of toxicosis were those caused by anticoagulants, bromethalin, cholecalciferol, strychnine, and zinc (DeClementi & Sobczak, 2012; Murphy, 2002). Ingested anticoagulant rodenticides are transported to the liver via the portal vein or chylomicrons, and while in the liver, they interfere with vitamin K1 hydroquinone recycling. The anticoagulant rodenticides exit the liver via the hepatic vein and are measurable in circulation, and they are eliminated through either urine or bile. In the case of biliary elimination, some anticoagulant rodenticides may undergo entero-hepatic circulation. Anticoagulant rodenticides can remain in the tissues of an animal even after successful treatment (Murphy, 2002).

Common intoxications have been widely reported in wildlife, especially in raptors such as the barn owl and mammals such as the polecat from the mustelid family (Elliott et al., 2014; Shore, Birks, Freestone, & Kitchener, 1996; Stone, Okoniewski, & Stedelin, 2003).

2. PHYSICAL METHODS

Trapping and hunting are labour-intensive methods that are unlikely to be cost-effective in countries where labour costs are relatively high, but such methods can replace chemical control in high-risk or environmentally sensitive areas. Pragmatically, the limited number of animals that can be trapped has been highlighted as well as the need for regular attention to control the traps. Trapping of non-target animals can occur, and trapping a mother leaves the nestlings without maternal care, which has welfare implications (Mason & Littin, 2003).

Sticky boards

Sticky boards are squares of wood, plastic or stiff cardboard coated with highly adhesive “rodent glue” that are placed on rodent runways. When the animal crosses the boards, it becomes stuck by the feet and fur. Animals are not killed by the trap itself, but they can die from hunger, thirst and they can also self-mutilate (Mason & Littin, 2003).

Snap traps

Snap traps are spring-based devices that kill by means of a rapidly descending bar. They are baited with chocolate, fruits, peanut butter and cooked meats all being effective lures, and they are potentially easier to monitor than live traps.

Electrocution traps

These devices consist of an open-ended box baited with dry food. The floor is made of two plates which are terminals; a rodent bridging these two plates receives a 2 min-long shock, transmitted via the feet, of approximately 2000 V. This causes the heart to fibrillate, and the respiratory muscles to become unable to function. The failure of these organs then causes death.

3. OTHER METHODS

Ultrasound

Ultrasounds are relevant to rodent communication by adults and young animals (Portfors & Perkel, 2014; Willadsen et al., 2014), but there is no scientific evidence that any of the available machines are effective for control (Clapperton, 2006; Smith & Meyer, 2015).

Biological control: predators

One problem related to biological control is that the predator species becomes a pest itself. Other theoretical problems with using vertebrate predators as biological control agents is that their generation time is usually substantially longer than that of their prey (Smith & Meyer, 2015). For example, mice have a shorter reproductive cycle, so cats have become pests on islands such as Australia or New Zealand.

Control of reproduction

Several strategies have been proposed in this sense, including disruption of reproductive behaviour or reproductive inhibitors/sterilants. However, there is no real alternative currently in the market (Smith & Meyer, 2015).

Pheromone baits

Few commercial pheromone lure products can be found in the market for rodents, including *Mus musculus* and *Rattus norvegicus.* Even if there is scientific evidence for sexual pheromone attraction to males and females in laboratory rodent's, pheromone lures can be less attractive than food lures (for review Clapperton et al, 2017). As a general approach they keep the same problems than other traps methods, like need of regular surveillance. Additionally, effects in other species shouldn't been underestimated (Apps et al, 2017).

OBJECTIVES

Controlling rodent populations has been a great challenge for humanity since humans began to store food following the development of farming and agriculture. Contradictorily, the greatest advances in biology, molecular biology, neuroscience and medicine came about through the domestication and use of rodents as research models, and it has been a love-hate story ever since.

The main aim of this thesis was to better understand predator and plant chemicals or mixtures that could be identified and used to manage of rodent populations, inhibiting their incursion into human resources and shelters.

The use of predator stimuli has been proved to elicit anti-predatory responses in rodents, but we have not identified many of these molecules or mixtures involved in these behaviours and physiological responses. As a first step, our aim was to investigate exploratory and foraging behaviour in the presence of predator chemical cues in the house mouse. We tested several mammalian carnivorous species and snakes, all of which are rodent predators. First, we validated a simple but robust test with three chambers using red fox (*Vulpes vulpes*) faeces, which triggered avoidance in mice as other authors have published. Later, we tested different snake, ferret, cat and dog samples to develop a more complex ethogram with specific behaviours associated with fear/anxiety (from a general perspective) and predator avoidance.

In another set of experiments, we aimed to complete the kairomone profile of the species most commonly used to obtain rodent predator samples, the domestic cat. First, we tested a hydrophilic solution of cat fur and skin containing high amounts of the main cat allergen Fel d 1. This molecule is largely emitted in the environment by cats and has been proposed as a putative pheromone, but there is no information in the literature about the effect of a solution containing this molecule on mice. For this reason, we developed a complex device with 8 different corridors and a central arena. Second, we tested the effects of an identified cat pheromone, commercially known as Feliway ®, on mouse exploratory behaviour and feeding. Simultaneously, and separate from the original objectives but arising as a result of adapting the environmental conditions and the tests to the biology of the study species, we developed a new illumination mode to reverse light cycles in laboratory rodents, allowing work to be performed during the dark phase of the cycle.

The next objective was to explore behavioural reactions to plant chemical cues that are ecologically meaningful for the rodents and to compare these reactions to those to rodent predator chemical cues. Finally, we aimed to provide a general overview from an ethical perspective and debate the lack of concern for animal welfare related to rodent pest control.

We have discussed alternatives including a semiochemical approach and framed it as a global strategy based in the ecology of the species.

The final aim of this thesis was to use this knowledge to add a new tool for rodent population control as part of an ecological and integrated pest management programme.

II RESULTS

Publications list

Peer reviewed publications

C.Grau; J.Leclercq, E.Teruel; C.Lecuelle; P.Pageat. *Behavioural responses to ferret olfactory cues and other mammalian and reptilian predators of the house mice* (Mus musculus). Accepted for publication in Chemical Signals in Vertebrates Vol 14. Edited by C.D. Buesching & C.T. Müller (expected publication mars 2019).

C.Grau, C. Bienboire-Frosini, C. Lafont-Lecuelle, P. Monneret, J. Leclerc, A. Cozzi, P. Pageat. *Does Fel d 1, the Main Cat's Allergen have a Kairomone Role in Mice?* To be submitted to Plos one

C.Grau, J. Leclercq, E. Descout, E. Teruel, C. Bienboire-Frosini, P. Pageat., 2019. *Ethanol and a chemical from fox faeces modulate exploratory behaviour in laboratory mice.* Applied Animal Behaviour Science. In press, Available at: https://doi.org/10.1016/j.applanim.2019.02.003.

International congress with peer reviewing

Oral presentations

C.Grau; *Ethical committees in animal research: law boundaries and beyond*. Proceedings of the European Congress of Animal Welfare and Behavioural Medicine (AWBM) Volume: Animal Welfare, ethics and law. Berlin, Germany, 27th September 2018

C.Grau; *Introduction to clinical ethology in ferrets*. Proceedings of the European Congress of Animal Welfare and Behavioural Medicine (AWBM) Volume: Behavioural Medicine. Berlin, Germany, 29th September 2018

C.Grau; *Effects of predator and plant olfactory cues in the exploratory behaviour of the house mouse*. Proceedings of the joint meeting: 6th International Conference of Rodent Biology and Management & 16th Rodents et Spatium. Potsdam, Germany, 3rd September 2018

Carlos Grau, Julien Leclercq, Estelle Descout, Cécile Bienboire-Frosini, Patrick Pageat. *Behavioural responses to a ubiquitous plant chemical and predator olfactory*

stimuli in the house mouse. Zbigniew Borowski, Wanda Olech, Agnieszka Suchecka (eds.). 11th European Vertebrate Pest Management Conference. September 25-29, 2017, Warsaw, Poland

C.Grau; *Effects of predator and plant olfactory cues in the behaviour of the house mouse*. Invited presentation. Congress of the UK Semiochemistry network. July 2018, Cambridge.

C.Grau; A.Cozzi; P.Pageat. *The humaneness of rodent control and alternatives to actual methods*. Proceedings of the European Congress of Animal Welfare and Behavioural Medicine (AWBM) Volume: Animal Welfare Science. Cascais, Portugal, 21th October 2016

C. Grau; C.Bienboire-Frosini ; A.Cozzi ; P.Pageat ; *Does Fel d 1 , the main cat's allergen have a kairomone role in mice.* Chemical Ecology of Vertebrates. Proceedings of the International Society of Chemical Ecology (ISCE) Stockholm, Sweden 30th June 2015

C. Grau; A.Cozzi; P.Pageat; *When rodents arrive to avoid predators: the role of olfaction*. Proceedings of the 4th annual meeting of the European College of animal Welfare and Behavioural Medicine -IRSEA congress, Apt, France, 2014

Poster

C.Grau, J. Leclercq, E. Descout, E. Teruel, C. Bienboire-Frosini, P. Pageat. Behavioural responses to a ubiquitous plant chemical and predator olfactory stimuli in the house mouse. Proceedings of the 11th European Vertebrate Pest Management Conference; Warsaw 09/2017

C.Grau; J.Leclercq, E.Teruel; C.Lecuelle; P.Pageat. Preliminary results in ferret olfactory cues (*Mustela putorius furo*) as a predator stimulus for the house mice (Mus musculus). Proceedings of the 14th meeting of the Chemical Signals in Vertebrates group (CSiV); Cardiff 08/2017

MJ Molina-Cimadevila (1), A Romero (2), T García-Robles (3), J Ramos (3), M Casado (4), Y Miralles (5), A García-Martínez (6), N Marín (7), O Fernandez (8), H

Paradell (9), JA García-Partida (10), Y Brito-Casillas (11), M Ricca (12), **C Grau** (13) , L Barrios (14) y E de-Madaria (15). *Eutanasia de roedores: Sobre cómo nos sentimos nosotros*. Proceedings Congreso de la Sociedad Española para las Ciencias del Animal de Laboratorio. Tenerife 06/2017

National congress with peer reviewing

Oral presentation

C.Grau *Avoiding danger : The Role of Olfaction in Rodents against Predators*
Journée de l'ecole doctorale SEVAB, Institut National Polytechnique de Toulouse
Mars 2015

First part

CHAPTER 1: INFLUENCE OF COMPLEX PREDATOR OLFACTORY CUES (NATIVE FORM) ON THE USE OF SPACE

1. PREAMBLE

We performed this study to evaluate the responses of laboratory house mice to several native olfactory stimuli from rodent predators, similar to that found in nature. For this purpose, we designed a three-chambered device with a central area, where the animal was released as a starting point, and two lateral areas, where the treatment was randomly assigned to one of the two sides. The mice did not have direct contact with the olfactory stimuli, and the stimulus was placed inside a metallic drilled tea ball to avoid visual or physical stimuli. As a first step, we performed a preliminary study to validate the use of the device. We compared avoidance behaviours between a positive control that has been previously described in the literature, red fox (*Vulpes vulpes*) faeces, and a negative control (medical gauze). Sixteen mice were tested, 8 mice (4 males, 4 females) were exposed to the fox faeces and 8 to the control.

Once we validated our 3-chambered device and the procedure, we carried out a test with 5 treatments from several predators including cats, snakes, dogs, ferrets, foxes and a control, and 8 animals were tested per treatment (n=48). We found significant avoidance effects for the ferret olfactory stimuli.

2. STUDY 1

Validation of a 3-chambered device for evaluating the avoidance of a predator stimulus, red fox faeces (preliminary study)

Introduction

Fox faeces were first described as a predator stimulus that elicited avoidance in rodents by Vernet-Maury (Vernet-Maury, 1980), and within this complex olfactory cue, she identified the putative kairomone trimethylthiazoline, which has been described in many studies as an olfactory stimulus that elicits anti-predator responses (including avoidance) (Rosen, Asok, & Chakraborty, 2015). For this reason, we decided to use this stimulus as a positive control to

validate our device and to determine if we could observe a significant difference in the level of avoidance of the stimulus against a negative control.

Materials and methods

The materials and methods are fully described in the next section of this chapter and within the paper accepted for publication in Chemical Signals in Vertebrates. To avoid unnecessary repetition of this information, I have only described the differences between this preliminary study and the final experiment.

Animals

Mice used for this preliminary study were 14-month-old RjOrl: Swiss mice (Janvier Labs, France); the mice were kept at the facilities of the Research Centre in Semiochemistry and Applied Ethology (IRSEA) according to the requirements of French and European Law (2010/63/EU). As a veterinarian specializing in laboratory animals, I supervised their health and housing conditions. Mice were naïve to fox faeces (encountered the stimulus for the first time during the experiments). Further details are explained in the following materials and methods section (page 88)

Olfactory stimuli

In this preliminary study, we used red fox (*Vulpes vulpes*) faeces from the south of France from a mixture of faeces from a domestic fox (kept in captivity) fed with commercial companion animal food and from conspecific wild animals that were attracted by its presence and left droppings in the vicinity. Fox faeces were poured over a medical gauze which was placed within a metallic, drilled tea ball (Leclerc, Apt, France); the control treatment was only the medical gauze. For this preliminary study, the tea ball was only placed in the treated area (with both positive and negative controls).

Statistics

The comparisons between the control and fox faeces according to the avoiding area duration and the close treatment area duration were carried out using a Student's t-test (ttest procedure) or a Wilcoxon two-sample test using the npar1way procedure of SAS 9.4 software depending on the normality of residuals (verified with the univariate procedure) and the homogeneity of variances (also verified with the ttest procedure).

Results

Mice spent significantly less time in the area treated with fox faeces than with the control (P=0.0136) (**Figure 1**); Student's t-test was used as parametric conditions were satisfied. In

the same sense, mice remained for significantly longer in the non-treated area (avoidance area) when the treatment was fox faeces compared to the control (**Figure 2**).

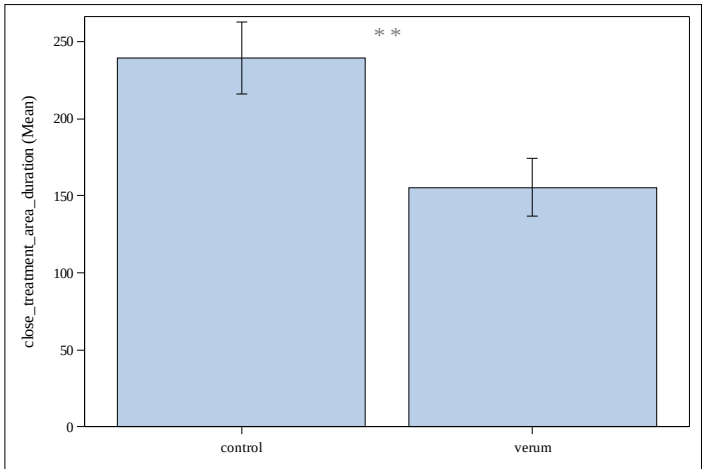


Figure 1 Total duration in treated area with the control (left) and fox faeces treatments (verum, right). Mean ±SE

The non-parametric Wilcoxon test was used to measure differences for the non-treated area (avoidance area), p=0.0171 (**Figure 2).**

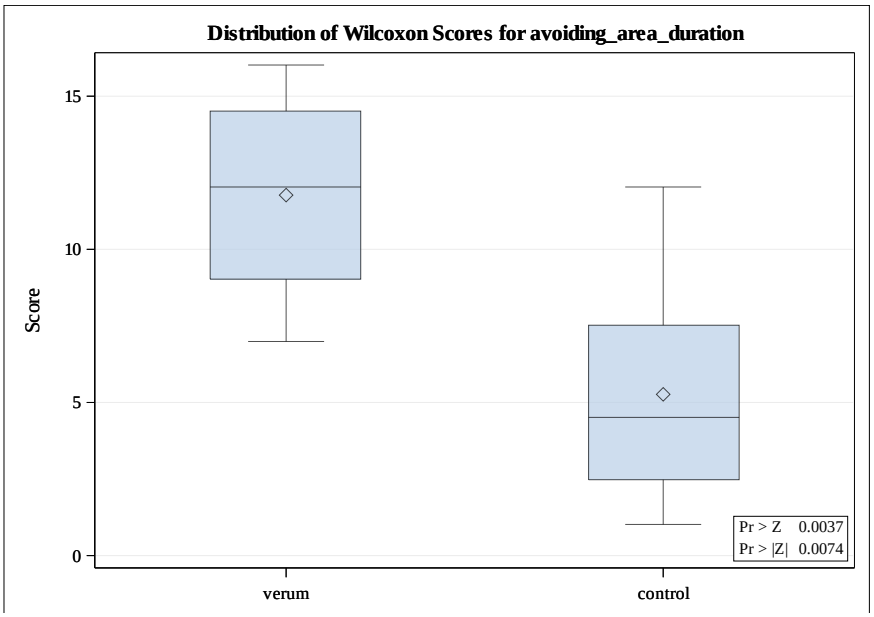


Figure 2 Wilcoxon test scores for total duration in the non-treated area, comparing fox faeces (verum, left) to the control (right)

Table 1 Descriptive statistics

Treatment	N	Variable	Mean	Std Dev	Std Error	Median	Minimum	Maximum
Control	8	Treated area total duration (s)	239.37	65.90	23.30	224.00	149.00	330.00
		Non-treated area total duration (s)	193.87	52.05	18.40	179.00	132.00	289.00
Fox faeces	8	Treated area total duration (s)	155.37	52.45	18.54	140.50	109.00	271.00
		Non-treated area total duration (s)	283.62	41.53	14.68	286.00	221.00	359.00

Discussion

Our results confirmed our initial hypothesis that fox faeces would elicit significant avoidance in laboratory mice. This preliminary study allowed us to validate this experimental device as it was capable of showing differences in the studied behavioural parameter, which would also be the main parameter for the following studies with this device.

Conclusions

Our experimental device was validated with this preliminary experiment as we were able to observe significant response differences between the positive control (fox faeces) and our negative control (medical gauze).

3. STUDY 2

Influence of different reptilian and mammalian predatory cues on the exploratory behaviour of house mice

Poster presentation at international congress with peer review

C.Grau; J.Leclercq, E.Teruel; C.Lecuelle; P.Pageat. Preliminary results in ferret olfactory cues (*Mustela putorius furo*) as a predator stimulus for the house mice (Mus musculus). Proceedings of the 14th meeting of the Chemical Signals in Vertebrates group (CSiV); Cardiff 08/2017

Poster

Preliminary results in ferret olfactory cues (*Mustela putorius furo*) as a predator stimulus for the house mice (Mus musculus)

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The house mice (Mus musculus) as other small rodents are in the base of vertebrate predator's trophic cascades. They are the most widespread mammal on earth after humans, which along with its fast sexual cycle and prolificity means large populations and a basic source of nutrients for a wide spectrum of predators.

As macrosmatic animals, mice use olfaction as a primary tool to avoid predators, however little is known about the predator olfactory cues and behavioral reactions linked to these stimuli. With this study we performed a preliminary approach to mammalian and reptilian olfactory predatory cues of the house mice. For this porpoise we carried out a choice test, where we measured for 10 minutes the total duration that mice remained in the nearby area or far end area from the predatory stimulus, mice had no physical access to the stimulus, and both parts were identical.

Our results showed that mice significantly avoided ferret olfactory stimuli from fur and faeces. These results are in line with a recent study that showed avoidance of hamsters to ferret urine

depending on the ferret's diet (Apfelbach, Soini, Vasilieva, & Novotny, 2015). However further research should delve in ferret's olfactory cues and semiochemicals as a significant rodent predator.

References

Apfelbach, R., Soini, H. a., Vasilieva, N. Y., & Novotny, M. V. (2015). Behavioral responses of predator-naïve dwarf hamsters (Phodopus campbelli) to odor cues of the European ferret fed with different prey species. *Physiology & Behavior*, *146*(June), 57–66.

Research paper accepted for publication in Chemical Signals in Vertebrates XIV

C.Grau; J.Leclercq, E.Teruel; C.Lecuelle; P.Pageat. Behavioural responses to ferret olfactory cues and other mammalian and reptilian predators of the house mice (Mus musculus). In Chemical Signals in Vertebrates Vol 14. Edited by C.T. Müller & C.D. Buesching. In press

House Mouse (*Mus musculus*) Avoidance of Olfactory Cues from Ferrets and Other Mammalian and Reptilian Predators: Preliminary Results

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Abstract

Like other small rodents, house mice are at the bottom of vertebrate predator-dominated food chain. After humans, house mice are the most widespread mammal on earth. With their short sexual-cycle and prolificity, they can quickly produce large populations that form a basic source of nutrients for a wide spectrum of predators.

As macrosmatic animals, mice use olfaction as a primary tool to avoid predators, however further research is still required to fully understand the main predator olfactory cues and behavioral reactions linked to these stimuli. This study offers a preliminary approach for examining the mammalian and reptilian olfactory predatory cues used by house mice. For this purpose, we carried out a choice test

where, during a 10 minutes period, we measured the total duration that mice remained in either the area closest to or farthest from the predatory stimulus; mice had no physical access to the stimulus, and both compared areas were identical. The stimuli tested were ferret fur and faeces, snake sheds, fox faeces, dog faeces, and cat urine.

Our results showed that mice significantly avoided ferret olfactory stimuli from fur and faeces. The other predator stimuli did not elicit significant avoidance. However, in some cases this may be due to specific genetic and phenotypic features of the mouse strain tested. These results are in line with previous work with ferret olfactory stimuli in mice. However, further research should examine the role of ferret olfactory cues and semiochemicals as good indicators of their presence that lead to avoidance by rodents.

Key words: allelochemicals, ferret olfactory cues, cat urine, fox olfactory cues, rodent predators, dog olfactory cues, snake olfactory cues.

1. Introduction

1.1 Predation

The detection of predator cues by prey constitutes a valuable tool for survival, making this feature a criterion for selection throughout evolution. Predators and prey run a constant arms race that leads to continuous evolution (Dietl & Kelley 2002) and that commensal species have continued in human habitats (Bull & Maron 2016; Lowry et al. 2013). Along with rodents, members of the orders Carnivora and Squamata (lizards and snakes) are macrosmatics, and olfaction and chemical communication play an overarching role in their lives. In the wild, and more recently in human environments, rodents and members of the orders Carnivora and Squamata have co-evolved (Abrams 2000) with each having a major influence on the other: the former as an important food resource and the latter two as major predator risks.

1.2 Ferret Olfactory Cues as a Predatory Stimulus

The ferret (*Mustela furo*) is a domestic mustelid (Church 2007; Alexander P. D 1951) whose probable wild ancestor species are the European polecat (*Mustela putorius*) and the Steppe polecat (*M. eversmanni*) (Church 2007); both are considered major rodent predators. The main prey of the European polecat are rodents followed by rabbits and anurans, depending on their abundance (Santos et al. 2009; Lodé 1997). The diet of the steppe polecat is similar, with a preference for small rodents (Lanszki & Heltai 2007). Ferrets and polecats are both obligate carnivores, even more so than cats, with a simple digestive tract (Bradley et al. 2006) that obliges them to feed several times per day, a fact which likely conditions their hunting habits.

Scent marking behavior in ferrets has been described with several behaviors: defecation and urination, anal drag, wiping, body rubbing, and chin rubbing (Clapperton 1985). Some experiments have demonstrated olfactory preference and sex identification in ferrets using olfactory cues. (Clapperton et al. 1989). Species, sex and age recognition has been confirmed later in wild relative species: *Mustela eversmanni* (Siberian Weasel) and *Mustela sibirica* (Steppe polecat) (Zhang et al. 2002) along with sex and individual recognition in ferrets (Zhang et al. 2005). These olfactory cues could be used by rodents to identify and avoid this predator.

Masini and collaborators found that rats avoided olfactory stimuli from ferret fur (Masini et al. 2005). However, Zimmerling et al (Zimmerling & Sullivan 1994) did not find any effect of anal gland secretion semiochemicals in feral populations of the deer mouse (*Peromyscus manuculatus*). The use of isolated ferret compounds or sibling species was tested recently by Sievert & Laska (2016). 2-propylthietane, a chemical identified in the anal gland secretion of several mustelids, and 3-methyl-1-butanethiol, a chemical identified in several species of skunks (Musteloidea superfamily), decreased general motor activity and elicited avoidance in cd-1 mice (Sievert & Laska 2016).

There are far fewer publications related to ferret olfactory stimuli as a predator stimulus for rodents than for the most commonly studied species in this area, the cat (*Felis catus*). This can likely be attributed to pragmatic reasons. First, cats outnumber ferrets as companion animals (AVMA 2012). Second, pet ferrets have been sold castrated and without anal glands in many countries, which means that their anatomy, physiology, secretions, and behavior have been altered and are therefore not suitable as models for chemical signals in the species.

In summary, there is some evidence that rodents use ferret cues by associating these stimuli to danger, and volatile putative kairomone compounds have been identified, but no heavier non-volatile compounds such as proteins have been established.

1.3 Snake Olfactory Cues as a Predator Stimulus

Olfaction is a primary sense for snakes, they use it for intraspecific communication and leave chemical messages produced by secretory glands in the skin or in the anal glands (Parker & Mason 2011; Mason & Parker 2010). Terrestrial snakes travel from one point to another by characteristic undulatory movements, with the ventral part in direct contact with the ground. During these movements, they can leave chemical compounds produced in the skin or the anal glands. These trails can be used by other conspecifics to identify their sex or mating status. These compounds have been identified mainly as fatty acids (Parker & Mason 2011), with the exception of some rare airborne pheromones, which are detected via direct contact (tongue flicking) with the Vomeronasal Organ (Shine & Mason 2012). These chemical cues could potentially be good kairomone candidates as they would indicate the relatively recent presence of snakes and where they have traveled, and rodents widely use sniffing behavior to detect heavy molecules like the Major Urinary Proteins (MUP).

Rodents and snakes have a long co-evolutionary history together, probably longer than with any other predator. Snakes and their clade Serpentes diverged approximately 35 million years before rodents (Graphodatsky et al. 2011; Reyes-Velasco et al. 2015); rodents have therefore shared habitats with snakes throughout their entire evolutionary history of 75 million years. Mechanisms and chemicals used to detect these predators were probably developed before speciation of actual species of rodents (Boursot et al. 1993) and snakes. Therefore, allopatric species (species not sharing the same geographic habitat) could demonstrate anti-predator behaviors based on ancient mechanisms originating from this long co-evolutionary history.

The order Squamata is the largest order within the Class Reptilia with 10265 living species, and its suborder Serpentes accounts for approximately 3600 species (www.reptiledatabase.org 2017). In this study we tested stimuli from three species. *Rinechis scalaris* and *Vipera aspis* are terrestrial medium sized species found in southern Europe with a diet composed largely of small mammals such as rodents from the genus Mus, Rattus or Apodemus (Pleguezuelos et al. 2007; Saviozzi & Zuffi 1997). *Trimesurus albolabris* is an arboricol species from southeast Asia that feeds partially on rodents, but probably in a smaller proportion than the other two species (Coborn 1991).

Chemical compounds used to detect predators can come from compounds that already have a chemical meaning in intra-specific communication or between competitor species (Banks et al. 2016). Some studies have provided evidence of anti-predator behaviors to snake olfactory stimuli in rodents or lizards, and in some cases, the species used were allopatric. However, this information is incomplete and sometimes contradictory (de Oliveira Crisanto et al. 2015; Papes et al. 2010). Due to the importance of snakes as rodent predators, we decided to test olfactory stimuli that could have an ecological meaning for rodents in avoiding these predators. The samples used were skin sheds as they probably contain the chemical compounds left by the snakes as they travel and are easily sampled and transported. In addition, skin sheds have already shown anti-predator effects in rodents (Papes et al. 2010).

1.4 Fox and Dog Olfactory Cues as a Predator Stimulus

Dogs (*Canis familiaris*) are a domesticated carnivorous mammal species descended from the wolf (*Canis lupus*). Along with the red fox *(Vulpes Vulpes),* both are considered generalist predators (Hanski et al. 1991). However, rodents represent a higher proportion of the diet in the red fox (*Vulpes vulpes*) (Leckie et al. 1998; Díaz-Ruiz et al. 2013) than the wolf, whose diet also includes rodents, but who prefers larger prey such as ungulates (Capitani et al. 2004; Wagner et al. 2012). The purpose of testing the faeces of these species was to compare two members from the Canidae carnivorous family with different dietary habits regarding rodents, one more focused on rodents or rabbits, and the other on larger prey, mainly ungulates.

1.5 Cat Urine Olfactory Cues as a Predator Stimulus

Cats are proteinuric, excreting large quantities of proteins (0.5-1.0mg/ml) in their urine, ~90% consists of cauxin, a carboxylesterase-like protein (Spotte 2014). Cauxin regulates and is directly correlated with L-Felinine, an amino acid excreted in cat urine (Miyazaki, Yamashita, Hosokawa, et al. 2006; Miyazaki, Yamashita, Suzuki, et al. 2006). Cat urine and the amino acid L-Felinine have shown some influence in rodent reproduction (Vasilieva et al. 2000; Voznessenskaya 2014). The role of cauxin has not been clearly identified (Spotte 2014), however we know that its production depends on the sex of the animal, with males secreting higher amounts than females (Miyazaki, Yamashita, Hosokawa, et al. 2006), as is also the case for Major Urinary Proteins in rodents. For this reason, we used a non-castrated male for cat urine sampling. We hypothesized that this protein or other chemical compounds in the cat's urine could be identified by the house mouse as a chemical cue signaling danger; and that it could influence exploratory behavior or locomotor activity.

1.6 Aims of the Study

The main aim of our study was to explore the behavioral reactions of mice to a complete repertoire of olfactory stimuli produced by ferrets, a rodent specialist predator. The stimuli were composed of male and female faeces and fur olfactory cues. We are aware of the loss of specificity with this approach, however this configuration likely provides a more realistic and complex set of stimuli.

In addition, we tested other predator olfactory stimuli that could play a significant role in the ecology of the house mouse. These species included snakes, the red fox, the domestic cat, and dog.

Our research hypothesis was that these stimuli could act as predator olfactory messages to elicit avoidance in house mice.

2. Matherial & Methods

2.1 Animals

Forty-eight (24 males and 24 females) RjOrl: Swiss 16-22 week old mice (Janvier Labs, France) free from infectious agents included in the FELASA health report (Mähler et al 2014) were kept at the facilities of the Research Centre in Semiochemistry and Applied Ethology (IRSEA) according to the requirements of French and European Law (2010/63/EU) and under the supervision of a veterinarian specializing in laboratory animals. The protocol and techniques described in this paper were approved by the IRSEA ethics committee (approval number AFCE20150501).

The breeding room was kept at a temperature of 22±2°C and 60±20% humidity. Animals were group housed until the beginning of the tests to avoid stress due to isolation. A 12-12h inversed (light: dark) light cycle regimen was used with the light cycle beginning at 12:00 PM (lights off). All the procedures were conducted between 12:00 PM and 5:00 PM, as the beginning of the dark cycle is one of the most active periods in mice. (McLennan & Taylor-Jeffs 2004).

