Thèse en Éthologie

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École Doctorale Galilée

Olfactory detection of human cancer by ants

Détection olfactive du cancer humain par les fourmis

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Part of the journey is the end

Tony Stark, Endgame

Contents

List of figures

Preface

The chapters of this thesis constitute a set of papers ready for submission, submitted or published in peer-reviewed journals.

Publications included in this thesis:

Piqueret, B., Sandoz, J.C., d'Ettorre, P. (2019) Ants learn fast and do not forget: associative olfactory learning, memory and extinction in *Formica fusca*. *Royal Society Open Science* 6, 190778.

Included as Chapter 1. B.P., P.d.E. and J.-C.S. conceived the project and designed the experiments. B.P. performed the experiments and analysed the data with the help of P.d.E. and J.-C.S. The manuscript was written by B.P. and revised by P.d.E. and J.-C.S. Final manuscript was approved by all authors.

Piqueret, B & d'Ettorre, P. (2021) Communication in ant societies, in Kaufman, A., Call, J., & Kaufman, J. (Eds.). (2021). *The Cambridge Handbook of Animal Cognition* (Cambridge Handbooks in Psychology). Cambridge: Cambridge University Press

Included as Annexe. B.P and P.d.E discussed together on the different sections of this chapter before the start of writing. The manuscript was written by B.P. and P.d.E added inputs and corrections. Both authors approved the final manuscript.

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Piqueret, B., Sandoz, J-.C., d'Ettorre, P. (2018) Appetitive associative memory is highly resistant in *Formica fusca* ants. *International Congress of the International Union for the Study of Social Insects IUSSI*. Guaruja, Brazil.

Piqueret, B., Sandoz, J-.C., d'Ettorre, P. (2018) Appetitive associative olfactory memory is highly resistant in Formica ants. Médiation chimique dans l'environnement – Ecologie Chimique (MediatEC), GDR 3658 - CNRS. Rennes, France.

Piqueret, B., Sandoz, J-.C., d'Ettorre, P. (2019) Ants learn fast and do not forget: olfactory associative learning and memory extinction in *Formica fusca*. Behaviour 2019. Chicago, USA.

Piqueret, B., Mechta-Grigoriou, F., Bourachot, B., Sandoz, J-.C., d'Ettorre, P. (2019) Ants are able to detect the odour of cancer cells. Congress of the International Union for the Study of Social Insects IUSSI – French Section. Avignon, France.

Piqueret, B., Sandoz, J-.C., d'Ettorre, P. (2019) Olfactory detection of human cancer by ants. Annual PhD day, organised by the Doctoral School "Sciences, Technologies, Santé – Galilée". Bobigny, France.

Poster presentations:

Piqueret, B., Sandoz, J-.C., d'Ettorre, P. (2018) Associative and appetitive learning within a specie of ant (*Formica fusca*): memory and extinction. *International Student Course in Behavioural Biology* organised by the Institut Francilien d'Ethologie IFE. Villetaneuse, France.

Piqueret, B., Sandoz, J-.C., d'Ettorre, P. (2018) Are *Formica fusca* the perfect student? *Club de Neurobiologie des Invertébrés*. Versailles, France.

Piqueret, B., Mechta-Grigoriou, F., Bourachot, B., Sandoz, J-.C., d'Ettorre, P. (2019) Ants are able to detect the odour of cancer cells. International Student Course in Behavioural Biology organised by the Institut Francilien d'Ethologie IFE. Villetaneuse, France.

List of abbreviations

CS: conditioned stimulus

CS+: rewarded conditioned stimulus (appetitive solution such as sugar solution)

CS-: punished conditioned stimulus (aversive solution such as quinine or salt solution)

US: unconditioned stimulus

UR: unconditioned response

CR: conditioned response

NS: neutral stimulus

N: new stimulus

ITI: inter-trial interval

SPME: Solid Phase Micro Extraction

GC-MS: Gas Chromatography–Mass Spectrometry

PER: Proboscis Extension Reflex (or Response)

ROS: Reactive Oxygen Species

VOCs: Volatile Organic Compounds

ORCs: Olfactory receptor cells

LTM: Long-Term Memory

CHX: Cycloheximide

MaLER: *Maxilla-Labium* Extension Response

Introduction

I) Cancer, a deadly disease

The burdens and origin of this disease

According to the world health organization, the number of patients with cancer has shown a steady increase over the last decade, going from 12.7 million people in 2008 to 19.1 million in 2020 (+50 % cases detected worldwide) (Ferlay et al., 2020). Current projections estimate 30.2 million cancer cases by 2040. If we look at the number of associated deaths, 9.9 million deaths were accounted in 2020. Worldwide, cancer is the second most common cause of death, just after heart diseases. Cancer kills more than all infectious and parasitic diseases (malaria, dengue, tuberculosis, HIV…), road injuries, intentional injuries (self-arm, conflicts…), drownings and fire accidents combined (Bray et al., 2018; Jemal et al., 2011; Wild et al., 2020).

Origins and risk factors of cancers are diverse. Cancer can arise due to genetic background (Joo et al., 2018), viral infection (Olusola et al., 2019) or be environmentally caused and linked to smoking behaviour (Alexandrov et al., 2016), alcohol consumption (Connor, 2017) or sunlight exposure (Nair-Shalliker et al., 2012).

Cancer is a disease, caused by the presence of tumours inside the organism. However, one can have tumours that are not cancerous (benign tumours). Along this thesis, the term *tumour* will refer as cancerous tumour, and not as benign, unless specified.

The hallmarks of cancer

Cancer is a genetic disease in which mutations impact cell metabolism. Cancerous cells differ from normal cells according to a number of criteria. These are called the hallmarks of cancer and six were initially described (Hanahan & Weinberg, 2011).

Through mitosis, a cell can divide into two new cells. A cell can be in two distinctive states: either a quiescence state (where no replication is ongoing) or a proliferative state (the cell enters the mitosis cycle and replicates). Access to the proliferative state is tightly controlled by the organism using growth factors, and only 'healthy' cells can enter this state. However, some mutations in the genome of a cell can give rise to oncogenes (cancerous genes) that can mimic growth factors. Cancer cells can then enter in a replicative state without being dependent on exogenous growth signals and will grow exponentially (1st hallmark of cancer cells).

Other defence mechanisms are present to stop the spreading of 'sick' cells. When a cell managed to enter in the mitosis cycle, it can be stopped by antigrowth factors, which can be classified as tumour suppressor genes. However, mutations in a cell can also suppress tumour suppressor genes, and cells then become insensitive to antigrowth signals (2nd hallmark). At this stage, the cancerous cell can go through replication without exogenous control.

Another protection against mutated cells is apoptosis (controlled cell death). When a cell has undergone a certain number of replications, it will trigger the apoptosis program and destroy itself. This ensures that errors in the genome that manage to pass previous defence mechanisms stop spreading at the next cell generation. However, cancer cells are able to resist cell death by promoting anti-apoptotic signals (3rd hallmark). Cells are then becoming virtually immortal and keep multiplying.

However, after each replication, the ends of chromosomes are destroyed, and part of the DNA can be damaged. To allow cells to replicate during a certain amount of time, chromosome ends are protected with telomeres: these genome regions are composed of repeated paired bases sequences that protect the DNA from degradation. With each replication, the telomeres become shorter and, at one point, the DNA can be destroyed. When DNA is damaged, crucial genes can be lost and at this point, the shortening of the chromosomes elicits massive mortality amongst the cells. In cancerous cells, an enzyme called telomerase is highly expressed. This enzyme repairs the telomeres, maintains, and elongates them, allowing the DNA of a cell to be protected from the damage of replication and be immortal (4th hallmark).

As cancerous cells keep growing and proliferating without control, they have a critical need for resources, otherwise they would not survive and expand to the whole organism. These resources (oxygen and nutrients) are supplied by the blood through the vascular system. Angiogenesis (development of the vascular system) is needed for the supply of cancerous cells. Mutations can trigger the expression of angiogenic inducing factors (5th hallmark). Angiogenesis is useful for bringing oxygen and nutrients, but also for evacuating metabolic waste and carbon dioxide. Once in the vascular system, waste compounds can then be transported and found in the blood, exhaled air, urine, sweat or faeces of the patient (Pauling et al., 1971).

Finally, cancer cells can migrate to other organs, invade distant tissues, and form metastases, thus spreading cancer to the whole body, eventually causing the organism's death (6th hallmark).

Later, four additional and emerging characteristics of cancer cells were described, bringing the total of hallmarks to ten (Hanahan & Weinberg, 2011): genome instability and mutation, tumour promoting inflammation, evasion from immune destruction and the reprogramming of energy metabolism. Oncogenes are acquired through mutations, that are not common. However, cancer cells can increase their rate of mutations by being more sensitive to mutagenic agents or by disabling DNA control mechanisms (7th hallmarks).

When cancerous cells are present in an organism, the immune system will attempt to eradicate the spread of the disease. T lymphocytes (also called T cells) are cells of the immune system that destroy foreign cells. However, cancer cells express a high level of a transmembrane protein that stops T cells. Cancer cells can thus evade the immune system (8th hallmark).

Reactive oxygen species (ROS) are well known inflammatory agents (e.g. H_2O_2), and when they accumulated in a cell, they usually kill it by promoting apoptosis. However, when they accumulate in cancer cells, inflammatory molecules induce tumorigenesis (9th hallmark), as ROS are also mutagenic agents. Inflammation can also provide growth factor (see 1st hallmark), limit the apoptosis of cancer cells (3rd hallmark), supply angiogenic factors (5th hallmark), and facilitate metastasis (6th hallmark) (Costa et al., 2014).

Finally, inflammation can elicit a shift in cells' energetic metabolism, known as the Warburg effect (10th hallmark). To produce energy (measured as the number of ATP molecules), cells can either use aerobic or anaerobic metabolism. The first one is highly efficient, uses oxygen, and produces 36 ATP molecules from a single glucose molecule. On the other hand, anaerobic metabolism is not efficient, produces only 2 ATP molecules, but can be done without oxygen. Why are cancer cells switching to the – less efficient - anaerobic metabolism, regardless of their access to oxygen? It is because the anaerobic metabolism is 10-100 faster than the aerobic one. Cancer cells are parasitic organisms, and do not necessarily need efficient metabolism. They simply need to divide and multiply as fast as possible.

All these characteristics, and the modified metabolism of cancer cells, result in the production, consummation, and globally an alteration of the molecules present in the environment of the cells. Specific molecule signature of cancer can be used as biomarkers, to detect and cure this disease.

Prevention and early detection

To reduce the burden of cancers, three actions are possible and must be implemented together: prevention, early detection, and treatment (Figure 1).

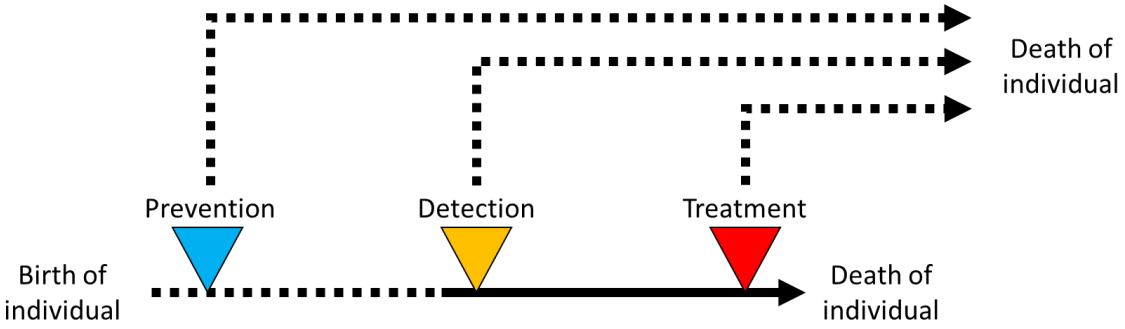

Figure 1 : Schema of the three actions possible to lower the rate of cancer related death.

The lines represent the life expectancy of an individual. Dotted lines indicate that the patient lives without cancer, while solid lines indicate that the patient lives with a tumour/cancer. To decrease the rate of people dying of cancer, we can (1 – blue triangle) prevent them when possible (e.g. vaccination or lowering the number of people smoking). It is also possible to detect tumours at an early stage (2 – yellow triangle) to remove them and/or treat them before they can spread to the rest of the body. Finally, treating the disease when symptoms are present (at a late stage of development) is the last option. Note that patients who underwent one of the three actions have a greater life expectancy than the ones that do not.

Prevention can help decrease the number of cases due to environmental causes or viral infection. Cancers related to smoking account for 25% of all cancer cases, despite the fact that it is one of the most evitable environmental exposures. However, for the passive smokers, or for the cancer with a genetic origin, screening procedures are critical to detect a potential tumour as soon as possible. Depending on the cancer, some populations are more at risk than others (e.g. the women above 50 for the breast cancer, and the men above 50 for the prostate cancer), and millions of people need to have access to efficient, rapid, and inexpensive screening procedures to ensure that the majority get screened as often as needed (e.g. every

two years for breast cancer screening). To detect tumours, surgeons can use magnetic resonance imagery (MRI). This method has the advantage of being extremely efficient for finding anomalies in the body (such as tumours), but it is extremely costly, time consuming, and cannot be used on a daily routine screening basis for whole populations (obese patients or with claustrophobia, or anyone with any metallic body parts cannot use MRI scanner).

Worldwide, breast cancer is the most prevalent type of cancer with 2.2 million cases detected in 2020 (Ferlay et al., 2020). Large screening campaigns are organized by countries for this cancer and relies on mammography. Even though this method is less expensive than MRI, not all populations have an equal access to it. For example, in Europe, Greece has 6.5 mammography units per 100.000 inhabitants, whereas France has less than 1. Furthermore, this method is invasive, and not all women at risk actually undergo this screening. This has led to a paradoxical situation in which almost half of the population at risk in rich countries (e.g. Germany and France, the two most wealthy European countries, and respectively 4th and 7th largest world economies) does not profit from this screening procedure (Figure 2).

The high cost of some early detection technics and/or the invasiveness of others are critical concerns for public health. Alternative screening methods, that are cheap, efficient, and non-invasive are urgently needed to fight against cancer.

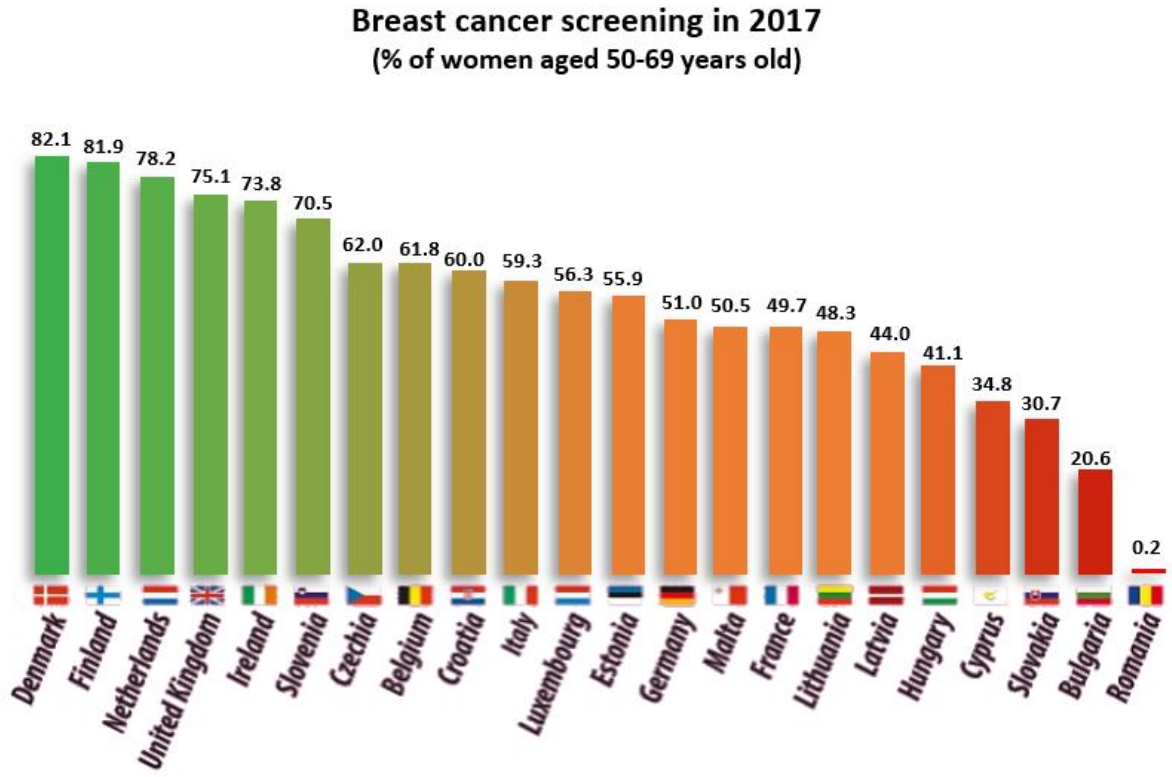

Figure 2. Breast cancer screening in the European Union

Percentage of women considered as risk (between 50 and 69) that underwent a breast cancer screening in the last two years in Europe in 2017. In some countries such as Denmark, more than 80% of this population underwent this screening. In other countries such as Romania, less than 1% of the population underwent it. This lack of screening is not directly linked to the economy of the countries. For example, in France and Germany, the two first economical European countries, half of the population at risk did not undergo screening procedure within the last two years.

II) Olfactory detection of volatile organic compounds

Olfaction is used by every species, from bacteria to vertebrates (Ache & Young, 2005). Compounds can be voluntarily emitted by individuals (such as pheromones: Wyatt, 2014), or linked to their metabolism. In order to live, organisms will consume resources and produce wastes. Linus Pauling (Nobel prize in Chemistry in 1954 and Nobel peace prize in 1962) and his team described the presence of more than 250 unique organic compounds in the breath of humans, and more than 280 in their urine (Pauling et al., 1971). This work led to the development of the field of metabolomics, defined as “the systematic identification and quantification of the small molecule metabolic products of a biological system (cell, tissue, organ, biological fluid, or organism) at a specific point in time”. Metabolic products can be linked to diseases (such as cancer) and used as appropriate biomarkers (Krilaviciute et al., 2015). Different conditions (not necessarily linked to bad health, for example pregnancy) have

different metabolomic signatures, and molecules that are specific to one condition can be used as biomarkers.

Linking biomarkers with diseases is not a novel idea *per se*. Hippocrates, an ancient Greek physician, could, solely based on a human's breath, make a diagnosis of its health condition. He described the *fetor oris* and *fetor hepaticus*. The *fetor oris*, "bad breath", was described in patients in bad general condition, whereas the *fetor hepaticus* was found in patients with hepatic diseases. Nowadays, one of the most common examples of the use of biomarkers is in the measurement of alcohol in the breath (and also in the blood) of car drivers: the breath analyser. During this test, ethanol reacts with other compounds to indicate the alcohol level.

Organic compounds such as ethanol in breath are volatile; otherwise, they will hardly be detected in expired breath. These compounds are called "Volatile Organic Compounds" (VOCs), due to their carbon basis and their volatility (vapour state) at ambient temperature. Detection of these compounds in breath to detect a modification of the patient's health is now used for the diagnosis of asthma, respiratory problems during anaesthesia, inflammations of the respiratory system or in intoxications due to carbon monoxide (reviewed in Paschke et al., 2010). Detection by use of VOCs is not restricted to breath. It is possible to use urine, faeces, saliva, sweat or blood to link VOCs to a disease (reviewed in Broza et al., 2015, Figure 3). Detection of these compounds relies on two major techniques: Gas Chromatography coupled with Mass Spectrometry (GC-MS) and the development of electronic noses ("e-noses").

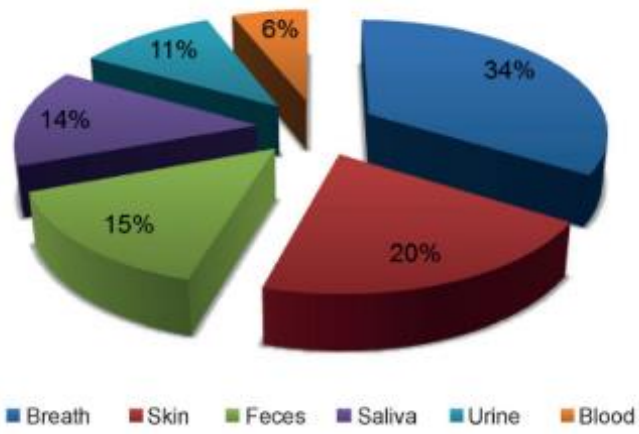

Figure 3. Percentage of VOCs detected in different products of the human and healthy body.
A total of 2577 compounds were measured with some only in specific products (e.g. 1-hexene in breath) whereas others were found in three or more (e.g. acetaldehyde is found in all fluids). From Broza, Mochalski, Ruzsanyi, Amann, & Haick, 2015.

Gas Chromatography–Mass Spectrometry (GC-MS)

During an analysis by GC-MS, the sample goes first into the gas chromatograph (GC), which separates the different compounds contained in the sample. With the help of an inert gas (helium for example), the compounds are transported along a capillary column while the temperature of the chamber in which the column is placed (oven) increased. Compounds that are more volatile reach the end of the column first, whereas less volatile compounds take more time to reach the end. Some compounds might also interact with the column, which interferes with their migration in a characteristic way, depending on the type of column. The GC thus separates chemical mixtures into their components. To identify the compounds, a mass spectrometer (MS) is coupled to the GC. Here, compounds are fragmented by ionization and, when going through an electric field, they can be analysed by their mass and charge. Each compound will give rise to a specific and unique fragmentation pattern. These characteristic patterns are included in databases, and can help identify mixture components, although the comparison with synthetic standards injected in the GC-MS is always recommended for correct identification.