Housing cages were Eurostandard type IIL (Tecnipast, Italy), (369*156*132mm), with a total floor surface of 435cm². Animals were group housed (except during the tests and the habituations) to minimize stress due to isolation. As mice are a social species, each cage housed three animals. Food was available ad libitum, with 2014 global rodent diet (Envigo, UK), and lignocel 3-4 (Envigo, UK) bedding was changed weekly. As enrichment material, each cage was equipped with a red plastic tube and craft paper and white paper as nesting material (Genobios, Laval, France), as mice prefer complex nests with more than one material (Hess et al. 2008).

2.2 Apparatus

Rectangular arenas with a 4mm thick 50x30cm glass base covered with a transparent plastic top were used for the replicates. The treatment was applied on a medical gauze (4*4cm) and placed on one of the two sides. The square of glass was marked underneath with electric tape to distinguish two laterals and a central area in the arena. Lateral areas were separated by a 1 mm thick opaque plastic PVC barrier measuring 24*30cm, which was attached to the top of the arena. A small square (4.5cm*4.5cm) was cut out in the center to allow the tested mouse to move freely (**Figure 1**).

This device was used as a modified open-field in order to measure the house mouse's exploratory and avoidance behavior, as well as specific anti-predatory and fearful responses to predator stimuli. The vertical plastic divisions had the role of reducing the passage of volatile compounds to adjacent areas and acted as a physical visual barrier.

2.3 Treatments and Treatment Application

The animals were naive to the tested stimuli having had no previous contact with any of them. Treatments were poured over a 4x4 cm medical gauze, which was placed inside a metallic drilled tea ball (Leclerc, Apt, France); the tea ball was placed on a circular recipient of the same diameter with a flat base to avoid rolling and set on a square of glass (8x8cm, 3 mm thick) to diminish contact with the arena. Another empty tea ball was placed on the other area in the same conditions. Treatment for each animal was chosen according to a randomized procedure. Treatment position was balanced, the treatment was placed on each side for half of the replicates to avoid bias. Treatment was considered as the only independent variable; 6 groups of 8 animals were tested for each treatment.

Ferret (*Mustela furo*): Samples were generously donated from two anonymous owners of pet ferrets in the south of France. The skin/fur olfactory cue sample was recovered by means of two cotton towels which remained in the cage of the male or the female for one month. The faeces/anal gland stimuli were recovered by taking fresh faeces from the ferret cages and were stored at -20°C until the tests. Animals were an adult male and female ferret, the treatment used as a complex general ferret olfactory cue was composed of 2 pieces of tissue (2.5*2.5cm) which remained in the cage for 1 month, 1 piece with the male; 1 piece with the female + 1.5g of faeces from the male and 1.5g from the female.

Snakes Ecdysis or shedding is the change of the most external keratinized part of the epidermis in snakes; snakes must regularly renew this layer to keep pace with continuous growth throughout their life span. Shedding also plays a role in sanitation and eliminating skin diseases such as mycosis (Tu et al. 2002; Jacobson 2007), and is the first barrier between snakes and their environment. Snake sheds are the most external part of these reptiles, and therefore probably contain the chemicals found in snake trails. For this reason, and because of the ease of sampling and transporting the material without the need to manipulate or stress the animals, we decided to use it as a snake olfactory predatory cue. Snake sheds were generously donated by the Centre d'Études Biologiques de Chizé (France). The sheds were recovered during the month of May after hibernation. They were sent to IRSEA between two and three weeks after being deposited and stored at -20° until their use for the behavioral tests.

Three 5*5cm pieces of snake shed, one for each species, *Rinechis scalaris (*ladder snake, unknown sex*)*, *Vipera aspis* (aspic viper, both sexes) and *Trimeresurus albolabris* (white-lipped pit viper, female) were used all together as a single predator stimulus. All three species were fed with mice.

Red fox (*Vulper vulpus*) Faeces of *Vulper vulpus* were taken from fresh droppings of wild animals in the south of France. The sex of the animals was unknown. They were stored at -20C° until the beginning of the experiments. 3g of faeces were used as a predator stimulus capable of eliciting avoidance according to previous research (Hacquemand et al. 2010).

Dog (*Canis familiaris*) Faeces of un-spayed female beagles (CEDS, centre d'elevage du domaine des souches, Mezilles, France) were sampled in the facilities of the IRSEA. The animals were 8 years old when the faeces were collected. 3g of female faeces were used as a generalist predator stimulus; this amount was based on previous studies with another member of the Canidae family (Hacquemand et al. 2010).

Cat (*Felis domesticus*) Cat urine from a European breed uncastrated male was collected in the facilities of the IRSEA from a non-absorbent bedding (Katkor, Rein Vet products, The Netherlands). Urine was frozen in aliquots after sampling and unfrozen the morning of the tests at room temperature. 1 ml of urine was used as a cat predator olfactory stimulus during the tests.

Blank A clean medical gauze was used as a control treatment as it was the physical support for all treatments.

During experimental manipulation, blinding was not possible as all of the conditions were easily visually recognizable before being hidden by the tea balls.

2.4 Behavioral Test

All the animals were habituated to the device for a period of 10 minutes on two days during the two weeks leading up to the experiment. No treatment was applied during these sessions, only the empty tea balls were present.

On the day of the experiment, the animals were transported from the holding cage to the testing room, placed in the arena using red PVC tubes in order to decrease stress from tail manipulation (Hurst & West 2010), and video-recorded for 10 minutes. The treatment was applied and its position in the arena was randomized for all the replicates.

Each treatment and control group were composed of 4 males and 4 females. Animals were not euthanized at the end of the experiments. The tests were conducted in the experimental room between 12:00 PM and 5:00 PM, with temperatures in the range of 21 ± 2°C, and humidity 50 ± 20%. The same operator manipulated the mice throughout all of the tests.

The glass base, the transparent cover, and the PVC separations were cleaned between replicates with Vigor surpuissant® disinfectant cleaner (eau écarlate, Ste Geneviève des Bois, France); they were then cleaned with white paper towels dampened with water, and finally dried with clean white paper towels. Two identical arenas were rotated between replicates in order to dissipate possible volatile traces of cleaner product. Each tea ball was used only for one treatment with its own glass square to diminish the risk of cross contamination. The external part of the tea ball and the metallic base were cleaned between animals with white paper wetted with alcohol.

2.5 Measures and Video Analysis

Each replicate was video-recorded with a video-camera placed 1 meter over the arena (JVC HD Everio 1920x1080 full HD model GZ-HM446), located at a 90° angle viewing position to the arena. This perspective allowed a complete analysis of avoidance behavior and locomotor activity. Video analysis was performed by two independent observers (CG and JL).

Video analyses were carried out blinded. The manipulators knew the treatment when conducting the tests, but had no notion of the experimental condition during the video analysis.

The avoidance behavior was measured using the dependent variables: treated area total duration, untreated area total duration. Avoidance behavior was interpreted when animals significantly increased the time they spent in the "untreated area," or decreased the time spent in the "treatment area."

2.6 Statistical Analysis

Data analysis was carried out using SAS 9.4 software Copyright (c) 2002-2012 by SAS Institute Inc., Cary, NC, USA. Bilateral situation; the significance threshold was fixed at 5%.

The Student t test was used with the ttest procedure as normality (analyzed with the UNIVARIATE procedure) was established.

3. Results

No difference was observed for the medical gauze alone, used as our blank (P=0.51), with an average of 201.62s in the treated area and 222.12s in the untreated area. This means that the medical gauze did not have any significant attractant or repulsive olfactory effect.

Mice spent significantly less time in the treated area than in the untreated area when they were exposed to the ferret's olfactory stimuli (P=0.0474), with a mean of 168.25s spent in the treatment area and 221.65s in the area farthest from the treatment (untreated area).

The other treatments didn't show any significant difference or tendency between the treated and the untreated area (**Figure 2**). However, regarding the descriptive data, animals exposed to fox faeces showed higher average times in the untreated area (233.25s) and the second lowest average time in the treated area (189.12s) when compared to all other treatment conditions. However, the t-test results are far from a significant (P= 0.25) when comparing times, animals remained in both areas for the fox faeces condition.

The treatment with dog faeces showed no significant difference between the two areas (P=0.59), with an average of 190s in the treated area and 215s in the untreated area.

Regarding the ophidian treatment, the snake sheds, mice showed no significant difference between the two areas (P=0.77), with an average of 218.5s in the treated area and 206.25 in the untreated area.

Finally, the treatment with cat urine showed no significant difference between the two areas (P=0.97), with an average of 203.43s in the treated area and 202.62 s in the untreated area.

4. Discussion

Our results show that an outbred strain of mice avoided a complex olfactory stimulus from the mustelid *Mustela furo* (Ferret), spending significantly more time in the farther end of the device than close to this predatory stimulus. No difference was observed for any of the other predator stimuli, including the cat, dog, fox, and snakes. Furthermore, no difference was observed between the control and the blank, which means that the medical gauze did not have an attractant or repulsive olfactory effect.

Our results on avoidance behavior to ferret olfactory stimuli appear to agree with previous studies. Masini et al found similar avoidance behaviors in Sprague-Dawley rats. Ferret fur stimuli elicited anti-predator behaviors and increased ACTH and plasma corticosterone. Fur also elicited the expression of c-fos (protein related to neuronal activation) in brain areas related to stress (Masini et al. 2005), and did not produce habituation to this predator stimulus in Sprague-Dawley-rats (Masini et al. 2006). Masini

et al did not find anti-predator responses to ferret faeces, urine or anal gland secretions, but these responses have been observed in other studies.

Apfelbach et al found that ferret urine influenced the estrous cycle of female Campbell's hamsters (*Phodopus cambelli*), (Apfelbach et al. 2001) delaying or inhibiting ovulation. In a similar study, the same authors found that Dwarf hamsters could distinguish ferret urine from animals fed with hamsters versus mice or chickens (as controls). Latency to approach the olfactory stimulus, the number of visits, and the total duration close to the stimulus was decreased when ferrets were fed with hamsters (Apfelbach et al. 2015). Ferret urine also decreased counter-marking in high marking male mice (Roberts et al. 2001). It was hypothesized that the hamsters were able to distinguish between the urines because of the different ratios of chemical compounds based on the diet composition of the ferrets, rather than the presence of new compounds. Specifically, pyrazines could play a role in the odor sensing and caution expressed in the presence of the ferret urine (Apfelbach et al. 2015), as it has been shown in prey species such as mice and deer (Osada et al. 2015).

Anal sac secretions of mustelids have been analyzed to identify some compounds that have been tested in mice for eliciting avoidance (C.Brinck et al. 1983; Zhang et al. 2002; Sievert & Laska 2016). As our ferret predator stimulus contained fecal material, the avoidance observed during our tests could be due to chemical compounds such as the sulfurous compound 2,2-dimethylthietane, which has already been identified as a putative kairomone (Sievert & Laska 2016). However, this compound can be considered as highly volatile and our samples were collected over a relatively long period. The most volatile compounds had most likely already volatilized, which could mean that this effect was produced by a heavier, long-lasting molecule. The heaviest and most resistant molecules in chemical communication are proteins (Wyatt 2014).

Lipocalins are the only protein family for which a kairomone effect has been identified in mammals; these include the major rat urinary protein MUP13, and the cat's lipocalin Fel d 4 (Papes et al. 2010). The ferret has a protein allergen whose structure and origin has not been identified (Díaz-Perales et al. 2013), however its size (17 KDa) is identical to mouse or rat lipocalins (Konradsen et al. 2015) and similar to other lipocalins, such as the cat allergen Fel d 4 (19.7 KDa). All the mammalian allergens belong to the lipocalins family, with the exception of the major cat allergen Fel d 1, so its allergenic property could be another indication of the similarity of these molecules. From these notions, we can hypothesize that one explanation for ferret olfactory stimuli avoidance in mice could be the presence of a lipocalin protein found in the ferret's fur, saliva and urine (Díaz-Perales et al. 2013). This protein would share properties and homology with other kairomone lipocalins, which would simplify the recognition of these different stimuli.

One possible critique of this hypothesis lies in the fact that, in our experiments, mice had no direct contact with the compounds (the drills in the metallic mesh measured 0.5mm which prevented any

contact); this may impede heavier molecules such as peptides or proteins from reaching the Vomeronasal Organ. However, the metallic balls were perforated, and the mouse's nose could approach the stimuli at a very short distance (0.16mm, the diameter of the wires), which may be close enough to absorb this small protein/peptide with the air stream created by sniffing.

Our results showed no avoidance behavior toward snake sheds in an outbred laboratory mouse strain; several factors may explain this result. The snake samples may not have been fresh enough to send a significant message of risk and this could diminish antipredator responses such as avoidance (Bytheway et al. 2013). The period of ecdysis can be especially rich in the production of pheromones in snakes and the skin shed has been shown to contain a large quantity of pheromones at this particular moment (Parker & Mason 2011). The sheds were transported at room temperature and the periods between some of the sampling and delivery were from 2 to 3 weeks, so there is some possibility that the lighter chemical compounds had evaporated and the heaviest had degraded. Regarding the relevance of the species used, *Rinechis scalaris* and *Vipera aspis* have a high proportion of small mammals in their diets, mainly rodents from the genus Mus, Rattus or Apodemus (Pleguezuelos et al. 2007; Saviozzi & Zuffi 1997). *Trimesurus albolabris* is an arboricol species and rodents are part of its diet, but probably in a smaller proportion than for the other two species (Coborn 1991). The use of predator cues from animals that specialize in hunting the species being tested is likely preferable but not mandatory for predator cue experiments. We find generalized prey responses to similar predatory species even if they are not the prey's own direct predator (Webb et al. 2010).

Snake sheds have already been tested with different results in mammals or other species, eliciting fear behaviors and avoidance in some studies and having no effect in others. Papes et al (Papes et al. 2010) found that snake sheds increased corticosterone in blood, elicited avoidance and specific antipredator behaviors including freezing and risk assessment, and produced a significant activation of the vomeronasal organ sensory neurons. The species used in this case was not specified in the paper, it was only identified as a "snake pet" and the sample was fresh. Pillay et al found that striped mice (*Rhadomys pumilio*) avoided the faeces of a predator snake (*Hemachatus haemachatus*), and the response was bigger when the snake had fed on striped mice (Pillay et al. 2003). In an older study, Weldon et al found responses only in female mice: an increased number of fecal boli and decreased consumption of food (Weldon et al. 1987). Regarding studies indicating negative or low responses in rodents to snake sheds as a predator stimulus, Wasko et al found no responses to faeces, and very limited responses to the live animals (Wasko et al. 2014) in 3 species of rodents.

Some studies have been performed in reptiles, Sullivan et al observed anti-predator responses in red-backed salamanders (*Plethodon cinereus*) to a distilled-water rinse of garter snakes (*Thamnophis sirtalis)* previously fed with this species of salamander (Sullivan et al. 2002). In another study, Webb and collaborators found anti-predator responses to snake chemical cues in velvet geckos (Webb et al.

2010) using "scented" cardboard which remained inside the cages of the snakes for two days; the snake species used to obtain the chemical cues were not common predators of the geckos.

Negative results regarding mouse avoidance to dog faeces can be explained by the fact that the diets of dogs and their wild ancestors don't contain rodents as a primary food source. Rodent consumption increased as dogs became domesticated due to their cohabitation in ecologically impoverished human environments. Unlike dogs, foxes are considered generalist predators that consume rodents in large quantities, and olfactory cues, such as the fox faeces compound TMT, have been extensively proven to elicit avoidance (Rosen et al. 2015). However, the TMT doses presented in faeces are lower than those used in almost every publication (Buron et al. 2007), which could explain the smaller responses seen in our study.

Our results showed no avoidance in mice to male cat urine. The existing literature provides no clear conclusions about rodent avoidance of cat urine. The traditional hypothesis states that predator mammalian urines could elicit anti-predator responses in prey due to the sulfurous compounds derived from meat metabolism (Nolte et al. 1994). 2-phenylethylamine (PEA) is a chemical compound found in carnivorous mammalian species urines that elicits avoidance and fires fear hard-wires in mice and rats, however its presence is low in cats compared with other carnivorous species (Ferrero et al. 2011). Pyrazines and compound analogues that have been found in wolf urine also elicited anti-predator responses and firing of fear pathways (Osada et al. 2015). In another study, urine from several felines and canids induced defensive behaviors, but those of cats and herbivores showed no influence (Fendt 2006). Taken together, these results seem to validate the idea that urine from canids and felines other than cats containing compounds such as PEA and pyrazines elicit anti-predator behaviors in rodents. The effect is not seen in response to cats because these compounds are absent or present in only small amounts in cat urine. Bramley and collaborators found unclear responses to cat urine in rats. In a first study with Norway rats, only one of two island populations of wild rats showed avoidance (Bramley et al. 2000). In a second study with the ship rat (*Rattus rattus*) and Polynesian rats (*R.exulans),* no responses to the predator stimuli were observed (Bramley & Waas 2001). On the other hand, as we stated previously, there is some evidence that cat urine and the amino acid Felinine itself are capable of altering reproductive parameters in rodents (Voznessenskaya 2014; Vasilieva et al. 2000). As a methodological critique, our urine samples were taken from only one cat, and ideally the sample tested should pool urine from several animals, so we cannot say that it is fully representative.

The mouse strain used for the experiments, RjOrl: Swiss, is a laboratory mouse outbreed strain, with a wider genetic repertoire than other laboratory strains. Nevertheless, it has a high degree of inbreeding and consanguinity (Wahlsten & Crabbe 2007). This implies some specific phenotypes that could affect predator chemical cue detection (Dell'Omo et al. 1994). Differences in vomeronasal organ receptors have been identified in other strains due to the process of inbreeding from the original wild species. This

may explain differences in responses between strains and between lab and wild animals (Stempel et al. 2016). In addition, laboratory mice strains have been selected to be tame and easily handled, and are less reactive in general to aversive stimuli, so this could also decrease the behavioral response to predator stimuli (Goto et al. 2013).

Finally, we must consider the statistical power and the number of animals used; in our study we observed a considerable degree of variability. So, the use of more sensitive animals, as is the case in wild animals or other strains probably would increase the behavioral response (but also the variability). Increasing the number of animals per group would augment the chances of observing statistical differences in further research.

5. Conclusion

Our results showed that mice significantly avoided ferret olfactory stimuli from fur and faeces. Additional research should further explore ferret olfactory cues as they likely present biologically meaningful messages for mice. The lack of avoidance behavior to the other stimuli tested may be attributed in some cases to phenotypic characteristics of the laboratory strain, statistical power, or aging of the samples. In our opinion, snakes remain a good candidate for finding the first reptile kairomone for rodents due to their ecological importance as predators and their long co-evolutionary history with rodents.

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TABLES

Table 1 Predator species tested as olfactory stimuli of the house mouse (*Mus musculus*)

Species	Vernacular name	Breed/subspecies	Sex	Olfactory cue/s
Canis familiaris	Dog	Beagle	Females	Faeces
Felis cattus	Cat	European	Males	Urine
Rinechis scalaris[1]	Ladder snake	N/A[2]	Unknown	Snake skin shed
Vipera aspis[1]	Aspic viper		Both sexes	
Trimesurus albolabris[1]	White-lipped pit viper		Female	
Mustela putorius	Domestic ferret	Furo	Both sexes	Cotton tissue in contact with the animals for 1 month + faeces[3]
Vulpes vulpes	Red fox	N/A[2]	Unknown	Faeces

[1]Stimuli tested together as snake olfactory cue [2] Not applicable [3]Stimuli tested together as ferret olfactory cue

FIGURES

Figure. 1. Experimental device used for measuring avoidance. A On the left side we see the treated area (1), central area (2) and starting point, right side: untreated area (3). **B** Close-up photography for the treatment containers, one of both sides contained the treatment and the other remained always empty as control.

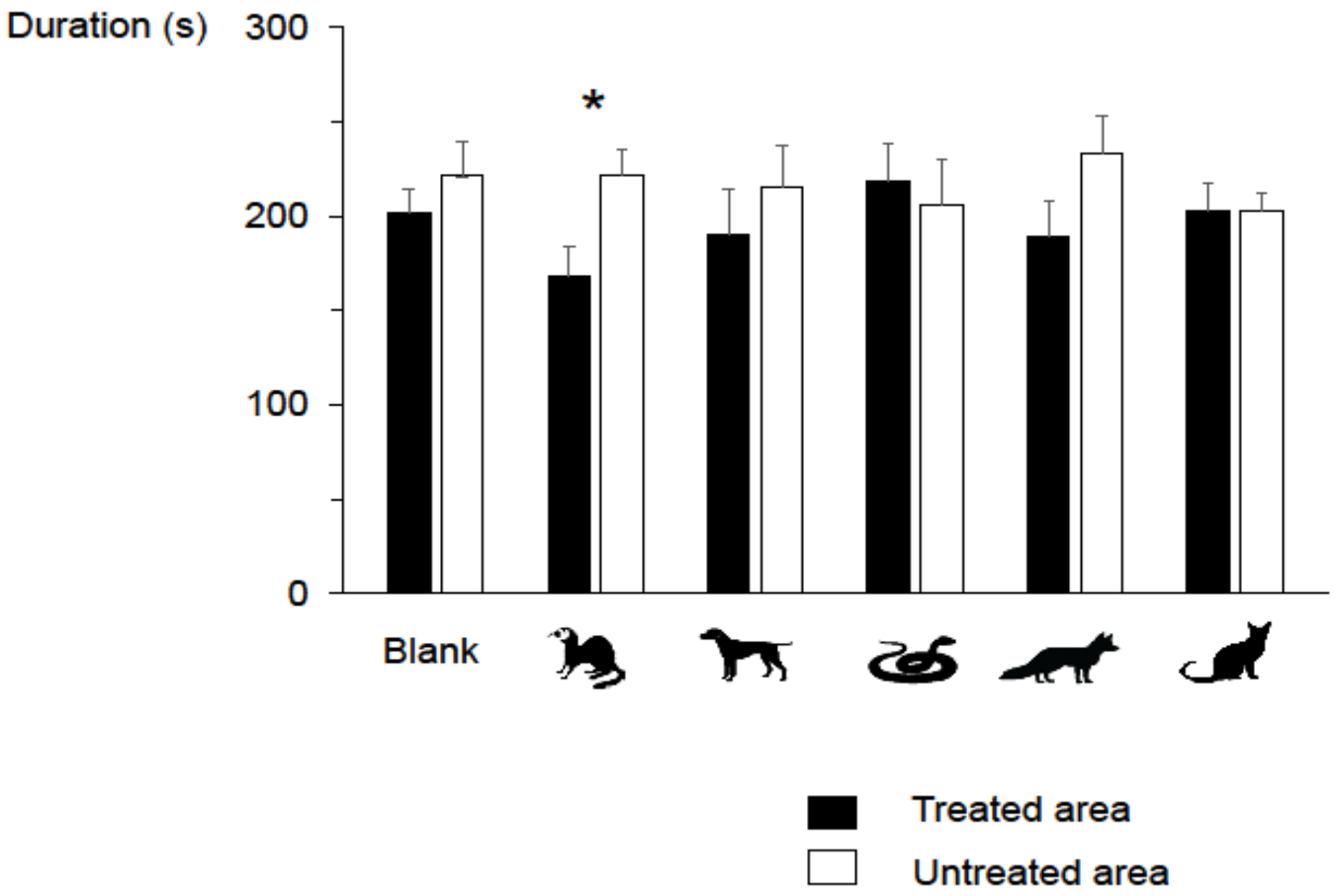

Figure. 2. Total duration of time remaining in the treated area and the untreated area. The multiple comparisons have been computed using the *T-test*. Data is shown as the mean ± standard error, as parametric conditions where accomplished. * P <0.05

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4 CONCLUSIONS OF STUDIES 1 AND 2

The ferret olfactory stimulus seems to be an interesting candidate for finding new chemicals with a kairomone effect in mice. Within the chemicals, the ferret allergen is probably an active part due to our sampling procedure and ageing of the samples. Little is known about this molecule; it is supposed to be a lipocalin protein and has been found in urine, but it has also been described in saliva and fur. In future research, a proteomic approach should identify this molecule, and bioinformatics tools and crystal structure identification would allow for a better understanding of its role and frame it as lipocalin or as part of another protein family. From the behavioural approach in this thesis, future experiments should test ferret native solutions containing the protein (from urine, saliva or fur) as well as the purified protein as a next step to test this hypothesis.

As previously discussed, some of the reasons why the mice did not show significant avoidance of the other predators could be due to ageing of the samples, a mouse strain with low sensitivity to predatory cues/fear stimuli and a lack of statistical power due to strain variability. However, it is also possible that the ferret olfactory stimulus was more representative of the olfactory profile of the species. Different samples were mixed, so molecules from different gland secretion areas could be present as stimuli. This feature would increase the value of the message to identify ferrets and thus the perception of risk by the mice.

Fox faeces elicited significant avoidance behaviour during our preliminary experiment, but we did not observe significant avoidance in the final experiment, which could have been due to age differences in the mice between both tests. The mice used in the preliminary study were almost 1 year older, and differences between young adults and old animals could modulate predator risk perception and explain the higher avoidance to fox faeces. Younger animals are considered to be bolder as they are in better physical condition, which could improve the chances of escape (Cooper, Jr. & Blumstein, 2015). In addition, during the transition between adolescents and young adults, some parts of the anatomy are slow to develop, such as the prefrontal lobe which also decreases the perception of risk (Chan et al., 2011). An ecological reason for these different behaviours is dispersion, especially for males, because young adults should search for new territories to establish new populations. Therefore, being less sensitive to risks is somehow necessary for survival purposes and the transmission of their genetic repertoire.

CHAPTER 2 : CAT MOLECULES AS RODENT PREDATOR OLFACTORY CUES

1 INFLUENCE OF CAT FUR HYDROPHILIC COMPOUNDS AND PURIFIED FEL D 1 ON THE FORAGING AND EXPLORATORY BEHAVIOUR OF MICE

1.1 Preamble to study 3

The cat protein Fel d 1 is a small protein that is considered the most important cat allergen to humans; it belongs to the secretoglobin protein family. This protein is considered closely related to androgen-binding proteins (Durairaj, Pageat, & Bienboire-Frosini, 2018), rodent proteins that play a role in sexual chemical communication.

Previous research in the IRSEA explored the molecular features, ligands and possible role of this molecule in intraspecific communication in cats. Fel d 1 is produced in large amounts by an important rodent predator, the domestic cat (*Felis catus*); it can be transported by air; its production is sex and behaviour dependent (Bienboire-Frosini et al., 2012); it is a long-lasting molecule in the environment (Wood, Chapman, Adkinson, & Eggleston, 1989); and cats show more interest in areas with than without this protein (Marcet et al., 2016). Our hypothesis was that this molecule had interesting features that make a rodent kairomone candidate, so it could elicit anti-predator behaviours in the house mouse (*Mus musculus*) and modify a basic self-maintenance behaviour, feeding.

To collect Fel d 1, we carried out fur and skin washes where this protein is abundantly found and pulled the samples to create a homogeneous stimulus. The techniques were based on a previous protocol developed for the thesis by Bienboire-Frosini (Bienboire-Frosini, Lebrun, Vervloet, Pageat, & Ronin, 2010).

1.2 Study 3

Oral presentation in international congress with peer reviewing

C. Grau ; C.Bienboire-Frosini ; A.Cozzi ; P.Pageat ; *Does Fel d 1 , the Main Cat's Allergen have a Kairomone Role in Mice.* Chemical Ecology of Vertebrates. Proceedings of the International Society of Chemical Ecology (ISCE) Stockholm, Sweden 30 June 2015

Does Fel d 1, the main cat's allergen, have a kairomone role?

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Different models of samples and animals have been used to obtain a predatory response under controlled conditions in mice. These experiments relied largely on the use of cats (Apfelbach et al. 2005). Previous studies have shown a kairomone role of Fel d 4 (Papes et al. 2010), a minor cat's allergen from the lipocalin family. Fel d 1 is the main allergen and long lasting molecule released in the environment by cats (Nicholas et al. 2008). It belongs to the secretoglobin family and is produced in large amounts in the sebaceous glands of the skin, especially in the cheeks' area. May et al (2012) found an effect of the cheeks rubbing marks of domestic cats decreasing the feeding behaviour in rats.

The aim of our study was to determine if a solution containing high amounts of Fel d 1 extracted from washes of chest and cheek zones of cats could alter feeding and exploratory behaviour in mice.

Six cats (males, females and castrated males) were used for sampling. The pooled sample contained 18.6 µg/ml of Fel d 1 and three Fel d 1 molecular forms, according to ELISA and Western-Blot analysis respectively.

Twenty-one mice RjOrl:Swiss (males and females) were used for behavioural essays. Tests were conducted in an 8 arm rectangular maze, during 10 minutes. Every arms contained flour wheat as an attractive stimulus and Fel d 1 or placebo solution on a gauze at their entrance.

No significant differences were observed for the number of entrances in tubes (P=0.42), feedings (P=0.97), or remaining time (P=0.76). No significant differences were observed between sexes. Our results suggested that Fel d 1 did not trigger a predatory response and so did not have a kairomone role for mice. Conversely, Fel d 1 may play a role in intraspecific communication.

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Research manuscript in process of submission to Journal of Chemical Ecology

C.Grau, C. Bienboire-Frosini, C. Lafont-Lecuelle, P. Monneret, J. Leclerc, A. Cozzi, P. Pageat. *Does Fel d 1, the Main Cat's Allergen have a Kairomone Role in Mice?*

DOES FEL D 1, THE MAJOR CAT ALLERGEN, HAVE A KAIROMONE ROLE IN MICE?

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Abstract -Cat odour has been extensively studied in lab and field studies as a model of a predator stimulus that elicits anti-predator responses in rodents. However, little is known about the compounds that mediate this interspecies communication.

Fel d 1 is the major cat allergen and the primary long-lasting molecule from cats found in their habitat. For the purposes of this study, a hydrophilic solution, known as cat fur extract (CFE), was prepared by rubbing the fur and the skin of several areas of 6 cats (flanks, cheeks, chin, and inter-digital areas). The solution was tested as a possible predator stimulus containing a high concentration of Fel d 1 (18.6 µg/ml) and 3 different molecular forms of Fel d 1. A behavioural test conducted in a multi-chamber arena in outbred Swiss mice (n=21) showed no effects of CFE in exploratory and feeding behaviour. Precisely, the statistical analyses did not show any significant effect of treatment for chamber entry latency (P=0.25), entry frequency (P=0.18), duration in treated chambers (P=0.93), food consumption frequency (P= 0.81), or first choice (P=0.86). In a second experiment (n= 28) purified Feld 1 was tested to avoid effects from other molecules, first results were confirmed as mice didn't showed a significant difference against its control (purified water). However, a statistical tendency was observed for number of faecal boli (P=0.079) and number of passages (P=0.064).

These results suggest that Fel d 1 from domestic cats does not play a clear kairomone role in mice. Nonetheless, the biological properties of Fel d 1 and the high amounts released in the environment strongly suggest a role in intra-specific communication and as a pheromone carrier, warranting future research in this direction.

Key Words-Fel d1, predator, kairomone, behaviour, mice.

INTRODUCTION

The detection of predator cues by prey constitutes a valuable tool for survival, making this feature a criterion for selection throughout evolution. Predators and preys run a constant arms race that leads to continuous evolution (Dietl & Kelley 2002) and that commensal species have

continued in human habitats (Bull & Maron 2016; Lowry et al. 2013). In the wild and in human environments, the domestic mouse (*Mus musculus*) and the domestic cat (*Felis catus*) are sympatric species that have co-evolved (Abrams 2000) with presumably high predation pressure from the feline species (Loss et al. 2013).

Chemical detection in animals is paramount, mediating in all aspects of the life cycle, from feeding, to reproduction and avoidance of predators (Wyatt 2003). In particular, rodents are macrosmatic animals, active mainly during the crepuscule and night, where dim light enhances the value of chemical messages in absence of visual acuity (Ripperger et al. 2015). The vomeronasal organ along with the main olfactory epithelium are the two main structures implicated in reception of chemical messages in mammals (Ihara et al. 2013; Tirindelli et al. 2009; Dey & Stowers 2016; Greer et al. 2016). Specifically, the vomeronasal receptors V2R have a high relevance in rodent's innate chemical communication and are specialized in detecting proteins like the well-known Major Urinary Proteins (Hurst & Beynon 2004; Cavaggioni & Mucignat-Caretta 2000; Hurst et al. 2001; Logan et al. 2008)

The anti-predatory effects of cat odour in rodents have been extensively studied under laboratory conditions (Apfelbach et al. 2005; McGregor et al. 2004; Papes et al. 2010). Nevertheless, these studies relied mainly in unspecific cat samples, odours, without chemical compounds identification. Indeed, to the authors' knowledge, only one isolated molecule from cats has succeeded in eliciting this defensive behaviour (Papes et al. 2010): the protein Fel d 4 belonging to the lipocalin family (Smith et al. 2004).

The major cat allergen Fel d 1 is a protein that is abundantly released by cats in the environment (Dabrowski et al. 1990). It belongs to the secretoglobin family, which is characterized by small dimeric proteins capable of binding hydrophobic molecules (Klug et al. 2000). It is mainly produced by the skin, sebaceous and anal glands (Dabrowski et al. 1990). The main reservoir for Fel 1 is in the fur and the skin of the cat, especially the cheeks (Carayol et al. 2000), as they are particularly rich in sebaceous glands. Of note, the cheeks area is also involved in the chemical communication in cats through the release of territorial marking

pheromones (Pageat & Gaultier 2003; Carayol et al. 2000). In addition, it has been suggested that fur-derived odours could provide more valuable information to rodent prey than urine or faeces (Apfelbach et al. 2005), since the fur-derived odours tend to dissipate faster (Blanchard et al. 2001).

In agreement with the anatomical origins and reservoirs of Fel d 1, May and colleagues (2012) found that cat rubbing marks had an effect on Sprague-Dawley rats, including decreased feeding behaviour in partially deprived animals, increased hiding behaviour, and decreased exploratory behaviour. Cat rubbing behaviour includes facial and lateral body marking (Feldman 1994).

Three assumptions can be made from the results of the previously described studies. First, cats' living areas have a high concentration of Fel d 1, as has been widely demonstrated in the literature on allergology and immunology (Custovic et al. 1998; Chew et al. 1999; Dabrowski et al. 1990; Grönlund et al. 2010; Konradsen et al. 2015). Second, cat rubbing marks showed a kairomone role in laboratory rodents (May et al. 2012). Third, the anatomical areas involved in cat rubbing coincide with the main reservoir and production areas of Fel d 1 (Carayol et al. 2000; Bienboire-Frosini et al. 2010). Given these observations, it was hypothesized that, as the main long-lasting molecule released in the environment by cats, Fel d 1 could have a kairomone role in mice. A behavioural preference assay was designed to elucidate the effect of natural Fel d 1, water-extracted from cat fur and skin, on mouse feeding and exploratory behaviour.

MATERIALS AND METHODS

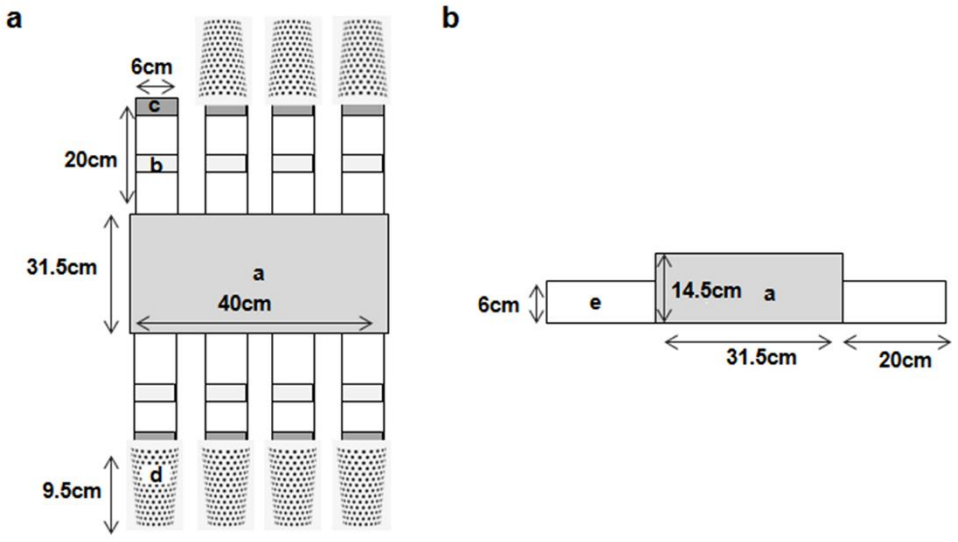
First experiment: Car fur hydrophilic extract influence on foraging behaviour

Animals. Twenty-one (11 males and 10 females) RjOrl: Swiss 9 week old mice (Janvier Labs, France) free from infectious agents included in the FELASA health report (Mähler et al 2014) were kept in facilities at the University of Avignon according to the requirements of French and European law (2010/63/EU) and under the supervision of a veterinarian specializing in

laboratory animals. The protocol and techniques described in this paper were approved by Research Institute in Semiochemistry and Applied Ethology ethics committee (approval number AFCE20150501). The housing room was kept at a temperature of 22±2°C and 60±20% humidity. Animals were group housed until the beginning of the tests to avoid stress due to isolation.

A 12:12-h (light: dark) light cycle regimen was used with the light cycle beginning at 8:00 a.m. Water and food (A-04 diet, SAFE, France) were supplied ad libitum. During the 3 days prior to the test, animals were habituated to powder food (whole wheat flour) which was available along with pellets. The night before the experiments, the pellets were removed, and wheat flour was restricted to 30% (1.5g per animal). Body weight was recorded the day before the experiment and two hours before the trials; weight loss was calculated in order to assess welfare.

Apparatus. A multi-chambered plastic device (**Figure 1**), hereafter referred to as "the arena" was used to perform the experiments. The arena consisted of eight cylindrical tubes (20 x 6 x 6 cm) and a central chamber (40 x 20 x 13 cm). The bottom of each tube was covered with a plastic cup; the cups were replaced after each video-recording.



arm

Figure 1 Top and lateral view of the arena.

Top (a) Lateral (b) *a*: central chamber; *b*: medical gauze; *c*: aluminium plate; *d*: plastic cup*; e*: tube height

Treatment: Cat Sampling. Six cats (2 castrated males, 2 males, 2 females) from the Research Institute in Semiochemistry and Applied Ethology (IRSEA) catteries (Saint Saturnin les Apt, France) were sampled as previously described (Bienboire-Frosini et al. 2010) with slight modifications. Animals were sedated with a combination of ketamine (2.5 mg/Kg, Ketamine 1000®, Virbac) and medetomidine (20 µg/Kg, domitor®, Pfizer) while atipemazole (10 µg/Kg, antisedan, Janssen Santé animale) was used to reverse the effects of the medetomidine. Sterile medical gauzes were moistened with 5 ml of the washing solution (ultrapure water, containing a cocktail of protease inhibitors, Sigma) and rubbed over the whole body surface, which was first moistened with the washing solution itself to solubilise the hydrophilic molecules (10 ml on each flank). The washing solution volume was decreased in comparison with Bienboire-Frosini et al (2010) in order to obtain a higher Fel d 1 concentration for the electrophoretic analyses. The cheek zone was rubbed particularly thoroughly due to its richness in sebaceous glands. Hair was also harvested by combing the cats and added to the gauze in a sterile sampling pot.

The “cat fur extract” (CFE) samples were triturated using a pipette tip to wring the gauze and the hair, and all samples were vortexed thoroughly and incubated overnight at room temperature under weak agitation on a wrist action shaker. The next day, the liquid obtained from the extracted samples was decanted and centrifuged (1300 g, 20 min, 4°C) in order to remove hair and contaminants. Supernatants were collected and kept at -20°C.

Biochemical Analysis of Cat Samples. Presence of significant amounts of Fel d 1 in all the supernatants was confirmed by ELISA (Fel d 1 ELISA kit, Indoor Biotechnologies, UK). The samples were then pooled to create a single sample, the “all cat CFE pool,” which was used during the tests. The pooled sample was again assayed using ELISA.

SDS-Page was performed in denaturing conditions (NuPage LDS sample buffer 4X from Life Technologies, France, 10 min at 70°C) using NuPage 4-12% Bis-Tris Gel (Life Technologies, France). The Mark12 Unstained standard (Life Technologies, France) was used as a molecular weight marker. The electrophoresis was followed either by staining with an Imperial Protein stain (Pierce, Thermoscientific, France) for 2h or by transfer to a nitrocellulose membrane (30 V constant, 1h). Immunodetection was carried out using the Western Breeze Chromogenic kit (Life Technologies, France) as described in Bienboire-Frosini et al (2010).

Treatments and Food Location. 300 µl of the CFE treatment was applied to 4 medical gauzes, while a solution of purified water with anti-proteases was applied to the four negative control (C) gauzes. The gauzes were then placed in the centre of the 8 tubes for each test. To avoid contamination, 4 tubes were used only for the CFE treatment and 4 tubes were used only for the control.

Aluminium plates (3.5 x 1.4 x 0.2 cm) were attached to the walls at the far end of each tube, away from the central chamber. Each of the 8 plates was supplied with 30 mg of whole wheat flour before each test.

Spilled food was recovered with fine brushes. The central chamber and tubes were cleaned between sessions with a protease-disinfectant cleaner (Aniosyme dd1, laboratories Anyos, France) and gently dried with paper towels. Aluminium plates were cleaned with ethanol.

Experimental Design and Treatment Randomization. The study followed a Randomized Complete Block Design (RCBD) without repetition where each mouse corresponded to a block, receiving each of the 2 treatments 4 times. The mean value of the 4 measures for each treatment was used for ulterior statistical analyses, as they were not real repetitions (Lellouch & Lazar 1993)

Treatment assignment was carried out at random on each branch of the device in order to avoid location effects. Lots were drawn for each experimental unit, randomizing the blocks, and the treatments within the blocks.

The entire procedure was blinded; the authors were aware only of the treatment locations, but not of their composition.

Behavioural Test. The tests were conducted in the experimental room between 11am and 4pm, with temperatures in the range 24 ± 0.5 °C, and humidity 50% ± 5. The same operator manipulated the mice throughout all of the tests. The transport of the animals from the holding cage to the arena was performed using a previously described method (Hurst & West 2010), which was slightly modified for the purposes of the experiment: plastic cups were used instead of tubes for transport in order to decrease stress due to tail manipulation. The centre of the arena was marked and mice were released from a plastic cup placed over the marking.

Experimental subjects were video recorded for 10 minutes, during the first two minutes the experimenters were present in the room with a physical visual barrier between them and the device, afterwards they left the room until the end of the test to diminish the observer effect.

Measures and Video Analysis. Video analysis was performed blindly by two independent observers. Controlled measures were: first treatment chosen (defined as the first time that the mice crossed over the medical gauze), food consumption latency, entrance frequency (average number of times that the mice entered the control or CFE tubes), consumption frequency (average number of times that the mice fed in control or CFE tubes), treatment duration (average amount of time that the mice remained inside control or CFE tubes) and general duration (total amount of time that the mice remained in all tubes or central arena).

Statistical Analysis. 9.4 SAS software (2002-2012 by SAS Institute Inc., Cary, NC, USA) was used for analyses. Before proceeding, dataset reliability between 2 independent observers was calculated with Pearson's correlation (Kappa coefficient for the first-choice variable) using corr and freq procedures, with an acceptable inter-observer reliability established at 0.9.

For all parameters, analysis was performed using a General Linear Mixed Model (mixed procedure of 9.4 SAS software) or Generalized Linear Mixed Model (glimmix procedure of 9.4 SAS software). Mixed model was performed in order to explore the main effects of treatment

and sex as fixed factors and their interaction. Animals were considered as a random factor. Statistical significance was established at $p < 0.05$.

Second experiment: Fel d 1 influence on exploratory behaviour

Animals

Twenty-eight C57BL/6JRj mice (14 males and 14 females, Janvier Labs, France) 8-10 weeks old were kept at the facilities of the Research Centre in Semiochemistry and Applied Ethology (IRSEA) according to the requirements of French and European Law (2010/63/EU) and under the supervision of a veterinarian specializing in laboratory animals. The protocol and techniques described in this paper were approved by the IRSEA ethics committee (approval number AFCE20150501).

The breeding room was kept at a temperature of 22±2°C and 60±20% humidity. A 12-12h inversed (light: dark) light cycle regimen was used with the cycle beginning at 12:00 PM (lights off). All the procedures were conducted between 12:00 and 5:00 PM as the beginning of the dark cycle is one of the most active periods in mice (McLennan & Taylor-Jeffs 2004). Animals were housed with the same cage conditions, but craft paper (Genobios, Laval, France) was added in addition to white paper to nesting material, as mice prefer complex nests with more than one material (Hess et al. 2008).

Apparatus

Rectangular arenas with a 4mm thick 50x30cm glass base covered with a transparent plastic top were used for the replicates, for details see Grau et al 2019.

Treatments and Treatment Application.

The animals were naive to the tested stimuli having had no previous contact with any of them. Treatment was poured over a 4x4 cm medical gauze, which was then placed over a square of glass (8x8 cm, 3 mm thick) to diminish contact with the arena and placed on one of the two

sides of the arena. Treatments and treatment position for each replicate were chosen according to a randomized procedure.

Fel d 1 (major cat allergen)

The cat protein and major allergen Fel d 1 was provided as purified natural Fel d 1 by Indoor Biotechnologies (Cardiff, UK) after extraction from cat hair and purification by affinity chromatography. The total amount applied on the medical gauze was 7 µg of Fel d 1 diluted in 1 ml of ultrapure water.

Behavioural Test

All the mice were habituated to the arena the day before the test for 10 minutes without any treatment. The tests were conducted in the experimental room between 12:00 PM and 5:00 PM, with temperatures in the range of 21 ± 2°C, and humidity 50 ± 20 %. The same operator manipulated the mice throughout all tests. The animals were transported from the holding cage to the arena using red PVC tubes in order to decrease stress from tail manipulation (Hurst & West 2010).

Animals were transported to a pre-test room at least 30’ before the experiments. They were then transported to the testing room, placed in the arena, and video-recorded for 10 minutes. The treatment was applied and its position in the arena was randomised for all the replicates. Every treated group was composed of 7 males and 7 females. Animals were not euthanized at the end of the experiments.

The glass base, the transparent cover, and the PVC separations were cleaned between replicates with Vigor surpuissant® disinfectant cleaner (Eau Ecarlate, Ste Geneviève des Bois, France); they were then cleaned with white paper towels dampened with water, and finally dried with clean white paper towels. Four identical arenas were rotated between replicates in order to dissipate possible volatile traces of cleaner product. The squares of glass where the treatment was applied followed the same cleaning procedure but were used only once each

day, at the end of the day they were exposed to a pyrolysis treatment, 500°C for one hour, to eliminate residues.

Measures and Video Analysis

Each replicate was video-recorded with a video-camera placed 1 meter over the arena (JVC HD Everio 1920x1080 fullHD model GZ-HM446), located at a 90° viewing angle to the arena. This viewpoint allowed for complete analysis of avoidance behaviour and locomotor activity. Video analysis was performed by two independent observers (CG and JL). Video analyses were carried out blinded. The observers knew which treatment was applied when conducting the tests but had no notion of the experimental condition during the video analysis (except for fox faeces treatment).

The avoidance behaviour was measured with the dependent variables: treatment area total duration, untreated area total duration, average duration per passage in treatment area, and average duration per passage in untreated area. Avoidance behaviour was interpreted when animals significantly increased the time they spent in the “untreated area,” or decreased the time spent in the “treatment area.” In the same way, in relation to this main avoidance parameter, we measured the average time per passage in the treatment area and the untreated area, and we interpreted avoidance behaviour when animals decreased the average time per passage in the treatment area and/or increased the average time per passage in the untreated area.

Locomotor activity was measured by the total number of passages (defined as the total number of passages between areas). An increase in the number of passages was interpreted as increased locomotor activity, and a reduced number of passages as decreased locomotor activity.

The number of faecal boli was noted as an independent parameter of the video analysis, after each replicate as a measure related to stress (Mönnikes et al. 1993).

Statistical test

For each variable, conditions of normality and homogeneity were verified with, respectively, the UNIVARIATE procedure and the General Linear Model. If conditions were established, *Student's test was* performed by using the T-test procedure. If normality was not established, the non-parametric alternative of Wilcoxon was used by means of npar1way procedure.

RESULTS

Biochemical Analysis of Cat Samples. The Fel d 1 concentration in the CFE pool measured by ELISA was 18.6 µg/ml. Imperial protein staining of SDS-page (**Figure 2a**) showed four protein bands with apparent molecular weights around 2 kDa, 21.5 kDa, 55 kDa, and between 116 and 200 kDa. The main band was the 21.5 kDa. Western-blot analysis with anti-Fel d 1 mAb (**Figure 2b**) confirmed three immunoreactive molecular species of approximately 21.5 kDa, 40 kDa and between 116 and 200 kDa. The first two correlated with the expected sizes of dimeric Fel d 1 and tetrameric Fel d 1 (Van Milligen et al. 1992; Kristensen et al. 1997).

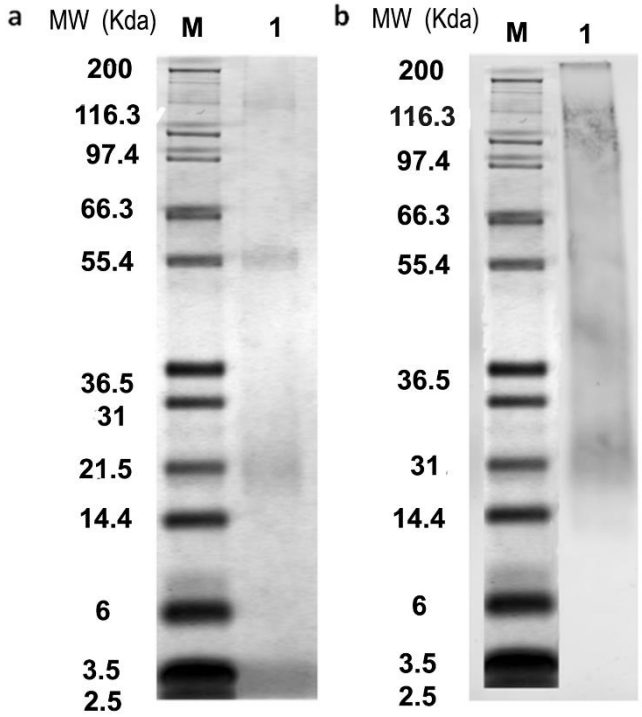


Figure 2 Biochemical characterization of cat CFE sample

a: SDS-Page analysis. *b*: Western-blot analysis with anti-Fel d 1 mAb (6F9). M: MW Marker Mark 12 (10 µL) 1: “all cat CFE pool” (15 µl) + NuPage LDS Sample buffer 4X (5 µl).

Behavioural Analysis. Reliability between the observers who carried out the video analysis was greater than 0.9 for all the parameters so the average of the two observers was calculated.

The analysis of the behavioural parameters with a Randomized Complete Block Design shown a significantly higher remaining time in the tubes (P<0.001, n=21) than in the central area without taking into account the treatment inside the tubes (**Figure 3**). However, the statistical analyses did not show any significant effect of treatment for any parameter (**Figure 4**), tube entry latency (P=0.25, n=21), entry frequency (P=0.18, n=21), duration in treated tubes (P=0.93, n=21), consumption frequency (P= 0.81, n=21), or first choice (P=0.86, n=21).

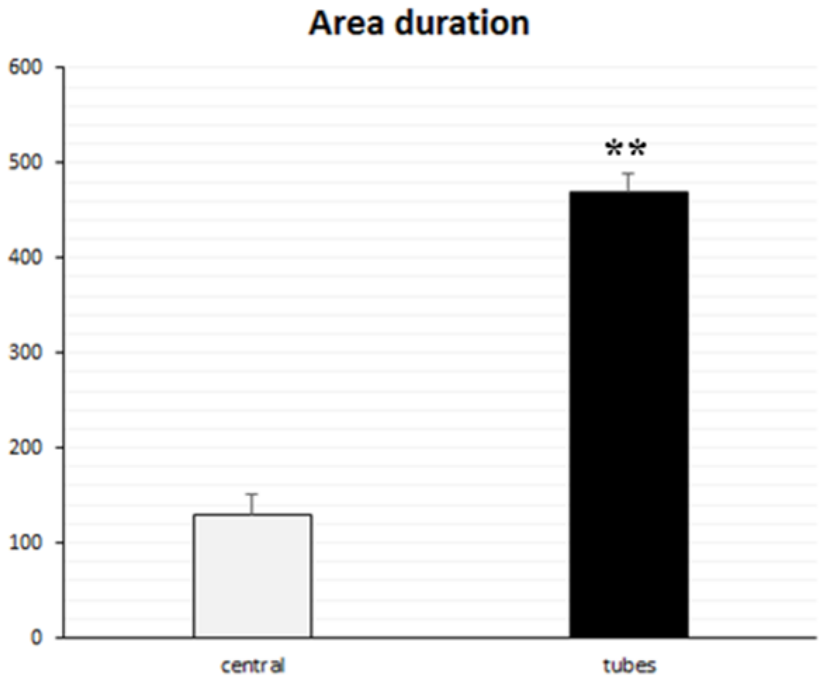


Figure 3 Time remaining in the central area against time in the feeding area

The results are expressed as the mean ± standard error, the mean values are the average of remaining time in the central area of the arena and the average of the total time remaining in the 8 tubes containing the food and the treatments. **P<0.001

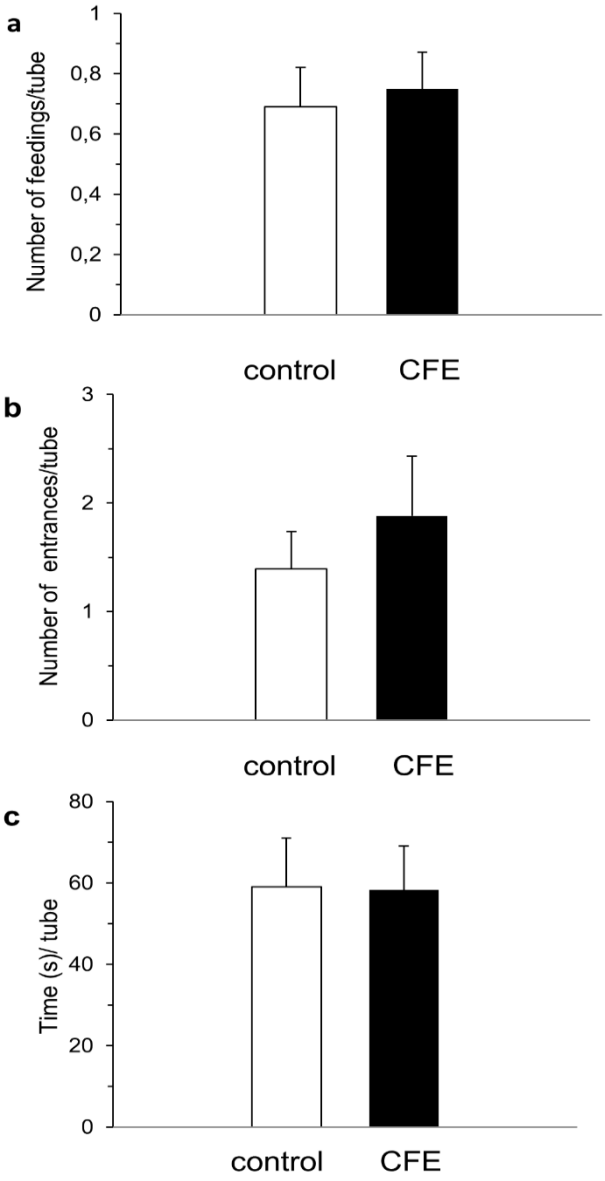


Figure 4 Behavioural parameters regarding feeding and exploratory behaviour

The results are expressed as the mean ± standard error, the mean value is the average for the four tubes for each treatment. *a:* mean number of times that the mice fed in control versus treated tubes, *b*: mean number of times that mice entered control versus treated tubes c: average time that mice remained in treated versus control tubes.

The analysis of sex and the interaction between treatment and sex revealed no significant effect (Table 1).

Table 1. Comparison between treatments, sexes and their interaction according to tube entry latency, entrance frequency, duration, consumption frequency, and first entry choice dependent variables

	Treatment effect(df=1)		Sex effect (df=1)		Treatment-Sex interaction (df=1)	
	F	*P* [a]	*F*	*P* [a]	*F*	*P* [a]
Tube entry latency	1.49	0.25	0.18	0.68	0.70	0.42
Entrance frequency	1.98	0.18	0.12	0.73	0.08	0.78
Duration	0.01	0.93	0.05	0.82	0.80	0.38
Consumption Frequency	0.06	0.81	0.90	0.36	2.93	0.10
First choice	0.03	0.86	0.00	0.98	0.03	0.86

n=21,11males and 10 females

[a] *P* values were calculated using a Mixed model

In terms of first tube choice, 52.38% of the mice chose the CFE, with a mean latency of about 43 seconds before entering the tube, and a food intake on average of 108 seconds after entering the tube. 47.62% chose the control tubes first with a mean latency of 66 seconds to enter, and 73 seconds for feeding.

Experiment 2

Reliability between the observers who carried out the video analysis was greater than 0.9 for all the parameters so the average of the two observers was calculated. No difference was observed between the blank and CFE for avoidance related parameters: treated area duration (**Figure 5a**), P=0.90; non-treated area duration (**Figure 5b**), P=0.70; average time per passage treated area (5c), P=0.26; average time per passage non treated area (**Figure 5d**), P=0.23

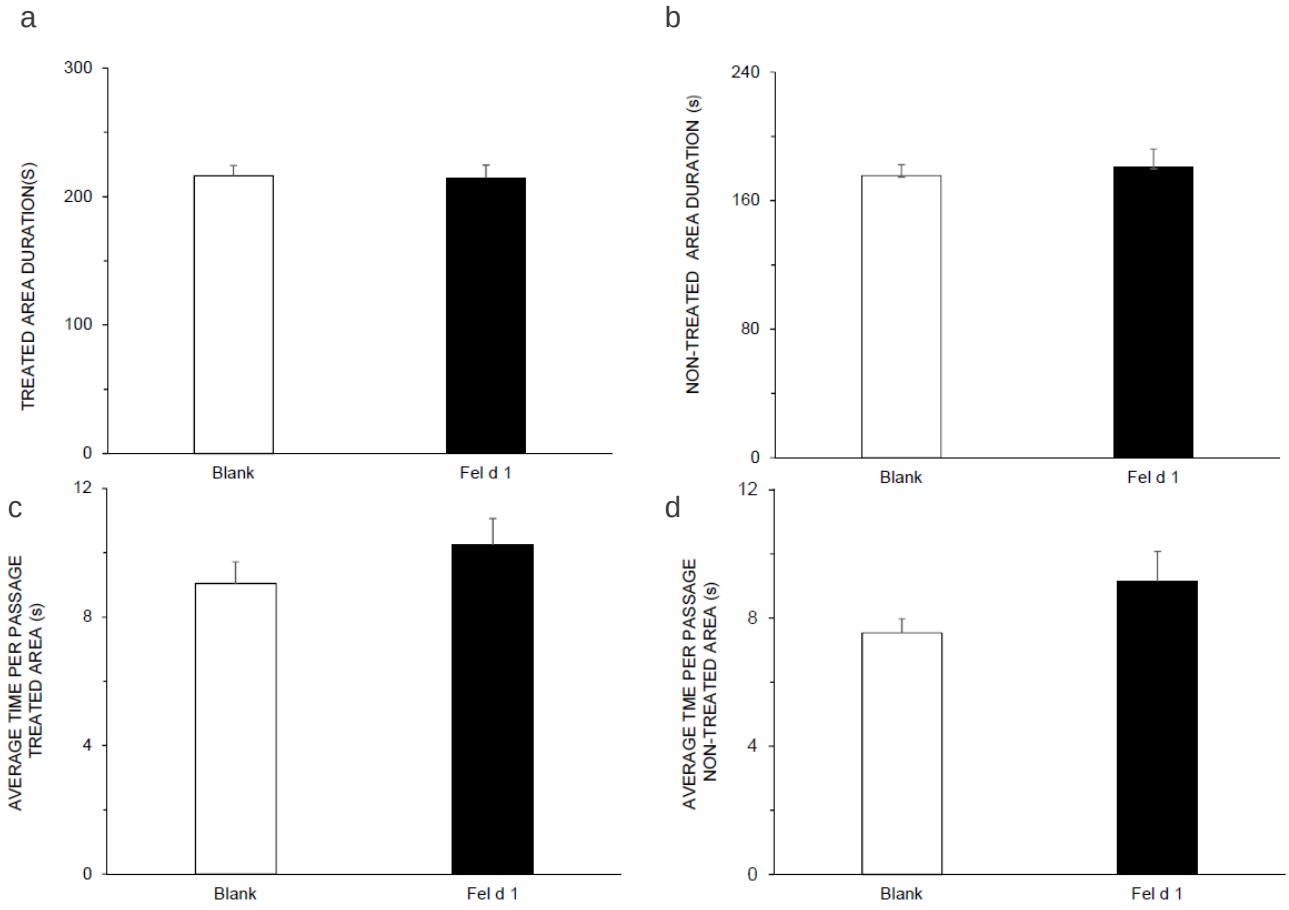
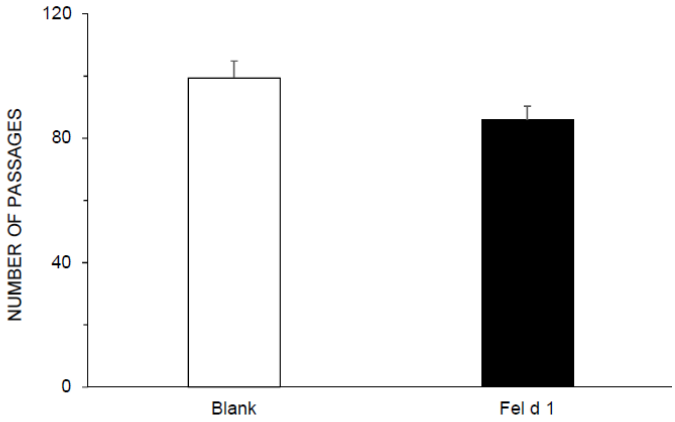


Figure 5 Behavioural parameters related to avoidance behaviour: a: treated area duration, b: non-treated area duration, c: average time per passage treated area, d: average time per passage non-treated area. Comparison has been computed with a Student Test.