One major drawback of this analytic technique is its price. It can cost up to 500.000 € for the most efficient GC-MS and the maintenance cost is also high due to the consumables and the need of highly qualified technicians.

Electronic noses or "e-nose"

The second major technique of analysis of VOCs is the use of electronic noses or e-noses. They are constituted of multiple sensors, which will react to the presented sample.

Different types of sensors are used, such as polymer or metal-based sensors, but the principle is the same. When VOCs are analysed with e-nose, they will bind to the sensors depending on their affinity. This binding will then change the resistance or conductivity of the sensors and form specific electric patterns. If a sample with abnormal VOCs is analysed, a different pattern will be detected, thus indicating an altered VOCs composition, and possibly a problematic health condition. E-noses are mostly low cost, and can provide fast results, however, the binding process can change the basal conductivity of sensors thus producing a drift effect. In

this case, sensors need to be replaced. Usually, sensors have a short lifespan. They can also be impacted by ambient humidity and temperature, and are less sensitive (higher detection threshold) than the gold standard of chemical analysis, the GC-MS.

Furthermore, by using e-nose, it is not possible to determine the exact chemical nature of the VOCs analysed. It is only possible to state that sample *A* is different from *B* due to the different patterns (review by Behera et al., 2019; Turner & Magan, 2004; Wilson, 2015). Pattern recognition is crucial here, and integration of machine learning, artificial neuronal networks, and multivariate statistics are required for the analysis (Figure 4). However, despite the exponential development of informatic solutions, it takes time to develop an automatic way to analyse patterns. Finally, for some applications such as oenology, the human nose is still more trustable and relevant than e-noses to determine the value and properties of wine (Rodríguez-Méndez et al., 2016).

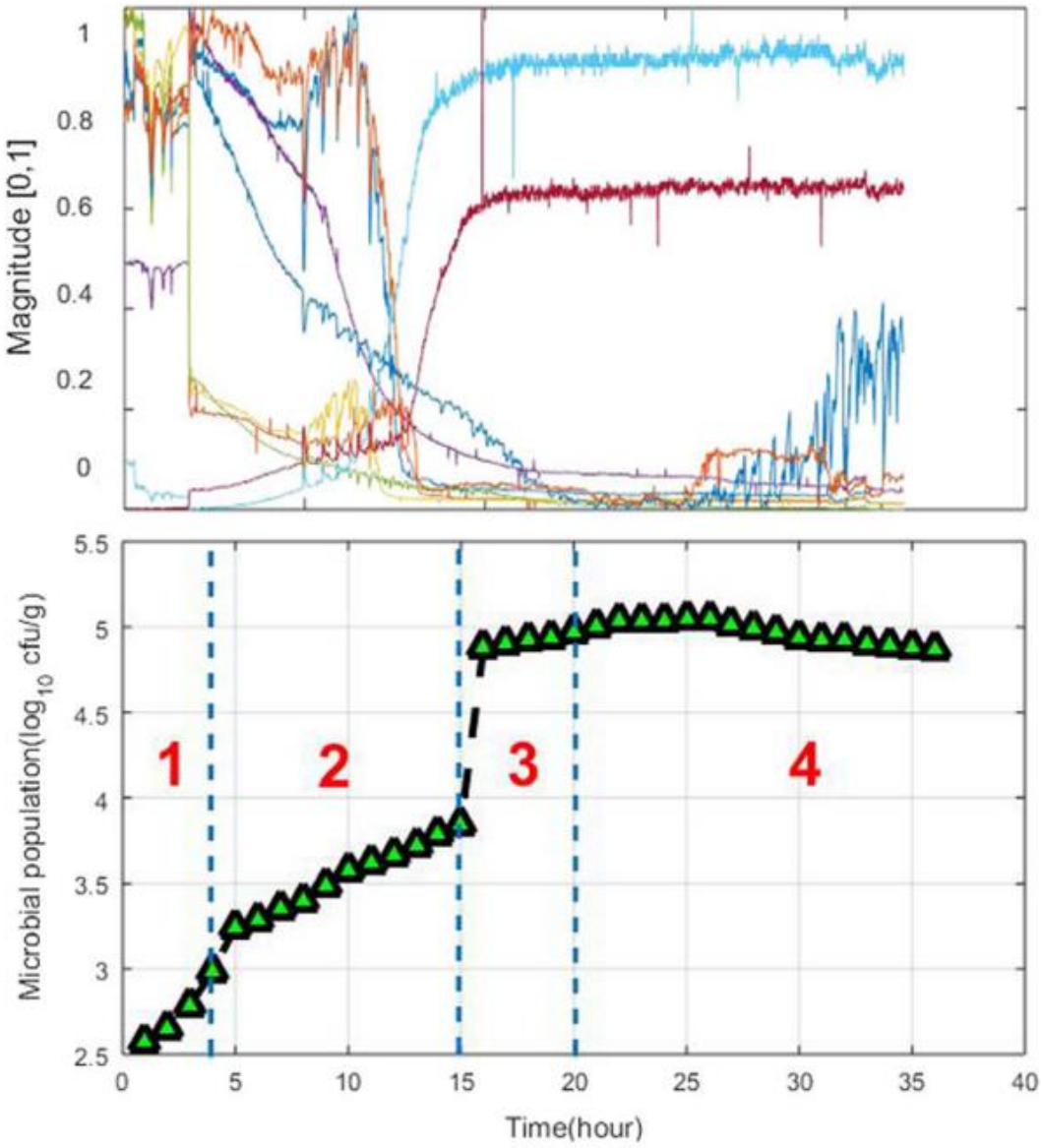

Figure 4. Example of a pattern obtained with an e-nose

A piece of fresh beef was left in a box for 36 hours at ambient temperature and monitored using an e-nose. On the upper panel, the electric activity of ten different sensors is represented using different colours. Each sensor was sensitive to one or several molecules. When the molecules were present in the room with the beef piece, the activity of the sensor was high. In parallel (bottom panel), the number of bacteria (microbial population) was measured. The piece of beef was considered as "excellent" (1), "good" (2), "acceptable" (3) or "spoiled" (4). After 20 hours, the meat was considered spoiled. In real conditions, humidity and average temperature change, thus producing noise in the response (conductivity) of each sensor. Figure obtained from Wijaya et al., 2018.

Use of animals for olfactory detection

To avoid analysis constraints, threshold detection and potentially high cost of GC-MS and e-nose, the use of animal olfaction is promising. Contrary to informatic tools that have been in development since the 1950's, animal olfaction has experienced millions of years of evolution,

tuning, setting, failures and ameliorations of its performances. Chemical communication is one of the principal communication channels used by living species, and it is also one of the most ancestral ways of communication (Wyatt, 2014). Chemical communication is found from bacteria to insects and mammals. Animal olfaction has been used as a tool by humans for thousands of years. For example, dogs (*Canis familiaris*) and humans shared a common history for at least the last 17.000 years (Vigne, 2018), and this is the most ancient form of domestication by humans. Through our common history, dogs and their smelling abilities have been used to protect humans from potential predators and for hunting.

Nowadays, dogs and their olfaction are used in various preventive actions such as drug detection (Francis et al., 2019), detection of flesh eating flies (Welch, 1990), surveillance of livestock parasites (Moser et al., 2020), human parasites (Kasstan et al., 2019), human viruses (such as COVID-19, Jendrny et al., 2020), and explosives (Furton & Myers, 2001). The detection of explosives (often landmines left after a conflict) is a major security concern and some vertebrate species, other than dogs, are used. The African giant pouched rat (*Cricetomys gambianus*) (Edwards et al., 2015) and African elephant (*Loxodonta Africana)* (Miller et al., 2015) are useful demining helpers. Invertebrates, like honeybees, moths or flies, even if not used as much as vertebrates for these specific tasks, show impressive abilities for detecting explosives (*Apis mellifera*: (Bromenshenk et al., 2003), *Manduca sexta*: (King et al., 2004), *Drosophila melanogaster:* (Marshall et al., 2010)). Parasitic wasps can also detect cocaine (*Microplitis croceipes* (Olson & Rains, 2014)). As stated at the beginning of the introduction, cancer is one of the major causes of death worldwide. Cancerous cells have an altered metabolism, and produce specific pattern of VOCs, that can be detected by olfaction. Notably, the olfactory abilities of dogs (Brooks et al., 2015; Mazzola et al., 2020; Pirrone & Albertini, 2017; Thuleau et al., 2019), honeybees (Schallschmidt et al., 2015), drosophila (Strauch et al., 2014), nematodes (Hirotsu et al., 2015), and mice (Matsumura et al., 2010) were challenged using cancerous samples.

Animal perception of odours

Olfaction is widely used by animals. For example, it is used for mating (Butenandt et al., 1959), foraging (Provecho & Josens, 2009), or recognition of nestmates (d'Ettorre & Heinze, 2005). Vertebrates and invertebrates, despite following different evolutionary paths, share common olfactory structure systems (Hildebrand & Shepherd, 1997). Olfactory compounds are

detected at the peripheral level, by olfactory appendices, such as the nasal chamber for vertebrates, and the antennae for invertebrates like insects. At the surface of these organs, olfactory compounds (such as VOCs) are detected by olfactory receptor neurons (ORNs) and elicit action potentials, that transmit the information to the central nervous system of the individual. Before entering the higher brain centers, the information will pass through glomeruli, which are the contact points between peripheric and central neurons. In insect, glomeruli are in the antennal lobe (AL), whereas in vertebrates, it is called olfactory bulb (OB). Odours will form specific patterns activation in the glomeruli, and the odour information will then be transmitted to higher integration centres, called lateral horn and mushroom bodies in insect, and amygdala in vertebrates (Figure 5).

The ORNs can be very specific, such as the ones used for pheromone detection. These ORNs project to the macroglomerular complex within the antennal lobe in insects. In vertebrates, the glomerul are called vomeronasal organ receptor cells.

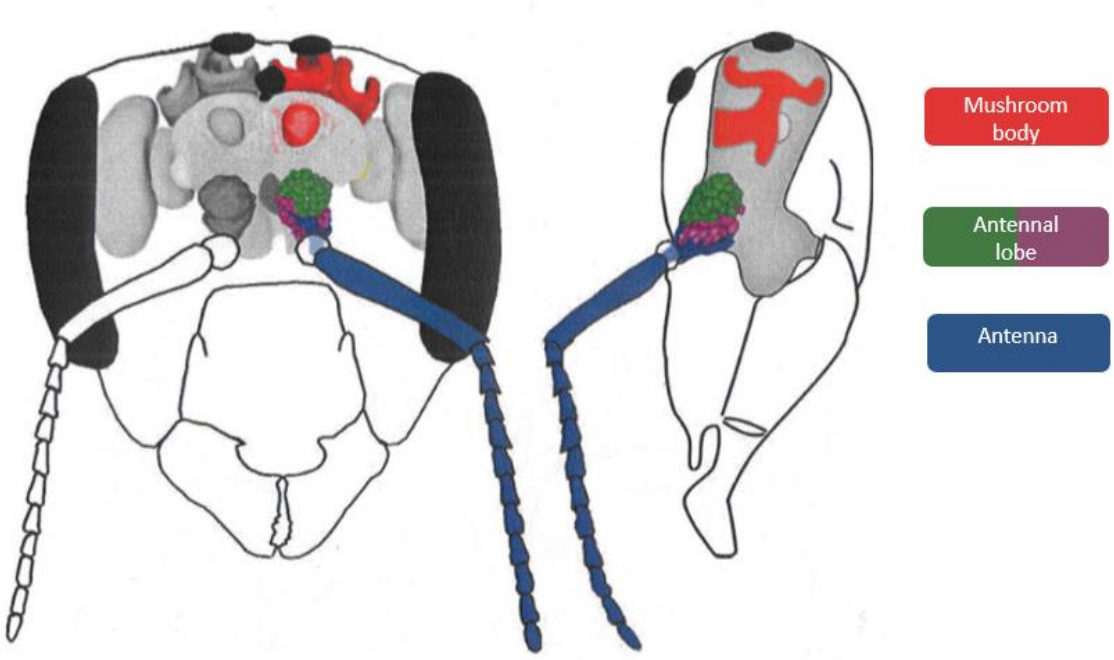

Figure 5: Brain structures of insects linked to olfaction

Overview of the honeybee olfactory system (front and side views), with the main olfactory organs and areas: the antenna (blue), antennal lobe (purple / green) and the mushroom body (red). In mammals, the anatomic equivalent of the antenna is the nasal chamber, the antennal lobe is the olfactory bulb, and the mushroom bodies are the amygdala. Adapted from Galizia & Rössler, 2010.

Depending on the stimuli perceived by the individual and its prior experience, a behavioural response could be elicited. Note that the physiological state of the individual such as its age, fertility or hunger, can impact its responses to stimuli (Anton & Rössler, 2020).

Through conditioning, it is possible for an animal to learn to respond and recognize any given odorant, even if it is not present in its natural habitat or does not have a biological role. The animal simply needs to have ORCs that are sensitive to this odorant and the concentration of molecules in the environment should be higher than the detection's threshold of the animal.

III) Associative learning

Classical conditioning

Animals live in a constantly changing environment, where a new source of food or a predator can arise at any moment. Behavioural flexibility can greatly improve the survival and reproductive success of individuals. One rapid way to adapt to these new situations is through learning. One of the simplest forms of associative learning is classical conditioning. Here, the individual learns to establish an association based on two stimuli: a first stimulus, initially neutral (that does not elicit a specific response from the individual), is paired with a second stimulus that elicits a reflex response. This learning was demonstrated by Ivan Pavlov (1849 – 1936) in 1927, who first described it in dogs, using the salivation reflex. When food is shown to a dog, it salivates. If a bell sound was associated with the food presentation several times, dogs were salivating just when hearing the bell. The sound acquired a predictive value of the reward, and the dog's behaviour was conditioned. The so-called neutral stimulus (the sound) is now a conditioned stimulus (CS) that is paired with an unconditioned stimulus (US, the food). When the CS is presented to the conditioned individual, it elicits a conditioned response (CR), the salivation (Figure 6).

However, for this conditioning to be optimal, several parameters must be considered, starting with the time elapsed between the presentation of the two stimuli (CS and US). If the neutral stimulus is presented too early before the US, then the individual will not associate both stimuli together, as the neutral stimulus will not be a good predictor of the US. Usually, the CS closely precedes the US and can partially overlap it (Giurfa & Sandoz, 2012). Paired stimuli (where CS was closely temporally paired with the US) elicit strong conditioning behaviour,

whereas unpaired stimuli (the CS and US did not have close temporal association) do not. Exception of this rule is when the US has an aversive value that, for example, induce sickness. Associating a flavour (CS) with water that is toxic (US), that will make the individual to throw up (UR) several hours later, can be used as conditioning. In this particular case, despite the long time elapsed between the CS and the outcome of the US, the animal will remember the displeasure associated and will not drink this flavoured water again (Welzl et al., 2001).

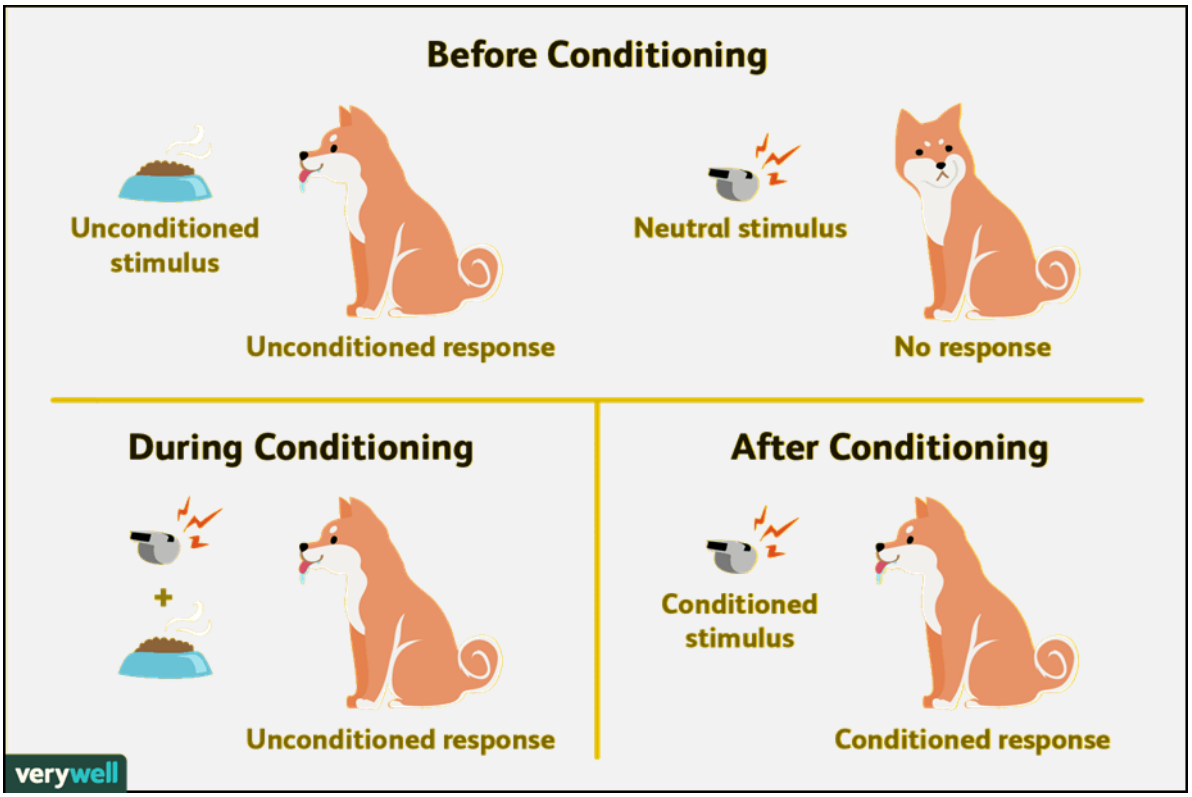

Figure 6. Canonic example of classical conditioning

Before conditioning, the unconditioned stimulus (US – food) elicits the unconditioned response (UR – salivation), while the neutral stimulus (NS – sound) does not elicit this response. During conditioning, both stimuli are presented in close temporal association. After conditioning, the presentation of only the NS elicits UR. This response is now called conditioned response (CR – salivation) and the NS is now a conditioned stimulus (CS – sound). The CS has become a predictor of the US.
Origin of picture: https://www.verywellmind.com/classical-conditioning-2794859

Depending on the species, the CS can be a visual pattern (Stach et al., 2004), colours (Buatois et al., 2017), a tactile stimulus (Scheiner et al., 2005), or odours (Bos et al., 2012). As long as the animal possesses both the relevant sensory system and the motivation for the US, it can be conditioned to it. Note that the motivation is particularly important for the conditioning, and can differs between individuals living in similar conditions (Perez et al., 2013).

Other important parameters to consider are the number of conditioning trials and the inter-trial interval (ITI). In principle, the higher the number of conditioning trials an individual underwent, the better it will remember the association. However, it seems that after a certain number of conditioning trials, some species are not as receptive as before, and learning is actually worse. For example, in honeybees, the strength of the association is positively correlated with the number of conditioning trials when they range between 1 and 6. After that, when honeybees are conditioned with 9 trials, the association is not as strong as before (Sandoz & Pham-Delègue, 2004). The ITI also has a major role in the learning process, especially for the formation of long-term memory. Multiple massed learning trials (with a short ITI) lead mostly to weak or short-term memory. Spaced learning trials (longer ITI) will produce a long-term memory more easily (reviewed in (Smolen et al., 2016)). However, some species demonstrated impressive learning abilities, and are able to remember for a long time an association (formation of a genuine long-term memory) after a single appetitive conditioning trial (*Drosophila melanogaster* (Weiglein et al., 2019) and *Apis mellifera* (Villar et al., 2020)). Conditioning can be performed using a single stimulus rewarded (food) or punished (electric shock). Here, the conditioning is called absolute. One can also use both reinforced stimuli, one is positively reinforced (CS +), and the other is negatively reinforced (or not reinforced) (CS -). In this case, the conditioning is called differential.

Operant conditioning

During operant conditioning (also called instrumental or Skinnerian conditioning in honour of Burrhus Skinner, who developed the first protocol), a reward (or punishment) is associated with a voluntary movement of the individual (Skinner, 1938). An animal left in a cage with a lever will randomly push the lever after a certain time. This voluntary movement will give food to the animal and, after several associations, the individual will learn that pushing the lever gives food. It is also possible to use a neutral stimulus, such as a light source, during operant conditioning. When the food is not available (pushing the lever does not give reward), the light is off. However, when the food is available, the light is on. The animal will then associate the neutral stimulus (light) to the food and will push the lever only when neutral stimulus is present. This neutral stimulus is now a conditioned stimulus. The major difference between operant and classical conditioning is that, for classical conditioning, the individual is passive

and just waits for the reward whereas, in the operant conditioning protocol, the individual must actively find the reward.

Often, learning paradigms are hard to categorize in a single conditioning procedure and, in some cases, researchers are not sure whether it is more of a classical conditioning approach or an operant conditioning (Dupuy et al., 2006). Here, they used a Y-maze and odours for conditioning ants. Individuals had to choose the right arm (with a reward) after the learning trials. This study can be classified as classical (odour is directly associated with the reward) or operant (the odour drives the choice of the right arm). However, it is not a major problem, as the parameters underlying both types of conditioning are similar, such as the ITI, the strength and value of the US and the time elapsed between the CS and the US.