No difference was observed neither, for parameters related to general activity, number of passages (**Figure 6a**), P= 0.064; and the parameter related to stress, number of faecal boli (**Figure 6b**), P= 0.079

a



b

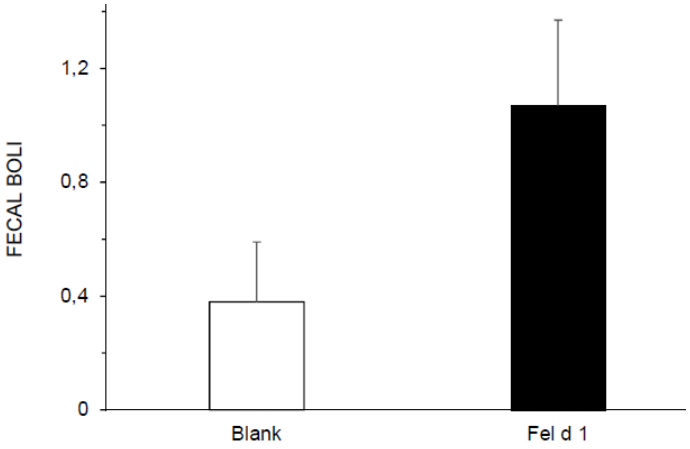


Figure 6 Behavioural parameter related to locomotor activity: number of passages (a) and parameters related to stress: number of fecal boli (b)

DISCUSSION

This study shows that a hydrophilic extract from washes of cat fur (CFE) containing high amounts of the major cat allergen Fel d 1 did not alter feeding or exploratory behaviour in RjOrl:Swiss mice. To the authors' knowledge, this is the first time that a solution with a known concentration of Fel d 1 and the purified molecule has been tested as a predator stimulus in rodents.

The initial hypothesis that the main long-lasting molecule released in the environment by cats could have a kairomone role in mice and thus an important evolutionary advantage was not confirmed by this behavioural study. The absence of changes in exploration behaviours both in terms of the time spent in close proximity to the predator stimulus (duration) and visiting frequency to the treated areas (frequency) suggest that mice cannot detect this stimulus as a dangerous signal or that it is of minor importance compared with the natural motivation to explore and feed. In contrast, the preference for narrow areas (tubes), close to the walls (thigmotaxis) with highly caloric food (78,27% of tested time) compared with opened exposed areas (21,73% of tested time) confirms the validity of the test and the interest of mice to explore and feed in the treated areas.

In the case of the soluble content of the CFE was indeed detected by the vomeronasal organ, the high motivation to feed might cause the risk of predation to be underestimated (Kavaliers & Choleris 2001). Preys coping with complex environments have to evaluate costs and trade-offs of their actions, so the risk of a detected predator cue is balanced against the benefits of feeding in the risky area, benefits that in our study were enhanced fasting the mice as is preconized in feeding choice tests (Crawley 2007). However, a recent study in fasting mice (48h) showed an ability to discriminate between an innate fear eliciting molecule and a control in feeding place preference (Isosaka et al. 2015).

Unlike these fear-eliciting molecules, Fel d 1 may not be detected by mice olfactory receptors. Fel d 1 is a protein and is unlikely to be volatile but could be involved in chemical communication most likely through contact-based detection. However, the majority of proteins that have shown evidence of a kairomone role belong to the family of lipocalins, such as MUP13 emitted in rat urine and Fel d 4 found in cat saliva (Papes et al. 2010). Based on current knowledge of the subject and the results of this study, one may suppose that the chemosensory receptor type for proteins in rodents, V2R (Tirindelli et al. 2009), is unable to recognize predator proteins of the secretoglobin family. Consequently, this kairomone function could be fulfilled by Fel d 4, as shown by Papes et al (2010). Fel d 1 closely resembles to the

mouse secretoglobine ABP from structural aspects, and as such might not be well discriminated (Durairaj et al 2018).

The “all cat CFE pool” sample did not contain a great diversity of proteins: only 4 bands were found in SDS-Page with the main band (21.5 kDa) being confirmed as the dimeric form of Fel d 1 by Western-blot analysis. This result suggests its relevance as the main protein released in the environment by cats. A second band between 116 and 200 kDa was observed both in SDS-Page and immunoblot with anti-Fel d 1 mAb: this may correspond to an undefined multimeric form of Fel d 1, persisting despite the denaturing conditions. This observation is not surprising given the prior evidence by other authors indicating that Fel d 1 is a particularly resistant molecule (Van Milligen et al. 1992; Bienboire-Frosini et al. 2010). The Fel d 1 molecular forms observed in this sample are in accordance with the existing literature (Bienboire-Frosini et al. 2010).

Mice were exposed to 5.6µg of Fel d 1 in the CFE treated medical gauzes for the first experiment and 7 µg of purified Fel d 1 for the second experiment, which corresponds to a realistic amount likely to be found in cat living areas (Custovic et al. 1998; Nicholas et al. 2008), and in comparison with the daily production of Fel d 1 by cats (Dabrowski et al. 1990). The concentration of Fel d 1 obtained from the ELISA test in the CFE pool was relatively high in comparison with previous publications (Bienboire-Frosini et al. 2012; Bienboire-Frosini et al. 2010). This is probably due to slight modifications in the CFE sampling and extraction methods, which resulted in more concentrated solutions.

It may be argued that the huge amounts of Fel d 1 released in the environment, along with their persistence over time, could transmit an unclear message about the presence of the predator: indeed, Fel d 1’s lasting presence within the environment (Custovic et al. 1998; Cain et al. 1998) after the predator’s passing may make Fel d 1’s putative message irrelevant for mice. Moreover, though Fel d 1 secretion is testosterone dependent, mice hunters are predominantly queens (Fitzgerald & Turner 2000) , a fact which may decrease the informative value of Fel d 1 as a predator signal.

Due to the protein conformation of Fel d 1, with its hydrophobic internal cavity one can expect a higher relevance of volatile ligands remaining inside the cavity(Kaiser et al. 2007). These may provide fresher and therefore more relevant signals about the presence of the predator than the protein itself, as has been suggested for other mammalian proteins [15,14,44].

Fel d 1 is particularly present in the facial area of cats, due to the high density of sebaceous glands [22,45]. May et al (2012) found an effect of cheek rubbing marks of domestic cats in rats: feeding behaviour was reduced compared to control, and avoidance behaviours increased. However, the authors did not identify the molecular content of the rubbing marks during their study, and rubbing marks were studied only from a single cat. Fel d 1 production can vary greatly between subjects (Nicholas et al. 2008), therefore it is not possible to confirm the presence of this protein in these experiments. Consequently, the anti-predatory response observed by May et al (2012) could have been elicited by compounds other than Fel d 1.

It is worth noting that differences may exist between laboratory mice strains leading to potential variations in their sensitivity to predator stimuli [46]. In this study, an outbred strain was selected with the aim of obtaining a wider genetic background and more representative results than with other widely used inbred strains, such as B57BL6.

In any case, the biological cost of Fel d 1 production strongly suggests a role in chemical communication. Feline marking (including rubbings, wood scratching and excrement marking) is an important element in intra-specific communication [25,47]; it provides information regarding individual and sexual identity, the amount of time spent at the location, and the reproduction cycle stage. Fel d 1 is released in large amounts by cats in the environment (Custovic et al. 1998; Dabrowski et al. 1990) and displays an immunological polymorphism linked to the cat's emotional state (Bienboire-Frosini et al. 2012). Its production is testosterone dependent [48,49] and varies according to sex [48]. In addition, it shares strong structural and genetic similarities with another member of the secretoglobin family, the mouse androgen binding protein (mABP), whose role has been suggested in chemical communication as a pheromone in itself, capable of carrying information on the basis of its own structure or as a

pheromone-binding protein that carries an informative ligand [50]. There is thus a sound basis to support its role in feline intra-specific chemical communication.

CONCLUSIONS

The results of this study point to a non-kairomone role of Fel d 1 in mice. The biological cost of its production, the properties of the molecule (lasting presence in the environment, airborne transport, and stickiness), and its testosterone dependence, indicate that it is likely to have a function in intra-specific communication.

In addition, the probable role of Fel d 1 as a carrier of hydrophobic compounds requires further investigation. As a carrier, the protein could fulfil a function in both intra-specific and interspecies communication.

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Supporting information

S1 Table. General information regarding the sex, age and Fel d 1 found in the CFE cat's samples

Sample	Animal Identification	Species	Sex	Age	Fel d 1 (ng/ml)
Fur Cat Extract	a	*Felis domesticus*	Male	5	2720
	b		Female	5	5360
	c		Male	3	64,8
	d		Male	5	260
	e		Female	8	1540
	f		Male	3	1600

S2 Video. Feeding and exploratory behaviour in the arena.

https://irsea-my.sharepoint.com/personal/c_grau_group-irsea_com/_layouts/15/guestaccess.aspx?docid=098dfd95b50c04597a0d6cf2ef1204115&authkey=AeKFZOQKucEb1og2qgGWmc8

S3 ARRIVE guidelines

https://irsea-my.sharepoint.com/personal/c_grau_group-irsea_com/_layouts/15/guestaccess.aspx?docid=001bcd9ae6e7c4ccf9483b681879fb166&authkey=AT09CjAcKqu6oBw2ZVrifpQ

1.3. Conclusions of study 3

The results of this study point to a non-kairomone role of Fel d 1 in mice. The biological cost of its production, the properties of the molecule (lasting presence in the environment, airborne transport, and stickiness), and its testosterone dependence, indicate that it is likely to function in intraspecific communication.

The results of some preliminary behavioural essays in cats point in this direction, as cats prefer to stay in areas in which Fel d 1 is present (Marcet et al., 2016). Another protein, the cat urinary protein cauxin, and specifically a peptide that is derived from this protein, felinine, can also be implicated in cat intraspecific communication. This peptide has features similar to Fel d 1 as its production is testosterone dependent and therefore higher in males than in females (Miyazaki et al., 2006), and it has a peptidic nature. This is the same case as for the major urinary proteins in mice and rats that are also excreted with urine in higher amounts in males than in females (Hastie, Held, & Toole, 1979; Hurst et al., 2001).

However, we cannot exclusively attribute the absence of anti-predator effects to the cat protein Fel d 1 in the first experiment; it must be attributed to the whole stimulus, which was the hydrophilic solution obtained after rubbing different parts of the cat (including the cheeks and the chest). Only one other protein was probably found in the sample, and its size coincided

with albumin. This species of protein has not been described as involved in the chemical communication in any species for, but it is known for its allergenic properties.

Our second experiment with purified Fel d 1, confirmed what was observed with CFE; mice did not avoid this cat protein stimulus or significantly alter their exploratory behaviour, which supports a non-kairomone role of this molecule for mice.

2. INFLUENCE OF A SYNTHETIC FACIAL CAT'S PHEROMONE IN MICE FORAGING

2.1 Preamble to study 4

May et al found that the scent found in cat cheek marking elicited antipredator behaviours, increased avoidance, decreased the number of contacts with the stimulus, and decreased feeding in Sprague-Dawley rats (May, Bowen, McGregor, & Timberlake, 2012). The third fraction of the cat facial pheromone was identified in the 1990s (Pageat & Gaultier, 2003), and it has been proved to have a role in chemical communication and modulating behaviour in cats (Griffith, Steigerwald, & Buffington, 2000; D. S. Mills, Redgate, & Landsberg, 2011). This pheromone is mainly secreted in the cheeks, so combining all the information, we hypothesized that this cat pheromone could be the compound that elicits anti-predator behaviours resulting from the cheek cat marking scents and could also modify feeding behaviour due to an increase in the perception of risk by the house mouse.

2.2 Study 4

Influence of a synthetic facial cat pheromone on the foraging behaviour of an outbred strain of laboratory mice

Carlos Grau, Philippe Monneret, Julien Leclercq**,** Céline Lafont-Lecuelle, Patrick Pageat

Introduction

Different models of samples and animals have already been used to obtain a predatory response under laboratory conditions. The most commonly used has been the cat, but there have also been studies with ferrets, red foxes, wolfs, dogs, least weasels, stoats, Siberian weasels, minks, brown rats, tigers, and others. The samples vary from the whole body of the animal (alive, dead, or anaesthetised) to the fur, faeces, urine, anal gland secretions, collars, bedding, and medical gauze rubbed on the necks of cats. Fur and skin odours dissipate quickly (Apfelbach et al., 2005) and are thus more reliable for predicting the presence of a predator than faeces, which dissipate very slowly along with the accompanying odours (Apfelbach et al 2006).

Defensive behaviours occur in response to threats to the life or body of an animal, and these threats can be divided into four categories: predators, aggressive conspecifics, threatening features of the environment (fire, water, lightning, high places) and heterospecifics that are

dangerous resource competitors (Apfelbach et al 2006). Cat odour has already been firmly demonstrated to elicit a predatory response in rodents, but this has been almost exclusively achieved with native odours, with only one study using a single isolated molecule, Fel d 4 (Papes et al., 2010). Based on our results, we can suppose that Fel d1 does not deter mice (at least an outbred strain, RjOrl:Swiss) from basic self-maintenance behaviour such as feeding or alter exploratory behaviour, but we cannot state that this molecule is not detected by mice. From another point of view, it could play a role in interspecific communication in cats.

However, chemical communication with proteins it is just one part of the message, these molecules can transmit information themselves but also help to slow the release of volatile molecules (Janotova & Stopka, 2009). The hydrophobic cavity of Fel d 1 can contain volatile compounds that are used by cats for intraspecific communication as well as interspecific communication, leading to an evolutionary advantage for rodents capable of detecting fresh signals from predator species such as these volatile compounds. In addition, cat rubbing odours decreased feeding behaviour in laboratory rats (May et al., 2012).

For these reasons, we decided to test different possible volatile compounds used by cats for intraspecific communication. The facial feline pheromone was a good candidate for this purpose as Feld 1 is released in large amounts from cat cheeks.

Objectives and research hypothesis

We carried out an experiment to determine if the presence of the cat facial pheromone, F3, altered a basic maintenance behaviour, feeding, or modified exploratory behaviour. For this test, we used F3 at a concentration of 10% with ethanol as a solvent.

The response to that kind of stimuli is context independent, so the response occurs when stimuli are solely presented on cotton gauze (Papes et al., 2010).

Our research hypothesis was that the third fraction of the cat facial pheromone, F3 could decrease feeding and foraging behaviour and modify the exploratory behaviour of house mice in a complex, multichambered device.

Materials and methods

Animals

Twelve mice (pre-test) +30 mice (test) RjOrl: Swiss mice, 12-16 weeks, from Janvier laboratories were kept under standard conditions of 22± 2 C° and 50 ± 20% humidity in a conventional laboratory animal facility in Saint Saturnin les Apt. A 12:12-h (light: dark) reversed light cycle regime was used with the light cycle turning off at 13:00 and on at 1:00. Food (Teckad global diets, 2014, Harlan) and water were supplied ad libitum. Orange LED lights

with a wavelength of 610 nm were used for the breeding and test rooms. This wavelength is outside the visible spectrum for mice but allows for good human visual acuity (**Figure 1**).

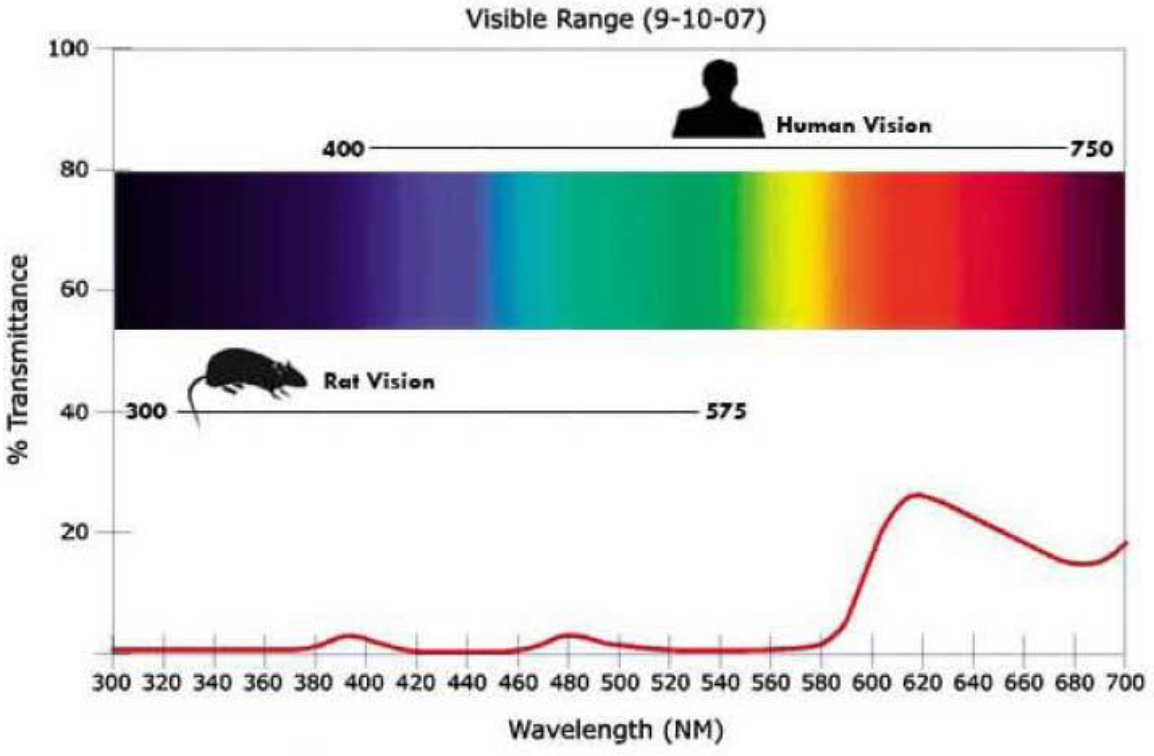


***Figure 1**:* Human vs. rodent visible range

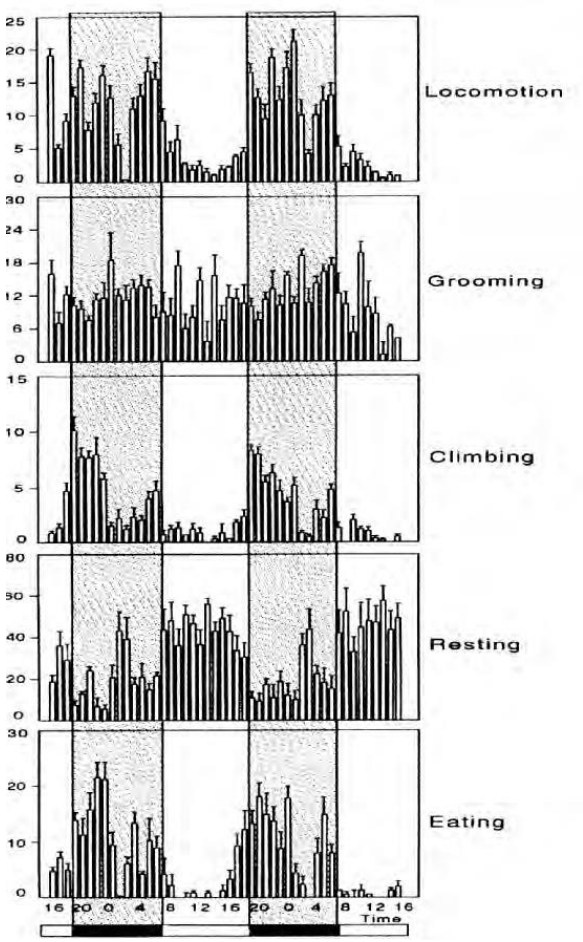


Figure 2 Circadian rhythms related to some mouse behaviours. The dark period is indicated by black bars (Schlingmann, Van De Weerd, Baumans, Remie, & Van Zutphen, 1998)

After arrival, the study mice had an acclimatization/quarantine period of 2 weeks, and during this interval, inspected the sanitary state of the animals. Animals were in single-sex groups within cages, with 3 or four per cage. The cages used during acclimatization before the experiment were polycarbonate Eurostandart type 2 L, ref: 1284 L, from the laboratory animal material company Tecniplast (Buguggiate, Italy). According to current European and French laws, the maximum of animals allowed in this kind of cage is 5 (> 30 g). Description: 365 x 207 x 140 mm – surface area of 530 cm^2 (**Figure 3**).

Figure 3 Breeding cages

The cages and the bedding material (Suralite 3-4, Harlan) were changed weekly, and the wire bar lid was changed monthly. A small amount of dirty bedding (mainly from the resting area) was kept in the new, cleaned cages to decrease the stress in the new environment by providing familiar odours. During the experimental weeks, the day to change cages was selected to avoid behavioural changes due to stress from the new cages. There was a period of at least 3 days between this change and the behavioural assays.

The conditions of the experimental room were the same as those of the maintenance room.

Apparatus

The experiment was carried out in a multichambered choice device with a central corridor, or central chamber, and 8 lateral arms (tubes) (**Figure 4**). There was a food reward, 14 5-mg pellets (test diets, St Louis, USA) inside each tube. To decrease possible contamination between tubes, 7*7-cm plastic curtains were fixed in the entrance of each tube.

Treatments and Food Location

All the procedures were performed blindly with treatments coded as A or B. One millilitre of the cat facial pheromone *F3* was applied to 4 pieces of medical gauze while 1 ml of purified water was applied to the four negative-control (C) gauzes (**Figure 5**). The gauzes were then placed at the end of the tube and fixed to the plastic cap with two magnets. To avoid cross contamination, different tubes were used for both treatments.

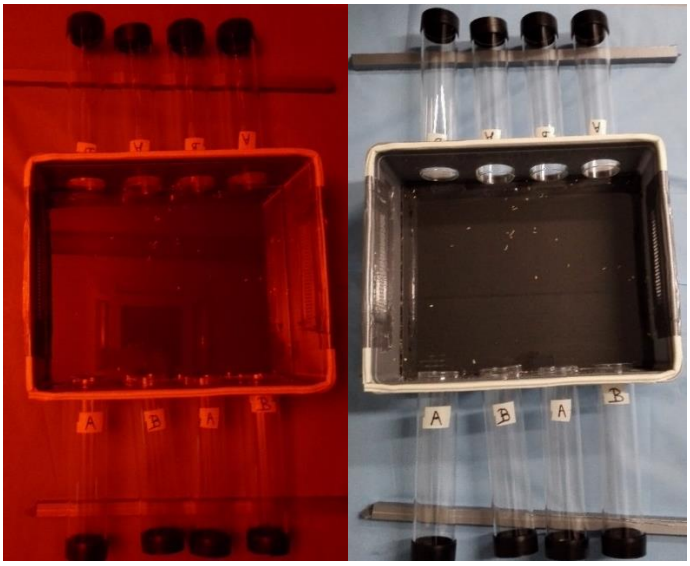


Figure 4 Experimental device: a rectangular maze with 8 arms. The left figure shows the light conditions used for the experiments during the dark phase: 600-nm orange LED lights. The right side shows whole-spectra lights for the light phase. Device measurements: tubes, 25 cm (long) x5 cm (wide) x10 cm (high); central area of the arena: 40x50 cm in diameter.

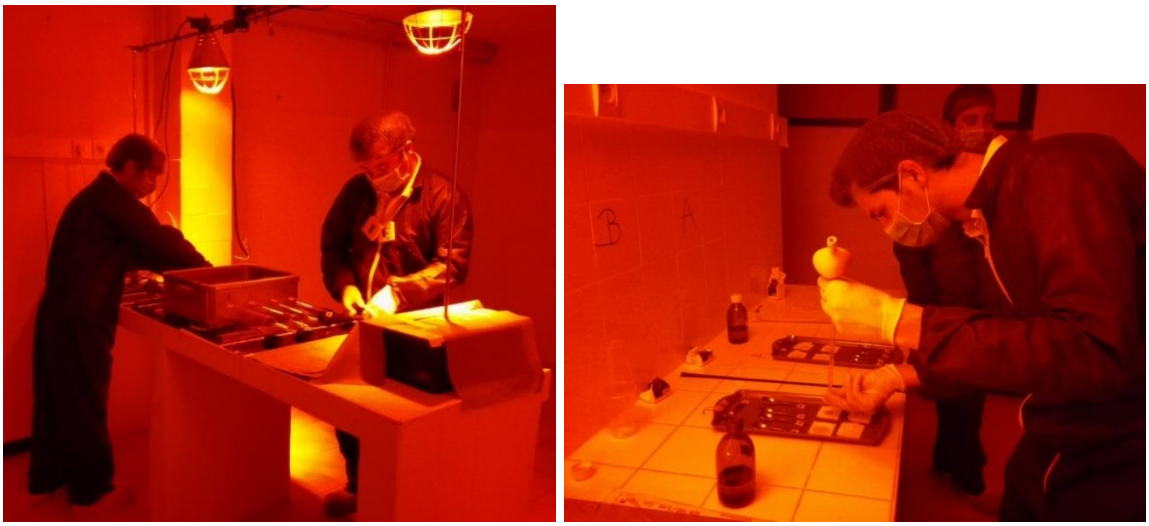


Figure 5 General perspective of the setup and the preparations before the tests with the camera at the top (left) and the application of the treatments to the medical gauzes (right)

Figure 6 Stainless steel spoons used to manipulate the food rewards on the left, and a detail of their placement in the experimental device

In the end of each tube, 14 5-mg pellets were placed directly over the surface of the tube approximately 4 cm from the end, and they were covered with plastic caps of the same diameter as the tubes (**Figure 6**).

Experimental Design and Treatment Randomization. The study followed a randomized complete block design (RCBD) without repetition, in which each mouse corresponded to a block and received each of the 2 treatments 4 times. The mean value of the 4 measures for each treatment was used for statistical analyses as they were not true repetitions (Lellouch & Lazar, 1993)

Treatments were assigned at random on each branch of the device to avoid location effects. Lots were drawn for each experimental unit; the blocks were randomized; and the treatments were assigned within the blocks.

The entire procedure was blinded; the authors were only aware of the treatment locations and not of their composition.

Behavioural Test. The tests were conducted in the experimental room between 13:30 am and 17:00 pm at temperatures in the range of 21 ± 2 °C and humidity of 50 ± 5%. The same operator manipulated the mice throughout all the tests, and the animals were transported from the holding cage to the arena following a previously described method (Hurst & West, 2010a) and using polycarbonate tubes for transport to decrease stress due to tail manipulation. The centre of the arena was marked, and mice were released from the plastic tube over the marking.

Experimental subjects were video recorded for 15 minutes; the experimenters left the room after placing the animal in the device. The analytical tests began at this point.

Measurements and Video Analysis. Video analysis was performed blindly by two independent observers, and the measurements included the first treatment chosen (defined as the first time that the mice crossed over the medical gauze), food consumption latency, entrance frequency (average number of times that the mice entered the control or F3 tubes), consumption frequency (average number of times that the mice fed in the control or F3 tubes), treatment duration (average amount of time that the mice remained inside the control or F3 tubes) and the overall duration (total amount of time that the mice remained in all tubes or the central arena).

Statistical Analysis. SAS software 9.4 (2002-2012 by SAS Institute Inc., Cary, NC, USA) was used for the analyses. Before proceeding, the dataset reliability between 2 independent observers was calculated with Pearson's correlation (kappa coefficient for the first-choice variable) using the corr and freq procedures, with an acceptable inter-observer reliability established at 0.9.

For all parameters, analysis was performed using a general linear mixed model (mixed procedure of SAS software 9.4) or a generalized linear mixed model (glimmix procedure of SAS software 9.4). Mixed modelling was performed to explore the main effects of treatment and sex as fixed factors and their interaction; the animals were considered a random factor. Statistical significance was established at 0.05. The response variables "first choice entry" and "first choice consumption" were analysed with a binomial test. The first choice of the mouse was counted for both treatments for both response variables.

A two-way ANOVA with one within-subject effect (treatment) and one between-subject effect (block).

A two-way factorial ANOVA with one within-subject effect (treatment), one between-subject effect (sex) and their interaction.

Results

No difference was observed between F3 and its control, ethanol, for any of the tested parameters, and sex also did not result in any significant difference in the tested parameters. However, the change in one of the parameters, tube entrance latency, was close to statistical significance between males and females alone but not when analysed with the treatment.

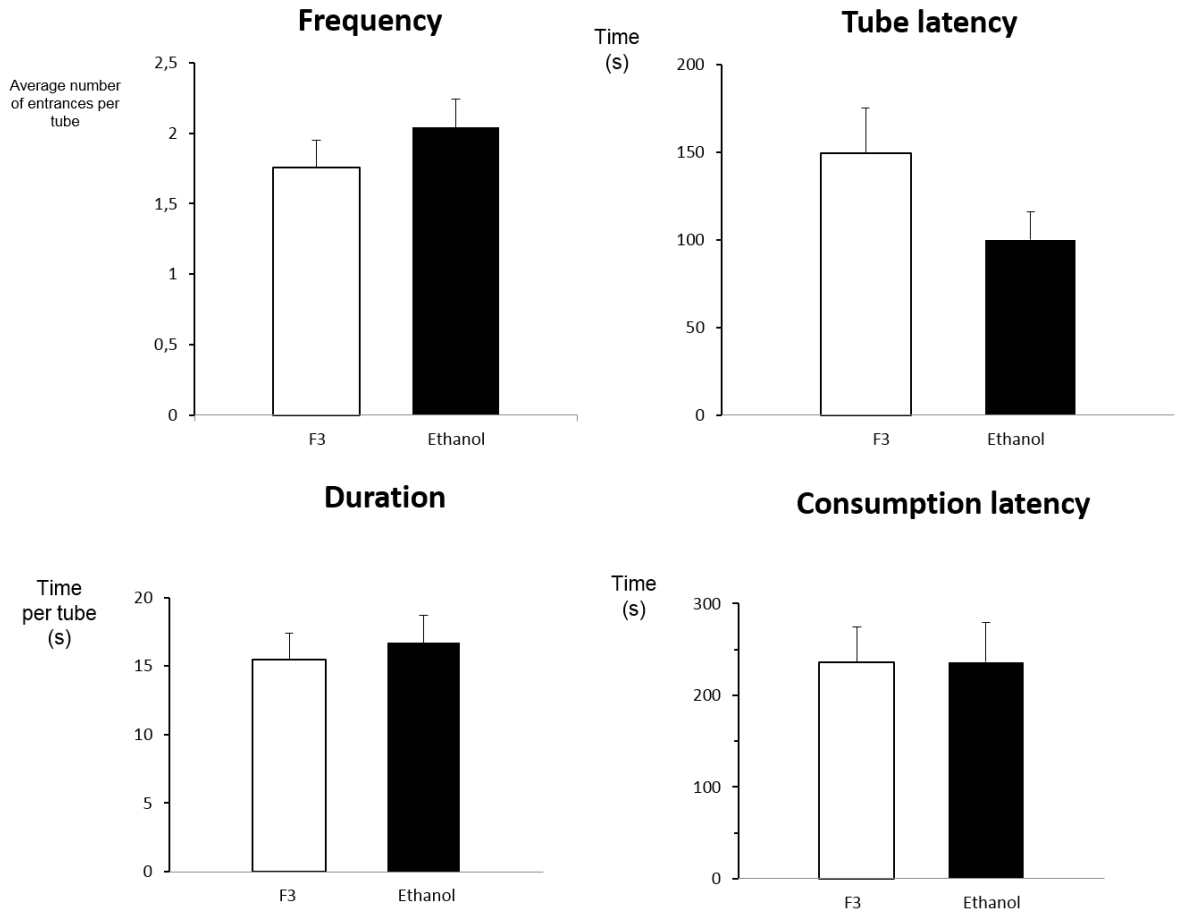
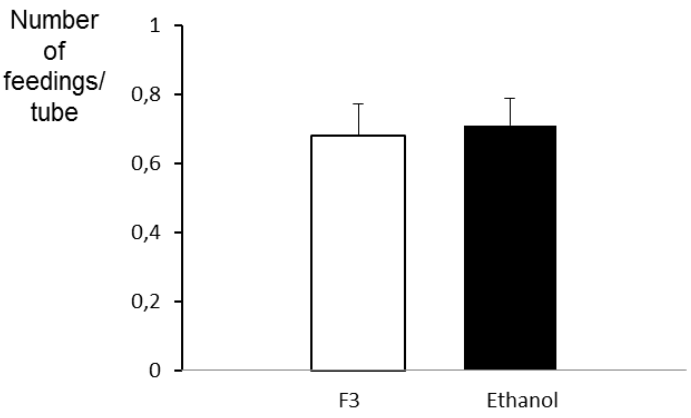


Figure 7 Behavioural parameters related to exploratory behaviour and avoidance

a



b

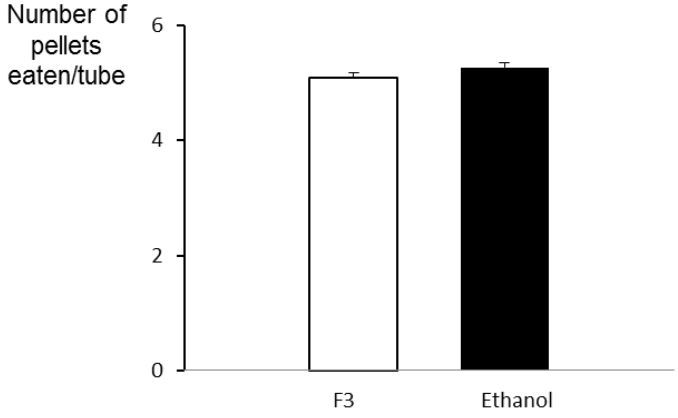


Figure 8 Behavioural parameters related to foraging and food consumption. a: number of feedings/tubes. b: number of pellets eaten/tube

Due to an incompletely charged camera battery, one of the animals was only video recorded for 10 minutes. This animal was excluded from the video analysis as it was not comparable to all other videos. He was replaced by one of the supplementary mice. One mouse was discarded according the exclusion criteria of stereotypies. This animal was not replaced, so the final number of animals tested was 29.

Ventilation areas situated lateral to the arena were covered with a metallic grid after some of the mice unexpectedly attempted to gnaw through the holes and escape.

	Effect of treatment (D.F.=1)		Effect of block (D.F.=28)	
Variable	*F value*	*Pr > F*	*F value*	*Pr > F*
Tube_latency	1.71	0.1911	26.27	0.5584
Frequency	0.89	0.3540	0.82	0.7016
Length	0.22	0.6395	1.35	0.2171
Consumption_latency	0.40	0.5334	7.94	**<0.0001**
Frequency_consumption	0.10	0.7521	1.68	0.0885

Food_consumption	0.06	0.8131	3.17	**0.0016**

Table 1 Summarized results of two-way ANOVA of animal (block)-treatment differences and sex-treatment differences.

	Block-Treatment model: Tukey's test of additivity						
	Effect of treatment (D.F.=1)		Effect of block			Effect of the interaction (D.F.=1)	
V*ariable*	*F value*	*Pr > F*	*F value*	*D.F.*	*Pr > F*	*F value*	*Pr > F*
Frequency	0.86	0.3622	0.79	28	0.7304	0.07	0.7881
Length	0.22	0.6454	1.30	28	0.2476	0.05	0.8327
Consumption_latency	0.39	0.5380	7.29	26	**<0.0001**	0.02	0.9035
Frequency_consumption	0.10	0.7545	1.65	28	0.0997	0.46	0.5037
Food_consumption	0.06	0.8112	3.24	28	**0.0015**	1.58	0.2194

Table 2. Summarized Tukey's test of additivity results for animal (block)-treatment interactions.

	Sex-Treatment model (D.F.=1)					
	Effect of treatment		Effect of sex		Effect of the interaction	
V*ariable*	*F value*	*Pr > F*	*F value*	*Pr > F*	*F value*	*Pr > F*
Tube_latency	0.69	0.4051	2.97	0.0847	1.73	0.1884
Frequency	0.04	0.8390	0.93	0.3345	0.00	0.9532
Length	0.18	0.6771	0.08	0.7749	0.06	0.8062
Consumption_latency	0.19	0.6611	0.00	0.9915	0.02	0.8769
Frequency_consumption	0.08	0.7829	0.03	0.8618	0.05	0.8284
Food_consumption	0.07	0.7874	0.33	0.5705	0.97	0.3338

Discussion

Facial rubbing by cats plays a role in sexual and visual communication; cats seem to perform this marking behaviour when a known individual approaches (Pageat & Gaultier, 2003). The F3 fraction of facial cat pheromones is used for spatial orientation and emotional stabilization functions. We hypothesized that this olfactory chemical cue could elicit avoidance in mice due to the important predation pressure that cats exert against rodents (Loss, Will, & Marra, 2013), so preys may have developed strategies to detect interspecific predator messages for their benefit (Papes et al., 2010).

No significant differences were detected in the studied parameters. A significant difference was only obtained between animals (independent of treatment) for the consumption latency and food consumption, which is logical due to normal variability between animals. No significant differences were observed between sexes. The tendency observed for a sex-related difference in tube latency could be due to behavioural sex differences as it has been stated that females are more cautious and exhibit more fear responses than males (Cahill, 2006).

In a high percentage of videos, the first entries to tubes were followed by behaviour indicating displeasure; mice moved to the rear and hesitated to enter. This response could be due to the use of ethanol as a solvent as the behaviour was seldom observed in the last part of the tests

(when the ethanol should have been partially volatilized and dispersed) and was observed in both treatments. Another possibility is that the origin was a cleaning product, but this is less likely as tubes were thoroughly rinsed with water before use.

May and colleagues (May et al., 2012) found that the rubbing marks of cats, recovered with pieces of medical gauze and heated prior to testing, could diminish feeding behaviour in Sprague-Dawley rats, but the authors used a non-choice test. This kind of test could show results in a clearer manner and is statistically very robust. Papes and colleagues (Papes et al., 2010) also observed anti-predator behavioural responses in mice using a non-choice test, in which just one stimulus was present at a time. However, non-choice tests are considered less realistic as these simplified situations seldom occur in nature. The absence of significant avoidance behaviours in our test could be due to the motivation for feeding being too high, a non-balanced ratio between positive motivation for feeding and the repulsive effect of F3, and the mice being incapable of detecting F3 as a predator stimulus.

There have been results evidencing an antipredator response to a cat stimulus in the two main mice strains used in research: cd-1 and C57BL/6Rj. However, for cd-1, I found research investigating responses to cat urine or TMT (fox faeces component) but not to cat fur or materials that have been in contact or rubbed against the skin/fur, which have mainly been tested in rats or in the C57BL/6Rj strain. The idea of using the cd-1 strain was to obtain more representative results as these mice have a wider genetic background. C57BL/6Rj is an inbred strain, meaning that the animals are almost genetically identical and are often used to generate transgenic animals.

Some practical aspects regarding the materials and procedure:

We highlight the quality of the data in this assay (as it was valued by our statistical service), which we observed to follow a normal distribution. In contrast, the only controversial parameter with more differences between observers was feeding.

Glass tubes were easily cleaned, so possible contamination was diminished. However, they were very fragile, and some of were broken during the experimental procedure. This mainly occurred during the early days when the manipulators had not yet had much experience. The walls were covered with plastic, which was somewhat difficult to clean, but the floor was kept cleaner because we used a glass square to cover the entire surface. However, the borders and corners of the square could accumulate faeces or other materials because they were not easy to clean.

Animals were tested within a narrow period of time which decreased variation due to changes in circadian rhythms and therefore daily activity patterns. We tested the mice during the hours

of high activity (first hours of the night) using reversed cycles with the help of orange LED lights. The change in the cycle was accomplished 3 weeks before the experiment, and a clear change in the activity pattern was observed in response to the new scheduled light cycle: moving from lights on from 8:00 AM to 1:00 AM and lights off from 8:00 PM to 1:00 PM.

2.3 Conclusions

In this study, we did not observe any significant difference between the facial feline pheromone F3 and its ethanol control, but we observed some avoidance behaviours with both treatments which helped us develop our next hypothesis. What could be the ecological meaning of ethanol? What is the interest of this molecule to other species, more specifically for rodents? Could we find differences in behavioural responses between this plant chemical cue and rodent predator olfactory cues? Through my bibliographical research, I developed the hypothesis that ethanol could influence the use of space by mice, eliciting avoidance from an olfactory cue from rotted fruits and seeds. Avoidance of ethanol in ecologically meaningful amounts for rodents was confirmed by our next study.

In retrospect, in this experiment, both treatments involved ethanol (F3+ ethanol and ethanol alone), so we cannot state that F3 does or does not affect exploratory behaviour or foraging by mice. To identify the effects of F3, further research should test this feline facial pheromone dissolved with another molecule. From this experience, I also came to appreciate why complex devices should be tested in later steps because, without a good baseline of mouse reactions to these olfactory stimuli, we could easily misinterpret the results.

References

WEB RESOURCES

The Mouse Phenome Database: www.jax.org/phenomene

Second Part: plant vs predator olfactory stimuli

CHAPTER 3: PREDATOR AND PLANT CHEMICAL CUES

1 PREAMBLE

Olfactory cues drive behaviours in rodents, informing them about food resources, predator risks or conspecifics. With this study, we aimed to answer the question of whether ethanol, a ubiquitous plant chemical cue, would influence the use of space by mice and elicit avoidance. In addition, we were interested if these responses could be different from those to rodent predator cues from the red fox, including its native form and its isolated kairomone chemical TMT.

2. STUDY NUMBER 5

Oral presentation

C.Grau *Effects of predator and plant olfactory cues in the exploratory behaviour of the house mouse*. Proceedings of the joint meeting: 6th International Conference of Rodent Biology and Management & 16th Rodents et Spatium. Potsdam, Germany, 2018, 3rd September 2018

Foraging behaviour and avoidance of predators cover basic needs for self-maintenance and survival. These basic behaviours are triggered by internal and external sources of information like blood glucose levels and olfactory cues. Plant olfactory cues are valuable for rodents as the house mouse because they can inform about the ripening state of fruits and risks associated to unripe or rotted fruits. Our research found that ethanol as olfactory cue elicited avoidance and decreased locomotor activity in mice, these results highlighted the relevance of ethanol as a probable cue for fruit ripening, in the wild, this chemical cue could convey primordial information about the ripening state of fruits.

Olfaction has also a main role in predator avoidance by mice, avoidance of physical encounters with the predator species, increases highly chances of survival. In another study, we found that mice avoided significantly olfactory cues from domestic ferrets (*Mustela Furo),* which probable ancestor is the European polecat *(Mustela Putorius)*, natural predators of rodents.

Future research should deeper our understanding in the interactions of predator and plant olfactory cues as they are part of the same olfactory dimension, and motivation for feeding and avoidance of predators are tightly linked.

Research paper in press in Applied Animal Behaviour Science

Ethanol and a chemical from fox faeces modulate exploratory behaviour in laboratory mice

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Abstract

Mice are macrosmatic animals that use olfaction as their main source of information to increase fitness; they process predator cues to assess risk, and plants and fruit cues to find nutritional

resources and assess their quality or toxicity. In this study, we examined the effects of ethanol as an olfactory stimulus related to fruit rotting, against 2,4,5-trimethylthiazoline (TMT, a fox faeces compound), its native origin, the fox faeces and a negative control on avoidance, locomotor activity, and stress related behaviour, measured by the production of faecal boli. Our results showed that mice clearly avoided ethanol (P=<0.0001) and decreased their locomotor activity (P=0.0076) when ethanol was present. The molecule 2,4,5-trimethylthiazoline (TMT) was the most avoided (P=<0.0001) and showed the lowest locomotor activity (P=0.0004). Both treatments, ethanol (P= 0.0348) and TMT (P= 0.0084), increased the number of faecal boli.

The clear avoidance and behavioural effects of ethanol in mice have direct implications in laboratory animal research, where it is used widely. This avoidance effect could elicit stressful situations and modify behavioural and physiological responses in mice housed in research facilities. In addition, this avoidance could be used as a non-lethal, inexpensive and non-toxic tool in rodent pest management. To explain these results, we suggest ethanol as a probable cue for fruit ripening, in the wild, this chemical cue could convey primordial information about the ripening state of fruits, allowing animals to avoid over-ripe, unhealthy fruits.

Key Words- Ethanol, olfaction, predators, allelochemicals, fruit ripening, avoidance

1. INTRODUCTION

Mice spend a great proportion of their time life exploring to find food and partners. Gathering information about predators and conspecific or allospecific competitors allow them to assess risks and benefits of their actions. During this life quest, olfaction provides the main environmental information to mice through detection of chemicals (Crawley, 2007; Latham and Mason, 2004; Mucignat-Caretta et al., 2012; Singleton and Krebs, 2007). Since foraging needs an important investment of resources and risk-taking for mice, plant volatile chemicals could modulate mice behaviour to minimize risk of intoxication and maximize benefits from finding highly nutritive foods with a minimal displacement (McArthur et al., 2014).

Mice are opportunistic animals which feed mainly from plants (grains, seeds, fruits) and invertebrates, depending on their availability and season (Shiels et al. 2013; Bomford 1987). Frugivorous mammalian species such as rodents eat fruits that are also consumed by microbial species, mainly fungi. Fungi rot fruits, and frugivorous species such as mice avoid rotten fruits (Cipollini and Stiles, 1993; Levey, 2004). As fruit ripens, the chemical profile of this feeding resource changes and, among other changes, a common molecule is produced: ethanol (Dudely, 2004; Levey, 2004). Ethanol is ubiquitous and the most common alcohol found in rotted fruits (Levey, 2004; Sánchez et al., 2006). In addition, ethanol has been defined as a key odorant, with a specific biological significance related to fruit ripening in the common fruit fly (*Drosophila melanogaster)* (Giang et al., 2017).

Avoidance of predators by olfaction is a primary motivation that guides rodent behaviour to improve chances of survival. Among predator signals, the molecule 2,5-dihydro-2,4,5-trimethylthiazoline is found in red fox faeces (*Vulpes vulpes*) and is commonly known as TMT (Vernet-Maury, 1980). A vast amount of research has proven its avoidance effect in mice and laboratory rats (Apfelbach et al., 2005; Galliot et al., 2012; Rosen et al., 2015; Staples and McGregor, 2006) . However, there is little previous research comparing the effects of the actual

native source (fox faeces) and TMT, and results were obtained only in a mice strain that is relatively uncommon in laboratory animal research (Buron et al., 2007; Hacquemand et al., 2013).

The stimuli secreted by plants and mammalian predators described so far both have a possible relevant message in the mouse's olfactory environment. Despite its wide use with laboratory animals, and biological and ecological importance, there is no clear evidence that ethanol as olfactory stimulus can alter the exploratory behaviour of laboratory mice by inducing avoidance or altering locomotor activity. For this reason, we developed a behavioural study to measure avoidance and influence in exploratory behaviour. Our initial hypothesis was that ethanol as olfactory stimulus could mediate and drive avoidance behaviours in laboratory mice and that this is probably related to the informative role that ethanol has about fruit rotting in the nature. The study aimed to determine whether these molecules or complex biological matrices could induce avoidance in mice and whether this effect could have an impact on locomotor activity and stress related responses, such as the production of faecal boli.

2. MATERIAL AND METHODS

2.1 Animals

Fifty-Six C57BL/6JRj mice (28 males and 28 females, Janvier Labs, France) of 8-10 weeks, free from infectious agents included in the FELASA health report (Mähler et al 2014), were kept at the facilities of the Research Centre in Semiochemistry and Applied Ethology (IRSEA) according to the requirements of French and European Law (2010/63/EU) and under the supervision of a veterinarian specialising in laboratory animals. The protocol and techniques described in this paper were approved by the IRSEA ethics committee (approval number AFCE20150501).

The housing room was kept at a temperature of 22±2°C and 60±20% humidity. A 12-12h inversed light-dark cycle was used (lights off at 12:00 PM) to perform behavioural tests during the naturally active period of mice (Latham & Mason 2004). Mice are crepuscular

animals and have a peak of activity during first hours of the dark period (McLennan & Taylor-Jeffs 2004). All the procedures were conducted between 12:00 and 5:00 PM.

The mice were housed in Eurostandard type IIL cages (Tecniplast, Italy), (369*156*132mm), with a total floor surface of 435cm². Animals were housed in single-sex groups per cage to minimize stress due to isolation, as mice are a social species; each cage housed three animals. Food was available ad libitum, with 2014 global rodent diet (Envigo, UK); the lignocel 3-4 bedding (Envigo, UK) was changed weekly. As enrichment material, each cage was equipped with a red plastic tube along with craft paper and white paper as nesting material (Genobios, Laval, France), as mice prefer complex nests with more than one material (Hess et al., 2008).

2.2 Apparatus

Rectangular arenas with a 4mm thick 50x30cm glass base covered with a transparent plastic top were used for the replicates. The square of glass was marked underneath with electric tape to distinguish two laterals and a central area in the arena. Lateral areas were separated by a 1 mm thick opaque plastic PVC barrier measuring 24*30cm, which was attached to the top of the arena. A small square (4.5cm*4.5cm) was cut out in the centre to allow the tested mouse to move freely (Figure 1). The treatment was applied on a medical gauze (4*4cm) and placed on one of the two sides.

This device was used as a modified open-field in order to measure laboratory mice exploratory behaviour, avoidance behaviour, and anti-predatory and fearful responses to olfactory stimuli. The vertical plastic divisions had the role of reducing the passage of volatile compounds to adjacent areas and acted as physical visual barrier.

2.3 Olfactory stimuli tested - Application

Animals were naïve to the tested stimuli having had no previous contact with any of them. Olfactory stimuli were poured over a 4x4 cm medical gauze, which was then placed over a square of glass (8x8 cm, 3 mm thick) to diminish contact with the arena and placed on one of

the two sides of the arena. Olfactory stimuli and its position for each replicate were chosen according to a randomised procedure. Olfactory stimuli were considered as the only independent variable; fourteen animals were tested for each of the four treatments. New gloves were used and changed between replicates.

2.3.1 2-5 Dihydro-2,4,5-Trymetilthiazoline (TMT)

2-5 Dihydro-2,4,5-Trymetilthiazoline is volatile compound present in fox faeces (Vernet-Maury, 1980) which elicits avoidance and fear responses in mice and rats. 8 µl of TMT (90% purity) containing no solvents (srqbio, Sarasota, USA) were used as the predator stimulus. This amount was based on previous literature where clear avoidance and fear responses were observed (Papes et al., 2010; Pérez-Gómez et al., 2015). TMT was considered as our positive control for avoidance behaviour.

2.3.2 Ethanol

Ethanol is a plant-based chemical produced in nature from fruit and cereal grain fermentation (Battcock and Azam-Ali, 1998; Dudely, 2004). 125 µl/cm² of ethanol were applied to the medical gauze (total amount 2 ml, 99%, Fisher Scientific, Hampton, USA). Ethanol evaporation was measured at 25% in our experimental conditions (temperature, humidity, liquid surface area exposed, duration). Ratio volume/volume of vaporized ethanol was 13.8 ppm for the whole device (36 l) and 46.29 ppm for the volume of the treated area (10.8 l), with a decreasing gradient from the treatment to the areas farthest from the treatment. Ethanol was used as a putative plant-based chemical cue for mice. This amount of ethanol is equivalent to 35g of overripe fermented fruits (eg: 6 average size grapes) with a 4.5% content in ethanol (Dudely, 2004). Mice did not have any previous direct contact and to the best of our knowledge, indirect contact with ethanol, therefore were considered naïve to this stimulus.

2.3.3 Fox faeces

Fox faeces are the natural origin of the chemical compound TMT and elicited antipredator responses in mice in previous research (Buron et al., 2007). Faeces from adult male and

female foxes were graciously donated by the “Laboratoire de la rage et la faune sauvage de Nancy” (Nancy, France). Droppings were collected within 24 hours after defecation and stored at -80 °C. Males’ and females’ faeces were sampled in equal amounts. We received the faeces frozen on dry ice and unfroze them to prepare aliquots. Each aliquot contained 2.5 g of male faeces and 2.5 g of female faeces; they were frozen again and kept at -20° until the day of the experiment when they were unfrozen at 4°C. Samples were kept at room temperature 1 hour before the experiments. Foxes were fed with extruded commercial dog food (Belcando, Germany). Male foxes were in their reproductive period during the faeces sampling.

2.3.4 Negative control

A medical gauze without any treatment was used as a negative control as it was the physical support for all treatments.

2.4 Behavioural Test

All the mice were habituated to the arena the day before the test for 10 minutes without any treatment. The tests were conducted in the experimental room between 12:00 PM and 5:00 PM, with temperatures in the range of 21 ± 2°C, and humidity 50 ± 20 %. The same operator manipulated the mice throughout all tests. The animals were transported from the holding cage to the arena using red PVC tubes in order to decrease stress from tail manipulation (Hurst and West, 2010).

Animals were transported to a pre-test room at least 30 minutes before the experiments. They were then transported to the testing room, placed in the arena, and video-recorded for 10 minutes. The treatment was applied and its position in the arena was randomised for all the replicates. Every treated group was composed of 7 males and 7 females. No differences were observed between sexes (Table S1) and interactions of sex versus treatments (Table S2), therefore, both sexes were pooled to increase the external validity of the study. The stage of estrus cycle was not identified for females. Animals were not euthanized at the end of the experiments.

The glass base, the transparent cover, and the PVC separations were cleaned between replicates with Vigor surpuissant® disinfectant cleaner diluted at 4% (chemical composition: water, 2-butoxyethanol, disodium metasilicate) (Eau Ecarlate, Ste Geneviève des Bois, France); they were then cleaned with white paper towels dampened with water, and finally dried with clean white paper towels. Four identical arenas were rotated between replicates in order to dissipate possible volatile traces of cleaner product. The squares of glass where the treatment was applied followed the same cleaning procedure but were used only once each day, at the end of the day they were exposed to a pyrolysis treatment, 500°C for one hour, to eliminate any possible residues.

2.5 Measures and Video Analysis

Each replicate was video-recorded with a video-camera placed 1 meter over the arena (JVC HD Everio 1920x1080 fullHD model GZ-HM446), located at a 90° viewing angle to the arena. This viewpoint allowed for complete analysis of avoidance behaviour and locomotor activity. Video analysis was performed by two independent observers. Both observers analysed all videos and were blinded for each other's scorings until the analysis were finished.

Video analyses were carried out blinded. Blinding was not possible for one of the treatments as fox faeces were clearly visible in the videos. The observers knew which treatment was applied when conducting the tests but had no notion of the experimental condition during the video analysis (except for fox faeces treatment).

The avoidance behaviour was measured with the dependent variables: treatment area total duration, untreated area total duration, average duration per passage in treatment area, and average duration per passage in untreated area (Table S1). Avoidance behaviour was interpreted when animals significantly increased the duration they spent in the "untreated area," or decreased the duration spent in the "treatment area." In the same way, in relation to this main avoidance parameter, we measured the average duration per passage in the treatment area and the untreated area, and we interpreted avoidance behaviour when animals

decreased the average duration per passage in the treatment area and/or increased the average duration per passage in the untreated area.

Locomotor activity was measured by the total number of passages (defined as the total number of passages between areas, treated area-central area, untreated area-central area and vice versa). An increase in the number of passages was interpreted as increased locomotor activity, and a reduced number of passages as decreased locomotor activity.

The number of faecal boli was noted as an independent parameter of the video analysis, after each replicate as a measure related to stress (Mönnikes et al., 1993).

2.6 Statistical Analysis

Data analysis was performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The significance threshold was fixed at 5%. Before proceeding, dataset reliability between 2 independent observers was calculated. When normality was established, Pearson correlation coefficient was used. Otherwise, Spearman correlation coefficient was preferred. The acceptable inter-observer reliability was fixed at 0.9.

For each variable, conditions of normality and homoscedasticity were verified with, respectively, the UNIVARIATE procedure and the General Linear Model (GLM) procedure. If conditions were established, *ANOVA* was performed by using the GLM procedure. If normality was not established, *Kruskal Wallis test* was used with the npar1way procedure. In the case where *ANOVA* was possible, multiple comparisons were done using the *Least Square Means (LSMEANS)* statement in the GLM procedure and adjusted with TUKEY. For *Kruskal Wallis test* multiple comparisons were carried out using *Wilcoxon test* pair by pair in the npar1way procedure. Bonferroni correction was applied with the MULTTEST procedure to maintain the significance threshold with the multiplicity of tests.

3. RESULTS

The *one-way ANOVA* and *Kruskal Wallis tests* showed significant differences between groups for all the parameters measured (Table 1).

3.1 Avoidance

3.1.1 Area Durations

Untreated Area Duration. Mice spent significantly higher durations in the untreated area during the ethanol, TMT and fox faeces conditions, showing significant differences against the negative control, and between the three (Figure 2a). TMT treatment showed the highest durations in the untreated area, followed by Ethanol, and fox faeces, respectively.

Treatment Area Duration. Mice spent significantly shorter durations in the area close to the ethanol and TMT when compared with fox faeces and the negative control (Figure 2b). The TMT showed a significantly shorter duration than all the other treatments, including ethanol, which validates its use as our positive control. The third shortest duration was seen with the fox faeces (Figure 2b), with a significant lower duration than the negative control. All three treatments showed significantly shorter durations against the negative control; these results were highly significant for TMT and ethanol (Table 2b).

i. Duration of Passages

Average duration of the passages in the untreated area. The average duration of passages in the untreated area showed a highly significant difference for the ethanol and TMT conditions (Table 2), with a shorter duration than fox faeces and the negative control, but without significant differences between the two (Figure 2c). In a second group of treatments we found no significant differences between the negative control and fox faeces (Figure 2c).

Average duration of the passages in the treated area. The average duration of passages in the treatment area was significantly lower for the TMT condition when compared to all other treatments (Figure 2d). TMT was followed by ethanol and fox faeces (Figure 2d).

Insert figure 2 here

3.2 Locomotor Activity

Number of passages. The total number of passages between areas was significantly lower with ethanol and TMT than with the negative control (Table 2). In addition, TMT and ethanol showed a significantly lower number of passages when compared to the fox faeces (Figure 3). The negative control and fox faeces showed the highest number of passages in this order (Figure 3); the statistical analyses did not show any significant differences between them (Table 2).

Insert figure 3 here

3.3 Faecal Boli

Animals exposed to TMT and Ethanol treatments produced a significantly higher number of faecal boli when compared to the negative control and the fox faeces (Figure 4), this difference was highly significant for the TMT (Table 2).

4. DISCUSSION

We found that ethanol elicited clear avoidance as animals remained for shorter periods near the ethanol, spent more time away from the stimulus, and performed shorter exploration visits when ethanol was present. In addition, ethanol reduced locomotor activity in laboratory mice and increased the number of faecal boli. TMT results confirmed the validity of the experimental device used in this study, as our positive control.

Ethanol is produced by sugar metabolism of fruits or seeds enhanced by yeasts and other fungi. This molecule can act as an olfactory cue for ripe fruits and their degree of ripening, acting as an attractant at low concentrations (Dudely, 2004; Ogueta et al., 2010), but also informing about the increasing presence of fungi and rotting fruit (Levey, 2004). Its high concentration in fruits at 1-2% has been shown as a deterrent for fruit bats, decreasing foraging of fruits with higher ethanol contents (Korine et al., 2011; Sánchez et al., 2006). A deterrent effect has been suggested in monkeys and apes, due to strong avoidance of overripe fruits,

probably due to ethanol plumes at high concentrations (Milton, 2004). Higher concentrations indicate that fruit is overripe or rotten and hosts larger populations of fungi, which could also be potentially toxic for frugivorous species such as small rodents. Ethanol vapour could produce irritation in the olfactory mucosa and stimulate the trigeminal nerve, also known as chemesthesis (Shusterman, 2002), eliciting avoidance, because ethanol, like other aliphatic alcohols, can be considered as a mild irritant (Cometto-Muñiz and Cain, 1990). From another perspective, oral ingestion of ethanol itself has been extensively proven to cause neurotoxicity and cytotoxicity (mainly in the liver but also in the cardiovascular and immune systems) in a dose-dependent manner (U.S. Department of Health and Human Services, 2000). In addition, it produces pharmacological effects with behavioural, motor and sensory perception changes. Inhalation of ethanol vapour has been studied to a lesser extent, but probably produces the same pharmacological effects as ingested ethanol through the capillary exchange in the lungs (Gilpin et al., 2009; Goldstein and Pal, 1971; MacLean et al., 2017). Taking together the different effects which ca be produced by ethanol, from olfactory perception to irritation, intoxication, and its connection with degraded food, we can associate ethanol in a broader perspective with the concept of disgust. The landscape of disgust has been described as the use of sensory information and use of the space to decrease risk of transmission of diseases and intoxication (Weinstein et al 2018).

The red fox faeces compound 2,4,5 Trimethylthiazoline (TMT) was avoided in a clear and highly significant manner. These results are in agreement with the literature, where TMT has been largely used as olfactory predator stimulus isolated from red fox faeces (Apfelbach et al., 2005; Ayers et al., 2013; Buron et al., 2007; Fendt and Endres, 2008; Fortes-Marco et al., 2013; Hacquemand et al., 2010; Rosen et al., 2015; Takahashi et al., 2005). In the same line, the fox faeces were avoided but in a lesser extent than TMT; These results are similar to the scarce published works where fox faeces were tested as a mice predator stimulus (Buron et al., 2007; Hacquemand et al., 2010). These differences could be due to a lower content in TMT in fox faeces samples than in the positive control tested here as proposed by Buron and

collaborators (Buron et al., 2007). The question of whether TMT has a pungent effect instead of eliciting a fear reaction has emerged often in the literature; recent research has demonstrated the activation of brain regions and pathways associated with fear from exposure to TMT (Hacquemand et al., 2013). No references were found regarding the use of fox faeces in the C57BL/6JRj mouse strain, which means that these results can be taken as a first approach to this strain's behavioural response to this olfactory predator stimulus.

Plant and animal chemical olfactory stimuli both have an ecologically relevant meaning for rodents such as mice, indicating the presence of unhealthy food and predators. The importance of plant chemical cues for rodents was recently highlighted (Hansen et al., 2016). However, the two signify different kinds of danger and therefore probably trigger different physiological responses. TMT was avoided and elicited stronger effects than ethanol for all the parameters. These behaviours agree with responses in nature, where predators mean an active danger which needs a clear response. On the other hand, rotted fruits can be avoided with more passive behaviours, and without the need of extreme responses. The number of faecal boli was increased with the ethanol and the TMT, however, we did not observe this increase with the fox faeces. Increasing of intestinal motility and defecation is related to emotional distress (Mönnikes et al., 1993), which can be the case of finding predator cues and in a lesser extent rotted food. The absence of increased defecation with the fox faeces could be due to the physical proximity of the predator stimulus, the faeces, and the inhibition of behaviours that could betray prey location to the predator. as may be the case for the faecal boli, which have been suggested as a prey cue for predators (Conover, 2007; Viltala et al., 1995).