Extinction and spontaneous recovery

Once the association between CS and US is established, if the CS occurs several times without being followed by the US, the association will start to fade because the CS is not seen as a reliable predictor of the outcome anymore. During his experiments, Pavlov found that the CS (sound of a bell) stopped eliciting the CR (salivation) when repeated too often without being paired with the US (food). Thus, this conditioned response had undergone an extinction procedure. However, this response is not extincted or erased from the memory. During the extinction procedure, a new association (CS - no US) takes place. The response of the animal will depend on the strength of both associations. If the first one (CS - US) is stronger than the late association (CS - no US), the individual will respond to the CS. However, with more extinction trials, the CS - no US association will gain strength and overcome the CS - US association. Here, the individual will stop responding to the CS that is no more a reliable predictor of the first learnt outcome.

As the CS - US association is not completely erased, if we give time to the individual after the extinction protocol, we might observe spontaneous recovery of the learnt response. This spontaneous recovery usually corresponds to a decay of the CS - no US memory, more labile than the CS - US memory (Rescorla, 2004). For example, bees that were trained to respond to an odour/reward association after one conditioning trial, shown ~90% of correct responses during the first extinction trial, performed one hour later (Sandoz & Pham-Delègue, 2004).

However, for the second extinction trial, their performance dropped to ~50%, and after the fifth one, they shown almost no response (~10%). Here, the CS – no US memory was stronger than the CS – US memory. Bees predicted that the odour will not be followed by reward, and did not express the conditioned behaviour. When tested for spontaneous recovery one hour after the extinction procedure, they shown ~60% of correct response. Here, the second learnt association (more labile) was losing strength, and, for the bees, the learnt odour was now predicting the reward. Interestingly, when bees were left alone for one hour and received a new conditioning trial after the extinction procedure, they shown very high spontaneous recovery. This response is not really a spontaneous recovery, but more a reminded memory, as a conditioning trial was done before. Nevertheless, the underlying mechanisms of extinction and spontaneous recovery / reminded memory are critical if one want to establish a reliable bio-detector based on conditioning.

Learning in invertebrates

Learning abilities are found in extremely diverse organisms, such as plants (*Mimosa pudica* (Abramson & Chicas-Mosier, 2016)) or unicellular organisms (without neurons) (*Physarum polycephalum* (Vogel & Dussutour, 2016)) but learning has been studied mostly in animals. Dozens of species have been studied, and vertebrates such as mammals (elephant (Miller et al., 2015), rodents (Armstrong et al., 2006) and dogs (Furton & Myers, 2001)), amphibians (*Dendrobates auratus*, (Liu et al., 2016)) and fish (*Danio rerio*, (Colwill et al., 2005)) demonstrated learning abilities. These abilities were not only found in « higher » vertebrates, but also in invertebrates like in a sea slug species, *Aplysia californica* (Sutton et al., 2002) where learning was demonstrated 50 years ago.

Following recent data and projections on the number of species that live on Earth, insects account for an important part of biodiversity (Mora et al., 2011). Studies on learning abilities in insect species are relatively recent and few species are represented, compared to the total number of insect species. However, for laboratory studies, insects are good model systems, as they can be easily collected in the field, if not, they cost very little to obtain and maintain, they can be reared easily, they are small and do not require large animal facilities, but they are big enough to study their organs. As such, they can be reared up to large numbers and hundreds of individuals can be conditioned at a time, when it is almost impossible to have this

number of individuals when using any other species. At least one hundred individuals were individually conditioned in different studies with bees (Cholé et al., 2019; Wang et al., 2016), ants (Desmedt et al., 2017; Perez et al., 2016), fruit flies (Weiglein et al., 2019), crickets (Mizunami et al., 2019), cockroaches (Sakura & Mizunami, 2001), and moths (Daly & Smith, 2000).

In the last decades, two insect species have become popular as model organisms for learning and memory research. Due to the ease of rearing and the advanced genetic knowledges, the fruit fly (*Drosophila melanogaster*) is now a major model used for learning protocols. Thanks to the genetic tools available and an easy access to the brain structures, it is possible to precisely characterize the biological bases of learning and how memory is formed and retained (Krashes & Waddell, 2008; Schwärzel & Müller, 2006; Tempel et al., 1983; Tully et al., 1994). The second model species in insects is the honeybee (*Apis mellifera*) that shows excellent learning abilities. One of the most simple and powerful conditioning procedures to implement in bees is the Proboscis Extension Response (PER). This protocol was first developed by Takeda in 1961 and later improved by Bitterman and its colleagues (1983). During this conditioning protocol, bees are restrained and can only move their head and antennae. An odorant (NS) is presented to the antennae of the bee and then, a sucrose solution (US) is presented to the antennae and to the proboscis, thus eliciting the PER. After conditioning, the presentation of the odorant (CS) alone elicits the PER. This technique puts the animal in a non-ecological and non-natural situation to respond to the CS but it allows for a strict control on the duration of presentation of the CS and the US and allows standardization of the ITI. Bees can also be conditioned using aversive stimulus, such as an electric shock or heat. Instead of triggering PER, the aversive stimulus will elicit the Sting Extension Reflex (SER, Vergoz et al., 2007).

One of the reasons honeybees became a model species is linked to their ecology. Bees can be solitary or eusocial species. They can form hive of thousands of individuals, with a high relatedness between individuals (as only a single bee, the queen, is producing offsprings) and similar environmental conditions. In eusociality, individuals cooperate for taking care of the youngs, several generations live together at the same time, and not all individuals reproduce. On earth, few species have achieved this true social way of life, and most of them are insect species. Among them, ants account for half of the insect biomass (up to 25% of all the terrestrial animal biomass in tropical forest) (Holldobler & Wilson, 1990; Schultz, 2000).

IV) The ants

Charles Darwin once wrote "*(…) the brain of an ant is one of the most marvellous atoms of matter in the world, perhaps more so than the brain of a man*" (Darwin, 1871). Darwin was not the first to be impressed and curious about ants. In ancient Greek mythology, Zeus created an army of obedient soldiers that fought under Achilles' commandment during the Trojan war. The soldiers, called myrmidons, came from an ant colony. Modern stories also depicted ants as amazing species, that can give incredible power to the ones that managed to understand them, such as Ant-man, a comic book super-hero. The place ants occupy in our culture, is strongly linked to their ecological success. Ants are present all over the world, in (almost) all terrestrial ecosystems (apart in extremely cold environment such as Antarctica or Greenland). Among the 14.000 described ant species, all of them are eusocial species. Their societies can be composed of up to hundreds of thousands of individuals. As they live in close communities, an excellent communication between individuals is critical to maintain the social group. In this domain, ants have access to different well-developed channels of communication. Ants can use tactile, visual, and acoustic communication (more details in Annexe – Communication in ant societies - Piqueret & d'Ettorre, 2021), but their use of chemical communication is not matched by the previous cited channels.

Ants used the olfaction for almost all situations in their life and have access to a remarkably large clade of odorant receptors (d'Ettorre, 2016). Olfaction is involved during individual recognition of affiliated group members (van Zweden & d'Ettorre, 2010), reproduction (Walter et al., 1993), foraging (David, 2009), alarm and enemy defence (Blum, 1985), or worker policing (Monnin et al., 2002). Using pheromones, ants may also modulate their nestmate recognition (Rossi et al., 2018) and learning behaviour (Rossi et al., 2020).

Despite the critical importance of olfaction for ants, few species were tested for their olfactory learning abilities. Recently, important effort was provided to gather information on this aspect of life of ants. Notably, two different learning protocol were performed. Ants were either tested when harnessed or using a free-walking paradigm.

Harnessed ants protocols are derived from the PER protocol used in honeybees (Bitterman et al., 1983). Here, ants are restrained in a holder, and only their antennae and mouthparts are free to move. The first protocol developed for harnessed ants was tested on *Camponotus*

aethiops (Guerrieri & d'Ettorre, 2010). Using a differential conditioning, ants had to associate a positively reinforced odour (CS +) to a sugar solution (US +) and a negatively reinforced odour (CS -) to quinine (US -) (see *Associative learning* part of this introduction). When the CS + was presented to ants, they expressed a behavioural response similar to the Proboscis Extension Reflex (PER): the *Maxilla-Labium* Extension Reflex (MaLER). Using the same protocol, *Camponotus fellah* ants demonstrated impressive learning abilities after twelve conditioning trials (six CS + and six CS -) where they could retain a learnt association after 72h (Guerrieri et al., 2011). The memory formed after the conditioning was a genuine long-term memory, which was proven using protein synthesis blocker (as a *de novo* protein synthesis is mandatory for genuine long-term memory (Menzel, 2001)). Absolute conditioning is also possible and produces high correct responses (Perez et al., 2015a, 2016). The MaLER protocol has the advantage of eliciting a simple binomial response (presence or absence of the behaviour), and the ITI can easily be controlled and changed. However, this situation is not natural and ecologically relevant for ants.

The utilisation of free walking paradigms has the advantage of putting the ants in a more natural situation. Using a differential Y-maze arena, Dupuy et al., (2006) studied the olfactory learning of *C. fellah* and *Camponotus mus*. After a series of 24 conditioning trials, ants were able to discriminate the positively reinforced odour from the other one minutes after the end of the conditioning. Later, using the same paradigm, *C. fellah* demonstrated the ability to build a memory that last up to 72h after the conditioning (Josens et al., 2009). Free walking paradigms can also be studied with circular arena. Using cuticular hydrocarbons profiles (or single hydrocarbon) as CS, *C. aethiops* preferred to spend more time near the CS odour during the memory tests than near a new odour (Bos et al., 2010, 2012). To date, *Camponotus* ants were the most studied genus of ants in individual olfactory learning. Workers in this genus are relatively large, and when they were first tested, they showed good learning abilities. Recently, species from other gena were tested, such as *Formica fusca* (see **Chapter 1**, Piqueret et al., 2019), *Lasius niger* (Czaczkes & Kumar, 2020; Oberhauser et al., 2019) and *Linepithema humile* (Rossi et al., 2020). *F. fusca* and *L. niger* demonstrated the ability to retain a CS - US association after a single conditioning trial.

In this thesis, we tested the individual olfactory learning abilities of *Formica fusca* ants (Figure 7). These ants are common in the northern hemisphere, and can be found in Europe, North

America, and also in Asia, including Japan (*Ant Web - Formica fusca*). This species is either monogynous (presence of a single queen) or polygynous (presence of several queens). Mature colonies can count thousands of workers that express a weak polymorphism. They are mostly found in forests, near dead trees, under moss or rocks, just below the surface. *F. fusca* are colonists and are dominant in the first years in a new environment. However, their population density decreases with years, when other competitors colonize the environment (Vepsalainen et al., 2000). Aggression behaviour (Wallis, 1963, 1964), worker policing and nestmate recognition (Helanterä & Sundström, 2007), and kin recognition (El-Showk et al., 2010) studies were performed on this species. Species of the genus *Formica* are considered as ones of the most advanced from a cognitive point-of-view in Hymenoptera and, in some tasks (such as maze learning), performed as well as dogs or mice (Reznikova, 2008). However, the individual learning abilities of *F. fusca* were not tested before this thesis.

Figure 7. Picture of *Formica fusca* ant
A *Formica fusca* worker is drinking a sugar solution. Note that the gaster (abdomen) of the individual is distended due to the ingestion of the solution. When this ant will be back to the colony, she will perform trophallaxis, and exchange food, but also odorant cues and hydrocarbons to nestmates. Photo by B. Piqueret.

Objectives of the thesis

The main objective of this research project is to test whether ants can be used as a bio-detectors tool of human cancers. For this, we aimed at developing a protocol that is efficient, fast, easy to implement and non-invasive for patients.

The first chapter focused on the learning abilities of our study species, the ant *Formica fusca*. We first tested if individuals from this species could be conditioned using single compound odorant (floral odours) and a free-walking paradigm. As this species was able to learn a stimulus-reward association, we investigated the limit of our conditioning protocol. To do so, we progressively reduced the number of conditioning trials to the minimum. The persistence of the memory was then investigated. First, we tested the maximum time ants could retained a learned information after the conditioning, by testing it at different time points. Later, using pharmacological approach, we tested if this memory was a genuine long-term memory or not. Finally, we explored the resistance of the memory using extinction protocol. **All these experiments allowed us to precisely quantify the olfactory learning abilities of this species, which are remarkable.**

In the second chapter, we tested the olfactory abilities of ants using cancer-related odours. Here, we used different cultured human cell lines (cancerous and non-cancerous) to test the olfactory discrimination of ants in a *real* screening situation. Using a *strong* conditioning (6 trials), we first examined if ants could detect the presence of cells in a sample using the culture medium alone as control. As this first protocol needed fresh cell lines and was time consuming, we looked for optimisations by testing the learning abilities of ants using frozen odour samples and a shorter conditioning. **Once the conditioning protocol was enhanced, we tested if ants could discriminate a cancerous cell line from a non-cancerous one. Finally, we investigated if ants could discriminate a cancerous cell line from another cancerous one.** The results of the behavioural experiments were also supported by the analyses of the composition of volatile organic compounds (VOCs) released by cell lines using solid-phase micro extraction (SPME) and gas-chromatography coupled with mass-spectrometry (GC-MS). The simple protocol developed in this chapter can be implemented everywhere, by anyone after few days of training, and does not require specific safety rules, as no cancerous human living cells are needed for the conditioning.

Finally, once the conditioning and the learning abilities of ants in a *real* screening situation were investigated, we challenged ants using whole organisms with tumours as a source of odours for the conditioning. Human tumours were graft in mice to reproduce the conditions in the human body. As we wanted to develop a non-invasive protocol for patients, we choose to use the urine as a source of odours for the conditioning. This allowed us to have access to a relatively important and reachable source of odours that can easily be frozen before the screening without alteration of its molecule composition. Ants were tested using the odours of mice before and after the graft, to see if they could detect the presence of the tumour. Interestingly, ants could learn to discriminate between the two odours only when trained with cancer-free samples. Using chemical analysis, we find evidences that the odour after the graft was less salient than the odour before, which could explain this learning asymmetry, observed after a short conditioning. **In this part of our research, we highlighted the potential of ants as a rapid, inexpensive, efficient, and non-invasive tool for cancer screening.**

Chapter 1:

Ants learn fast and do not forget: associative olfactory learning, memory and extinction in *Formica fusca*

Ants learn fast and do not forget: associative olfactory learning, memory and extinction in *Formica fusca*

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Abstract:

Learning is a widespread phenomenon that allows behavioural flexibility when individuals face new situations. However, learned information may lose its value over time. If such a memory endures, it can be deleterious to individuals. The process of extinction allows memory updating when the initial information is not relevant anymore. Extinction is widespread among animals, including humans. We investigated associative appetitive learning in an ant species that is widely distributed in the Northern hemisphere, *Formica fusca*. We studied acquisition and memory between one hour and one week after conditioning, as well as the extinction process. Ants learn very rapidly, their memory lasts up to three days, decreases slowly over time and is highly resistant to extinction, even after a single conditioning trial. Using a pharmacological approach, we show that this single-trial memory critically depends on protein synthesis (long-term memory). These results indicate that individual ant workers of *F. fusca* show remarkable learning and memory performances. Intriguingly, they also show a strong resistance to updating learned associations. Resistance to extinction may be advantageous when the environment is stochastic and individuals need to switch often from one learned task to another.

Key-words: conditioning, learning and memory, olfaction, protein synthesis inhibitor, social insects

Abbreviations: US – Unconditioned stimulus, CS – Conditioned Stimulus, N - Novel odorant, CHX – Cycloheximide, LTM – Long-term memory

Introduction

Behavioural flexibility offers significant fitness advantages especially in environments where resource distribution or threats are characterized by stochasticity. One way to achieve this flexibility is via learning, defined as a change in behaviour occurring as a result of experience. Many learned behaviours can be further modified to suit changing environmental conditions. The ability to learn and memorize allows animals to respond to environmental stimuli in an adaptive way, for instance by either ignoring them or by giving them a specific value, positive or negative. This helps in predicting the environment when facing new but similar situations (Dukas, 2004).

Storing information is costly, therefore only essential pieces of information should remain available for the individual. For instance, with time, a stimulus which used to predict a certain resource in the environment (e.g. the presence of food), might lose its significance and be no longer associated with the resource. It is beneficial to learn rapidly that such a stimulus is not reliable anymore. Extinction is the process in which a conditioned response gradually decreases through repeated experience with the stimulus in the absence of its outcome. Extinction generally involves the formation of a new inhibitory memory rather than the destruction of the previous memory (Eisenhardt & Menzel, 2007). Knowledge about the extinction process has important clinical applications, for instance for the treatment of drugs addiction and abnormal fear of a past event (e.g. war trauma) in humans (Luo et al., 2015).

The extinction phenomenon was first described by Pavlov in 1927 in experiments with dogs using classical conditioning, the association of an unconditioned stimulus (US, for example a reward) with an initially neutral stimulus that becomes a conditioned stimulus (CS) producing the response in absence of the US. After a successful conditioning (CS - US association), Pavlov observed the conditioned responses stopped after a few unrewarded CS presentations,

leading to the extinction of the conditioned behaviour. Extinction does not erase the old memory. It is rather a new learning (creation of a CS – no US association). Therefore, two memories coexist. When time passes after successful extinction, the original behaviour may reappear (called spontaneous recovery or relapse), through a decay of the extinction memory (Eisenhardt & Menzel, 2007).

Associative learning and extinction are widespread in the animal kingdom and have been intensively studied in several vertebrate species such as mice (Myers et al., 2006) or zebrafish (Williams et al., 2002) and also in invertebrates species such as snails (Alexander et al., 1984), crabs (Hepp et al., 2010) or nematodes (Nishijima & Maruyama, 2017). Among invertebrates, insects like fruit flies became key model species for learning and memory (Chabaud et al., 2009; Isabel et al., 2004; Krashes & Waddell, 2008). Insects are well suited for laboratory studies because they are relatively easy to keep, they have short reproductive cycles and offer easy access to brain structures (e.g. crickets, Matsumoto & Mizunami, 2000). Learning and extinction have also been investigated in social insects, including bumblebees (Leadbeater & Chittka, 2007; Loukola et al., 2017) and honeybees (Eisenhardt & Menzel, 2007; Sandoz & Pham-Delègue, 2004). Among social insects, ants are the most diverse group with more than 14.000 described species, which represent up to 25% of the total animal biomass on earth (Schultz, 2000). Visual learning in ants has been intensively studied, also at the individual level, in the context of spatial orientation and navigation (Collett & Collett, 2002; Freas & Schultheiss, 2018; Narendra et al., 2007). Individual olfactory learning has been less investigated (Bos et al., 2012; di Mauro et al., 2015; Dupuy et al., 2006; Guerrieri et al., 2011; Guerrieri & d'Ettorre, 2008; Oberhauser et al., 2019; Perez et al., 2016; van Wilgenburg et al., 2012). Carpenter ants are very efficient in discriminating between different odorants (Guerrieri & d'Ettorre, 2008; Perez et al., 2016) and even between different concentrations of the same compound (di Mauro et al., 2015). Recently, workers of *Lasius niger* were shown to be able to learn odour-reward associations after only one training trial, while more trials were required when using spatial cues instead of odours (Oberhauser et al., 2019). However, in this study the dynamics of memory formation was not investigated. We know that individual ants can form long-term olfactory memories after six CS-US presentations (Guerrieri et al., 2011), but whether fewer conditioning trials lead to LTM is unclear. Furthermore, data about extinction of olfactory learned associations are very scarce in ants (Josens et al., 2009).

In the present work, we present the results of a laboratory study on individual associative olfactory learning, memory and extinction in the ant *Formica fusca*. Among ants, the genus Formica was described as one of the most advanced from a cognitive point-of-view (especially concerning communication and learning) (Reznikova, 2008). *F. fusca* is widely distributed and lives in a variety of environments with a large range of temperatures, resources, predators and competitors. Colonies are populous (hundreds of individuals) and grow well in laboratory conditions. We investigated the acquisition performance of individual ants by changing the number of conditioning trials (from one to six). We tested ants' memory abilities by subjecting them to a memory test between one hour and one week after training. We then categorised the memory using a pharmacological approach by administrating a protein-synthesis inhibitor. Finally, we studied the extinction phenomenon in individual ants, by measuring their behaviour after unrewarded presentations of the CS.

Materials & Methods

Insects and origin of colonies:

Formica fusca is a relatively common ant species found in the Northern hemisphere. This species can be monogynous or polygynous and colonies may contain several hundred individuals (Seifert, 1996). Nine queenright colonies were collected in the forest of Ermenonville (France, 49°09'51.5" N, 2°36'49.2" E) in September 2013 (n = 5) and 2017 (n = 4) and kept under laboratory conditions (25 ± 2°C, 50 ± 10% relative humidity, 12 h/12 h: day/night). Tested ants were foragers and were coloured on the abdomen or thorax using oil-based paint. Each ant was used only once in the conditioning and testing procedure.

Odorant stimuli

Hexanal and 1-octanol (Sigma Aldrich, respectively U.S.A and Germany, purity > 99%) were used as conditioned stimuli. These compounds are found in floral emissions (Knudsen et al., 2006) and therefore may be ecologically relevant for *F. fusca* ants who feed on extra-floral nectar. Ants did not show a spontaneous preference for any odorant (details in Supplementary Materials).