Laboratory mouse strains have displayed different reactions in the literature to dangerous olfactory stimuli such as predator stimuli (Dell'Omo et al., 1994; Staples and McGregor, 2006). However, C57BL/6 shows a high degree of sensitivity to novel/dangerous olfactory stimuli when compared with other laboratory mouse strains (Dell'Omo et al., 1994), as is the case in wild mice (Blanchard et al., 1998). In addition, C57BL/6 mice showed the

same expression as wild mice in Vomeronasal receptors, however many other laboratory strains showed an altered expression (Stempel et al., 2016). This example supports the existence of inter-strain differences in behavioural reactions to biologically meaningful olfactory stimuli, and this phenomenon can likely be generalized to other olfactory receptors. In addition, C57BL/6 is the most used laboratory mouse strain, which implies a direct application of our results in research facilities.

Ethanol is a common chemical used for laboratory animal procedures. As cleaning (Buccafusco, 2009), disinfection (Weir et al., 2002) or as a solvent in behavioural olfactory procedures, because it volatizes fast. Once volatilised it can be perceived without direct contact by olfaction. However, its influence as olfactory stimulus has not been stated clearly, especially in naïve animals, because ethanol studies in rodents have been focused from an anthropocentric perspective as models of human alcoholism. According to our results, this molecule should be used cautiously as it has a clear behavioural impact in mice with ratios of vaporized ethanol that could easily be found in the lab. These concerns have been described in another common solvent, the Propylene Glycol (Inagaki et al., 2010). While its physical properties include rapid volatilisation, this quality should be considered carefully, as ethanol in its gaseous state could act as a chemical cue eliciting avoidance and related stress. It would be of interest for further research to test different volumes and purities in confined and open spaces to more precisely determine avoidance parameters in different conditions and the impact of ethanol on foraging behaviour. In addition, it would be of interest to test reactions of wild mice to this olfactory stimulus.

5. CONCLUSION

These results provide evidence that mice avoid ethanol in quantities similar to those found in overripe and rotten fruits and in common procedures with laboratory animals. The implications of these results should be considered both in laboratory animal research and wild animals, as it is a common molecule in both scenarios. This avoidance effect could elicit stressful situations and modify behavioural and physiological responses in mice housed in research facilities. In

addition, in the wild, this chemical cue could convey primordial information about the ripening state of fruits, triggering avoidance of overripe unhealthy fruits. Taking this in consideration, ethanol could be an inexpensive and less toxic tool than rodenticides in the management of rodent pests.

6. FUNDING

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

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FIGURE CAPTIONS

Figure.1 Experimental device designed for measuring avoidance and locomotor activity (dorsal view)

Figure.2 Behavioural parameters related to avoidance behaviour. Data is shown as the mean ± standard error when the parametric test *ANOVA* was used (b) and as the median when the nonparametric test *Kruskal-Wallis* was used (a, c, d). Treatments with the same symbol correspond to an insignificant difference and treatments with a different symbol correspond to a significant difference. The multiple comparisons have been computed using the *lsmeans* statement when *ANOVA* was applied (b) and using *Wilcoxon test* with the Bonferroni correction when *Kruskal-Wallis* test was applied (a, c, d). The significance threshold is fixed at 5%.

Figure.3 Behavioural parameter related to locomotor activity: number of passages. Treatments with the same symbol correspond to an insignificant difference and treatments with a different symbol correspond to a significant difference. The multiple comparisons have been computed using the *lsmeans* statement as *ANOVA* was applied. Data is shown as the mean ± standard error. The significance threshold is fixed at 5%

Figure.4 Parameter related to stress: number of faecal boli. Treatments with the same symbol correspond to an insignificant difference and treatments with a different symbol correspond to a significant difference. The multiple comparisons have been computed using *Wilcoxon test* with the Bonferroni correction when *Kruskal-Wallis* test was applied. Data is

shown as the median as the non-parametric test *Kruskal-Wallis* was used. The significance threshold is fixed at 5%

FIGURES

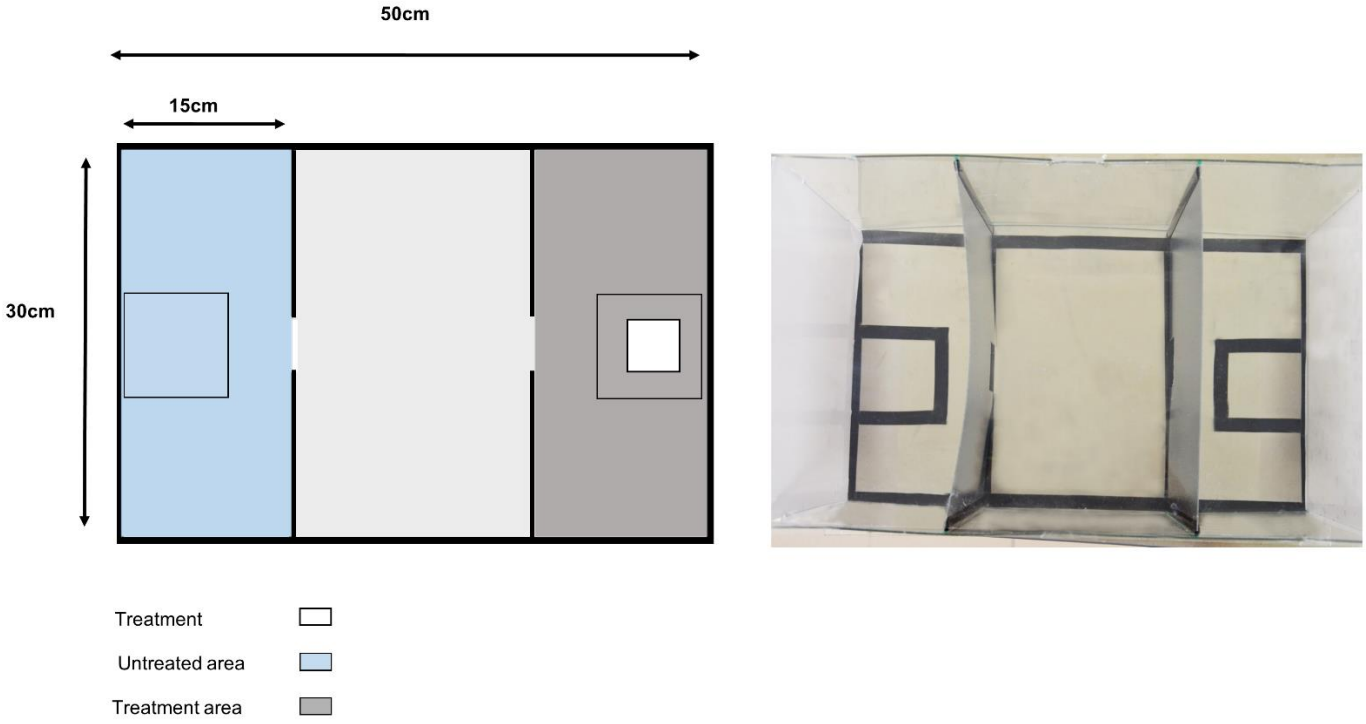

Figure.1 Experimental device designed for measuring avoidance and locomotor activity. On the left side is a schema and on the right side is a picture of the device, both from a dorsal view

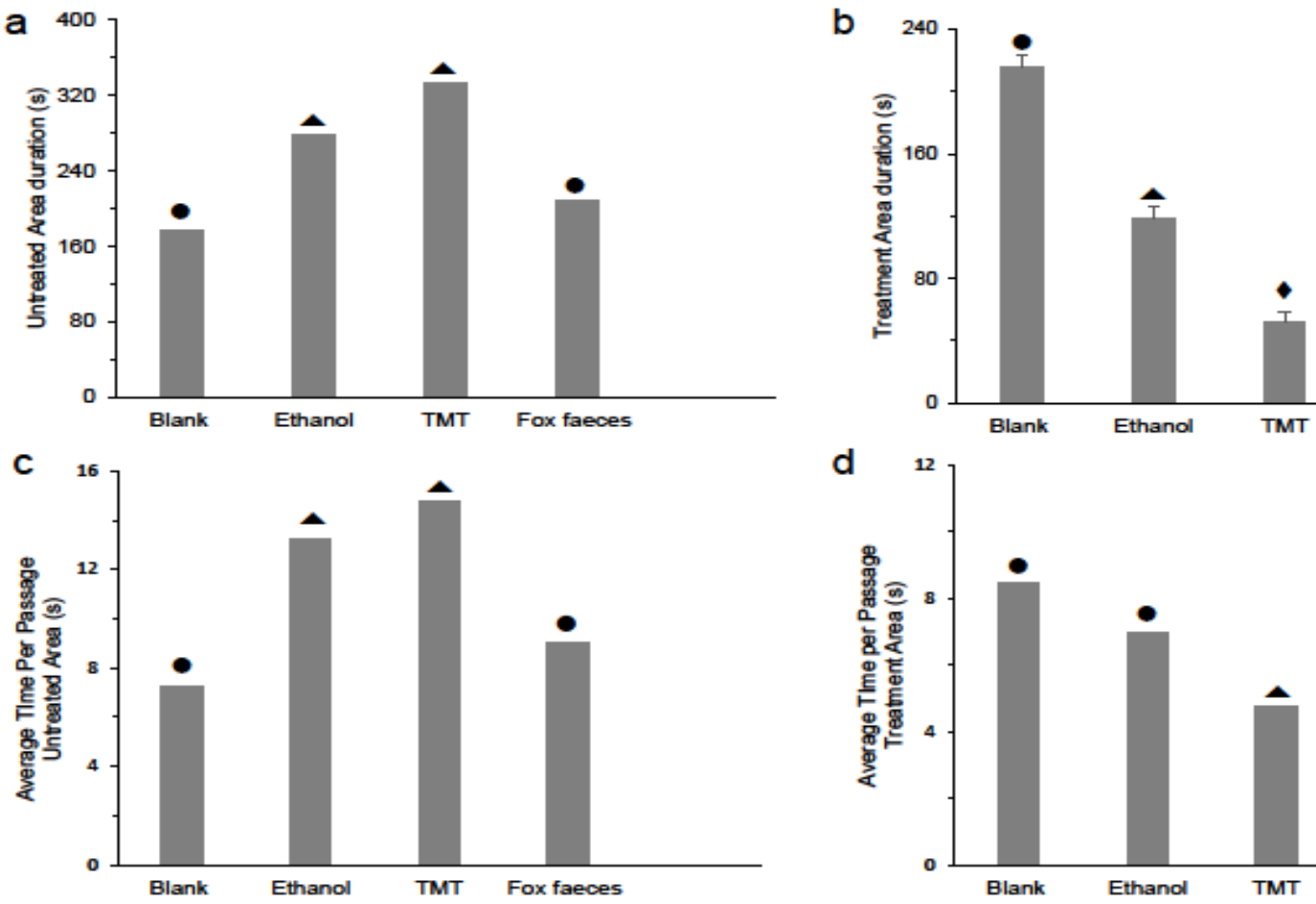

Figure.2 Behavioural parameters related to avoidance behaviour. Treatments with the same symbol correspond to an insignificant difference and treatments with a different symbol correspond to a significant difference. The multiple comparisons have been computed using the *lsmeans* statement when *ANOVA* was applied (b) and using *Wilcoxon test* with the Bonferroni correction when *Kruskal-Wallis* test was applied (a, c, d). Data is shown as the mean ± standard error when the parametric test *ANOVA* was used (b) and as the median when the nonparametric test *Kruskal-Wallis* was used (a, c, d). The significance threshold is fixed at 5%.

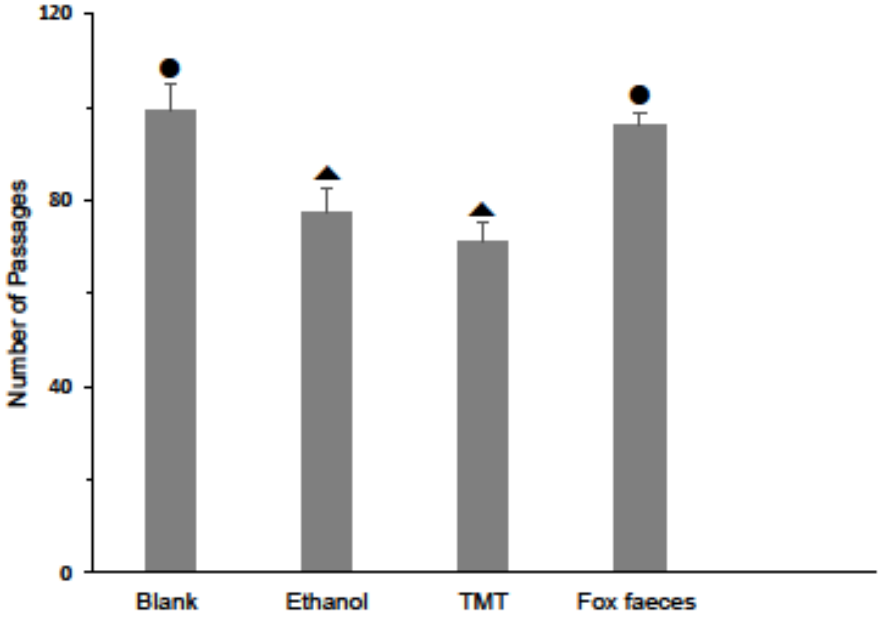

Figure.3 Behavioural parameter related to locomotor activity: number of passages. Treatments with the same symbol correspond to an insignificant difference and treatments with a different symbol correspond to a significant difference. The multiple comparisons have been computed using the *lsmeans* statement as *ANOVA* was applied. Data is shown as the mean ± standard error. The significance threshold is fixed at 5%

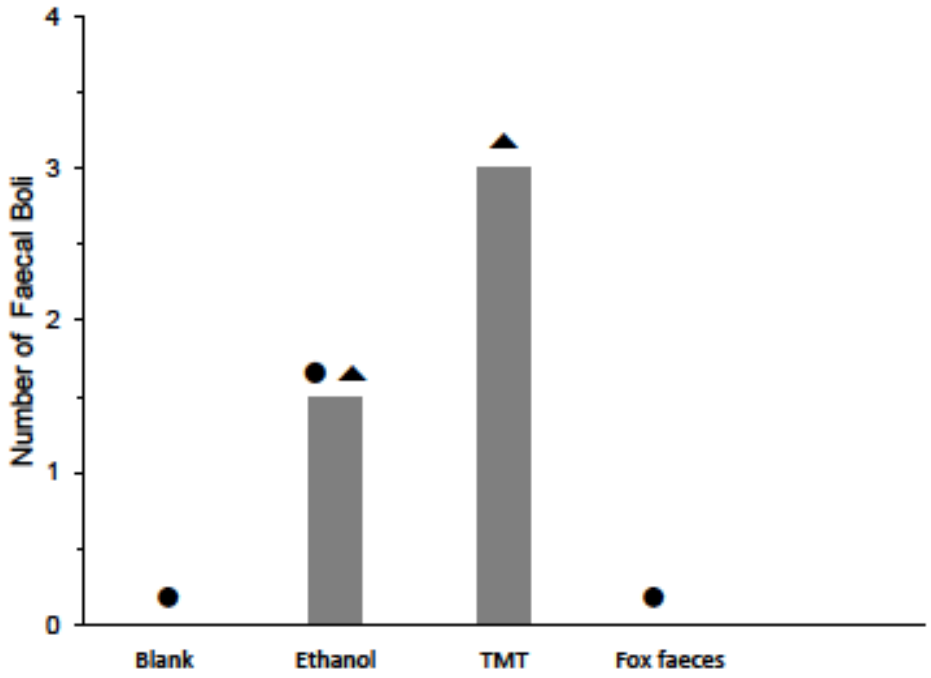

Figure.4 Parameter related to stress: number of faecal boli. Treatments with the same symbol correspond to an insignificant difference and treatments with a different symbol correspond to a significant difference. The multiple comparisons have been computed using *Wilcoxon test* with the Bonferroni correction when *Kruskal-Wallis* test was applied. Data is shown as the median as the non-parametric test *Kruskal-Wallis* was used. The significance threshold is fixed at 5%

TABLE 1. BEHAVIOURAL RESPONSES TO ETHANOL AND PREDATOR OLFAC
THE HOUSE MICE AND STATISTICAL ANALYSES USED FOR VAI

	Blanc	**Ethanol**	**TMT**	**Fox faeces**	***X²/F*** [a]	
Total TA duration[b] (s)	216.18 (7.83)	118.29 (7.83)	52.11 (5.96)	172.11 (8.34)	75.79 (F)	<.
Total UA duration[c] (s)	176.50	277.00	332.75	207.50	46.20	<.
Average time per passage TA[c] (s)	8.50	7.00	4.75	7.25	36.07	<.
Average time per passage UA[c] (s)	7.25	13.25	14.75	9.00	34.27	<.
Number of passages[b]	99.39 (5.43)	77.43 (5.35)	71.21 (4.33)	96.21 (2.87)	6.92 (F)	0.
Faecal boli[c]	0.00	1.50	3.00	0.00	18.80	0.

[a]

Differences between groups were calculated with the help of *One Way ANOVA* or *Kruskal Wallis test*. When (F) is indicated, parametric conditions were found and the test used was *ANOVA*, otherwise non-parametric conditions were found and data was analyzed with *Kruskal Wallis* test and expressed as X^2. The total length of the tests was 600s.[b] Values are expressed as the mean (standard error) in parametric conditions, for time spent in the treatment area (TA duration) and number of passages as the total frequency of crossings between areas.[c] Values are expressed as the median in non-parametric conditions for time spent in the area furthest from the treatment, expressed as untreated area duration (UA), average time per passage TA, average time per passage UA, and Faecal boli as the total number of faecal boli found after each replicate.

TABLE 2 SUMMARY OF THE STATISTICAL DIFFERENCES OBSERVED BETWEEN TREATMENTS

Treatments	*Total TA duration*[a]	*Total UA duration*[b]	*Average time passage TA*[b]	*Average time passage UA*[b]	*Numbe passag*
	P values (*Df*=3, n=14)				
Blank vs Ethanol	**<.0001*** **	**0.0006** **	0.0837	**0.0006****	**0.0076*
Blank vs TMT	**<.0001*** **	**0.0006** **	**0.0006** **	**0.0006** **	**0.0004*
Blank vs Fox faeces	**0.0007** **	**0.0382** *	0.2504	0.1352	0.9615
Ethanol vs TMT	**<.0001*** **	**0.0382** *	**0.0008** **	0.5495	0.7763
Ethanol vs Fox faeces	**0.0001** **	**0.0042** **	0.2504	**0.0042** **	**0.0285*
TMT vs Fox faeces	**<.0001*** **	**0.0006** **	**0.0006** **	**0.0006** **	**0.0019*

P <0.001 ***, 0.001 to 0.01**, 0.01 to 0.05 *; [a] Parametric data: multiple comparisons with *LS means* statement of the GLM procedure [b] Non-parametric data: multiple comparisons with *Wilcoxon test* using the npar1way procedure, Bonferroni correction with the MULTTEST procedure; TA (treatment area), UA (untreated area)

TABLE S1. EFFECTS OF SEX IN BEHAVIOURAL RESPONSES TO ETHANOL AND PREDATOR OLFACTORY STIMULI IN LABORATORY MICE

	Females	Males	Effects of sex	
			X²/F [a]	***P***
Total TA duration[b] (s)	141.19 (13.45)	138.14 (12.32)	0.16 (F)	0.69
Total UA duration[c] (s)	223.25	236.50	0.49	0.48
Average duration per passage TA[c] (s)	7	7	0.03	0.84
Average duration per passage UA[c] (s)	9.25	10.00	0.03	0.84
Number of passages[b]	82.94 (8.94)	89.17 (4.35)	1.79 (F)	0.18
Faecal boli[c]	1.00	0.50	0.12	0.72

[a] Differences between groups were calculated with the help of *Two Way ANOVA* or *Sheirer-Ray-Hare test*. When (F) is indicated, parametric conditions were found and the test used was *ANOVA*, otherwise non-parametric conditions were found, and data was analyzed with Sheirer -Ray-Hare test and expressed as X^2. The total length of the tests was 600s.[b] Values are expressed as the mean (standard error) in parametric conditions, for time spent in the treatment area (TA duration) and number of passages as the total frequency of crossings between areas.[c] Values are expressed as the median in non-parametric conditions for time spent in the area furthest from the treatment, expressed as untreated area duration (UA), average duration per passage TA, average duration per passage UA, and Faecal boli as the total number of faecal boli found after each replicate.

TABLE S2. EFFECTS OF SEX-TREATMENT INTERACTIONS FOR BEHAVIOURAL
PREDATOR OLFACTORY STIMULI IN LABORATORY

		Negative control	Ethanol	TMT	Fox faeces	Effects of sex -treatn interactions	
						X²/F [a]	
Total TA duration[b] (s)	f	221.92 (13.11)	112.07 (9.41)	53.07 (7.55)	166.5 (14.04)	0.53 (F)	0.6
	m	210.42 (9.08)	124.5 (12.80)	51.14 (9.82)	177.71 (9.63)		
Total UA duration[c] (s)	f	171.50	269.00	330.50	204.50	0.15	0.9
	m	188.50	308.00	335.00	208.50		
Average duration per passage TA[c] (s)	f	9.00	7.00	5.00	8.00	0.94	0.8
	m	8.00	7.00	4.50	7.00		
Average duration per passage UA[c] (s)	f	8	13.50	14.00	9.00	0.42	0.9
	m	6.5	13.00	16.50	9.00		
Number of passages[b]	f	92.28 (8.94)	76.42 (4.93)	70.00 (4.88)	93.07 (3.43)	0.37 (F)	0.7
	m	106.50 (5.54)	78.42 (9.95)	72.42 (7.54)	99.35 (4.54)		
Faecal boli[c]	f	0.50	2.00	2.00	0.00	0.64	0.8
	m	0.00	1.00	3.00	0.00		

[a] Differences between groups were calculated with the help of *Two Way ANOVA* or *Sheirer-Ray-Hare test*. When (F) is indicated, parametric conditions were found and the test used was *ANOVA*, otherwise non-parametric conditions were found, and data was analyzed with Sheirer -Ray-Hare test and expressed as X^2. The total length of the tests was 600s.[b] Values are expressed as the mean (standard error) in parametric conditions, for time spent in the treatment area (TA duration) and number of passages as the total frequency of crossings between areas.[c] Values are expressed as the median in non-parametric conditions for time spent in the area furthest from the treatment, expressed as untreated area duration (UA), average duration per passage TA, average duration per passage UA, and Faecal boli as the total number of faecal boli found after each replicate.

TABLE S3 BEHAVIOURAL PARAMETERS USED TO EVALUATE AVOIDANCE, LOCOMOTOR ACTIVITY AND STRESS AND THEIR INTERPRETATION

General parameter	Dependent variables		Result interpretation
Avoidance behaviour	Total duration	Treated area	Lower duration→Avoidance
		Untreated area	Higher duration→ Avoidance
	Average duration per passage	Treated area	Lower duration→ Avoidance
		Untreated area	Higher duration→ Avoidance
Locomotor activity (LA)	Total number of passages		Higher number of passages→ increased LA
			Lower number of passages→ decreased LA
Stress	Number of faecal boli		Increased number of faecal boli→ increased stress (Mönnikes et al., 1993)

3. CONCLUSIONS

Plant and predator olfactory cues can elicit avoidance in rodents due to their ecological meaning. Plant chemicals carry important messages that would modulate foraging behaviour and many other related behaviours. Predator chemical cues will provide information about risks associated with these predators, and the global outcome (behaviour) will be the result of all these external stimuli, the physiological status of the animal receiving these messages, the social environment and previous experiences, all of which can modulate neural connections (plasticity) and even modulate the behavioural features of future generations (epigenetics)(St-Cyr, Abuaish, Welch, & McGowan, 2018; St-Cyr & McGowan, 2015).

Plant metabolites have been explored as modulators of rodent behaviour to a lesser extent than predator stimuli. Studies of their effects have mainly focused on secondary metabolites related to plant defences against herbivorous species and metabolites related to immature stages of fruits, but rotting of fruits has sanitary, toxic and nutritional consequences for plant foragers. We showed that ethanol, as an olfactory stimulus, elicited clear avoidance, which could be due to its ecological meaning related to rotting in nature. Differences were observed between predator and plant chemical cues, probably due to different risk consequences for rodents, who receive these messages and the doses of chemical cues (TMT vs fox faeces).

Future studies should more deeply investigate the interactions among these cues (plant +predator chemicals) since different risks would create a more realistic context that could have cumulative predator risk and rotted food avoidance effects.

Third part: Ethics in rodent control

CHAPTER 4: THE HUMANENESS OF RODENT CONTROL AND ALTERNATIVES TO CURRENT METHODS

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1 PREAMBLE TO STUDY NUMBER 6

In this part of my thesis, I aimed to review rodent control methods from an ethical perspective and evaluate their humaneness, understood as the quality of producing fast, painless deaths in the case of lethal control methods or of avoiding stressful events in non-lethal methods.

This bibliographical research revealed that the main rodent control methods, which are dominated by anticoagulant rodenticides, can be considered inhumane since they result in slow deaths with high levels of pain. In addition, they can poison other non-target species, including wild or domestic animals, triggering similar signs to those found in rodents, which would exacerbate ethical concerns.

2 STUDY NUMBER 6

Oral presentation at an international congress with peer review

C.Grau; A.Cozzi; P.Pageat. *The humaneness of rodent control and alternatives to actual methods*. Proceedings of the European Congress of Animal Welfare and Behavioural Medicine (AWBM) Volume: Animal Welfare Science. Cascais, Portugal, 21th Octobre 2016

SPOKEN PRESENTATION, ANIMAL WELFARE SCIENCE

The Humaneness of Rodent Control and Alternatives to Actual Methods

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Many millions of rodents die per year because of human pest control methods. The human-driven inconsistent treatment and rights between laboratory versus wild rodents calls for a better regulation and ethical approach towards wild animals (Mason & Littin 2003).

Current rodent pest control methods are mainly based on the use of inhibitors of Vitamin k-1 metabolism and the coagulation cascade which produces continuous haemorrhages for long periods and finally the death, or sequelae at sub-lethal doses (Buckle & Smith 2015). These methods have been described as inhumane as they produce a slow death, cause distress, disability and/or pain (Yeates 2010). The control of pain, suffering and general welfare in laboratory rodents has been proposed as a model for control of wild populations. These standards and the use of the 3Rs (Meerburg et al. 2008), a legislated obligation in research animals, should guide future research and development in rodent pest control.

The use of chemical communication as a non-painful, non-toxic and ecologically acceptable method, along with preventive methods such as physical barriers and resource control, could fulfil these demands.

Buckle, A.P. & Smith, R.H.., 2015. *Rodents pests and their control* 2nd ed, Oxfordshire: CABI.

Mason, G. & Littin, K.E., 2003. *Animal Welfare*, 12:1–37.

Meerburg, B.G., Brom, F.W.A. & Kijlstra, A., 2008. *Pest management science*, 64:1205–1211.

Yeates, J., 2010. *Pest Management Science*, 66(3):231–237.

Results and Discussion

The development of an animal welfare conscience in western societies has rapidly evolved during recent decades, which is a great advance, but pest species are the last on the list in this humane conscience of pain and suffering in animals. This is due to a utilitarian perspective of animal welfare that is consequential and outcome based (Morton, 2017). Animals that are valuable to humans (as pets, for human research, and as food) have rights and strong social support for respectful treatment, but pest species, which do not provide value to society and on the contrary are considered destructive, do not have rights and can be killed by any available method without moral or legal consequences. As an example of social concern, I searched for keywords on the website of the non-profit organization PETA (People for the

Ethical Treatment of Animals) in April 2018. I found 26400 posts for the keyword “dog”, 10400 posts for “cat”, 8660 posts for “farm”, 5400 posts for “slaughter”, 1365 posts for “laboratory animals” and 244 posts for “pest” in PETA’s historic database (PETA, 2018). This organization represents the opinions of a population that is supposedly highly concerned with animal rights and welfare, but this means that even activists have less concern for pest species compared with pet, farm or laboratory animals. Within societies, we can find differences between different groups. Some of the factors that have been to correlate positively with concern for animal welfare are human-human empathy, being female or companion animal ownership (Taylor & Signal, 2005).

Animal care by veterinarians is ruled by deontological ethics, of which the pioneer document was the veterinary regulation written in the 18th century by the French lawyer and veterinarian Claude Bourgelat (Bourgelat, 1777; Degueurce, 2012; Harris, 2011). The actual version of the deontological ethics in his article R.242-48, which is devoted to general veterinarian duties in France, states that veterinarians should ensure attenuation of animal suffering, which is inconsistent with control methods that relieve pest animals from pain. Rodent control methods are regulated in Europe by the Regulation 528/2012 (European Union, 2012) for the availability and use of biocidal products, but the same species receive a much different treatment in European Directive 2010/63/EU (European Union, 2010), in which their ethological and physiological needs are carefully considered and (when possible) relieving pain and distress and ethical evaluation are mandatory. As criteria for granting biocidal authorisation, (EU) 528/2012 states that the biocidal product can have no unacceptable effects on the target organisms, particularly unacceptable resistance or cross-resistance, or result in unnecessary suffering and pain for vertebrates. The criteria to avoid unnecessary suffering and pain is a death synchronous with the loss of consciousness or immediate death or a gradual reduction in vital functions without signs of obvious suffering. The word welfare appears two times in the biocide regulation and 54 time in the 2010/60/EU for laboratory animals. In Article 44, the text recognizes that the use of biocidal products of certain types might give rise to animal welfare concerns, and state members could derogate products based on these criteria but “without compromising the internal European market”, which means that the first criterion would be effective even if welfare concerns arise without further consideration. Products already on the market should be reviewed to satisfy the criteria demanded by the new regulation; the anticoagulant family has already been approved within these new laws. However, as it has been reviewed, rodent anticoagulants do not satisfy this criteria (Mason & Littin, 2003; Meerburg, Brom, & Kijlstra, 2008), but their actual status is approved within the whole EU (Table 1). This fact presents a clear contradiction between the regulation for biocide products

and the approved chemicals since they produce genetic resistance; death occurs long after intake; and there is clear evidence of suffering and death does not synchronously occur with the loss of consciousness. All these parameters are specified to be avoided in the biocide directive.