Experimental protocol

i) Acquisition

Our protocol is a modified version of that used by Bos et al. (2012) to study perceptual similarity among cuticular hydrocarbons. In absolute olfactory conditioning, a single initially neutral odorant (Conditioned Stimulus - CS) is associated with a reward (Unconditioned Stimulus - US). For the conditioning trials, the ant was placed in the centre of a circular arena (Ø = 12 cm, height = 3.5 cm) with clean filter paper at the bottom. The arena had two holes in the wall facing each other. Eppendorf tubes were inserted into the holes with their openings towards the centre of the arena. The tube presenting the CS contained a piece of filter paper (1 cm^2) soaked with 1 µL of hexanal or 1-octanol, while the other tube contained a clean piece of filter paper (this tube without odour represents the control for visual and tactile cues). On each side, a cotton plug allowed passive diffusion of the odorant when present but prevented the ants from entering in direct contact with the filter papers. Small plastic discs (Ø = 6 mm) placed at 1 cm in front of each tube received 1 µL of sugar solution (30% w/w) on the CS side and 1 µL of distilled water on the other side (Fig. 1A). Due to the presence of these two drops of liquid, the two stimulus sides were visually indistinguishable. The time needed by the ant to find the sugar solution (US) was recorded during each conditioning trial. The ant was allowed to drink the drop of sugar solution and was then returned to the colony, where the ant could perform trophallaxis. During the trophallaxis, ants exchange the sugar solution drunk during the conditioning trial with nestmates. Without trophallaxis, the crop of tested ants would be full in only a few conditioning trials and the ants would not be motivated to find more food. Trophallaxis thus ensures high and stable motivation for the tested ants. Tested ants were left for about three minutes in the colony (inter-trial interval, 186 ± 18 s, mean ± standard deviation), during which they terminated trophallaxis and came back to the foraging arena. During this interval, the filter paper at the bottom of the arena and the plastic discs were replaced with clean ones to avoid the use of any possible chemical cue left by the ant. The orientation of the arena and the position of the experimenter were also modified between trials to limit the possible use of visual or other spatial cues. Three independent groups of ants were subjected to one, three or six conditioning trials (Fig. S1).

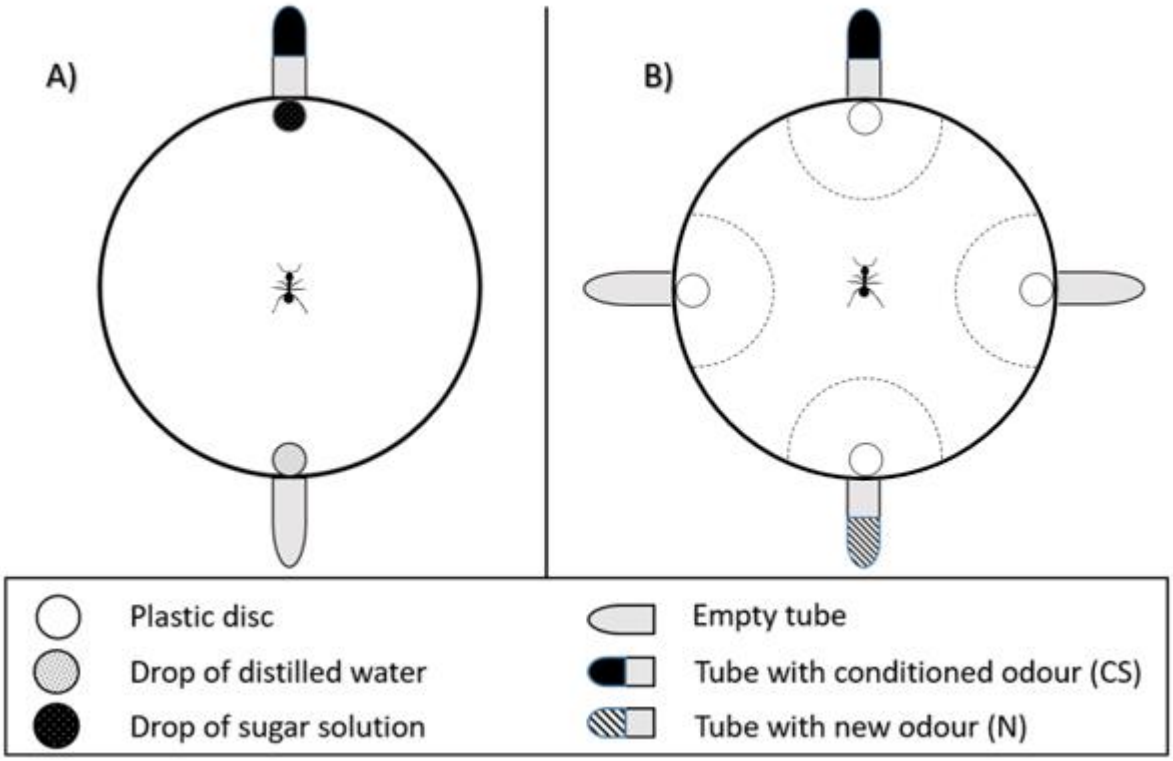

Figure 1: Schema of the experimental device (the arena). During conditioning, the setup on the left was used (A). During memory test and extinction trials, the setup on the right was used (B). The time spent by the ant in the areas around the discs was recorded during two minutes. Each area measured 35.5 cm² (dashed lines). The orientation of the arena in the experimental room was changed between trials so that ants could not learn spatial cues.

ii) Memory tests

For the unrewarded memory tests, we used an arena with four holes in the wall (instead of two), which were connected to four Eppendorf tubes. Four empty plastic discs were placed in front of them. There were two tubes with odorants (the CS and the novel odour, N, never encountered during conditioning) and two tubes without odorants (Fig. 1B). For ants conditioned with hexanal, 1-octanol was used as N, and conversely for ants conditioned to 1-octanol, hexanal was N. A circular area was drawn around each tube which allowed us to record the time spent by the ant in the vicinity of each odorant for two minutes with the software Ethoc (v1.2, CRCA, Toulouse, France). Independent groups of ants were subjected to the memory test 1 h, 24 h, 72 h or 168 h after conditioning. Each ant underwent only one memory test.

iii) Extinction

The extinction protocol was performed with the same arena as the memory test and started 1 h after the end of the conditioning. Each extinction trial was similar to the unrewarded memory test: the ant had the choice between CS and N for two minutes. All ants underwent at least six extinction trials. In the case where we tested the resistance to extinction after one conditioning trial, a sub-group of ants underwent six additional extinction trials, for a total of 12 extinction trials. Between two trials, each ant was returned to the colony for three minutes (inter-trial-interval). During this time, the filter paper and plastics discs of the arena were replaced with new ones. Spontaneous recovery was tested 24 h after the end of the extinction protocol, by performing a last unrewarded test (Bouton, 2004). After this test, we offered each ant a drop of sugar solution to check whether they were motivated to feed. All ants (N = 88) but one drank the sugar solution. The one that did not was discarded from the analyses.

Pharmacological treatment

Memory is divided in different categories depending on its duration and the molecular cascades it involves. For example, a long-lasting memory involving de novo protein synthesis will be qualified as long-term memory (LTM) (Krashes & Waddell, 2008; Menzel, 2001). To test if ants' olfactory memory depends on protein synthesis, additional groups of ants were given cycloheximide, a protein-synthesis (translation) inhibitor (CHX, Sigma Aldrich, U.S.A, purity > 99%). To prevent the drug from spreading in the colony, we created sub-colonies consisting of groups of 40 ants in a nest box with five or six larvae. A maximum of ten of these 40 ants were tested. Each experimental ant was individually confined in a small cylinder placed inside the sub-colony and received either 1 µL of sugar solution (30% w/w) containing 1 µg of CHX (treatment) or 1 µL of sugar solution (control), similarly to Guerrieri et al. (2011). After two hours, the ant was released, allowing interaction with nestmates. One hour later, therefore 3 hours after receiving the treatment, the experimental ant was subjected to one conditioning trial and was then placed back into its sub-colony until the memory test. This memory test was performed either 1 h or 72 h after the end of conditioning. We verified that CHX did not affect ants' health (Supplementary materials and table S4).

In total, for all experiments, 496 individual ants were conditioned, of which 467 (94%) underwent a memory test or an extinction protocol. Twenty-nine ants were excluded because

they took too long to find the sugar solution during conditioning (more than 10 min for the first trial or more than 2 min for the following ones), they did not drink the CHX or control solution, or they died between conditioning and test procedures.

Statistics

Data were analysed using R software (v 3.5.2, R Core Team, 2018). Significance was fixed at $\alpha = 5$ %. All data were analysed using Linear Mixed Models (LMM, package "lme4", Bates et al., 2015), details in Supplementary materials. Post-hoc differences were observed by using LMMs with reduced dataset and the alpha level was adjusted using Holm-Bonferroni Correction (Holm, 1979).

i) Acquisition

We analysed the effects of two independent (predictor) variables: *conditioning odorant* (factor with 2 levels, hexanal or 1-octanol) and the number of conditioning trials (continuous variable up to 6, named *trials*) on the dependent variable *time* (continuous variable, the time before finding the reward). We looked at the interaction *conditioning odorant* × *trials* to detect possible differences in ants' responses depending on the odorant used.

ii) Memory tests

We analysed the effects of four independent variables: *stimulus* (factor with 2 levels, CS and N), the time elapsed since conditioning (factor with 4 levels, 1h, 24h, 72h and 168h, called *elapsed time*), the number of conditioning trials (factor with 2 levels, 1 or 6 conditioning trials, called *conditioning groups*) and the conditioning odorant on the dependent variable *time* (continuous variable, the time spent in the vicinity of a stimulus). We used post-hoc tests to see whether the time spent in the CS or N area varies according to the time elapsed since conditioning. We tested whether ants spent more time in the CS or N area in function of the elapsed time since the conditioning.

For the memory tests performed in the pharmacological experiment (CHX), we tested if ants spent more time in the CS or N area 1h or 72h after the conditioning trial as a function of treatment (CHX or control).

iii) Extinction

We analysed the effects of four independent variables: *stimulus*, the number of conditioning trials (factor with 3 levels, 1, 3 or 6 conditioning trials, called *conditioning groups*), the number of extinction trials (continuous variable from 1 to 12, called *extinction trials*) and the conditioning odorant on the dependent variable *time* (continuous variable, the time spent in the vicinity of a stimulus). At each extinction (or spontaneous recovery) trial, we tested if ants spent more time in the CS or in the N area.

Results

Acquisition

During the acquisition phase, the time spent by ants to find the reward decreased significantly over trials (LMM: $F = 72.45$, $df = 5$, $p < 0.001$) (Fig. S2). The observed decrease in the time to find the reward suggests that ants have associated the odorant (CS) with the reward (US). To validate that learning occurred, ants underwent memory tests without reward.

Memory tests

The number of conditioning trials (one or six) did not influence the performance (time spent near the CS) of the ants in the memory tests, as indicated by the non-significant interaction *stimulus* × *conditioning groups* ($F = 0.52$, $df = 1$, $p > 0.05$). However, the time elapsed since conditioning had a significant effect on ants' behaviour (Fig. 2), as indicated by the significant interaction *stimulus* × *elapsed time* ($F = 7.89$, $df = 3$, $p < 0.001$). Post-hoc tests showed that the time spent in the stimuli areas was not significantly different when memory tests were performed 1h or 24h after conditioning (*stimulus* × *elapsed time*, $p > 0.1$). A tendency was observed between 72h and 168h ($p = 0.069$): ants performed better, *i.e.*, spent more time in the CS area, at 72h than at 168h.

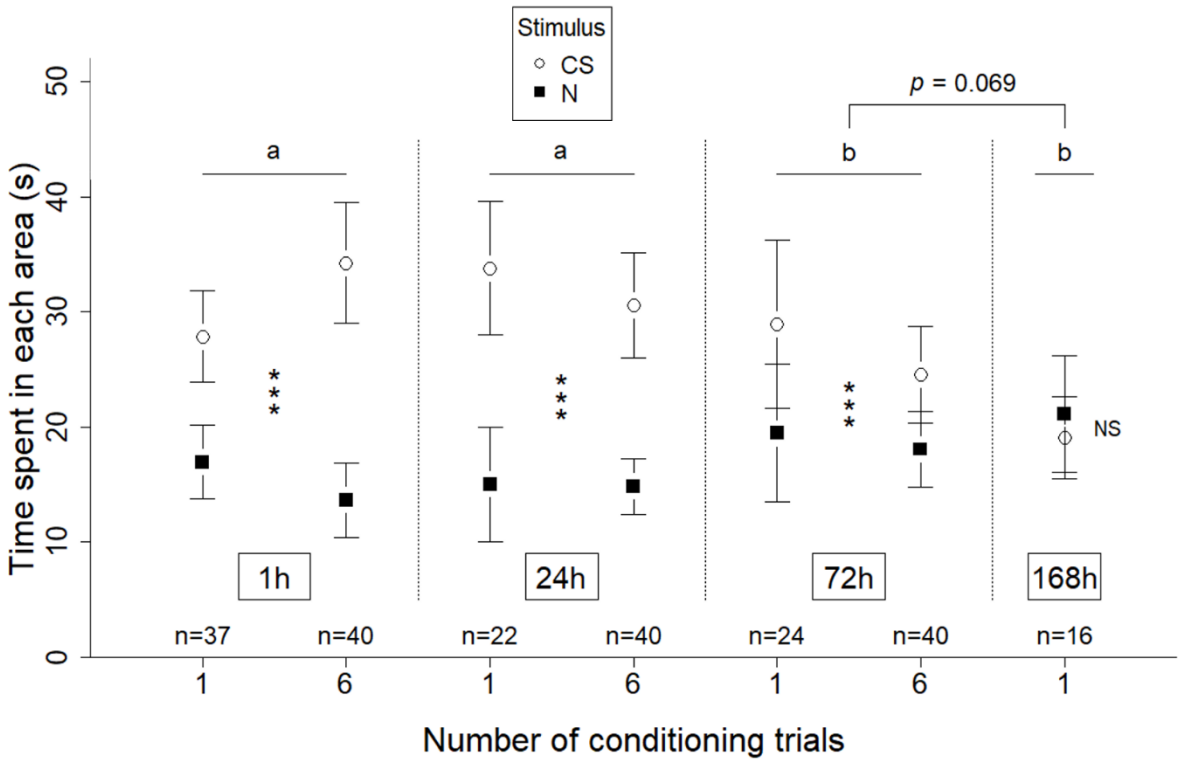

Figure 2: Time spent by individual ants in each area during the memory tests (white circle: CS area; black square: N area). Memory tests were performed 1 h, 24 h, 72 h or 168 h after the end of the conditioning. Ants underwent one or six conditioning trials. Circles and squares represent the mean and error bars are confidence intervals (95%). Significant differences within a group are noted with asterisks (***: $p < 0.001$; NS: $p \geq 0.05$). Significant differences between groups (1 h, 24 h, 72 h and 168 h) are noted with letters.

Instead, there was a significant difference in the ants' performance between 1h and 72h, with ants performing better at 1h than at 72h (*stimulus* × *elapsed time*, $p < 0.05$), between 24h and 72h (better performance at 24h, $p < 0.05$), between 1h and 168h (better performance at 1h, $p < 0.001$) and between 24h and 168h (better performance at 24h $p < 0.001$).

Finally, we observed that ants spent significantly more time in the CS than in the N area for the memory tests performed at 1h, 24h and 72h (for the three cases, $65.6 > F > 13.1$, $df = 1$, $p < 0.001$), while no significant difference was found at 168h ($F = 0.49$, $df = 1$, $p > 0.1$, Fig. 2), indicating that the ants do not prefer the CS to the N anymore. These results show that ants are capable of forming an appetitive associative memory that lasts for at least three days even after a single conditioning trial.

Pharmacological treatment

After a single conditioning trial, the olfactory memory of *F. fusca* appears to be very strong. We hypothesized that, even with such short training, ants built a LTM. To investigate this, we tested the susceptibility of this single-trial memory to a protein-synthesis inhibitor (CHX). During these memory tests, control ants (sham treated) spent more time in the CS area than in the N area, no matter whether the test was performed 1 h ($F = 4.16$, $df = 1$, $p < 0.05$) or 72 h after conditioning ($F = 6.03$, $df = 1$, $p < 0.05$), thus showing intact memory retention after three days. CHX-treated ants, however, displayed a preference for the CS area at 1 h ($F = 8.62$, $df = 1$, $p < 0.01$) but not at 72 h after conditioning ($F = 0.02$, $df = 1$, $p > 0.05$) (Fig. 3). This show that ants establish an appetitive olfactory LTM (Menzel, 1999) after a single conditioning trial.

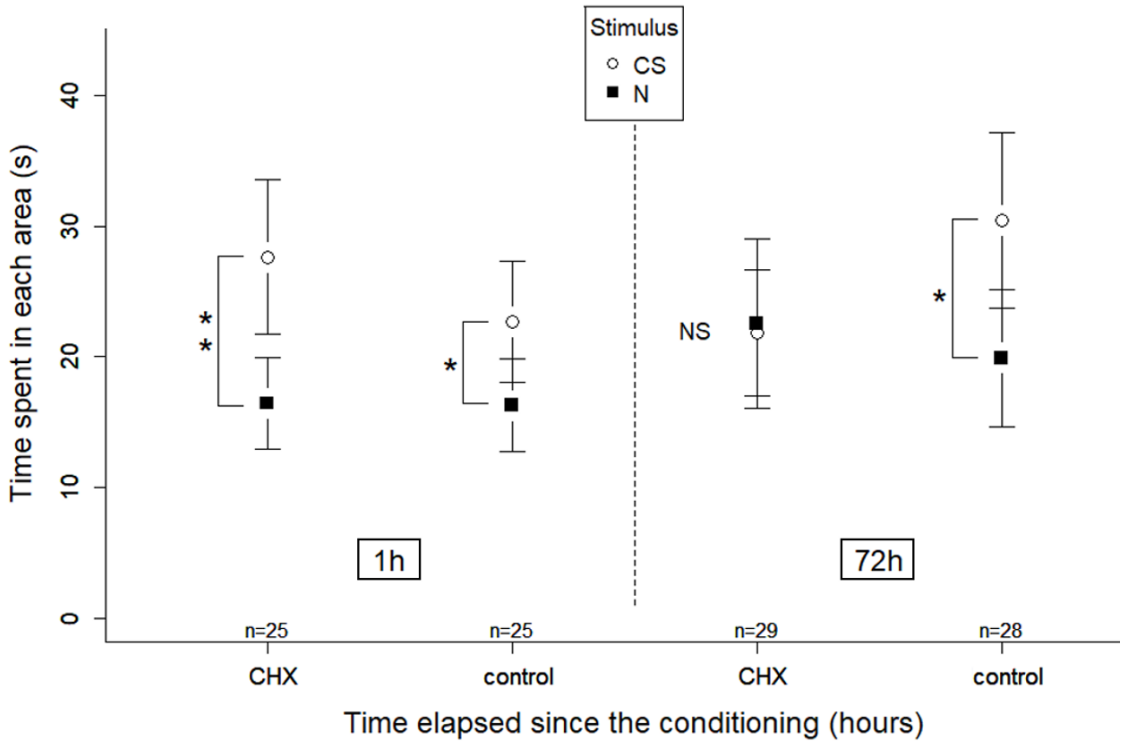

Figure 3: Time spent by individual ants in each area during memory tests. Ants received a drop of sugar solution 3 h before a single conditioning trial (control). In the experimental group (CHX), ants received 1 µg of CHX with the sugar solution. Memory tests were performed 1 h (left) or 72 h (right) after conditioning. Significant differences within a group are noted with asterisks (**: $p < 0.01$; *: $p < 0.05$; NS: $p \geq 0.05$). Circles and squares represent the mean and error bars are confidence intervals (95%).

Extinction

i) After six conditioning trials

Ants did not show any significant extinction in the course of the six-test procedure (Fig. 4A). Indeed, the ants' performance was stable over time, as indicated by the non-significant *stimulus* × *extinction* trials interaction ($F = 1.40$, $df = 5$, $p > 0.05$). When comparing the ant performance in the last extinction trial and in the spontaneous recovery test, no significant difference was found ($F = 0.10$, $df = 1$, $p > 0.05$) (Fig. 4A). In all the extinction trials and in the spontaneous recovery test, ants spent more time in the CS than in the N area ($142.7 > F > 50.54$, $df = 1$, $p < 0.001$). Therefore, with six conditioning trials, ants showed high resistance to extinction.

ii) After three conditioning trials

Given that extinction did not occur after six conditioning trials, we decided to subject ants to only three conditioning trials before the six extinction trials. Again, we found that the *stimulus* × *extinction trials* interaction was non-significant, indicating that ants' performance was stable during the extinction procedure ($F = 2.14$, $df = 5$, $p > 0.05$) (Fig. 4B). When comparing the last extinction trial with the spontaneous recovery test, we did not find any significant difference ($F = 0.01$, $df = 1$, $p > 0.05$). In all the extinction trials and in the spontaneous recovery test, ants spent more time in the CS than in the N area ($71.7 > F > 15.15$, $df = 1$, $p < 0.001$). Here again, ants show very high resistance to extinction.

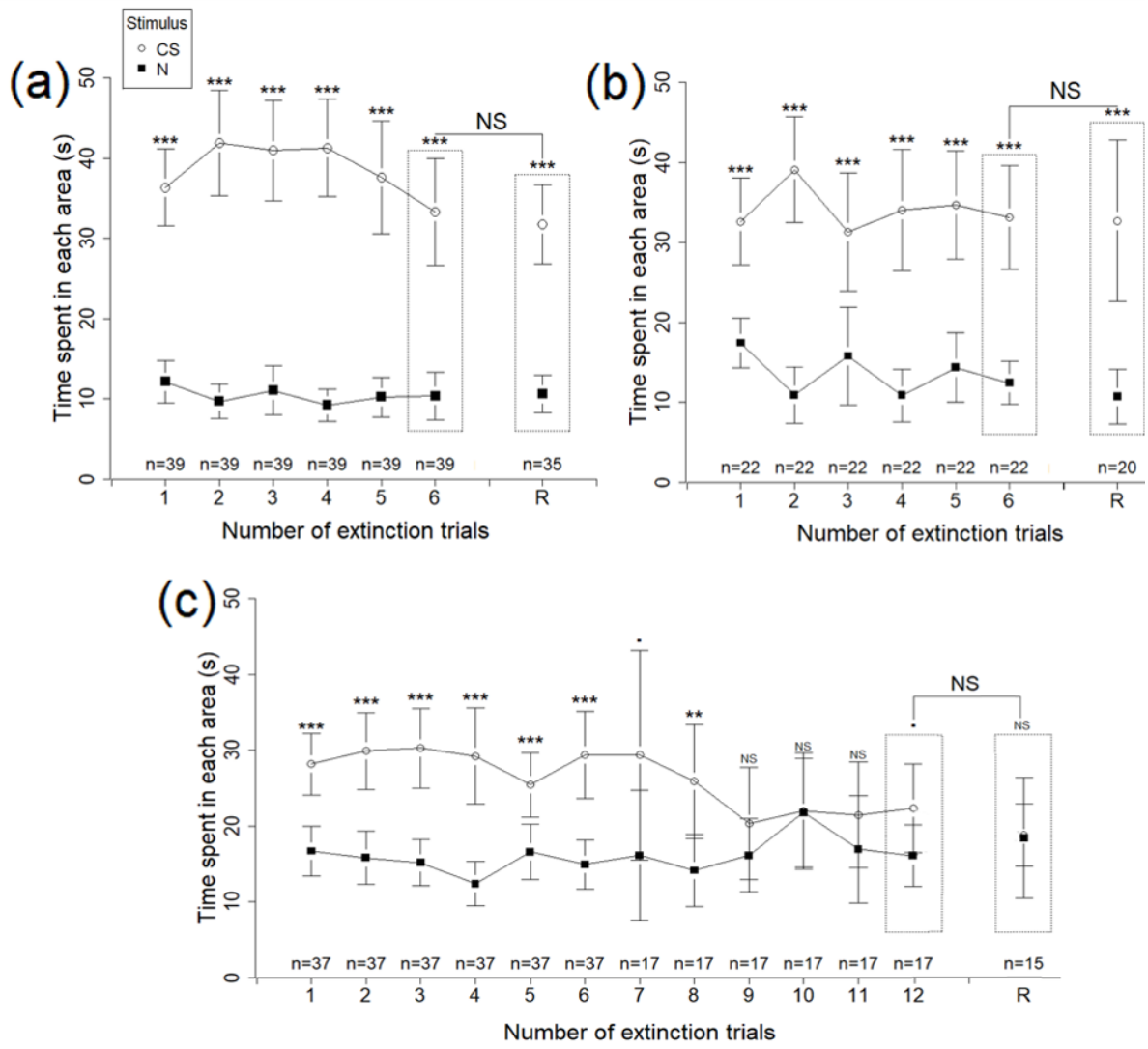

Figure 4: Time spent by ants in the CS and the N area during an extinction protocol that began 1 h after the last conditioning trial. Y-axis: time spent in the CS (white circles) and the N area (black squares). X-axis: number of extinction trials (six to 12). The test for spontaneous recovery (24 h after the last extinction trial) is represented by the letter "R". Different panels represent different groups of ants: A) ants that underwent 6 conditioning trials and six extinction trials (n=39), B) ants with three conditioning trials and six extinction trials (n=22) C) and ants with one conditioning trial and six (n=20) and 12 extinction trials (n=17). Significant differences within extinction trials or between the last extinction trial and the test of spontaneous recovery are noted with asterisks (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; NS: $p \geq 0.0.5$). Circles and squares represent the mean and error bars are confidence intervals (95%).

iii) After one conditioning trial and up to 12 extinction trials

Even with three conditioning trials, no extinction was found after six extinction trials. We then further reduced training strength and subjected the ants to a single conditioning trial before the extinction procedure. If we look at the first six extinction trials, the ants' performance did not decrease along the trials (Fig. 4C) as indicated by the non-significant interaction *stimulus* × *extinction trials* ($F = 0.67$, $df = 5$, $p > 0.1$). For these ants, which underwent one conditioning trial and six extinction trials, there is no significant difference between the 6th extinction trial

and the test of spontaneous recovery ($F = 0.17$, $df = 1$, $p > 0.1$). For the first six trials and the test of spontaneous recovery, ants spent significantly more time in the CS than in the N area ($28.38 > F > 16.68$, $df = 1$, $p < 0.001$) (Fig. 4C). Part of the ants that received only one conditioning trial underwent six additional extinction trials. Overall, the ants' performances (*i.e.*, the time spent near the CS) decreased along the 12 tests, indicating extinction (Fig. 4C), as shown by the significant *stimulus* × *extinction trials* interaction ($F = 1.83$, $df = 11$, $p < 0.05$). To detect at which trial extinction started, we tested if ants spent more time in the CS or N area during the last six extinction trials. For the 7^{th} trial, a tendency is observed ($F = 3.69$, $df = 1$, $p = 0.063$), for the 8^{th} trial, ants spent more time in the CS area ($F = 8.74$, $df = 1$, $p < 0.01$) but at the 9^{th}, 10^{th} and 11^{th} trials, ants did not show any significant preference for one of the stimuli ($0.88 > F > 0.001$, $df = 1$, $p > 0.1$). For the last extinction trial, a tendency was observed ($F = 4.14$, $df = 1$, $p = 0.051$). Finally, no significant difference was found between ants' performances in the 12th extinction trial and in the spontaneous recovery test ($F = 0.75$, $df = 1$, $p > 0.05$). During this spontaneous recovery test, ants did not spend more time in the CS area compared to the N area ($F = 0.26$, $df = 1$, $p > 0.1$), indicating that spontaneous recovery did not occur within 24h.

Discussion

By using a simple conditioning paradigm, our study shows that *Formica fusca* ants are very efficient in individual olfactory learning, and reveals unconventional characteristics in their learning ability. These ants learn an odour-reward association very quickly (within a single trial) and thereby build a highly-stable memory form (genuine LTM, dependent on protein synthesis), which was undescribed in ants. Moreover, the established odour-reward association is highly resistant to contradictory information, being subject to extinction only after many unrewarded trials.

Formation of a long-term memory after one conditioning trial

During conditioning with three and six conditioning trials, ants showed fast acquisition: the time to find the reward rapidly decreased after the first conditioning trial, suggesting that memory is already formed after a single conditioning trial. When ants that underwent one or six conditioning trials were tested with an unrewarded memory test (1h, 24h or 72h after conditioning) no significant differences in ants' performances were found between the one-conditioning trial group and the six-conditioning trials group. Typically, in insects as diverse as fruit flies, crickets and honeybees, a single conditioning trial results in the formation of a short-term memory (lasting 1h – 24h), whereas, several conditioning trials are needed to form a LTM (lasting more than 24 h) (Davis, 2011; Matsumoto et al., 2006; Menzel, 2001). In ants, a recent study showed that one training trial is sufficient for olfactory learning, but if this leads to LTM was not known (Oberhauser et al., 2019). To test whether the memory formed after a single conditioning trial is genuine LTM, we treated ants with a protein synthesis inhibitor (cycloheximide) before conditioning and we observed that, 72h later, treated ants could not retrieve any memory. This confirms that the memory formed by *F. fusca* ants after a single conditioning trial is true LTM (Guerrieri et al., 2011; Krashes & Waddell, 2008; Wittstock & Menzel, 1994). Formation of a LTM after one single appetitive conditioning trial is not common in insect, and has been found until now only in the fruit fly, Drosophila melanogaster (Colomb et al., 2009; Krashes & Waddell, 2008).

To investigate the limit of this memory, we tested ants one week after this one single conditioning trial and no trace of memory was found. We did not perform this test at one week for ants that underwent six conditioning trials, but we assume that they would behave similarly since their performances were not different from ants that underwent one conditioning trial when tested after 1h, 24h and 72h.

Our study is original in that it shows both single-trial olfactory learning and the formation of a highly-stable memory form after this single learning. Single trial visual learning has been shown in individual foragers of desert ants, for example *Melophorus bagoti*, but it is unclear whether this short training leads to LTM (Narendra et al., 2007). In the case of olfactory learning, previous studies found that ants can learn rapidly or retain memories for a long time, but both abilities were rarely found together. Moreover, these studies were performed at the colony level (not at the individual level) and/or involved very young individuals in an imprinting

context. Workers of the desert ant *Cataglyphis fortis* can collectively learn to associate one odorant with food after one trial, and about half of the ants remember this association for up to 26 days afterwards (Huber & Knaden, 2018). Leaf-cutting ants, *Atta colombica*, feed their symbiotic fungus with freshly collected leaves. Field colonies learn to avoid plants that are dangerous for their fungus (e.g. experimentally treated with fungicide) and show robust memory for plant unsuitability lasting up to 18 weeks (Saverschek et al., 2010).

Young workers of *Formica polyctena* that were reared just after emergence with cocoons of an alien species for 15 days will take care of cocoons of this species when encountering them six months later, while they will eat conspecific cocoons that they never encountered (Jaisson, 1974). This is an imprinting-like phenomenon occurring during a critical period after emergence and that has been shown in ants several times (review in Jaisson, 1991). Imprinting-like phenomena also occur with environmental odorants. If young ants are reared from eclosion in a nest with a specific plant odorant (*i.e.* thyme), and are then kept in a nest without odorant, they will prefer a nest with the odour experienced during their young age when given the choice (Jaisson, 1980). Young individuals can possibly form long-term olfactory memories that persist for weeks or even months. Indeed, high retention abilities were found when training cricket nymphs to discriminate an odorant associated with water or saline solution (Matsumoto & Mizunami, 2002), and in very young mice trained to associate an odorant with milk (Armstrong et al., 2006), especially after a period of deprivation from these resources. In the present case, we documented ants' adult learning abilities at the individual level and show that they build long lasting olfactory memories within a single rewarded trial.

Formica fusca ants are particularly resistant to extinction

The second part of our study involved testing resistance to extinction. Based on the social insect literature, we expected to observe a rapid decrease of performances along extinction trials. In honeybees, using the proboscis extension response conditioning paradigm, 80 % of the bees show a conditioned response after six-conditioning trials. After two extinction trials, 70 % of these bees display the conditioned response, and after five extinction trials 60 % still responds. However, with a single conditioning trial, only 10.7 % of bees show the conditioned response after five extinction trials (Sandoz & Pham-Delègue, 2004). In *Myrmica* ants tested

at the colony level, two extinction trials are needed to extinguish an olfactory association established with a 12 conditioning-trials procedure (Cammaerts, 2004). In our first extinction protocol, with ants that underwent six conditioning trials and six unrewarded extinction trials, no extinction was observed. This result in itself is surprising and was replicated with ants that underwent three or only one single conditioning trial. We could observe extinction only in ants that underwent a single conditioning trial and more than 6 extinction trials, demonstrating an exceptionally high resistance to extinction in these ants. During extinction, the memory formed after conditioning (CS – US association) is not erased, but a new learning usually takes place (CS – no US association) (Bouton, 2004). The behaviour of the individual will reflect the relative strength of each memory. With more extinction trials, the CS – no US association will become stronger than the CS - US memory and individuals will stop responding to the CS. As the CS – US memory is not erased, if we test the behaviour of an individual at a later time after the end of the extinction protocol, positive response to the CS may increase again. This phenomenon, called spontaneous recovery (Rescorla, 2004) usually corresponds to a decay of the CS - no US memory. Here, we did not observe any spontaneous recovery when extinction took place (*i.e.* one conditioning trial and 12 extinction trials). This absence of spontaneous recovery could be due to the time elapsed between the end of the extinction trials and spontaneous recovery test, which may have been too short for the decay of the CS - no US association. In honeybees, spontaneous recovery usually appears within a few hours (1h for Freas & Schultheiss, 2018, 35 min for Bitterman et al., 1983). In any case, the lack of spontaneous recovery in these ants confirms the stability of the associations they form: just like their CS - US association is highly resistant to time and extinction, their CS - no US association also appears to be highly resistant to time.

Why do ants show such fast learning and high resistance to extinction? In ants, as in other social insects, individuals are usually specialized in a particular task according to their age. Young workers will avoid threats, stay in the nest and take care of the brood, whereas old workers will go out foraging, and therefore be exposed to biotic and abiotic threats (Gordon, 1996). In undisturbed natural colonies, as workers keep the same job for weeks or even months, it is not relevant to learn rapidly and to be resistant to extinction. However, if a category of workers suddenly decreases in number (e.g. due to predation or raids from slave-making ants, of which *F. fusca* is a common host species (Mori et al., 2000)), task switching

may occur and workers with the ability to learn quickly will be very advantageous for the colony. Being resistant to extinction is an advantage when the environment is extremely stochastic and workers need to switch often from a current task to another, previously learned, task. In *F. fusca*, there is no clear specialization of individuals working outside the nest, which may engage in different tasks such as foraging, scouting, guarding (Novgorodova, 2015). An ant could act as forager one day, guard another day and then forager again. In this scenario, learning quickly and building strong memories of a previously learned task (*i.e.* being resistant to extinction) is advantageous because it allows ants to exploit optimally their environment without spending time to learn again.

After documenting the unconventional olfactory learning and memory abilities of *Formica fusca*, we are left wondering if this species is really exceptional among ants, and more generally among social insects. While visual learning is well documented in ants, especially in the context of navigation (Freas & Schultheiss, 2018), data on individual olfactory learning and memory abilities in ants are relatively scarce and future comparative studies taking into account the ant phylogeny may be useful to provide answers to this question. Furthermore, given that the insect brain shares many similarities in its architecture with the vertebrate brain (Giurfa, 2003), a better understanding of the neural mechanisms underlying such a stabilized memory and resistance to extinction might help improving treatment of maladaptive behaviours.

Data accessibility: Supplementary Methods and Results, Tables S1–S5 and Figure S1-S2 are available in annexes.

Authors' contributions: BP, PdE and JCS conceived the project and designed the experiments. BP performed the experiments and analysed the data with the help of PdE and JCS. The manuscript was written by BP and revised by PdE and JCS. Final manuscript was approved by all authors.

Competing interests: None

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Supplementary Information:

Ants learn fast and do not forget: olfactory associative learning, memory and extinction in Formica fusca

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Methods

Odorant stimuli

Due to their low molecular weight, hexanal and 1-octanol are volatile at ambient temperature. We tested whether ants might show a spontaneous preference for these odorants, but this is not the case. When individual ants were presented in a circular arena (Fig. 1A) with hexanal and 1-octanol at two opposite sides, the ants did not show any preference (Linear Mixed Model: $F = 0.10$, $df = 1$, $p > 0.1$, $N = 10$). The two odorants are clearly different from a chemical point of view and were shown to induce low behavioural generalisation in honey bees (Guerrieri et al., 2005). Therefore, they are expected to be perceived as clearly different odorants by the ants (Dupuy et al., 2010).

Pharmacological treatment

To exclude a possible effect of CHX on the ants' health, we carried out a control experiment in which ants received CHX (or only sugar solution) 72h - instead of 3h - before a single conditioning trial. We then performed a memory test 1h after conditioning. No difference was observed between treated (CHX) and control ants, demonstrating that CHX does not affect the physical conditions of ants (Linear Mixed Model: *stimulus* × *treatment*: $F = 2.20$, $df = 1$, $p > 0.1$, $N = 17$ treated and $N = 16$ control ants, table S4).

Detailed statistical methods

Data were analysed using R software (v 3.5.2, R Core Team, 2018 (R Core Team, 2018)). Significance was fixed at $\alpha = 5\%$. Data were transformed with neperian logarithm or square root, depending on which transformation was the best to approach normality. Homogeneity of variance was checked for all the full models. All data were analysed using Linear Mixed Models (LMM, package "lme4", (Bates et al., 2015)). To allow repeated measurements and adjust for colony origin, individual identity was coded as random factor nested into colony origin. F and p-values from the LMM were calculated using a Wald-test with Satterthwaite's correction ("car" package, (Fox & Weisberg, 2011)). Post-hoc differences were observed by using LMMs with reduced dataset and the alpha level was adjusted using Holm-Bonferroni Correction (Holm, 1979).

i) Acquisition

We analysed the effects of two independent (predictor) variables: *conditioning odorant* (factor with 2 levels, hexanal or 1-octanol) and the number of conditioning trials (continuous variable up to 6, named *trials*) on the dependant variable *time* (continuous variable, the time before finding the reward). We looked at the interaction *conditioning odorant* × *trials* to detect possible differences in ants' responses depending on the odorant used. The conditioning odorant did not influence the acquisition dynamics (LMM: $F = 0.91$, $df = 5$, $p > 0.05$). We then ran a simplified model to test the effect of the conditioning trials on acquisition. We also ran a post hoc analysis to compare each conditioning trial with the others; all the 15 possible comparisons (trial 1 vs trial 2, trial 1 vs trial 3…) were tested and the Holm-Bonferroni correction was applied (Table S1).

ii) Memory tests

For the memory tests, we analysed the effects of four independent variables: 'stimulus' (factor with 2 levels, CS and N), the time elapsed since conditioning (factor with 4 levels, 1h, 24h, 72h and 168h, called *elapsed time*), the number of conditioning trials (factor with 2 levels, 1 or 6 conditioning trials, called *conditioning groups*) and the *conditioning odorant* (factor with 2 levels, hexanal or 1-octanol) on the dependant variable *time* (continuous variable, the time spent in the vicinity of a stimulus). We looked at the triple interaction between *stimulus* × *elapsed time* × *conditioning group*, which was not significant (LMM: $F = 1.77$, $df = 4$, $p > 0.05$).

A simplified model was run where we looked at the interactions between *stimulus* × *elapsed time* and *stimulus* × *conditioning groups*. We then used post-hoc tests to see whether the time spent in the CS or N area varies according to the time elapsed since conditioning. We finally tested whether ants spent more time in the CS or N area in function of the elapsed time since the conditioning.

For the memory tests performed in the pharmacological experiment (CHX), we tested if ants spent more time in the CS or N area 1h or 72h after the conditioning trial as a function of treatment (CHX or control).

iii) Extinction

We analysed the effects of four independent variables: *stimulus* (factor with 2 levels, CS and N), the number of conditioning trials (factor with 3 levels, 1, 3 or 6 conditioning trials, called *conditioning groups*), the number of extinction trials (continuous variable from 1 to 12, called *extinction trials*) and the *conditioning odorant* (factor with 2 levels, hexanal or 1-octanol) on the dependant variable *time* (continuous variable, the time spent in the vicinity of a stimulus). In the full model, we observed that the interaction *stimulus* × *conditioning group* was significant (LMM: $F = 35.36$, $df = 2$, $p < 0.001$), so we ran different models for the different conditioning groups (1, 3 or 6 conditioning trials). For the simplified model, we looked at the interaction *stimulus* × *extinction trials* to detect extinction.

To test for spontaneous recovery, we ran a model with a subset of data consisting only in the last extinction trial and the test of spontaneous recovery and looked at the *stimulus* × *extinction trials* interaction. Finally, at each extinction (or spontaneous recovery) trial, we tested if ants spent more time in the CS or in the N area.