Table 1 List of rodenticides approved in the European Union or under review in November 2017 (European Chemicals Agency, 2017)

Substance	CAS-Number	Status
Alphachloralose	15879-93-3	Approved
Aluminium phosphide releasing phosphine	20859-73-8	Approved
Brodifacoum	56073-10-0	Approved
Bromadiolone	28772-56-7	Approved
Carbon dioxide	124-38-9	Approved
Chlorophacinone	3691-35-8	Approved
Coumatetralyl	5836-29-3	Approved
Difenacoum	104653-34-1	Approved
Difethialone	104653-34-1	Approved
Flocoumafen	90035-08-8	Approved
Hydrogen cyanide	74-90-8	Approved
Powdered corn cob		Approved
Warfarin	81-81-2	Approved
Cholecalciferol	67-97-0	In progress (new active biocide product)

Based on the results of this thesis, ethanol could be an inexpensive, non-toxic and ethically suitable chemical for use as an olfactory cue. In the European Union, ethanol has been approved as a product type 1, disinfectant for human hygiene (applied to the skin or scalp),

and as a product type 2, disinfectant and algaecide that is not intended for direct application to humans or animals. It is also under review as a product Type 4, products used for the disinfection of equipment associated with food or feed for humans and animals. However, it has not been included as pest control product, where it would be classified as a product Type 19, repellents and attractants.

In the 21st century, some ethical concern over pest species control has emerged in the research community and society. Mason and Littin (Mason & Littin, 2003) evaluated the humaneness of rodent pest control methods according to four main parameters: the degree of pain, discomfort or distress; the length of time for which rodents are conscious and exhibiting clinical signs of poisoning; the effects on individuals who escaped and survived; and finally, the effect on non-target species.

Another approach proposed by Sharp and Saunders (Sharp & Saunders, 2011) evaluates control methods according to the five freedoms: water or food restriction (1), environmental challenge (2), disease, injury or functional impairment (3), behavioural or interactive restriction (4), and anxiety, fear, pain or distress (5). They use binomial scoring boards, accounting for the impact on animal welfare (from no impact to extreme) and the duration (from seconds to weeks). They also evaluate the modes of death of lethal methods, considering the time to insensibility and the level of suffering.

Yeates (Yeates, 2010) proposes a different perspective using the experience of laboratory animals as s model. The 3 R principles, refinement, reduction and replacement, can be translated to the pest control environment. Lethal methods should be replaced by non-lethal alternatives when possible. This should apply to integrated and preventive management, and lethal methods should be restricted to acute sanitation emergencies. In terms of reducing, rodent control methods should affect as few animals as necessary to achieve the desired purpose and be specific for the desired species. The last R, refinement, means causing the least pain, suffering, distress or lasting harm, which would favour methods with less severe or shorter effects. The term endpoint, which is usually applied to research animals, is presented, and he proposes an adapted meaning by which rodent control should be stopped or modulated to maintain effectiveness once we achieve a pre-planned objective, e.g., no rodents in a sensitive area or population density of X in a defined area (**Figure 1**).

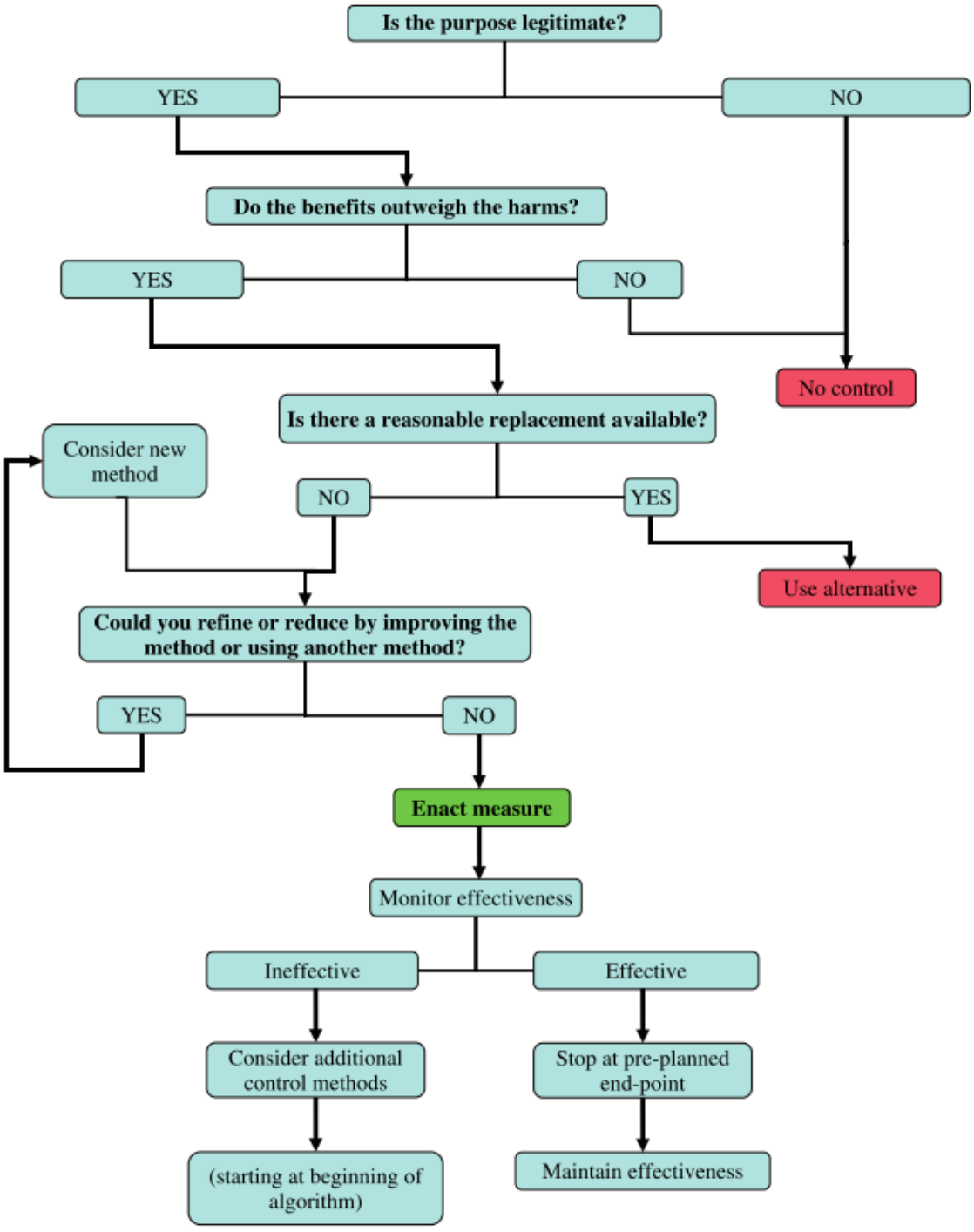

Figure 1. Ethical decision-making algorithm for the application of pest management principles. Modified from Yeates (Yeates, 2010).

Actual control methods mainly rely on the use of anticoagulant rodenticides, of which the main compounds are coumarins and warfarins that mainly act on vitamin K metabolism but also on other related proteins (Murphy, 2002). These substances were discovered in the 1940s, and the development of resistance in rodents stimulated the need for a second generation.

Warfarins are inhibitors of vitamin K-1 metabolism, and the coagulation cascade that produces continuous haemorrhaging for long periods and finally death, or sequelae at sub-lethal doses (Buckle & Smith, 2015). Warfarins are by far the most common means of rodent control, accounting for 95% of the methods in the USA and 92% in the UK (Mason & Littin, 2003). However, there is an increasing problem with genetic resistance, mainly to first-generation warfarins (Oldenburg et al., 2014). If we apply the four parameters of the classification proposed by Mason and Littin (Mason & Littin, 2003), we observe the following.

1. Degree of pain: High degree of pain. Haemorrhages in the deep tissues of the thorax including gastrointestinal, orbital, and intracranial that can produce severe pain. The degree and duration of the suffering depend on the site and severity of the haemorrhages. The signs depend on the dose, the compound and individual predispositions (e.g., resistance).

2. Length of signs: From a few hours to an average of 1-3 days with a maximum of 3-5 days. Paralyzed animals lay prostrate for a mean of 11.4h prior to death.

3. Effects in surviving animals: Surviving animals usually have sequalae and long-lasting problems.

4. Non-target species intoxication: Risk of carnivore intoxication that is higher with second-generation warfarins (Murphy, 2007).

The commonly used physical control methods are traps. Snap traps are used most frequently, but sticky boards and electrocution traps are also employed. All these methods have welfare concerns. Snap traps produce distress by confinement, and potentially severe pain and injuries linked to dehydration; they are also dangerous to other species. Electrocution traps are relatively fast but potentially very painful until the animal dies. Finally, sticky boards are probably the worst from a welfare perspective. They produce severe distress, trauma, dehydration and starvation, and the time to death depends on the management of the traps, which are often not monitored, causing prolonged starvation and pain until death (Mason & Littin, 2003).

Replacement of lethal methods can be achieved by through a better understanding of rodent biology and ecology (M. D. Gomez, Provensal, & Polop, 2008; Krijger, Belmain, Singleton, Groot Koerkamp, & Meerburg, 2017; McArthur, Banks, Boonstra, & Forbey, 2014; Singleton, Hinds, & Leirs, 1999). For example, the removal of local competitor species by nonspecific lethal methods could produce an unwanted increase in pest species such as the house mouse (M. D. Gomez et al., 2008). The use of non-lethal methods considering the characteristics of

the species and the environment can lead to the reduction of pest populations in sensitive areas without producing pain or unavoidable stress. Managing the olfactory environment can greatly influence rodent behaviour without the need to killing or trap animals (with the accompanying risk of starvation and dehydration). Perceiving predator olfactory cues can decrease the reproductive performance of animals (Voznessenskaya & Malanina, 2013), and these events can influence future rodent generations through epigenetic changes; pups can be more sensitive to these stimuli and be smaller in size with decreased energetic efficiency (Broad & Keverne, 2012; St-Cyr et al., 2018; St-Cyr & McGowan, 2015). In the same way, plant cues can influence the use of space by rodents, inducing avoidance with an avoidable source of stress (Hansen, Stolter, Imholt, & Jacob, 2016a). Plant chemical cues provide information about the toxicity of plants, fruits or seeds, and the presence of secondary pathogen agents will discourage the exploration of these areas (Hansen et al. 2016b, Grau et al, submitted 2018). In summary, plant or predator chemical cues are part of the environment of rodents, by understanding these messages and their significance for rodents, we can manage their behaviour to benefit human interests.

Along with olfactory management, physical management of the environment can effectively control rodent access (Gómez Villafane et al., 2001). Barrier methods and good maintenance of buildings and human structures considerably decrease rodent pest entry. Plant cover and bushes are other elements that provide protection and enable rodents to move securely, so management should consist of cleaning areas surrounding sensitive areas, buildings, farms, etc. (Dickman, 1992). Light also influences the perception of predation risk because preys are conspicuous to predators; the moon cycle is known to influence this perception of risk and thus foraging behaviour in rodents. Artificial lights have proved to decrease foraging and the time spent in these areas (Farnworth, Innes, & Waas, 2016). The perception of risk could be combined with several factors that would probably further prevent rodent foraging or exploration, having an accumulative effect. Illuminated areas, without plant/bush cover and predator/plant chemical cues could be perceived as highly risky.

3 CONCLUSIONS

In this part of my thesis, I attempted to reflect the great problem that is inhumane treatment in rodent control. The development of an animal welfare conscience in western societies has quickly evolved during recent decades, which is a great advance, but pest species are the last on the list in this humane conscience of pain and suffering in animals.

Actual rodent control methods are employed too late, after rodent populations have established. The main effort should be based on prevention and ecological management that manages environmental resources, increases the perception of risk and decreases the food resources. Integrated pest management considers a global strategy for rodent control, but we are currently focused in lethal methods with toxic effects and welfare concerns. Instead, the first approach should be prevention and the use of non-lethal and non-toxic methods that account for the ecology of the target species.

III General Discussion

CHAPTER 1: OBJECTIVES, ORGANIZATION, RESEARCH PLAN AND MAIN RESULTS

The house mouse is considered one of the main pest species of humans, and it, along with other commensal rodents, has been associated with human settlements for 12000 years, since humans first began providing food and cover (Pialek, 2012b). Rodent pest species cause serious damage in terms of agriculture, infrastructure, houses and other human goods, and they present a major sanitary problem as vectors through direct transmission of diseases to humans or domestic animals or through indirect transmission by contaminating food and materials. They also play an important epidemiological role as disease reservoirs.

Methods to control pest rodent populations have been developed for millennia, but success has always been challenging. In the 20th century, the discovery of anticoagulants and their applications for rodent control, initiated a new stage (Gomez-Outes et al., 2012; Murphy, 2007). They have shown some advantages over older, traditional methods, but as time has passed, many problems have emerged. The toxicity of these substances to non-target species (domestic animals and wild fauna), the development of genetic resistance and, lastly, human concerns over animal welfare have challenged researchers to find new alternatives.

Understanding the behaviour of the target species is a logical basis for successful control strategies. Motivations guide behaviour in mammals, and olfaction drives a main sensory network and neural pathways that can trigger a motor response in mice. Based in this rationale I organized the research in this thesis.

The main objective of this thesis was to better understand the influence of olfactory chemical cues that mice can find in their environment and their possible applications in rodent pest control. For this purpose, we decided to mainly study two basic behaviours, exploratory behaviour and foraging. Behaviour in mice is mainly guided by survival and the desire to find reproductive partners. Survival includes feeding and thus the search of this food, which is foraging, but it also includes the avoidance of risks, of which predation is an outstanding danger for small mammals such as mice.

Research plan

Our first research axis was based on the study of exploratory and foraging behaviour by the house mouse in the presence of predator chemical cues. We developed a modified open field with three chambers to analyse the effects of several predator olfactory stimuli. The tested species were ferrets (*Mustela putorius)*, snakes (several species), dogs (*Canis familiaris*), foxes and cats (*Felis domesticus*), and we found that mice avoided olfactory stimuli from ferrets but not from the other species.

In another research stage focused on cats as rodent predators, we demonstrated that water-soluble molecules from cat fur and skin did not modify mouse foraging or exploratory behaviour; the major cat allergen Fel d 1 was the main protein found in these samples. To verify the effects of this major cat allergen, we tested purified Fel d 1 in a simpler environment to avoid the complicating effects of food in our tests. Our first results were confirmed since mice did not present avoidance or significantly alter their exploratory behaviour when Fel d 1 was present. To more further explore the effects of cat chemical cues in mice, we tested the cat facial feline pheromone, F3, in a complex environment in the presence of food. We did not observe differences in the control of exploration or foraging behaviours, but we observed avoidance behaviours in response to both olfactory cues. The only common chemical compound between F3 and its control was ethanol.

These observations initiated the development of a new hypothesis and the second axis of the research, which was understanding the ecology of this molecule in the rodent environment and how it could influence the exploratory behaviour of the house mouse. Would mice show different responses to predator and plant chemical cues? Ethanol has a plant-based chemical origin and could be considered a signaller of the ripeness of fruits or in some conditions, cereal grains. Information on ripeness is needed to avoid intoxication from rotted food and wasting energy for unnecessary locomotor activity. Our results showed that mice clearly avoided ethanol as an olfactory stimulus in amounts that could be easily found in nature, and these data support the hypothesis of the transmission of a meaningful message to mice through volatized ethanol that is possibly related to ripening. From another point of view, plants use chemical communication with secondary metabolites to avoid herbivory (Hansen et al., 2016a; Schupp, Jordano, & Gómez, 2010) and to manage seed dispersal in the optimal seed state, which will guarantee the best conditions for seed germination. If seed dispersal were to occur too early, the seeds would not have completed development or they would not encounter ideal seasonal conditions, thus compromising germination (Howe & Miriti, 2004). By the same logic, if seed dispersal occurred too late, the same effects might be observed.

This research uncovered differences between plant and predator olfactory cues, which should be the result of different strategies to avoid risks linked to intoxication and feeding as well as predators.

As humans are capable or perceiving animal suffering and modern societies have developed high moral standards for animal-human relationships, we performed a bibliographical research to review and evaluate the humaneness of rodent pest control methods. This research found that the main rodent pest control methods can be considered inhumane and inflict unnecessary suffering and pain to the animals (Mason & Littin, 2003). Some concern related to animal welfare in vertebrate pest control has been shown in academia (B. Jones, 2003), but this concern is much less prominent compared with current concerns over the welfare of farm and laboratory animals. In view of these facts, the use of olfactory messages to guide and influence rodent preferences and the search for resources should mean advancing to achieve higher humane standards for rodent control.

The use of chemical communication for rodent control is included in a larger perspective that is ecologically integrated pest management (IPM). This paradigm was first developed to manage insect pests for crop protection. IPM is a decision support system for the selection and use of pest control tactics that are applied either singly or are harmoniously coordinated into a management strategy based on cost/benefit analyses that consider the interests of and impacts on producers, society and the environment (Kogan, 1998, from (Koul, Dhaliwal, & Cuperus, 2004). The use of different and coordinated pest control strategies based on the ecology of the target species and the features of the environment considerably increases the possibility of success.

CHAPTER 2: METHODOLOGICAL CRITIQUE

1.CHOICE OF THE STUDIED SPECIES

1.1 Species and strain

Rodent pest species cause enormous damage to agriculture and human property, and they are related to an important number of diseases in humans, domesticated species and wildlife. In fact, it is logical that they are involved in so much "damage" and "disease". From an ecological and evolutionary standpoint, rodents are probably the most successful mammalian order, meaning that they occur almost everywhere in great numbers, which implies that they interact with the environment and other species (such as viruses or bacteria). From an anthropomorphic, utilitarian perspective, species without any utility to humans are considered pests or of no interest; this limited perspective does not account for the value of species in their ecosystems.

Many rodent species are considered pests, but only 3 species have a worldwide distribution: *Mus musculus* (the house mouse), *Rattus norvegicus (*Norway rat*)* and *Rattus rattus (*black rat*).* Of these three species, mice probably have the widest distribution, and due to their smaller size, they are relatively inconspicuous to humans and require less food resources than rats, which increases the number of possible habitats.

The choice of *Mus musculus* for the experiments performed during this thesis research was based in its importance and wide distribution as a pest species. Furthermore, practical reasons were considered; the smaller size of the mice meant greater affordability in terms of space and materials, and this PhD thesis was initiated without laboratory animal facilities. My previous experience with rodents was also slightly greater with mice than rats, which could also have influenced the choice of the species.

Rattus norvegicus and *Rattus rattus* are also extremely important pest species that have a noteworthy impact in human environments, but there are other important commensal species. Some receive less attention because they are located in developing countries, which is the case of the lesser bandicoot rat (*Bandicota bengalensis*) in South Asia, the Polynesian rat (*Rattus exulans*) in South and Southeast Asia and the multimammate rat (*Mastomys natalensis*) in central and south Africa.

Species

We chose laboratory mouse strains as models of the wild house mouse. The benefits of using laboratory strains are that they are genotypically and phenotypically (including behaviourally) standardized animals, and suppliers ensure a homogeneous microbiological and sanitary status (including behaviour) as well as breeding conditions (cages, food, temperature, humidity). All these factors increase the internal validity of the research done under these conditions, and the possibility of reproducing the same conditions between laboratories greatly increases the reliability of the results and the possibility of comparing them while theoretically decreasing the influence of external factors on the experiment. On the other hand, while laboratory strains are models of wild rodents, the process of domesticating laboratory mice impacted their behavioural, physiological and anatomical features, among others, thus differentiating them from their species of wild origin.

Once this decision was made, the choice of the strain was studied carefully, as there are hundreds of strains of laboratory mice. However, we can differentiate between two large groups: inbred and outbred strains. Outbred strains are mice with a defined phenotype and a certain variability, as we can find in other domesticated species, and their phenotypic features have been selected by humans for easy handling, reproductive parameters, size, physiology, etc. They can be considered more representative of wild species as they have greater genotypic variety, but they are still only a model. In our case, we chose outbred mice for one group of experiments (foraging) as we considered them more representative due to the greater genetic variability compared with inbred strains.

For a second group of experiments, we selected an inbred strain. These strains are characterized by a very homogenous genetic repertoire can be considered less representative of a wild population. However, outbred strains also have a high degree of inbreeding and consanguinity (as can be found in other domestic species), and they remain a model. So the point is not just whether we have greater genetic diversity, it is also whether our phenotypes are a good model for our subject of research, and this is a key point (that is also controversial as a specialist in rodent genetics was not able to tell me which strain, outbred or inbred, I should chose) that probably improved our data in the second group of experiments. We find the same examples in biomedical research; model mouse strains for hepatic diseases should have similar genes, biochemical pathways and physiologies to those implied in humans for the same processes. It does not matter if 92% of the genes are identical to the future target species of our research if our model lacks the enzymes, protein receptors or any other characteristics that is fundamental for our study.

C57BL/6Rj mice are more behaviourally reactive to general stimuli than other outbred and inbred strains, and they are more sensitive to predator stimuli (Dell'Omo, Fiore, & Alleva, 1994), as is the case for wild animals. This means that the strain can be considered a good mouse model for olfactory stimuli that could elicit avoidance due to its sensitivity. We understand that this is a model, so it is not the same as the wild species, but we can obtain useful results from animals that are genetically and phenotypically very similar. One easily perceived difference in reactivity between mice strains is how they respond in laboratory cages. CD1 mice usually remain inside the cage once the cover grill is removed for inspection and are easily handled. In contrast, C57BL/6Rj mice will jump from the cage, and handling is more difficult. They can eventually jump from the hand; similarly, wild mice will jump from the cage and handling is very complicated (V. Voznessenskaya, 2017, personal communication, CSiV XIV).

1.2 Age

The animals used in our tests were young adults because the development of the olfactory system completes at 8 weeks of age (Tirindelli et al., 2009). House mice of this age are sexually active and have a crucial role in foraging and reproduction. Mice are altricial animals, which means that they are born without a completely developed nervous system, including sensory organs such as the main olfactory epithelium and the vomeronasal organ. Major changes in olfaction occur during the first weeks of age along with other sensory systems such as vision or hearing, but a gradual decline in the sensitivity of olfactory capabilities can be expected with ageing (Doty & Kamath 2014). For all these reasons, young adults were chosen for the tests, and it was expected than their olfactory sensitivity and thus their behavioural responses to these stimuli would be maximal at this stage.

Nevertheless, other stages such as new-born, sexually immature and aged animals are also of importance in understanding the development and senescence of olfactory sensory epitheliums and neural pathways and the associated brain structures. There is some evidence that predator olfactory stimuli could affect reproductive parameters in rodents, such as prolificity or the survival of nestlings (Vasilieva, Cherepanova, von Holst, & Apfelbach, 2000), and future research should provide stronger evidence for these phenomena because they could indirectly modify rodent populations.

1.3 Sex

We used both sexes in our studies because testing only one sex would bias the results and would be less representative of the species; additionally, both sexes have important roles in the ecology of the house mouse (Wahlsten & Crabbe, 2007). Taking all these factors into

consideration, we do not think that the use of only one sex is justified for experiments studying predator or plant olfactory cues in rodents.

Our results did not reveal significant differences between the sexes, which is consistent with other studies of the responses to predator olfactory stimuli, such as the putative cat kairomone Fel d 4 (Dey et al., 2015). Predation risk perception is highly valuable information that is independent of sex and the stage of the oestrous cycle. However, as shown by Dey and collaborators, the VNO changes its receptivity to male pheromones depending on whether the female is in oestrus or dioestrus (Dey et al., 2015). Food consumption and activity levels can vary with the oestrous cycle (Dixon, Ackert, & Eckel, 2003), and there are differences in the brain between both sexes (Cahill, 2006). During our tests, the oestrous cycle was not considered in the analyses, so the female mice could have been in any state of the cycle; ideally this information would be valuable for foraging and activity behaviour. However, it is less probable that oestrous influences the risk perception of rotted fruits as they should have negative effects in any state of the cycle.

1.4 Ethics

The research with animals in this thesis was carried out according to high laboratory animal welfare standards. We applied up-to-date knowledge on the enrichment, handling and transport of the animals as well as validated methods for euthanasia recommended by European legislation (Directive 2010/63/EU) and specialized societies including the Federation of the European Association of Laboratory Animals (FELASA) and the American Veterinary Medical Association (AVMA). Enrichment was assured with two different nesting materials, shredded paper strips and white tissue paper, that enabled material manipulation and temperature self-regulation (Hess et al., 2008). Instead of tail handling for mice manipulation, the handling methods were refined with the use of tubes; this technique has been proven to significantly decrease stress due to human manipulation (Hurst & West, 2010a).

House mice are mainly nocturnal animals with activity peaks during crepuscular hours, including the first hours of the night and the last hours before sunrise. This circadian rhythm has welfare implications, animals in research facilities experience light cycles adapted to human working hours, which implies that they are usually disturbed during their resting period for cage changing, sanitation or research procedures, which causes stress and prevents the completion of natural circadian rhythms (McLennan & Taylor-Jeffs, 2004). Except the experiment in which we tested the hydrophilic content of cat fur and skin with special interest in the cheeks, the other studies were performed under a reversed light cycle, and the procedures with animals were carried out during the dark period. This was not possible for the

cat fur and skin experiment because the laboratory animal facility was shared with the University of Avignon, and the light cycles were centralized.

Animal research procedures during this thesis were considered of low severity. This is justified because no painful procedures were performed on the animals, and the behavioural experiments and exposure to predator or plant-based olfactory stimuli allowed for natural avoidance. Working with olfactory stimuli instead of directly with predators also decreases the perception of imminent danger.

1.5 Number of animals

The number of animals in the first experiments related to feeding and exploratory behaviour with outbred mice was based on the literature; the considerable variability observed during the first experiment with cat fur and skin hydrophilic molecules was notably decreased during the second experiment. These improvements were probably due to better control of the environmental conditions (decreased environmental noise, light, and a controlled temperature) that was not possible in the previous facilities. We also improved the cleaning procedure to allow washing with water and less porous materials (glass, steel), which decreased the presence of undesirable materials/chemicals between replicates. The number of animals used for the experiments performed with inbred strains was also based on the literature as well as our previous experiences with outbred strains and preliminary results with inbred strains.

1.6 Use of the animals at the end of the experiments

Following our first study, the animals were euthanized after the behavioural tests due to the sanitation policy of the university. A common policy in laboratory animal research facilities is that animals that leave the “controlled” environment of the laboratory vivarium are not allowed to enter the facility again nor are they allowed to remain outside. Therefore, the only possibility is euthanasia after the experiments or fostering, which is usually more difficult with smaller animals such as mice.

At the end of the other experiments, animals were not euthanatized and instead were used to establish other behavioural tests or devices.

If the use of naïve animals is a standardized procedure in the literature to avoid the consequences of learning/habituation/dishabituation/sensitization or other processes, we consider it worthy to discuss whether these standards have a sound basis.

2. PERTINENCE OF OBSERVED BEHAVIOURS

The final aim of this research was the use of chemical messages to influence the use of space by rodents. For this reason, a first approach to determine the effects of the olfactory stimuli under laboratory-controlled conditions seemed appropriate. We studied this general approach using several behavioural parameters and two different devices. The test conditions can be classified as those with the presence of food (in addition to the olfactory stimuli), in which animals were partially fasted, and tests exclusively involving the olfactory stimuli, in which animals were fed ad libitum (as is common practice in laboratory rodent facilities). The behavioural parameters used in this thesis were based on a previous study of the literature and the results and observations obtained during this period. Fasting animals increases the need for foraging, thus increasing the search of food and food intake. This method is considered a good practice for evaluating the effects of olfactory cues in foraging decisions by many authors (Bursztyka, 2015; Lima, 1998b).

2.1 Behaviours related to the use of space

Animals use space according to perceived risks and benefits, and they make their decisions and evaluate trade-offs based in this information (Lima, 1998b).The use of space and how stimuli can influence it has been broadly described in the literature (Barbosa & Castellanos, 2005; Lima, 1998a). More specifically, avoidance behaviours have been used to determine the effects of predator olfactory stimuli (Apfelbach et al., 2005) or plant volatiles (Hansen et al., 2016a) such as secondary metabolites.

Behaviour can be analysed manually or with automated software. We used semi-automatic behavioural analysis with Excel files because behavioural software was not available. Some authors argue that human analysis yields more accurate results than software with fewer mistakes (Armario, 2013, personal communication).

Avoidance is a highly reliable parameter; in our research, avoidance behaviour exhibited the lowest inter-observer variability in every behavioural test. We highlight that avoidance is a nonspecific behaviour that could be the consequence of any dangerous or noxious stimulus. This lack of specificity allows this parameter to be used to evaluate responses with different stimuli and biological meanings (predators and plant-related chemicals) as well as a single and important final consequence, the use of space.

Locomotor activity is decreased in the presence of danger, such as predator olfactory cues. Animals begin to approach the stimulus to obtain potencially valuable information (Parsons et al., 2017) about predators, food, conspecifics or co-occurring species once, and they estimate the trade-offs and risks and benefits of their actions. If the assessment indicates that it is risky

to remain close to the stimulus, they will search for shelter or covered areas, and once there, decreased activity and avoidance of additional visits to the risky area would limit exposure to dangers (Lima, 1998b). For these reasons, we estimated locomotor activity as a valuable parameter in the behavioural response to olfactory stimuli. In addition, this parameter also showed high reliability, with low inter-observer differences.

In our studies, we also performed a preliminary analysis of more specific behaviours to determine specific anti-predator responses including freezing, risk assessment and flight (Apfelbach et al., 2005; Papes et al., 2010). These results were not included in the results section because the minimal inter-observer reliability fixed for the statistical analysis was not achieved. Other behaviours that were analysed and discarded were hesitation and latency, which had the same problem. Specific anti-predatory behaviours have often been used in studies related to fear and the relevant neural pathways, but such research has lacked an ecological rationale for the species and have mainly focused on translational neuroscience. At the same time, their validity and reliability of these behaviours are more controversial than avoidance behaviour because they are subtle and complex and occur in a few tenths of a second. This feature promotes inter-observer differences and therefore poor reliability.

2.2 Rodent thermodynamics, consequences for foraging behaviour and an evolutionary overview

As with other small rodents, the house mouse has a high external surface/body weight ratio, which from the thermodynamics perspective means a large surface area over which to exchange energy with the environment (Schmidt-Nielsen, 1984). As the body temperatures of the mice are always higher than the temperatures in the environment, the animals constantly lose a large amount of energy, which means that they require substantial, constant nutritive resources to fulfil these needs. This pattern is observed in nature; rodents consume a greater proportion of their weight in food than larger animals, and have a rapid metabolism is needed to replace the lost energy. The heart can pump 600 times per minute to provide nutrients and oxygen to the organism and to warm the blood. Thus, rodents such as the house mouse need to feed often and in proportionally large quantities, and their morphology and related physiology conditions their behaviour and generates the need to feed constantly.

In our first study, we used whole wheat powder due to its high caloric content and because cereal grains are an important part of the diet of small rodents such as the house mouse. The use of powder and not grains can be discussed since the former would not be the form found in nature. However, this method improves the hygienic conditions of the feed and allows the

nutritive stimuli to be standardized; the amounts were easily measured with precise balances. In our second study measuring food consumption, we used 5-mg micro-pellets with a composition similar to that of food pellets used for breeding. This method allowed the amount of food consumed to be easily and precisely measured without weighing.