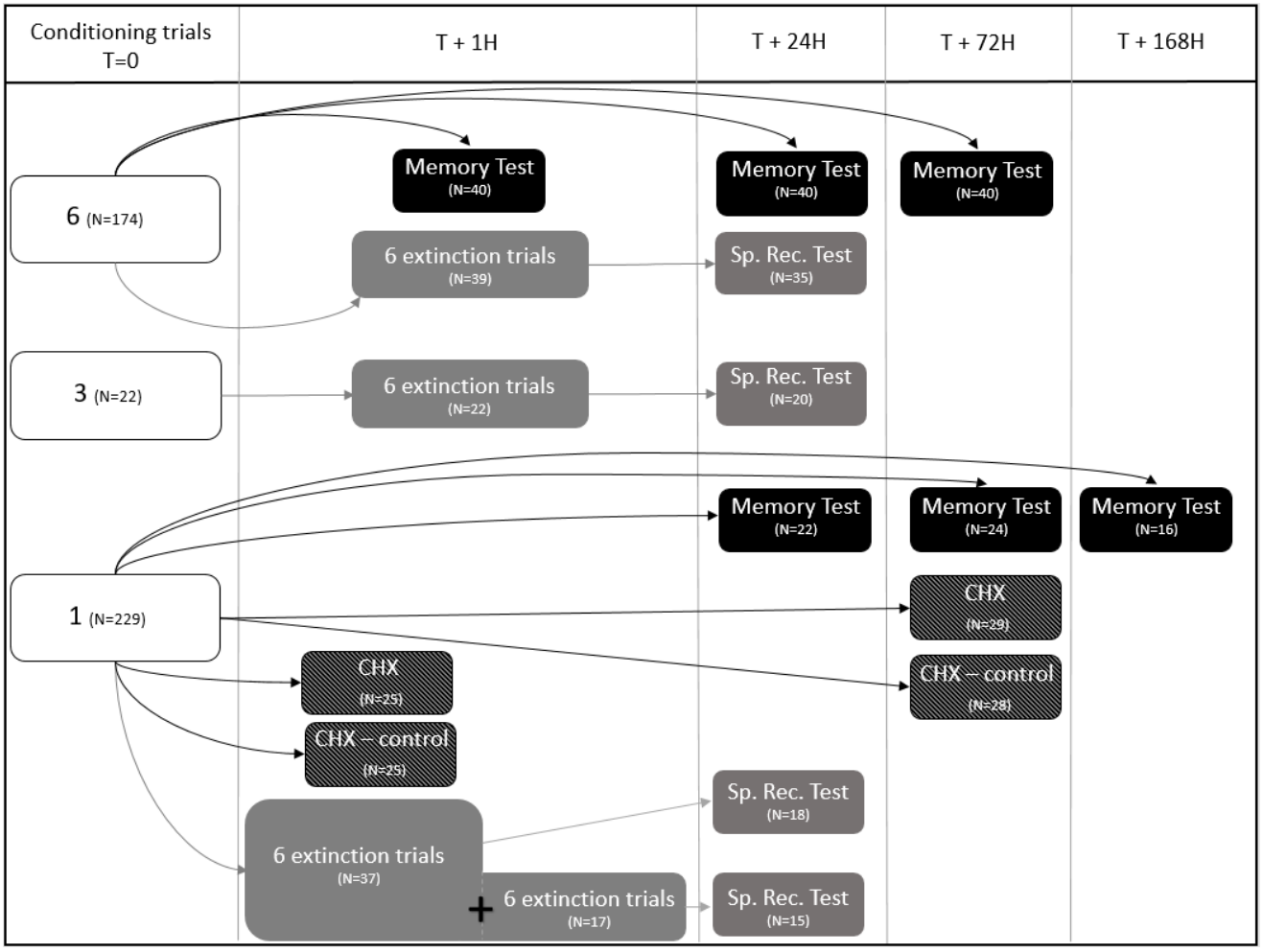

Figure S1: Overview of the conducted experiments. The left column shows the number of conditioning trials ants were subjected to (white box). Three different experiments were performed. Independent groups of ants underwent a memory test (black boxes), an extinction protocol (grey boxes) or were treated with CHX before performing a memory test (black striped boxes). Tests were conducted 1 h, 24 h, 72 h or 168 h after the end of conditioning. For the ants that underwent an extinction protocol, a test of spontaneous recovery (Grey boxes: Sp. Rec. Test) was performed 24h after the last extinction trial. N= number of ants.

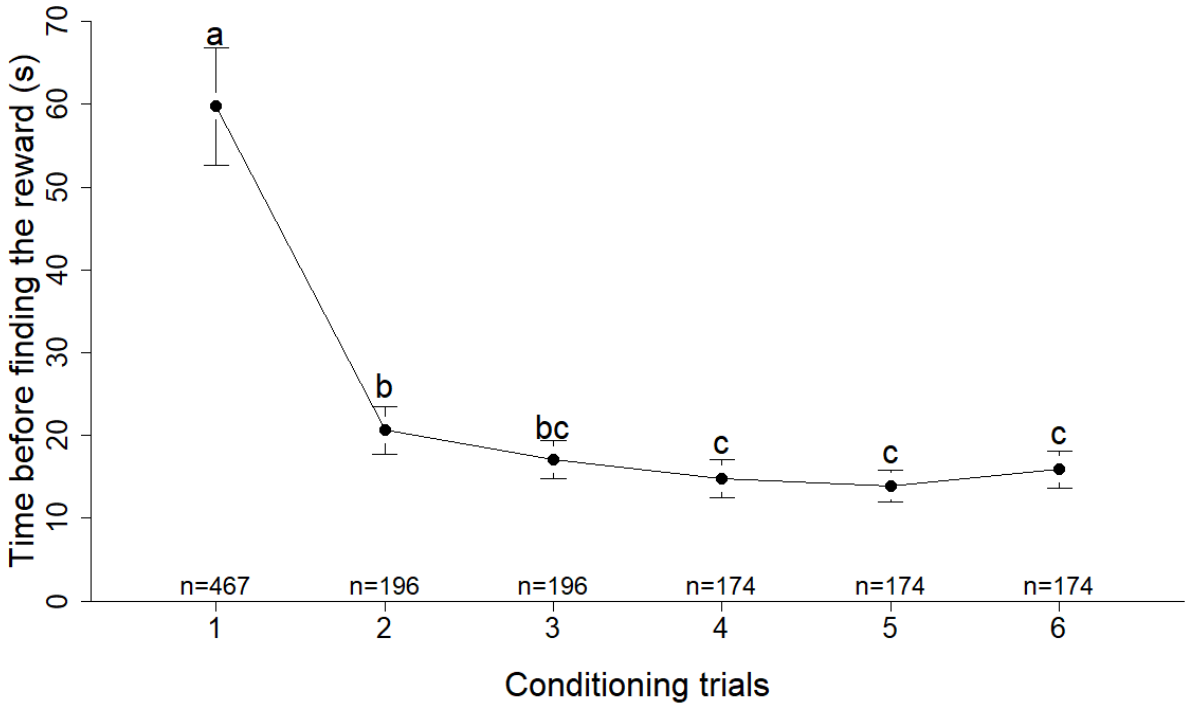

Figure S2: Time (mean + 95% confidence interval) spent by the ants to find the reward along the conditioning trials (N = 174 for six trials, N = 22 for three trials, N = 271 for one trial). Letters indicate difference between trials after Holm-Bonferroni correction. In particular, the first trial was different from all the other trials, in which the time to find the reward was significantly shorter (post-hoc, $p < 0.001$ for all comparison, after Holm-Bonferroni correction). The second and third trials were not significantly different ($p > 0.05$); however, the second trial was different from all the other trials ($p < 0.05$ in all cases). There was no difference from the third trial to the sixth ($p > 0.05$ in all cases).

Table S1| Result of the different model used during the analyses of the ant conditioning (Fig S3): Time was always the dependent variable. Significant effects are given in bold.

Focus on the factor(s)	Df	F value	p value
Conditioning odorant × trials × Conditioning groups	2	0.9271	0.396
Conditioning odorant × trials	5	0.9101	0.473
Trials	5	72.4502	**<0.001**
Trials (1 vs 2)			**<0.001**
Trials (1 vs 3)			**<0.001**
Trials (1 vs 4)			**<0.001**
Trials (1 vs 5)			**<0.001**
Trials (1 vs 6)			**<0.001**
Trials (2 vs 3)			0.287
Trials (2 vs 4)			**0.007**
Trials (2 vs 5)			**<0.001**
Trials (2 vs 6)			**0.048**
Trials (3 vs 4)			0.685
Trials (3 vs 5)			0.270
Trials (3 vs 6)			1.000
Trials (4 vs 5)			1.000
Trials (4 vs 6)			1.000
Trials (5 vs 6)			0.680

Table S2 | Results of the model used during the analyses of the memory tests (Fig 3): Time was the dependent variable; *p*-value are adjusted with Bonferronni-Holm correction when needed. Significant effects are given in bold. Tendency effects are underlined.

Interaction or factor analysed	Post-hoc	Df	F value	p value
elapsed time × conditioning groups × stimulus		4	1.7707	0.133
conditioning groups × stimulus		1	0.5172	0.472
stimulus × elapsed time		3	7.8924	**<0.001**
stimulus × elapsed time	1h/24h	1		0.716
stimulus × elapsed time	1h/72h	1		**0.022**
stimulus × elapsed time	1h/168h	1		**<0.001**
stimulus × elapsed time	24h/72	1		**0.012**
stimulus × elapsed time	24h/168h	1		**<0.001**
stimulus × elapsed time	72h/168h	1		<u>0.069</u>
Stimulus	1h	1	62.997	**<0.001**
Stimulus	24h	1	65.597	**<0.001**

Stimulus	72h	1	13.141	**<0.001**
Stimulus	168h	1	0.488	0.495

Table S3 | Results of the model used during the analyses of the effect of the cycloheximide (CHX, given 3 hours before the conditioning trial) on memory retention (Fig 4): Time was the dependent variable. Significant effects are given in bold. Tendency effects are underlined.

Interaction or factor analysed	Post-hoc	Was CHX given?	Df	F value	p value
Treatment × Stimulus			1	0.783	0.378
Stimulus	1h	No	1	4.167	**0.046**
Stimulus	1h	Yes	1	8.627	**0.005**
Treatment × Stimulus			1	2.907	0.091
Stimulus	72h	No	1	6.039	**0.017**
Stimulus	72h	Yes	1	0.025	0.872

Table S4 | Results of the model used during the analyses of the effect of the cycloheximide (CHX, given 72 hours before the conditioning trial) on memory retention: Time was the dependent variable. Significant effects are given in bold.

Interaction or factor analysed	Post-hoc	Df	F value	p value
Treatment × Stimulus		1	2.204	0.143
Stimulus	CHX	1	24.468	**<0.001**
Stimulus	Control	1	12.590	**0.001**

Table S5 | Results of the model used during the analyses of the extinction procedure (Fig 5): Time was the dependent variable. Significant effects are given in bold. Tendency effects are underlined.

Interaction or factor analysed	Post-hoc	Df	F value	p value
stimulus × conditioning group		2	35.621	**<0.001**
		Six conditioning trials		
stimulus × extinction trials		5	1.397	0.224
Stimulus	Trial 1	1	106.180	**<0.001**
Stimulus	Trial 2	1	127.940	**<0.001**
Stimulus	Trial 3	1	102.750	**<0.001**
Stimulus	Trial 4	1	142.700	**<0.001**
Stimulus	Trial 5	1	74.979	**<0.001**
Stimulus	Trial 6	1	50.542	**<0.001**
Stimulus	Trial Recuperation	1	66.753	**<0.001**
stimulus × extinction trials	Trial 6/ Recuperation	1	0.104	0.746
		Three conditioning trials		
stimulus × extinction trials		5	2.140	0.061
Stimulus	Trial 1	1	29.489	**<0.001**
Stimulus	Trial 2	1	71.702	**<0.001**
Stimulus	Trial 3	1	15.145	**<0.001**
Stimulus	Trial 4	1	35.551	**<0.001**
Stimulus	Trial 5	1	28.973	**<0.001**
Stimulus	Trial 6	1	49.574	**<0.001**
Stimulus	Trial Recovery	1	19.645	**<0.001**
stimulus × extinction trials	Trial 6/Recovery	1	0.008	0.926
		One conditioning trial		
Stimulus × extinction trials		11	1.825	**0.046**
Stimulus	Trial 1	1	16.689	**<0.001**
Stimulus	Trial 2	1	23.354	**<0.001**
Stimulus	Trial 3	1	28.386	**<0.001**
Stimulus	Trial 4	1	27.947	**<0.001**
Stimulus	Trial 5	1	12.617	**<0.001**
Stimulus	Trial 6	1	22.053	**<0.001**
Stimulus	Trial 7	1	3.687	0.063
Stimulus	Trial 8	1	8.742	**0.005**
Stimulus	Trial 9	1	0.883	0.354
Stimulus	Trial 10	1	0.001	0.975
Stimulus	Trial 11	1	0.874	0.356
Stimulus	Trial 12	1	4.139	0.051
Stimulus	Trial Recovery 6	1	22.597	**<0.001**
Stimulus	Trial Recovery 12	1	0.262	0.612
Stimulus × extinction trials	Trial 6/ Recovery 6	1	0.177	0.675
Stimulus × extinction trials	Trial 12/ Recovery 12	1	0.749	0.390

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Chapter 2:

Ants detect cancer cells through volatile organic compound

Ants detect cancer cells through volatile organic compounds

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Abstract:

Cancer cells possess specific features such as a deregulated cellular energetic metabolism, the ability to self-sustain themselves with proliferating signals or the formation of tumour-promoting inflammation factors. Their metabolism produces volatile organic compounds (VOCs) that can act as biomarkers for cancer diagnosis. Animals, especially dogs, have been used to detect human VOCs via olfactory associative learning. However, training dogs is costly and time-consuming. Other animals, such as insects, have a refined sense of smell and can be easily trained with olfactory conditioning.

Here, we show that individual ants need only a few training trials to learn, memorize and reliably detect cancer cells. Using ovarian cancer cells (IGROV-1), we first found that ants can

learn to detect VOCs from IGROV-1, when compared to culture medium alone. In addition, by comparing normal mammary cells (MCF-10A) to breast cancer (MCF-7, MDA-MD-231) cell lines, we demonstrated that ants are able to distinguish cancer from normal cells, as well as to discriminate between the two breast cancer cell lines. These performances are due to cell line-specific VOC patterns, as shown by headspace chemical analysis via gas-chromatography/mass-spectrometry. Our findings demonstrate that using ants as living tools to detect biomarkers of human cancer is feasible, fast, and less laborious and expensive than using other animals.

Keywords: *Formica fusca*, conditioning, VOCs, human cancer cells, GC-MS

Introduction

Cancer cells differ in their metabolism from non-cancerous cells by producing a unique pattern of metabolites (Haick et al., 2014; Hanahan & Weinberg, 2011). These are then excreted into the host circular blood system and will impact the chemical composition of urine (Khalid et al., 2015), breath (Filipiak et al., 2014) or skin secretions (Thuleau et al., 2019). The metabolites characterising cancer cells, and present in the body-fluids of affected individuals, are idiosyncratic volatile organic compounds (VOCs). VOCs can be used as biomarkers of cancer cells (Filipiak et al., 2016) and their analysis can lead to the development of non-invasive detection methods as promising tools to achieve early diagnosis (Nakhleh et al., 2017).

Electronic noses (E-noses) have the potential to fulfil this task. They consist of electronic sensors that are activated by a specific category of molecules. The activation of different sensors by the VOCs of a sample will produce a unique response pattern. E-noses have the advantage of being transportable but, as they represent a relatively new technology, improvements are still needed (Behera et al., 2019). E-noses can have a low sensitivity, are particularly sensitive to water vapour (producing false diagnosis), their sensors have a short lifespan, and they are less sensitive than the gold standard of chemical analysis, gas-chromatography coupled with mass spectrometry (GC-MS). GC-MS is a powerful technique for the detection and identification of small amounts of molecules. To collect cancer's VOCs, the current standard is the use of Solid Phase Micro Extraction (SPME) of the headspace of cells cultures (Lavra et al., 2015; Schallschmidt et al., 2015; Silva et al., 2017; Thriumani et al.,

2018), urine (reviewed in da Costa & Spinosa De Martinis, 2020) or breath samples (reviewed in Jia et al., 2019). However, the lack of standardized protocols for the chemical analysis of VOCs, the large range of different compounds present in body fluids (aldehydes, ketones, alcohols, acids, esters…) (De Lacy Costello et al., 2014) implying different analysis protocol choices, the purchase and maintenance costs of a GC-MS and the specialised personnel needed to operate it, represent disadvantages for using this technique on a large number of patient samples to efficiently detect cancerous tumours.

Millions of years of evolution have produced finely-tuned systems, which are ideal for detecting small odorant concentrations, have the computational power for discriminating among differently composed odorant mixtures and are portable: animals' olfactory organs. Amongst animals, dogs are the most widely used, by means of olfactory associative learning, for the detection of various types of VOCs, such as drug related substances (Francis et al., 2019), explosives (Furton & Myers, 2001), parasites in livestock (Moser et al., 2020) and in humans (Kasstan et al., 2019), or human viruses (Jendrny et al., 2020). Dogs' detection abilities are also used for cancer-specific VOCs found in body-fluids (Mazzola et al., 2020; Thuleau et al., 2019; reviewed in Pirrone & Albertini, 2017) or in the headspace of cell cultures (Schallschmidt et al., 2015). Dogs' olfactory abilities provide good results, but training dogs in standard associative learning paradigms is time consuming and expensive. The conditioning phase, in particular, takes several months and hundreds of trials are needed before the dog is operative. Consequently, sample sizes (both in terms of individual dogs and numbers of tests) are often low. For instance, a recent study reached ~90% correct identification using two dogs, 5 months of training and 1531 conditioning trials to perform only 31 memory tests (Thuleau et al., 2019).

Dogs are not the only animal species that have been considered for detecting cancer VOCs. Nematodes (*Caenorhabditis elegans*), for instance, can express chemotaxis to cancer odours (Hirotsu et al., 2015). Amongst insects, fruit flies and honeybees were used as bio-detectors for cancer. Different cancer cell line odours evoked specific olfactory receptor activity patterns in the fruit fly antenna (Strauch et al., 2014), as revealed by in vivo calcium imaging, a complex and expensive technique. Honeybees were conditioned to detect lung cancer cells (Schallschmidt et al., 2015), expressing a simple and easily readable behaviour when exposed to the learned odour (extension of the proboscis) (Giurfa & Sandoz, 2012). Compared to dogs,

insects have the advantage of being small and relatively easy to rear in controlled conditions, they have a well-developed olfactory system (Rössler & Stengl, 2013) and they can detect and discriminate, via olfactory associative learning, small concentrations of odorants (di Mauro et al., 2015; Menzel et al., 2007; Olson & Rains, 2014; Wright & Smith, 2004). Remarkably, insects can be easily conditioned with very few trials, and in large numbers (N = 132, Czaczkes & Kumar, 2020; N = 2048, Guerrieri et al., 2005; N = 250, Oberhauser et al., 2019; N = 496, Piqueret et al., 2019; N = 900, Wycke et al., 2020). All these characteristics make insects particularly promising candidates as bio-detectors of cancer.

Here, we used individual workers of a common ant species, *Formica fusca*, for the detection of cancer (ovarian and breast) cell lines. With a simple, fast and easily reproducible conditioning paradigm, ants can be trained to associate an odour with a food reward. They retain memory for days and this memory is highly resistant to repeated testing (Piqueret et al., 2019). Ant colonies, composed of hundreds of individuals, can be collected in the wild and they are not expensive to keep in the lab. Ants are also relatively easy to manipulate. Here, we demonstrated that i) ants are able to detect the presence of cancer cells via their VOCs, ii) they can discriminate a cancerous cell line from a healthy one and iii) they can discriminate between two different cancer cell lines. We also showed by solid phase microextraction (SPME) and gas-chromatography coupled with mass-spectrometry (GC-MS) that the cell lines could be chemically discriminated and we identified the chemical differences that match ants' behaviour.

Material and methods

Insects and origin of colonies

Formica fusca is a common ant species found in the Northern Hemisphere. Colonies are headed by one queen (monogynous) or several queens (polygynous) and contain several hundred individuals. Fifteen queenright colonies were collected in the forest of Ermenonville (France, 49°09'51.5" N, 2°36'49.2" E) and kept under laboratory conditions (25 ± 2 °C, 50 ± 10% relative humidity, 12 h/12 h: day/night) at the Laboratory of Experimental and Comparative Ethology (LEEC, University Sorbonne Paris Nord). Tested ants were foragers (ants that leave the nest to search for food) and were individually marked with a dot on the

abdomen or thorax using oil-based paint (Mitsubishi Pencil) the day before the experiment. Each ant was used only once, undergoing one conditioning phase and one testing phase.

Cell cultures

Ovarian cancer (OC), breast cancer (BC) and immortalized (non-tumorigenic) breast cell lines were cultivated at the Curie Institute ('Stress & Cancer Lab', Paris, France). Four human epithelium cancer cell lines were derived from adenocarcinoma ovarian or breast cancers: IGROV-1 (ovarian cancer), MCF-7 (breast cancer, Luminal-A), MDA-MD-231 (breast cancer, triple-negative), and MCF-10A (non-transformed breast cells). Each cell line identity was tested by Short Tandem Repeat (STR) DNA profiling (Promega, #B9510) and tested for absence of mycoplasma contamination. Cells were propagated in DMEM (Dulbecco modified Eagle's minimal essential medium - GE Healthcare Hyclone SH30243.01) supplemented with 10 % foetal bovine serum (FBS - Biosera, #1003/500), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco #15140122). Cells were placed in an incubator at 37 °C and 5 % CO_2. The medium was renewed twice a week.

Cells were cultivated in Petri-dishes with 10 mL of DMEM for the propagation. Before the medium collection, 0.8 to 1 million cells (depending on the cell line) were plated in 10 cm dishes. After four days, the medium was transferred to falcon tubes and then centrifugated (5 min, 1200 rpm, at RT). The supernatant (not containing any cells) was transferred to 4 mL and 15 mL glass vials for the behavioural experiments and chemical analysis respectively. All the samples were frozen at -20 °C before being used.

Experimental protocol

A preliminary experiment showed that ants significantly discriminate the odour of living cancer cells against the culture medium (Supplementary Methods, Supplementary Tables 1 and 2, Supplementary Figs. 1 and 2). We then used frozen supernatant samples extracted from cell cultures as described above.

1. Behavioural experiments

a. Conditioning

The protocol is a modified version of that used by Piqueret et al. (2019) to study individual learning and memory abilities in *Formica fusca* ants. We used olfactory conditioning, in which a single initially neutral odorant (CS – conditioned stimulus) is associated with a reward (US – unconditioned stimulus). Ants were individually placed in a circular arena (Ø = 12 cm, height = 3.5 cm) with clean filter paper at the bottom (Fig. 1). Two holes were presented at the ground level of the arena (10 cm between the holes). Two glass vials (4 ml, Supelco, Bellefonte, PA, USA) were each placed below one of the holes with the opening toward the arena. One vial was filled with 4 mL of the supernatant (extracted as explained above, representing the CS), whereas the other was empty. As cells were cultivated at 37 °C, they were kept at this temperature throughout the experiment by inserting the vials in a water bath (1.8 L, diameter = 15.5 cm x 14 cm, height = 10 cm, temperature sensitivity of 0.2 °C at 37 °C, Edvotek, Washington D.C., USA) placed just below the arena. To avoid a possible effect of heat on ants' learning and memory performances, polystyrene foil (insulating material) was placed between the water bath and the arena. The portion of filter paper above the vials was pierced with an entomological pin to allow natural diffusion of the odour. Small plastic discs (Ø = 6 mm) were placed above each hole, and received a 1 µL drop of sugar solution (30% w/w) for the CS odour and of distilled water for the other, unscented vial (Fig. 1A). Due to the presence of these two drops of liquid, the two stimuli were visually indistinguishable. During each conditioning trial, we recorded the time needed by the ant to find the sugar solution (US). The ant was allowed to drink the drop of sugar solution and was then returned to the colony, where it could perform trophallaxis (mouth to mouth exchange of liquid food) with her nest-mates. Without trophallaxis, the crop of tested ants would be full in only a few conditioning trials and the ants would not be motivated to find more food. Tested ants were left for about 3 min in the colony (inter-trial interval), during which they terminated trophallaxis and came back spontaneously to the foraging arena, where they were picked up for the next training trial (Piqueret et al., 2019). During this interval, the filter paper at the bottom of the arena and the plastic discs were replaced with clean ones to remove any possible chemical cues left by the ant at the previous trial. The orientation of the arena and the position of the experimenter were also

modified between trials to limit the possible use of visual or other spatial cues. Each ant underwent three consecutive conditioning trials.

b. Memory tests

To test whether ants have learned that the CS is a predictor for reward, we performed memory tests in which the reward was absent. For this unrewarded memory test, four glass vials were used, which were inserted on the four cardinal points of the arena (Fig. 1B). One vial contained the CS odour and, on the opposite side, a second vial contained a novel odour (N). On the two vacant positions, the additional glass vials were empty and acted as controls. Empty plastic discs (Ø = 6 mm) were placed above the glass vials, and circular areas were drawn around each plastic disc, allowing us to record the time spent by the ant in the vicinity of each stimulus for 2 min. Ants underwent two consecutive unrewarded memory tests 15 and 20 min after the end of the last conditioning trial.

Ants' behaviour was scored using a behavioural transcription tool (software Ethoc v. 1.2, CRCA, Toulouse, France). All experiments were also video recorded with a camera (Canon, Legria HFR806) placed above the experimental arena.

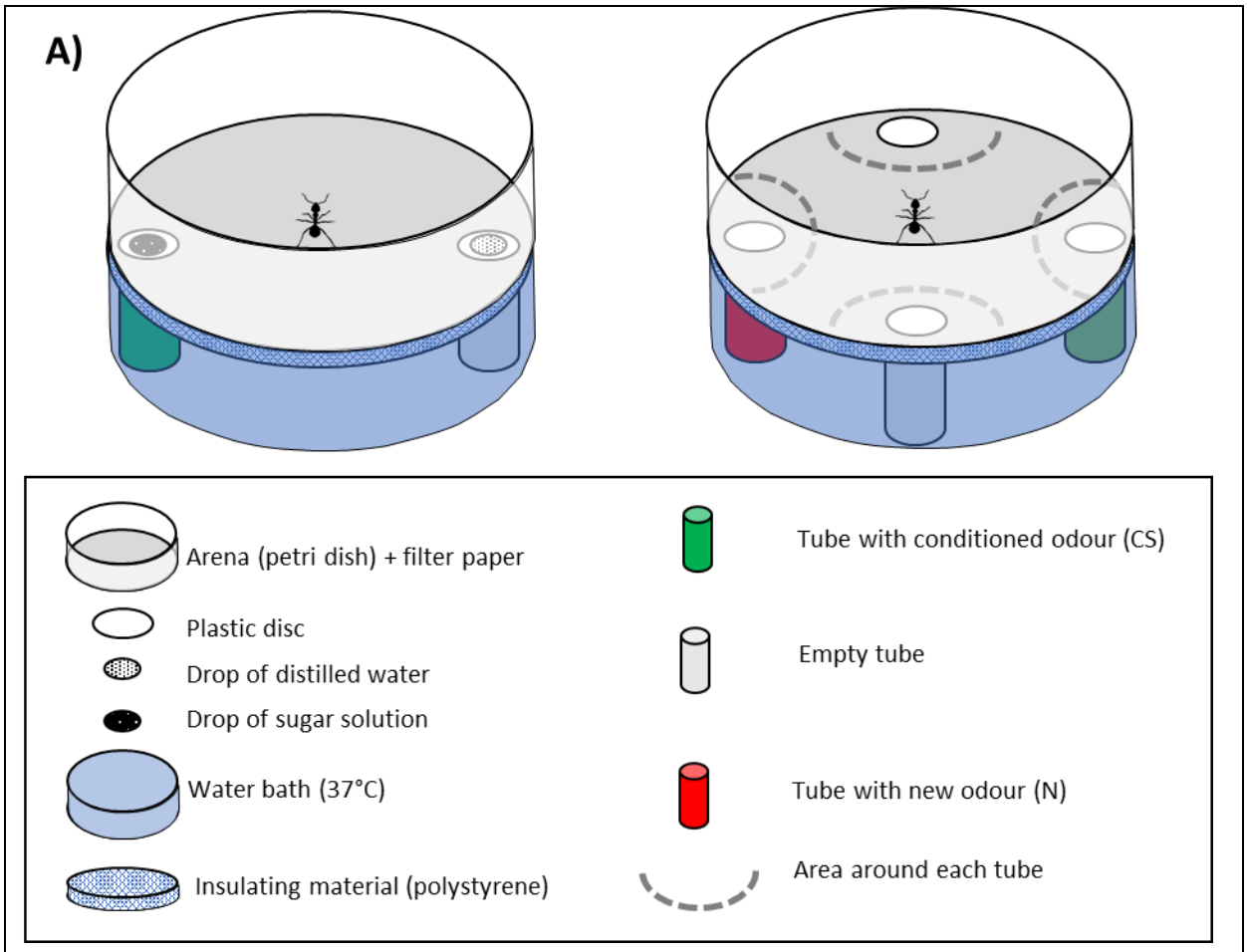

Figure 1: Schema of the experimental arena. During conditioning, the setup in (**A**) was used. We recorded the time needed by the ant to find the reward. During memory tests, a slightly different setup was used **(B)**. The time spent by the ant in the vicinity of the odours (areas, dashed lines) was recorded for two minutes. Each area measured 35.5 cm². As cells grow at 37°C, the vials containing stimuli were kept in a water bath, so that cell medium remained at 37°C. To avoid the possible effect of heat on ants' learning and memory performances, polystyrene foil was inserted between the water bath and the arena. Small holes in the filter paper above the tube allowed the odour to diffuse. The orientation of the arena in the experimental room was modified between trials so that ants could not use spatial cues.

2. Chemical analysis

VOCs emitted by the supernatant of all cultured cell lines used for behavioural experiments were determined using chemical analysis. Cell metabolism produces compounds that can be found in the culture medium. Cells of different origins do not consume and expel the same compounds, thus producing a unique pattern of VOCs. For all conditions, we used the medium that had previously contained the cells as source of VOCs. In the case of the DMEM, the medium was incubated in the same conditions but not in contact with any cells. For IGROV, MCF7, MDA-MD-231 and MCF10, the medium was in contact with these cell types for four days (see cell cultures for details). For each sample, a 15 mL glass vial was filled with 10 mL of the supernatant (or clean medium in case of the DMEM analysis) and placed at 37 °C using a

water bath. A SPME fibre (50/30 DVB/CAR/PDMS, Supelco) was introduced through the PTFE/silicone 1.5 mm cap for 50 min (Hanai et al., 2012). After that, the fibre was immediately inserted into an Agilent Technologies 7890A gas-chromatograph, equipped with a HP5MS GC column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, Les Ulis Cedex, France). The carrier gas was helium (1 $mL.min^{-1}$), and the injection was split less (250 °C). The oven temperature was programmed at 40 °C for 5 min, then increased to 220 °C at 7 $°C.min^{-1}$, and to 300 °C at 15 $°C.min^{-1}$ and was held for 3 min. The GC was coupled with a 5975 C mass-spectrometer (Agilent Technologies). Mass spectra were recorded with electron impact ionization at 70 eV. Peak areas were integrated with MSD ChemStation software version E.02.01.1177 (Agilent Technologies). Peaks were identified by comparing their ion spectrum to the NIST library (NIST v2.2, 2014) and to standards injected with the same temperature programme (decane, benzyl alcohol, acetophenone, undecane, nonanal, dodecane, decanal, benzene, 1,3-bis(1,1-dimethylethyl), and decanol, all from Sigma Aldricht, Saint-Louis, MO, USA). We found high consistency between the spectra of the standards and those of the compound extracted from our cell samples (Supplementary figure 4).

Statistics

1. Behaviour analysis

Data were analysed using R software (v. 4.0.0, R Core Team, 2020). Significance was fixed at $\alpha = 5\%$. All data were analysed using linear mixed models (LMM, package 'lme4', Bates et al., 2015). The identity of individuals and the colony were included as nested random factors.

a. Conditioning

We analysed the effect of the number of conditioning trials (named *trials*) on the dependent variable *time* (continuous variable, the time to find the reward). For the experiment in which several odours were used for training, we analysed the effect of the *conditioning odorant* (factor with two levels, MCF-7 *vs* MCF-10A or MCF-7 *vs* MDA-MD-231). We also looked at the interaction *conditioning odorant* x *trials* to detect possible differences in ants' responses depending on the odorant used.

b. Memory tests

First, we checked whether ants spent more time near the vials with odours or near the control unscented vials, by analysing the effect of the independent variable *presence of odour* (factor with two levels, Yes or No) on the dependent variable *time* (continuous variable, the time spent near the odours or near the unscented vials not). Then, in all experiments, we analysed the effect of the independent variable *stimulus* (factor with two levels, CS or N) on the dependent variable *time* (continuous variable, the time spent in the vicinity of an odour) during the memory tests. Finally, using data subsets, we also analysed the first and the second memory test in each experiment.

2. Chemical analysis

Contaminants (silicate-derived molecules originating from the GC column) were discarded from the analysis (e.g. compounds at 12.25 min, Fig. 3A). The areas of 25 regularly occurring peaks were standardized by calculating the ln (Pi /g(P)) (Aitchison,1986), where Pi is the area of a peak and g(P) is the geometric mean of all the peak areas of the individual. We then reduced the number of variables by running a principal component analysis (PCA) on the standardized peak areas and retained the first eight principal components (PCs). These new variables (PCs), which together explained 90.40% of the total variance (Supplementary Table 3), were used to construct a heatmap. Combined to that heatmap, the standardized area of the 25 peaks were used in a Hierarchical Cluster Analysis using Ward's classification method to classify cell samples. The significance ($p < 0.05$) of each node in the cluster was determined by multiscale bootstrap clustering with 10.000 iterations using the 'pvclust' package (Suzuki et al., 2019). The results are visualized as a heat map, where positive PCA scores are in blue, and negative ones are in red. The relative abundance of each peak is also displayed in Supplementary table 4.

Results

1. Behavioural experiments

a. Conditioning

During the acquisition phase of the olfactory conditioning, the ants learned the different stimuli in a similar way, as shown by the absence of any significant interaction between *conditioning odorant* and *trial* (Linear Mixed Models (LMM): $0.54 \leq F \leq 2.51$, d.f. = 2, $p > 0.05$, Fig. 2B, N = 47, and Fig. 2C, N = 49). In all experiments, the time spent by the ants to find the reward decreased significantly across training *trials* (LMM: $4.43 < F < 6.27$, d.f. = 2, $p < 0.05$, see Supplementary Table 1 for details, N = 36 - 49). This suggests that ants learned that the CS (conditioned odorant) predicted the US (reward) (Fig. 1A-C). Memory tests were then performed to verify this in the absence of reward.

b. Memory tests

In all our conditions, ants spent more time near the odorant vials than near the unscented ones ($22.1 < F < 186.1$, d.f. = 1, $p < 0.001$, Supplementary Table 2, N = 36 - 49), meaning that ants were not conditioned to the presence of a vial or a disc, but to the odour. When focusing on the time spent near the vials with odours, ants spent more time in the CS area than in the N (novel odour) area during the memory tests ($8.91 < F < 11.19$, d.f. = 1, $p < 0.01$, Fig. 2D-F). This indicates that ants learned to differentiate different cell-based stimuli after a short learning phase (three training trials). Thus, ants could distinguish the VOCs of an ovary cell line from the culture medium (IGROV *vs* DMEM, N = 36, Fig. 2D), differentiate a cancerous cell line from a healthy one (MCF-7 *vs* MCF-10A, N = 47, Fig. 2E) as well as discriminate between two breast cancer cell lines (MCF-7 *vs* MDA-MD-231, N = 49, Fig. 2F). Using data subsets (Supplementary Table 2), we observed that for the second memory test, ants always spent more time near the CS than the N odour ($7.32 < F < 10.86$, d.f. = 1, $p < 0.01$). For the first memory test, a tendency to spend more time in the CS is observed when using a single cell line ($F = 3.57$, d.f. = 1, $p > 0.06$, IGROV-1 *vs* DMEM, N = 36), but no significant differences was observed when using two different cells lines ($1.56 < F < 2.21$, d.f. = 1, $p > 0.1$, MCF-7 *vs* MCF-10A, N = 47, and MCF-7 *vs* MDA-MD-231, N = 49).

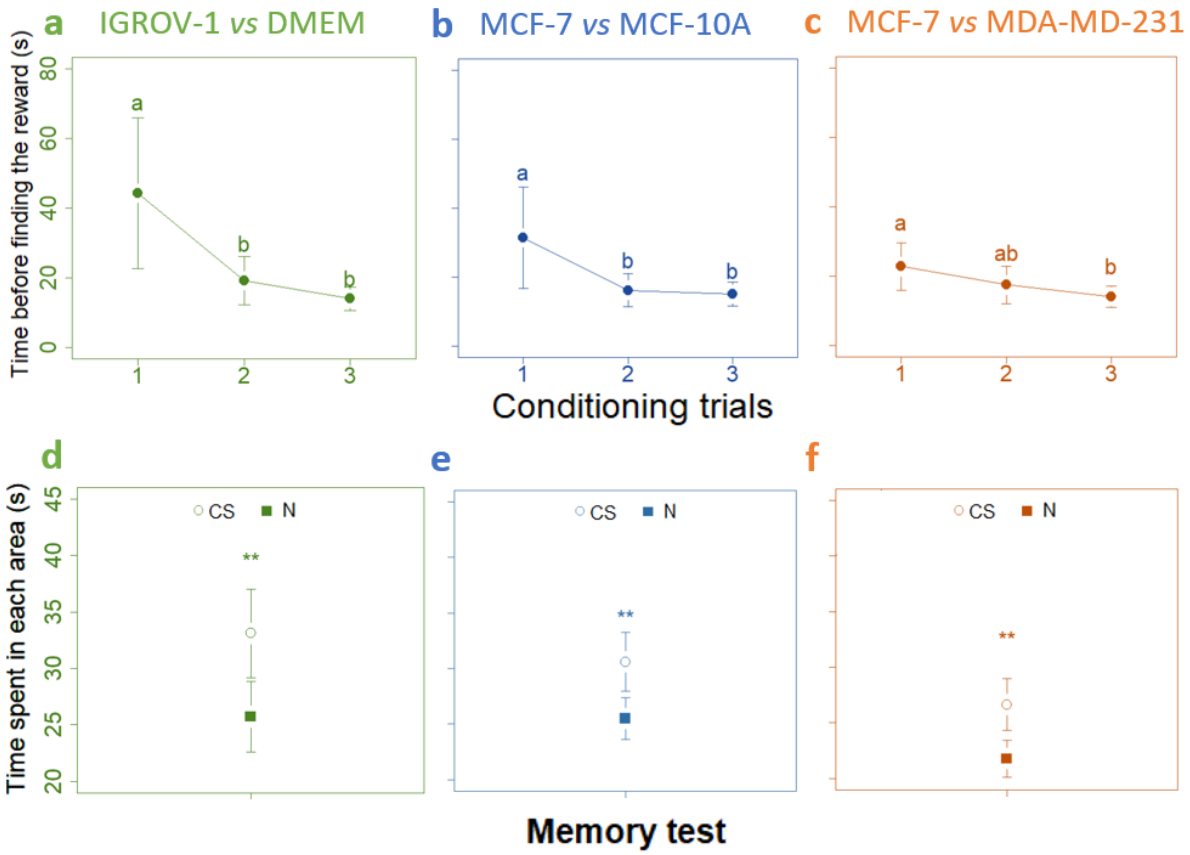

Figure 2: Ants' performances during acquisition **(A, B, C)** and memory tests **(D, E, F)** in an appetitive conditioning experiment with cancer cell line odour. **A-C:** Ants were conditioned to associate a reward (sugar solution 30%) with the conditioned stimulus (CS) represented by cell line odour of IGROV-1 (**A**, N = 36), MCF-7 (N = 25) or MCF-10A (N = 22) **(B)** and MCF-7 (N = 25) or MDA-MD-231 (N = 24) **(C)**. The graph shows the time needed by ants to find the reward in the circular arena. Three conditioning trials were done with an inter-trial interval of three minutes. Significant differences between conditioning trials are indicated with different letters. **D- F:** time spent by individual ants in the vicinity of each stimulus during the pooled memory tests (circle: CS area; square: Novel odour area). Circles and squares represent the mean while error bars show confidence intervals (95%). Significant differences between stimuli are noted with asterisks (**: $p \leq 0.01$).

2. Chemical analysis

The Principal Component Analysis (PCA) was based on the 25 common peaks across samples (Fig. 3a). Eight principal components (PCs) were extracted, that together explain 90.40% of the variance. The different cell lines were well separated, as shown by a plot of the first two principal components (Fig. 3b). A hierarchical cluster analysis based on the 25 peaks showed a clear separation of all the sample types (Fig. 4). In this analysis, the node separating MCF-7 and MCF-10A samples was significant ($p < 0.05$), as were the nodes grouping MCF-7 samples on the one hand, and MCF-10A samples on the other. The IGROV-1, MDA-MD-231 and corresponding medium (DMEM) samples were each clustered in different groups with well-

supported nodes (p-value < 0.1). In this analysis, all the individual samples were correctly clustered, showing their distinctive VOC compositions.

On the heatmap (Fig. 4) based on the first eight PCs, we observed that the first PC discriminated all the cell lines (IGROV-1, MDA-MD-231, MCF-7 and MCF-10A) from the culture medium (DMEM). By comparing the factor loadings showing a coefficient higher than 0.6 (Supplementary Table 3) with the heatmap, we observed that, styrene (1), oxime-, methoxy-phenyl (2), unidentified hydrocarbon (15), dodecane (18), unknown VOCs (21), and benzene, 1,3-bis(1,1-dimethylethyl)- (22) are all more abundant in cell lines. On the contrary, benzaldehyde (3), unknow aromatic compound (5), unidentified VOC (17), decanal (19) and decanol (23) are more present in DMEM than in cells, which suggests that they are consumed by the cells. The next 2 PCs differentiated among the different cell lines. While the second PC discriminated MCF7 and IGROV-1 from MDA-MD-231 and MCF-10A, the third PC separated MCF10A and MCF7 from IGROV-1 and MDA-MD-231. Phenol (4), benzeneacetaldehyde (9), nonanal (16) were more abundant in MCF-7 and IGROV-1, whereas a hydrocarbon (10) and an unidentified VOC (24) were more present in MDA-MD-231 and MCF-10A. Benzyl alcohol (8) is found in lower relative proportion in MCF7 and MCF-10A compared to the other cell lines as indicated by the third PC.