From an historical and evolutionary point of view, the domestication of cereals and vegetables, and the development of agriculture are correlated with human sedentarism (E. Jones et al., 2012) and enormous changes in the global landscape up to the present, so much so that it is debated whether this moment marks the start of a new geological era, the Anthropocene. In this anthropogenic environment, rodents began to proliferate as commensal species, and they have been coping with predator species that are also adapted to the human landscape, such as cats. However, a key point is evident, human settlements stocked food, such as cereals and fruits, as they continue to do, so the ability to detect these sources of food (as also occurs in nature for rodents) and their degree of ripeness or toxicity would be of paramount importance. This importance of this phenomenon is increased if we consider that ripening and fermentation are not homogeneous in nature, so if a good fruit or cereal grain cannot be found on one area, others can be selected. However, humans have selected fruits and cereals to produce homogeneous products, so they are stored in a homogeneous state. Therefore, the value of detecting these properties is probably even greater for commensal species.

2.3 Overview of stress physiology and related behaviours

Living organisms survive by maintaining a complex dynamic and harmonious equilibrium or homeostasis, but this equilibrium is constantly challenged by intrinsic or extrinsic forces or stressors that is termed stress (Chrousos & Gold, 1992). Releasing faecal pellets is directly related to stress since it influences intestinal motility and the relaxation of anal sphincters. Stress reactions induce the release of corticotrophin releasing factor (CRF) (Tsigos & Chrousos, 2002), and this peptide, among many other effects, acts on the central nervous system to accelerate colonic motility and transit through the activation of the vagal and sympathetic pathways innervating the proximal and distal colon (Browning, Travagli, & Sciences, 2014). Our aim was to register a non-invasive, reliable, stress-related behaviour. Other methods to measure acute stress responses exist, including the measurement of ACTH and corticosterone in the blood, saliva or urine. However, some of these methods are invasive, and the additional animal manipulation needed for the sampling could influence the results because it is stressful for mice (Madetoja, Madetoja, Mäkinen, Riuttala, & Jokinen, 2009). Other non-stressful methods that do not require manipulation of the animals include hair corticosterone (Meyer & Novak, 2012) or faecal cortisol (Touma, Palme, & Sachser, 2004)

analyses, but these are not good indicators of acute stress events and are instead useful for detecting long-lasting stress.

3 BEHAVIOURAL TESTS/DEVICES

The chosen behavioural test allowed mice to express natural behaviours without excessive spatial constraints. The three-chamber avoidance test was designed as a modified open field, in which the intermediate, treated and non-treated areas were physically separated with a fixed opaque barrier. Symmetry between both ends of the device was carefully studied since the treated area in other studies with predator olfactory cues is open while the shelter is covered, which could bias the results (Inagaki et al., 2014; Papes et al., 2010) because the conditions are not comparable and would increase avoidance due to the natural fear of open space. Some studies measuring avoidance or fear reactions have preferred to use standard mice rearing cages, arguing that there is no need for habituation to the devices as the mice are already in the housing environment (Papes et al., 2010). However, this kind of test does not allow the subject to search for shelter and physical or visual barriers between it and the dangerous stimulus. These situations seldom would occur in nature, where the complexity of the environments allow shelters to be found, escape or the search for other food.

The eight-arm device was designed to measure the use of space, exploratory behaviour and foraging by laboratory mice in a more complex environment than the three-chambered device. However, it basically remains a two-choice test with repetitions (4 tubes with one treatment and 4 tubes with the other treatment). It could be considered similar to radial mazes, which are more standardized devices, with a larger central area and rectangular instead of rounded. One possible risk would be cross contamination due to the close proximity of the entrance of each arm, but Fel d 1 is a non-volatile protein (it could be carried by an airstream or wind, but this was not possible in our test conditions). In fact, it would have been very difficult for the protein to pass from one arm to another. In this sense, the test with the facial cat pheromone could be more delicate because the pheromone and its solvent were volatiles. For this reason, the entrances to each arm were covered with plastic “curtains” to decrease this possible contamination.

4 OLFACTORY STIMULI

Olfactory stimuli used during this thesis can be classified into two main groups: isolated chemical compounds or putative semiochemicals and native olfactory cues.

4.1 Isolated chemical compounds

Isolated chemical compounds or putative semiochemicals are substances with different scientific statuses:

-Compounds with already characterized biological roles in mice such as TMT, which has been identified to have a kairomone role in mice (Vernet-Maury, 1980). We used this chemical as a positive control for our studies due to the large bibliography in which it elicited avoidance and fear reactions in mice.

- Compounds whose roles have been elucidated in other species but not in mice:

The cat protein Fel d 1. There is evidence for a putative role of this protein in cat chemical communication (Bienboire-Frosini, 2009, Durairaj et al. 2018), but before the research done for this thesis, there was no information about the chemical ecology of this molecule in mice, to the best of my knowledge.

The chemical ecology of ethanol has been studied in insects (Schneider et al., 2012) and one mammalian frugivorous species, the Egyptian fruit bat *(Rousettus aegyptiacus*) (Sánchez et al., 2006), but the ecology of this chemical in rodents has not been discussed elsewhere, to my knowledge. This is probably because laboratory rodent studies using ethanol have focused on a major health concern, human alcoholism.

Depending on their molecular weight and volatility, these chemicals were also classified as volatile (F3, Ethanol, TMT) or non-volatile (purified Fel d 1), and the test protocols and cleaning procedures were adapted accordingly.

4.2 Native secretions, complex olfactory cues (mixture of volatile and non-volatile chemical compounds)

Fox faeces were tested as a control for TMT because some research has indicated that this compound (identified in fox faeces) would produce trigeminal irritation and not chemical communication per se (McGregor et al., 2004). Therefore, we considered it important to test it in its natural form and compare it with TMT. We observed a significant effect of fox faeces as an olfactory stimulus that induced avoidance but to a lesser extent than the tested TMT concentrations. These results agree with those of previous studies comparing TMT and fox faeces, and it has been proposed that lower amounts of TMT should be tested that are comparable to what would occur in nature (Buron et al., 2007), where mice cope with fox faeces as olfactory cues, to obtain more realistic results.

Snake, cat, ferret and dog olfactory cues were chosen because these animals are all significant rodent predators, and the native forms are closer to the stimuli rodents would encounter in nature. Testing the cat body extract was our first approach before testing purified Fel d 1. The extract was tested with other hydrophilic compounds (presents in the native solution), but Fel d 1 was identified in large quantities through immunosorbent assays (ELISA). Also, Fel d 1 molecular variants were the most prominent protein molecules found in the cat body extract according to SDS-page and Western blot's results. There have been contradictory results regarding the role of snake sheds or other snake substances as fearful stimuli for mice. Some studies have found evidence of the activation of behavioural and neural pathways (Papes et al., 2010), whereas others have found no reaction (de Oliveira Crisanto et al., 2015), as in our results. A probable reason is differences in the sensitivity of the laboratory mice strains; Papes (Papes et al., 2010) used C57 mice, which are more responsive to novel/fearful stimuli than Swiss mice. Ferret olfactory stimuli chosen to be representative of the main cues left by ferrets included anal drags associated with defecation and wiping, belly crawling, body rubbing, and chin rubbing, all of which are related to several skin glands (abdominal glands, around the urogenital opening, and tubular and sebaceous glands over the whole body (Clapperton, 1985)). For this reason, we used faeces and tissues that had remained in contact with the main parts implied in ferret chemical communication.

Cat urine has been previously tested as a predator stimulus, and its compound, felinine, exhibited some evidence of influencing rodent reproduction (Voznessenskaya, 2014). However, to our knowledge, no evidence of avoidance behaviour has been published. In addition, these cat urine results were not published in any peer-reviewed journal, and they have only been observed in one laboratory, even after several years. In our results, Swiss mice did not exhibit avoidance, which could have been related to the absence of biological meaning from the urine, the reception of the stimulus but the inability to elicit an avoidance behavioural response, or the inability of the specific strain phenotype to receive this chemical message. Finally, our conclusions regarding this stimulus are limited due to cat sampling as we used only one male.

CHAPTER 3: DISCUSSION OF RESULTS AND COMPARATIVE PERSPECTIVE WITH THE LITERATURE

Rodents have greatly influenced history and human endeavours. They constitute 42% of the known mammalian species (Macdonald, 2007), but less than 5% are considered pest species (Singleton, Hinds, Krebs, & Spratt, 2003). These species have adapted to agricultural and urban human environments, where they cause substantial damage. Rodent behaviour is largely guided by motivations and emotions, and these two concepts are closely related and belong to the most ancient parts of mammalian brains, such as the amygdala and the hypothalamus. Motivations and emotions are triggered by different sources of information: external and internal. Internal sources inform the need for food, water or rest, and external sources guide foraging or the avoidance of dangers. These sources of information influence behaviours to fulfil needs and increase chances of survival.

Through the questions developed from our research hypotheses and results, I have organized the general discussion into three main parts: predator-prey interactions, plant-rodent interactions and the plant-animal olfactory landscape and finally, ethics in rodent control.

1 PREDATOR-PREY INTERACTIONS

Predator-prey interactions are a major driving force in nature, and in addition to the direct effects on killed prey, non-lethal effects from predation can greatly influence prey ecology (Lima, 1998a). In our tests, mice avoided olfactory cues from ferrets, and these results agree with the observations by Masini and collaborators (Masini, Sauer, & Campeau, 2005; Masini, Sauer, White, Day, & Campeau, 2006) with Sprague-Dawley rats. In another study, they found that the combined chemical perception of ferret olfactory cues by the VNO and olfactory epithelium increased corticosterone levels in rats. Together, olfactory epithelium inactivation by $ZnSO_4$ and VNO ablation decreased corticosterone levels in the presence of ferret cues, but corticosterone did not decrease with only one of the two methods, suggesting that a combination of volatile and non-volatile compounds from ferret fur elicited fear responses in rodents. Our samples were not frozen immediately after sampling, which probably decreased the presence of highly volatile compounds. As volatile and non-volatile compounds were capable of triggering a stress response, this suggests a role of non-volatile compounds to be investigated with our tests. As a next step, using the fur washes from ferrets would be a logical approach to test the hypothesis that non-volatile chemical compounds act as predator olfactory cues. Fur washes have been used in other studies with cats to collect proteins (Bienboire-

Frosini et al., 2010; Carayol et al., 2000), including the Fel d 1-related studies conducted for this thesis. Such a compound is probably a lipocalin as some preliminary research related to ferret allergies has suggested (De Olano et al., 2009); this protein could also be identified in urine and saliva (Díaz-Perales, González de Olano, Pérez-Gordo, & Pastor-Vargas, 2013). However, more research should be performed to clearly identify this peptide and its possible role in interspecific communication as a kairomone or in intraspecific communication between ferrets.

Another difference between our study and the results of Massini is the combination of faeces with the fur olfactory stimuli. Faeces contain anal gland secretions that are used for intraspecific communication in ferrets (Clapperton, 1985; Cloe, Woodley, Waters, Zhou, & Baum, 2004). Based on our results, we cannot determine if the avoidance effect was from the fur or faeces, so further research should also test anal gland secretions, which are eliminated with droppings (Clapperton, 1989). In another study, ferret urine and its compound, quinoline, elicited a reduction in the exploratory responses of house mice (Zhang, Sun, & Novotny, 2007), but the ferret urine was mixed with mice urine in some tests, so in my opinion, this makes it difficult to reach clear conclusions about the effects of ferret urine alone.

Through this thesis, I have presented the first results of the effects of the cat protein Fel d 1 presented within a native cat fur extract solution and as an isolated compound on the behaviour of mice. Based on these results, we do not have any evidence to state that mice would identify this compound as a predator stimulus and thus increase the perception of risk. However, the absence of avoidance or significant differences in the behaviours measured in our ethogram do not necessarily indicate an inability to detect the molecule. Fel d 1 is closely related to androgen-binding proteins (ABPs) (Durairaj et al., 2018), which play an important role in sexual communication in rodents. If mice did not develop mechanisms to identify Fel d 1 as a kairomone, they could partially identify Fel d 1 as a chemical related to their ABPs, despite slight structural differences, without triggering intraspecific sexual motivations due to its similarities. This hypothesis could be tested through immunofluorescence (c-fos protein or others) or by brain imaging techniques (two-photon or three-photon calcium imaging), which would allow the brain areas activated with each molecule to be identified (Horton et al., 2013; J. W. Wang et al., 2003).

Olfactory information has the advantage of being less risky than direct encounters with competitors or predators. Finding predator chemical cues can elicit the non-lethal effects of predation in prey animals; these are less obvious than lethal effects, but they modulate the ecology and behaviour of species (Lima, 1998a). As previously introduced, this avoidance can be modulated by internal and external factors, and this combined information will balance the

trade-offs and consequences of the reaction by the prey. Because the perception of predation risk is not unique, it can be modulated by different factors: environmental, social, physiological or pathological. The presence of cover significantly decreases the perception of risk; small rodents such as mice and rats use this cover to avoid attacks by predators. The presence of conspecifics can also decrease the perception of predation risk (Sullivan, Maerz, & Madison, 2002). The ontogeny and pathology of sensory organs, the musculoskeletal system, or the central nervous system could also influence this perception and the reactions of rodents to these stimuli. In mammals, vomeronasalitis has been described as the inflammation of the vomeronasal organ (Asproni et al., 2015), and this pathology has been linked to modified behavioural reactions in affected animals, probably due to a decreased capacity to detect conspecific chemical messages. The VNO and the olfactory epithelium are both implied in the detection of predator olfactory cues, so changes in these sensory organs could influence the associated rodent behavioural responses. In mice, the VNO is a frequent target of viral attack; the virus associated with VNO and olfactory rhinitis in neonatal mice can result in failure to suckle (Percy & Barthold, 2007). In addition, age and musculoskeletal development or pathology will condition responses to risky olfactory cues. In laboratory tests, we use healthy, young animals that are probably bolder than older animals because their physical condition improves their chances of escaping predators or other risks (Cooper, Jr. & Blumstein, 2015), so by the same logic, injured animals or those with physical disabilities will be more cautious when exploring or foraging. Maturity of the central nervous system would also influence these responses because risk perception changes between the pre-adult and adult stages. In the brain, the prefrontal cortex area modulates the perception of risk, and this area undergoes a belated maturation that conditions increased boldness during this life stage (Chan et al., 2011). This behaviour could especially influence pre-adult males who have achieved sexual maturity and have dispersed to find their own territories; these animals could be less cautious when encountering predator olfactory stimuli. An interesting point about the influence of fear on foraging is personality; animals will behave differently when encountering the same fearful stimulus depending on if they are bold or shy. A bold animal will take more risks, which will have the benefit of finding better feeding resources in terms of nutritional quality and lower toxicity, whereas shy animals will take fewer risks and will probably have access to less-valuable resources with higher toxicity (McArthur et al., 2014). The costs of fleeing are mainly linked to losing opportunities to feed, to engage in social activities such as courtship, mating and territorial defence and to perform other activities that increase fitness (Cooper, Jr. & Blumstein, 2015).

Avoidance of predators is especially important in stable areas (Edmunds, 1974). Selection for defence against or surviving predations is higher where the selection pressure from the environment is lower, and human environments provide this stability in terms of food and environmental conditions. However, commensal rodents have evolved, developing anticoagulant-resistant mutations. The constant and massive use of anticoagulants to control pest rodents could be understood as predation pressure (i.e., the will of another species to kill the rodents).

2 PLANT-RODENT INTERACTIONS AND THE PLANT-ANIMAL OLFACTORY LANDSCAPE

The primary function of fleshy fruits is to attract seed dispersers, but the timing of this attraction seems critical. Fruits present a variable chemical profile during fruit development that will encourage or discourage consumption from the early stages of development to fruit drop. The avoidance of ethanol as an olfactory stimulus observed in this thesis is relevant to the last part of this cycle, rotting.

Some secondary metabolites are chemicals in leaves and fruits that prevent or decrease consumption by herbivorous species; they are considered a chemical defence for plants and can be detected by rodent species (Hansen et al., 2016a). Their study has mainly been focused on the chemicals in leaves that protect against herbivory, but they also play a role in fruits. Whitehead and colleagues proved an antifungal effect of secondary metabolites in fruits (Whitehead & Bowers, 2014), other microorganisms and insects. However, the production of secondary metabolites for defence seems to incur a high metabolic cost for the plant, which could explain the decreasing concentrations of these chemicals with ripening. At this stage, plants are favoured by seed dispersal and therefore fruit consumption. Plant secondary metabolites decrease with advanced ripening and rotting, and this fact combined with other physical changes (fruit skin) allows fungi to grow. The consumption of toxic/unhealthy fruits by seed dispersers would have a detrimental effect for the plants as animals would associate their fruits with these qualities and would search for other fruits without these constraints. Generalist species such as house mice or commensal rats do not have the adaptive mechanisms that allow specialists (e.g., the koala) to metabolize toxics or plant secondary metabolites that can negative affect their physiology.

Plants and predation risk seem like distant concepts, but they are closely connected in terms of the use of space and the internal physiology of rodents and other species. Predation risk influences foraging because predation costs will balance the reward of finding foraging

patches. Plant chemical messages are on the other side of this balance and will provide information about the nutritional or toxic properties of food, and together with predation costs, they constitute a trade-off balance by which the animal will decide to invest in the foraging patch if the benefits are greater than the risks. Considering physiological responses, both types of information are stressors. The toxins or pathogens associated with rotted fruits may trigger the HPA axis stress response and the release of glucocorticoids (Grau et al., 2019), and predator cues will also activate the HPA axis.

Generalist foraging species such as the house mouse or the Norwegian rat have different strategies than specialists to cope with predation risk. Theoretically, generalist species will be bolder when foraging because they have fewer resources to metabolize toxic compounds from less risky foraging patches in the landscape (those with lower predation pressure). However, integrating both concepts, low-quality foraging patches (with high toxicity and possible pathogens) with high predation risk would create an extremely avoidable area from the point of view of rodent ecology and chemoreception. The application of this idea would be an integrative approach to elicit avoidance and decrease interest in foraging and exploring in human resources or facilities from two main perspectives, predation risk and food intoxication/contamination/related diseases. High predation pressure would be balanced with a great need to forage to avoid starvation, while low-quality food patches with low predation pressure would be foraged to avoid having to cope with predators. Integrating both inhibiting factors, low quality/toxicity and high predator risk would result in no interest to forage or explore with rare exceptions involving extremely dangerous alternatives, e.g., physical attack by other predators.

Modulating chemical olfactory perception from areas of interest to rodent pests to protect human interests would probably be a useful tool, but if rodents lose interest in such areas due to chemical olfactory modulation, they will search other areas. Therefore, the non-lethal effects of predation should continue to play a role (Lima, 1998). The negative effects of predation risk on reproduction can decrease populations, and with the help of environmental control, access to resources, increased physical perception of risk through different approaches (increase illumination and decrease vegetation cover/bushes) (Navarro-Castilla & Barja 2014; McDonald et al. 2016), commensal rodent populations can be kept outside sensitive areas and in manageable numbers without the need for further actions.

If this integrative approach is considered, the need for acute lethal interventions should be considerably reduced. Cases where the need would outweigh the costs could include acute zoonotic epidemics; in these cases, rodent corpses should ideally be burnt. However, even in these extreme cases, humane lethal methods should be the priority.

From the perspective of the evolution of olfactory perception and the associated receptors, commensal rodent species could have two important components in their genetic repertoire. The first and more important would be conserved receptors and mechanisms from before the Anthropocene, or the advent of commensalism, which could include the ability to detect species of predators or plants that shared habitats with these rodent species or their ancestors hundreds or thousands of years ago. Second, receptors and mechanisms that evolved within these human environments (Anthropocene) with specific characteristics, predation pressures, and food resources (Steffen et al. 2011). This would seem to be a short period in terms of evolution, but it seems probable that some olfactory adaption developed during this period. There are several examples of species evolving to adapt to human environments that occurred in short periods of time (Johnson & Munshi-South 2017); one is warfarin resistance. Rodents have spread this trait within a few decades (Rost et al. 2009). Therefore, environmental selection of olfactory mutations with adaptative advantages in human environments would be highly valuable.

3 ETHICS IN RODENT CONTROL AND INSIGHTS INTO WELFARE FROM THE EXPERIMENTAL WORK

Ethics in rodent control

The most common method for rodent control is the use of anticoagulant warfarins that produce painful symptoms until intoxicated animals die due to anaemia, haemorrhaging and other related disorders, or if sublethal doses are ingested, the animals live with severe sequelae (Mason & Littin 2003). There is no real ethical alternative available, even if some efforts have been made. Non-lethal human traps require that captured pest rodents to be released in other areas, but there is a high risk of starvation if the traps are not checked often. In addition, mice would probably be captured during the night when they have higher nutritional and water needs (Ritskes-Hoitinga & H.Strubbe, 2007; Tobin, Stevens, & Russell, 2007). These traps also pose a sanitation risk as humans will manipulate the cage and could come into direct contact with mice and their fomites (urine, saliva, faeces) (J. N. Mills & Childs, 1998). However, the main problem with these methods probably lies in the absence of analyses related to the ecology of the target species; the environment remains unchanged and thus equally attractive to mice or other rodents. Using the terminology of the 3 Rs, humane traps may replace glue traps or snap traps but will not have any effect in the long term. The reduction and replacement of rodent control should be managed using an integrated approach based on the ecology of the species.

Olfactory cues that are indicators of rotted/toxic plant fruits/seeds or predation risk could be classified as ethical rodent control methods. Their effect would be based on the perception of risk without the need for death, pain or suffering because animals could choose other areas to explore. Using the classification proposed by Mason and Littin (Mason & Littin, 2003) and taking the degree of pain, discomfort and distress as well as the length of time with clinical signs into account, predatory cues would only produce distress due to the perceived increased risk of predation. These acute effects should only last briefly, but they are part of the natural stimuli that mice would use in any environment to decide its use of space. This means that we are not adding new stimuli with noxious effects but only managing information that already exists in the environment. Stress from the perception of ethanol related to rotted fruits or seeds should be lower as these are passive risks that do not pursue rodents as is the case with predators, which have direct and indirect effects (Creel, 2011).

From an applied perspective for research facilities, ethanol is often used in close or direct contact with laboratory rodents in many protocols, and it is commonly used for cleaning, disinfection, or as a solvent for other molecules. Considering our results, these procedures with mice or rats could be affected by this volatile molecule, as it elicited significant avoidance during our tests. An example is the use of ethanol to disinfect laboratory gloves when changing cages with the intention of decreasing cross contamination. This procedure is performed to minimize time, and there is no interval between disinfection with ethanol and contact with the mice. Furthermore, mice handling when changing cages is usually performed by tail manipulation, a procedure that has been described as highly stressful (Gouveia & Hurst, 2013; Hurst & West, 2010a); in addition, the effect of an aversive olfactory stimulus such as ethanol, which mice would avoid in free-exploring conditions, becomes unavoidable during manipulation. The avoidance of ethanol during manipulation by mice could be masked by their avoidance behaviour in response to tail handling. As suggested by Hurst and colleagues (Hurst & West, 2010b), the use of tubes for these manipulations would decrease stress and exposure to ethanol because handling the mice with gloves is greatly reduced, and the mice are able to escape into a safe zone (tube). The ethanol concentration and allowing time between changing cages for the vaporisation and dispersion of the ethanol should be considered to refine this and other procedures in which ethanol is used. In some cases, it could be exchanged for other molecules with similar disinfectant or solvent features.

During this thesis, I developed a new light system to reverse the rodent circadian cycle. The human and murine diurnal rhythms are out of phase, and in conventional mouse houses the deep sleep of the mice is often disrupted, welfare monitoring of the mice is limited by their inactivity, and the obtained scientific data is from the naturally inactive period. The vision of

mice and humans differs in the wavelengths that stimulate their visual receptors, which allows the use of wavelengths within the human spectra that are outside rodent vision. In the past, red lights have been the gold standard for reversing light cycles in laboratory rodents, but during the last 10 years, sodium lamps with a narrow light spectrum of 586 nm (yellow-orange) have replaced some of the ancient red lights as they offer a better acuity for the human eye (McLennan & Taylor-Jeffs, 2004).

For the studies in this thesis (except one), we used orange LED lights (600 nm) to illuminate the dark periods of the reverse cycle. After two weeks of acclimation, the animals changed their activity patterns according to the new light-cycle schedule (Grau 2015, personal observation), exhibiting increased activity during the dark phase (illuminated with orange LEDs) and decreased activity during the light part of the cycle. This lighting system allowed for less disturbing manipulation of the animals according to their natural activity patterns. This wavelength is further from that of the sodium lamps in the rodent visible spectra, which should be reflected in less disturbance of their cycle and thus increased welfare. Additionally, this wavelength permitted good human visual conditions with better stimulation than red wavelengths, which are within the limits of human vision. In addition, working in reversed cycle conditions seems easier and less expensive than using sodium lamps. Finally, these methods could be improved by using LED technology to mimic spectra transitions during sunset and sunrise, which would reproduce more natural and physiological conditions during circadian rhythms.

CONCLUSION AND PERSPECTIVES

In a globalized world, invasive pest species are expected to be an increasing problem. From viruses or bacteria to insects, plants or vertebrates, exponential increases in human transport and world trade open doors for alien species to enter new biotopes. This was how the story of rodent pests began, and it is a story that has been repeating ever since with severe consequences for human health, goods, agriculture and biodiversity.

Human-animal interactions with pest species should be framed within an ethical perspective. The moral consequences of inflicting pain or suffering have been modulated according to the value of these animals to human societies that has been translated into permissive legislation and policies. However, ethical standards should apply equally across different situations including laboratory animals, pets, farms or “pest species”. The human understanding of property includes the entire surface of the earth, and from this perspective, any unexpected

species is against the law. This anthropocentric premise results in aggressive strategies and reactions, but urban, peri-urban or agricultural environments can be shared with other species if resources, motivations and access are correctly managed.

The definition of pest itself should probably be reviewed; this term has legal consequences because is used as a basis for pest control legislation and applies once a problematic species has spread widely. The term invasive species is more meaningful, and the use of indicators to classify and prevent potential risks before new populations become established seems a wiser and cheaper strategy than global-scale treatments. Economic studies have highlighted the importance of early intervention in pest outbreaks (Williams et al., 2010); the economic costs of control will increase exponentially, especially with new invasive species and fragile ecosystems such as islands. Early intervention should be combined with education to recognize invasive presence because the possibilities of detecting the early stages of invasion would otherwise be scarce.

Rodent pest control represents a huge global market, in which only a few companies, including some of the largest pharmaceutical corporations control the production and distribution of rodenticides. There are no viable alternatives to these products in generalist markets, and producers have taken advantage of permissive legislation (Eisemann, Fisher, Buckle, & Humphrys, 2018). Therefore, we face the paradox of preventing the transmission of rodent diseases using highly toxic chemicals that will produce pathological symptoms or death in non-target species. We can observe evident parallelisms with invertebrate crop pest management or herbicides (Begon, Townsend, & Harper, 2006); highly toxic products have been used for decades and have severely impacted treated environments (Isman, 2000).

Integrated pest management (IPM) combined with a deep understanding of the ecology of the pest species involved and the web of interconnected populations should be the preferred approach to deal with established pest populations. Non-specific lethal methods have severe consequences for non-target species, including pets or wildlife, and in some cases, they can have an effect opposite the intended use as killing the predators of the pest species leaves a vacant niche, allowing the pests to quickly increase their populations (faster than the predator species) (Krebs, 2018). The goal of IPM is to maintain populations below a significant level of economic injury, so understanding and controlling key factors in these populations would allow tolerable levels of self-regulated populations.

Chemical information should be a main factor within this integrated management as it plays a key role in the lives of pest species. In particularly, we know that it modulates many key aspects of rodent pest populations, predator-prey interactions, foraging and sexual communication

(Karn, 2013; Wyatt, 2014). The perception of chemical information in the environment modulates the use of space by commensal species. However, it would be naïve to state that chemical information would solve the problem alone. As discussed above, managing the availability of food resources and increasing the perception of risk from several angles (decrease cover and increase predator cues, lights, and the amount of toxic food) seems a more robust and long-lasting strategy. In terms of populations, our studies addressed two main paradigms: predator-prey interactions and plant-forager interactions. In the first case, our results demonstrated that animals that were naïve to ferret olfactory cues avoided these stimuli. Fur washes similar to methods that we previously used with cats could be applied to obtain fewer complex stimuli, which could allow us to identify putative candidate kairomones for screening tests. One of these candidates might be the ferret allergen for instance, which is supposed to be a lipocalin but is largely unknown. The lipocalin family includes the only two kairomone proteins known in mice, Fel d 4 from cats and MUP13 from rats (Papes et al., 2010). In the second case, plant-forager interactions, ethanol elicited clear avoidance in mice. Due to its ubiquity and presence related to the rotted stages of fruit ripening and seed production, it seems an ecologically pertinent chemical for managing the use of space by rodents. Tests with different concentrations of ethanol and rotted fruits with a known concentration of ethanol and other putative plant chemical cues from rotting, would be of interest to complete our results.

Combining olfactory stimuli from different origins such as plants and predators, almost represents a new area of exploration, which I consider of special interest for more strongly influencing rodent behaviours: risky/low-quality foods+ high predator pressure. I presume stronger and less variable avoidance reactions because predator stimuli or low-quality/risky foods olfactory cues alone could be overwhelmed by other realities, such as if food resources are scarce.

Our tests with plant and predator chemical cues, were conducted under controlled conditions to appreciate the behavioural consequences of this chemical information and to avoid or decrease the interference from other factors. Semi-controlled or field studies should be future directions to evaluate more realistic consequences of ferret chemical cues or plant fermentation chemicals such as ethanol.

Developing successful strategies to manage commensal pest species should account for ongoing evolution and the ecology of human environments (Johnson & Munshi-South, 2017). Pest species management is often planned using idealized models of populations without human influence, but there is an increasing evidence that human environments influence the evolution of commensal species and that adaptation can occur rapidly, as we have seen with rodenticide resistance (Rost et al., 2009) or the classical model of darker moth morphs (*Biston*

betularia) being favoured by environmental pollution (Kettlewell, 1956). *Felis catus* has been an important rodent predator within these human environments since the beginning of agriculture and grain storage, and for the first time, our results showed responses of laboratory rodents to the cat protein and major allergen Fel d 1. The absence of avoidance to this important cat protein could be partially explained by its close similarity to androgen-binding proteins (Durairaj et al., 2018), which are rodent proteins that are implied in sexual communication.

Behaviour is the final consequence of many factors, but to complete and develop the results from this thesis, other approaches will be of interest for future research. Elucidating neural pathways implied in the avoidance of ferret olfactory cues would be of interest for comparison with results published for other predatory cues (mainly cats, the best-studied model). The effects of animal age and the related pathology of animal olfactory subsystems in the avoidance of plant or predator olfactory cues are largely unknown. Our studies and related research from other authors are based on young adults due to their importance in developing new populations, but to completely understand the ecology and behavioural responses of this species, we should cover the entire life cycle including immature and aged animals. With a bioinformatics approach, we could find common areas in the already identified rodent kairomones, such as Fel d 4 and MUP13, and look for other possible predator proteins with these conserved regions through the identification of isomers and ligand-protein testing.

The consequences of stress or anxiety related to olfactory cues from predators or the presence of unhealthy food can alter other parameters that influence rodent populations: effects on the endocrine system and hormone release, reproduction, and the immune system; these indirect effects can be explored based on our results using ferrets and ethanol as olfactory cues. The research hypothesis used for our work with mice also applies for other rodent pest species, and a logical path would be tests with *Rattus norvegicus* (Norway rat) and *Rattus rattus* (black rat), which are the other two globally distributed rodent pest species.

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