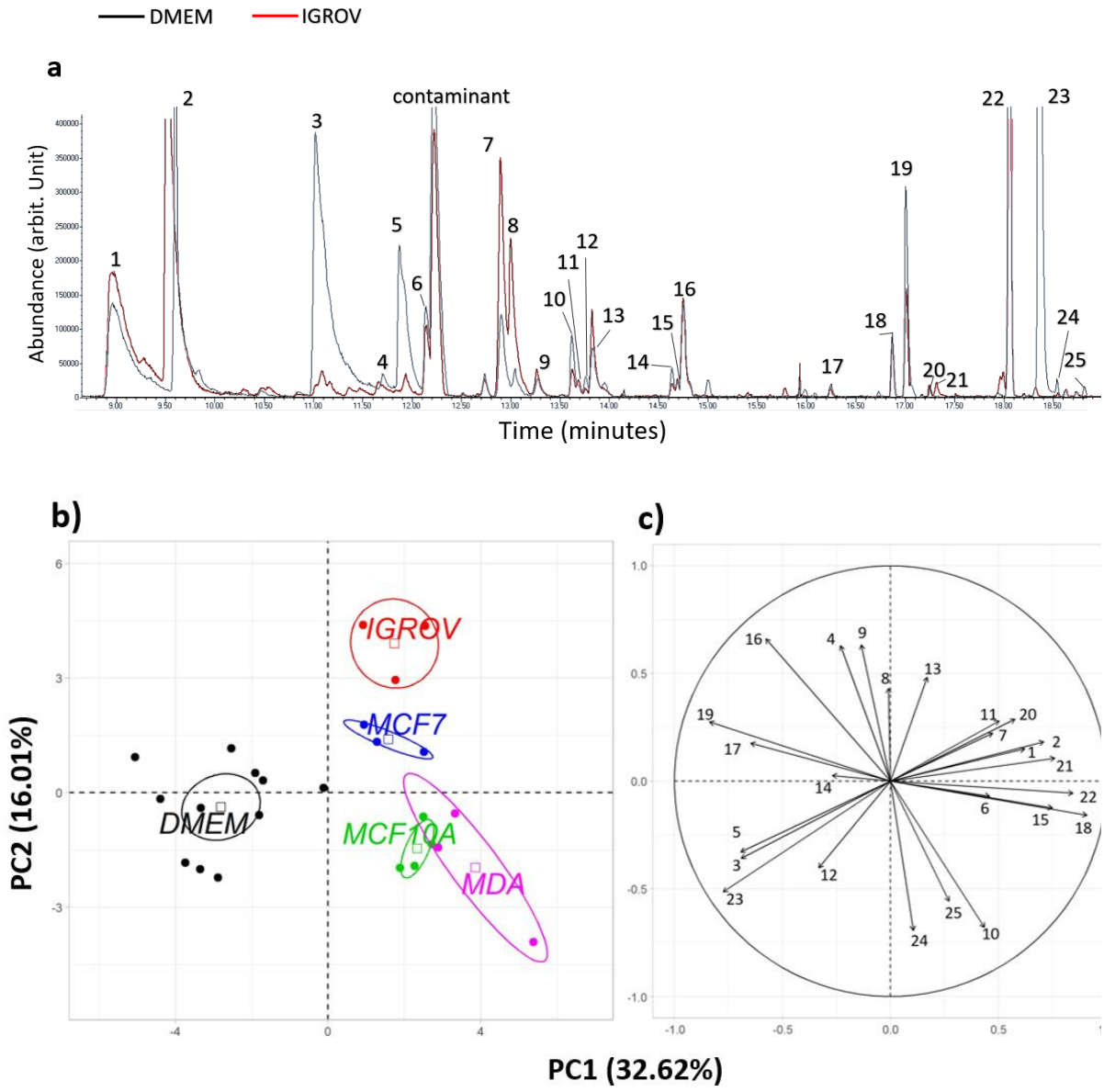

Figure 3: (a): comparison of two chromatograms (DMEM in black, IGROV in red) showing the analysed molecules (see below for compound identification). Some peaks were present in relative high abundance compared to others. In order to see all the peaks, the present version was cut. For the original chromatogram, see supplementary figure 3.
b) Plot of the first two Principal Components (PC), explaining 48.63% of the total variance. Cell line samples are well separated by the Principal Component Analysis (PCA) **c)** Circle of variables used in the PCA showing the correlation between the first two PC and the original variables. The angle between the vectors represents the correlation between the variables. The length of the vector line and its closeness to the circle indicate how well the variable is represented in the plot, and consequently its contribution to the discrimination of cell types. Identification of the VOCs: (1) styrene, (2) oxime-, methoxy-phenyl, (3) benzaldehyde, (4) phenol, (5) aromatic compound, (6) decane, (7) 1-hexanol, 2-ethyl-, (8) benzyl-alcohol, (9) benzeneacetaldehyde, (10) hydrocarbon, (11) decane, 4-methyl-, (12) hydrocarbon, (13) acetophenone, (14) undecane, (15) hydrocarbon, (16) nonanal, (17) unidentified VOC, (18) dodecane, (19) decanal, (20) benzaldehyde, 3,4-dimethyl, (21) unidentified VOC, (22) benzene, 1,3-bis(1,1-dimethylethyl)-, (23) decanol, (24) unidentified VOC, (25) 2-undecanone. Spectra of the compounds can be found in Supplementary Fig. 4.

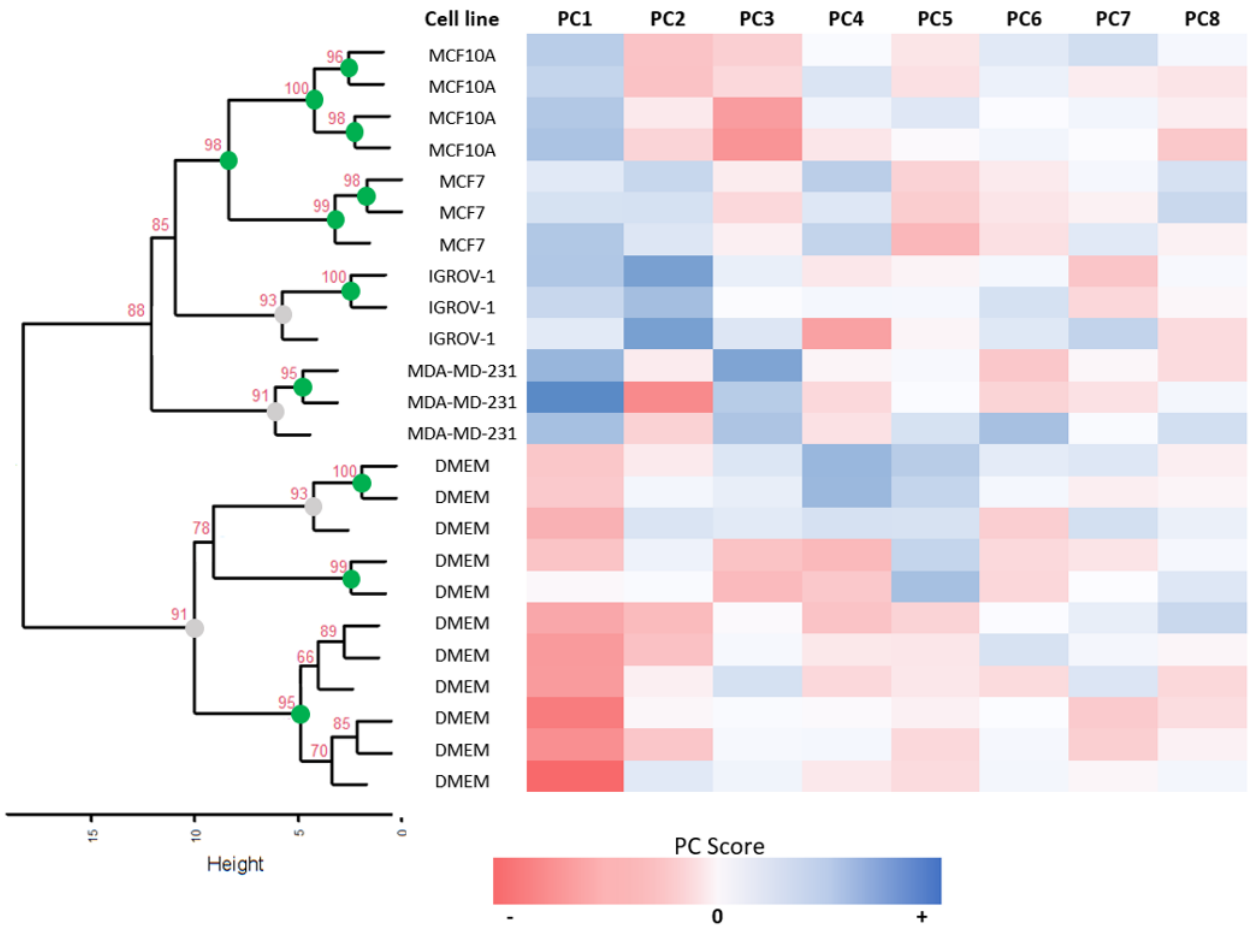

Figure 4: Hierarchical Cluster analysis and heat map of the contribution of each principal component (PC) to the discrimination of samples. PC scores are represented from a red (negative scores) to a blue spectrum (positive scores). The significance ($p < 0.05$) of the sample marker clusters was determined by multiscale bootstrap clustering with 10 000 iterations. Green circles represent nodes with $p < 0.05$, grey circles are for nodes with $p < 0.1$.

Discussion

Using a simple conditioning protocol, we show here that *Formica fusca* ants can detect the VOCs emitted by cancer cells. A conditioning protocol based on only three training trials was sufficient for ants to associate cell-derived VOCs with a reward. Ants were able to i) perceive the presence of cells in a medium, ii) differentiate cancerous VOCs from non-cancerous ones, and iii) differentiate between two cancerous samples based on VOCs. Chemical analyses via SPME and GC-MS demonstrated that the different cell lines used in the behavioural study can be chemically characterised and discriminated from each other based on their VOCs.

Discrimination abilities of ants

Formica fusca ants learn fast and retain a learned association after only three trials. Such remarkable learning abilities were recently described in this species (Piqueret et al., 2019) and in other ant species such as *Lasius niger* (Czaczkes & Kumar, 2020; Oberhauser et al., 2019), *Camponotus spp.* (Dupuy, Sandoz, Giurfa, & Josens, 2006; Guerrieri & d'Ettorre, 2008; Josens, Eschbach, & Giurfa, 2009) and *Linepithema humile* (Rossi et al., 2020). In all our experiments, ants were able to discriminate between the chemical samples, even when the task was potentially arduous (discrimination of two cancerous samples, Fig. 2F). As several ant species displayed robust learning abilities using the same conditioning protocol (Piqueret et al., 2019; Rossi et al., 2020), comparative studies using several species would be of interest to investigate their discriminative abilities in the context of cancer VOCs detection. If no significant difference emerges, we suggest using a commonly available species, which is not aggressive, and large enough to be easily individually followed, such as *Formica fusca*.

Advantage of using ants

Dogs are the animals most commonly used as bio-detectors of cancer. They notably show high discrimination abilities. They were first tested using cell line samples, as in the present study, but were also submitted to body-fluids odours, which are more complex (reviewed in Brooks et al., 2015 and Pirrone & Albertini, 2017). Here, we used ants as bio-detectors. Ants are available in great numbers, and collectively, choose the right odour with a very high probability ($p < 0.01$ in all our experiments) and thus equivalent to dogs in terms of detection abilities. In some respects, ants surpass dogs with an extremely shorter training time (1 h compared to 6 – 12 months for a dog), a reduced cost of training and maintenance (honey and frozen crickets twice a week). Our simple protocol can be implemented by everyone, after a training time of about 3-days (personal observation). Individual *Formica fusca* ants can also be used more than once. With a single trial, ants could be tested up to 9 times before response extinction (Piqueret et al., 2019). Ants therefore represent a fast, efficient, inexpensive and highly discriminant detection tool for VOCs biomarkers.

GC-MS analysis

Using SPME and GC-MS, we provided chemical support for ants' behaviour. Analysed VOCs, can be used to discriminate one cell sample from another (and from the medium alone, see Fig. 4 and Supplementary Table 3 for identification). Cell lines were also well separated from each other by an unsupervised cluster analysis (Fig. 4), thus supporting the results of the ants' behaviour based on olfactory conditioning. We detected several VOCs that were also found in other studies on cancer biomarkers. In particular, we identified styrene, oxime-methoxy-phenyl, benzaldehyde, phenol, decane, 1-hexanol, 2-ethyl, acetophenone, nonanal, dodecane, decanal (Altomare et al., 2013; Amal et al., 2015; Bajtarevic et al., 2009; Brooks et al., 2015; Filipiak et al., 2014; Hanai et al., 2012; Lavra et al., 2015; Liu et al., 2019; Silva et al., 2017; Wang et al., 2014). We found that, in the MCF-7 cell line, dodecane was more abundant compared to the DMEM alone, whereas the relative proportion of benzaldehyde was lower in the cells than in the medium. This is consistent with results of Silva et al., (2017), which also focused on MDA-MD-231 cells and found that MCF-7 expressed more dodecane and less benzaldehyde. Benzaldehyde was also found in lower quantity in MCF-7, MCF-10A and MDA-MD-231 cells in another study (Lavra et al., 2015).

In the IGROV-1 cell line, we found that styrene and dodecane were more present compared to the DMEM alone. These VOCs were also found in higher abundance in the breath of patients with ovarian cancer (Amal et al., 2015). In this study, they also noted that decanal was less abundant, which is not the case in our study. However, we lack a real control for IGROV-1, such as a non-cancerous ovarian cell line.

Conclusion

The results of this first controlled study using ants as cancer bio-detectors are clear and highly promising. The next step will be to use body odours of patients as stimuli, instead of VOCs from cell cultures. Body odours add noise, due to patients' variability in diet, sex, age, or the presence of other medical conditions. Our study clearly shows that ants have the potential to become an inexpensive, fast and efficient tool for the early detection of human tumours. By 2040, current projections (www.who.it, last access on the 09 December 2020) estimate that there will be up to 30 million cases of cancer worldwide. The development of new tools, such

as the use of VOCs as biomarkers, that could help in the detection of cancer should be intensively studied to help reduce the burden of this deadly disease.

Authors' contributions

B.P., P.d.E. and J.-C.S. conceived the project and designed the experiments. The conditioning and memory test arenas were built by P.D. with inputs from B.P and B.B. F.M.-G. & B.B. proposed the different cell lines, which were cultivated by B.B. and B.P. B.P. performed the behavioural experiments and analysed the data with the help of P.d.E. and J.-C.S., and F.M.-G. discussed about experiments and results all along the study. C.L. oversaw the chemistry data acquisition. Chemistry data were analysed by B.P. with help of C.L. and P.d.E. The manuscript was written by B.P. and revised by P.d.E., J.-C.S., B.B and F.M.-G. Final manuscript was approved by all authors.

Competing interests.

We declare we have no competing interests.

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Supplementary Information:

Ants detect cancer cells through volatile organic compounds

Piqueret, B., Bourachot, B., Leroy, C., Devienne, P., Mechta-Grigoriou, F., d'Ettorre, P., Sandoz, J.C.

Supplementary Table 1| Result of the different linear mixed models (LMMs) used for analyzing ants' conditioning performances (Fig 2A-C): Time was the dependent variable. Significant effects ($p < 0.05$) are indicated in bold.

Focus on the factor(s)	Df	F value	p value
Fresh cells experiment			
IGROV-1 *vs* DMEM			
Trials	5	9.8983	**<0.001**
Trials (1 *vs* 2)	1		**0.024**
Trials (1 *vs* 3)	1		**0.006**
Trials (1 *vs* 4)	1		**<0.001**
Trials (1 *vs* 5)	1		**<0.001**
Trials (1 *vs* 6)	1		**<0.001**
Trials (2 *vs* 3)	1		1
Trials (2 *vs* 4)	1		1
Trials (2 *vs* 5)	1		0.506
Trials (2 *vs* 6)	1		1
Trials (3 *vs* 4)	1		0.586
Trials (3 *vs* 5)	1		0.343
Trials (3 *vs* 6)	1		1
Trials (4 *vs* 5)	1		1
Trials (4 *vs* 6)	1		1
Trials (5 *vs* 6)	1		1
Frozen cells experiment			
IGROV-1 *vs* DMEM			
Trials	2	5.9852	**0.003**
Trials (1 *vs* 2)	1		**0.033**
Trials (1 *vs* 3)	1		**0.009**
Trials (2 *vs* 3)	1		1
MCF-7 *vs* MCF-10A			
Conditioning odorant × trials	2	2.5129	0.08658
Trials	2	6.2705	**0.003**
Trials (1 *vs* 2)	1		**0.010**

Trials (1 *vs* 3)	1		**0.006**
Trials (2 *vs* 3)	1		1
MCF-7 *vs* MDA-MD-231			
Conditioning odorant × trials	2	0.5389	0.585
Trials	2	4.4332	**0.014**
Trials (1 *vs* 2)	1		0.051
Trials (1 *vs* 3)	1		**0.015**
Trials (2 *vs* 3)	1		1

Supplementary Table 2 | Results of the linear mixed models (LMMs) used for analyzing ant's performances in the memory tests (Fig 2D-F): Time was the dependent variable. Significant effects ($p < 0.05$) are indicated in bold.

Focus on the factor(s)	Df	F value	p value
Fresh cells experiment			
IGROV-1 *vs* DMEM			
Presence of odour	1	22.064	**<0.001**
Stimulus	1	16.046	**<0.001**
Frozen cells experiment			
IGROV-1 *vs* DMEM			
Presence of odour	1	103.01	**<0.001**
Stimulus	1	10.749	**0.001**
Stimulus Test 1	1	3.567	0.063
Stimulus Test 2	1	7.323	**0.008**
MCF-7 *vs* MCF-10A			
Presence of odour	1	186.12	**<0.001**
Stimulus	1	8.909	**0.003**
Stimulus Test 1	1	2.209	0.141
Stimulus Test 2	1	7.381	**0.008**
MCF-7 *vs* MDA-MD-231			
Presence of odour	1	81.165	**<0.001**
Stimulus	1	11.234	**<0.001**
Stimulus Test 1	1	1.563	0.214
Stimulus Test 2	1	10.859	**0.001**

Supplementary Table 3| VOCs found in samples with identification. Compounds in bold were compared with standards injected using the same protocol. The spectra of other VOCs were compared with a reference database (NIST v2.2. 2014). The area under each peak was normalized according to Aitchison transformation, and a Principal component analysis was performed of the transformed peak areas. The factor loadings of the compounds in each Principal Components (PCs) are indicated. The Eigen values, the percentage of explained variance, and the cumulative percentage of explained variance are also presented at the end. Together, the eight PCs accounted for more than 90 % of the total variance. The most important VOCs (contribution >0.6) for each PCs are highlighted in blue or red, depending on their positive or negative impact on each PC.

Peak no.	Compounds	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
1	Styrene (C_8H_8)	0,62	0,15	-0,1	0,18	0,12	-0,41	0,38	-0,33
2	Oxime-, methoxy-phenyl ($C_8H_9NO_2$)	0,71	0,18	0,56	-0,01	0,04	0,03	0,1	-0,2
3	Benzaldehyde (C_7H_6O)	-0,69	-0,36	0,44	0,17	0,17	-0,3	0,08	0,05
4	Phenol (C_6H_6O)	-0,23	0,63	0,06	0,36	-0,11	-0,46	-0,1	-0,04
5	Aromatic compound	-0,69	-0,33	0,21	-0,1	-0,26	0,08	0,06	-0,27
6	**Decane ($C_{10}H_{22}$)**	0,46	-0,07	0,16	0,53	-0,23	0,25	-0,31	-0,36
7	1-Hexanol, 2-ethyl- ($C_8H_{18}O$)	0,47	0,22	-0,53	-0,34	0,48	-0,04	0	-0,24
8	**Benzyl alcohol (C_7H_8O)**	-0,01	0,43	-0,87	0,07	-0,11	0,11	0,02	-0,06
9	Benzeneacetaldehyde (C_8H_8O) (Phenylethanal)	-0,13	0,63	0,39	-0,07	0,35	-0,18	-0,06	0,32
10	Hydrocarbon	0,43	-0,68	0,06	-0,02	0,48	0,26	0,04	0,03
11	Decane, 4-methyl-	0,5	0,28	-0,4	0,54	-0,09	0,12	-0,11	0,33
12	Hydrocarbon	-0,33	-0,4	-0,43	0,4	0,44	0,28	0,26	0,08
13	**Acetophenone (C_8H_8O)**	0,17	0,48	0,52	-0,02	0,12	0,36	0,35	0,06
14	**Undecane**	-0,27	0,03	0,32	0,8	0,04	0,09	-0,21	-0,12
15	Hydrocarbon	0,75	-0,13	0,36	0,39	0,12	-0,04	0,11	0,02
16	**Nonanal ($C_9H_{18}O$)**	-0,58	0,66	0,37	-0,06	0,14	0,06	-0,02	0,07
17	Unknown VOCs	-0,65	0,18	0,34	-0,14	-0,25	0,44	0,11	-0,08

18	**Dodecane ($C_{12}H_{26}$)**	0,91	-0,16	0,18	-0,1	0,02	0,02	0,07	0,14
19	**Decanal ($C_{10}H_{20}O$)**	-0,84	0,27	-0,02	-0,26	0,2	0,14	-0,03	0,06
20	Benzaldehyde, 3,4-dimethyl- ($C_9H_{10}O$)	0,58	0,29	-0,05	-0,52	-0,51	0,11	-0,06	0
21	Unknown VOCs	0,76	0,11	0,07	-0,02	0,1	0,1	-0,4	0,23
22	**Benzene, 1,3-bis(1,1-dimethylethyl)- $C_{14}H_{22}$ =** 1,3di-tert-butyl benzene ($C_{14}H_{22}$)	0,84	-0,06	0,24	-0,38	-0,09	-0,06	0,12	0
23	**Decanol ($C_{10}H_{22}O$)**	-0,77	-0,51	-0,12	-0,11	-0,14	-0,17	0	0,07
24	Unknown VOCS	0,11	-0,69	0,25	-0,38	0,2	-0,12	-0,44	-0,03
25	2-Undecanone ($C_{11}H_{22}O$)	0,27	-0,56	0,03	0,22	-0,54	-0,1	0,29	0,37
Eigen value		8.15	4.00	3.06	2.57	1.76	1.18	1.00	0.88
Variance		32.62	16.01	12.25	10.27	7.03	4.71	4.00	3.51
Cumulative Variance		32.62	48.63	60.88	71.15	78.17	82.88	86.89	90.40

Methods and results of the experiment with fresh cell lines

In total, fifteen queenright colonies of *Formica fusca* were used. They were collected in 2015 (N = 3), 2017 (N = 4), 2018 (N = 1), 2019 (N = 4) and 2020 (N = 3). When testing whether ants can learn the odour from fresh cell samples, sub-colonies of ants were used. They were composed of 30 individuals (plus 3-5 larvae), that were kept in circular boxes (Ø = 12 cm, height = 8 cm) with black paper on the walls to limit stress (Franks et al., 2003). The fresh cell experiments were performed at the "Stress and Cancer" Laboratory (Curie Institute, Paris, France), and sub-colonies were brought from the LEEC twelve hours before the start of the conditioning phase, so that ants could acclimatize to local conditions.

Origin of odours

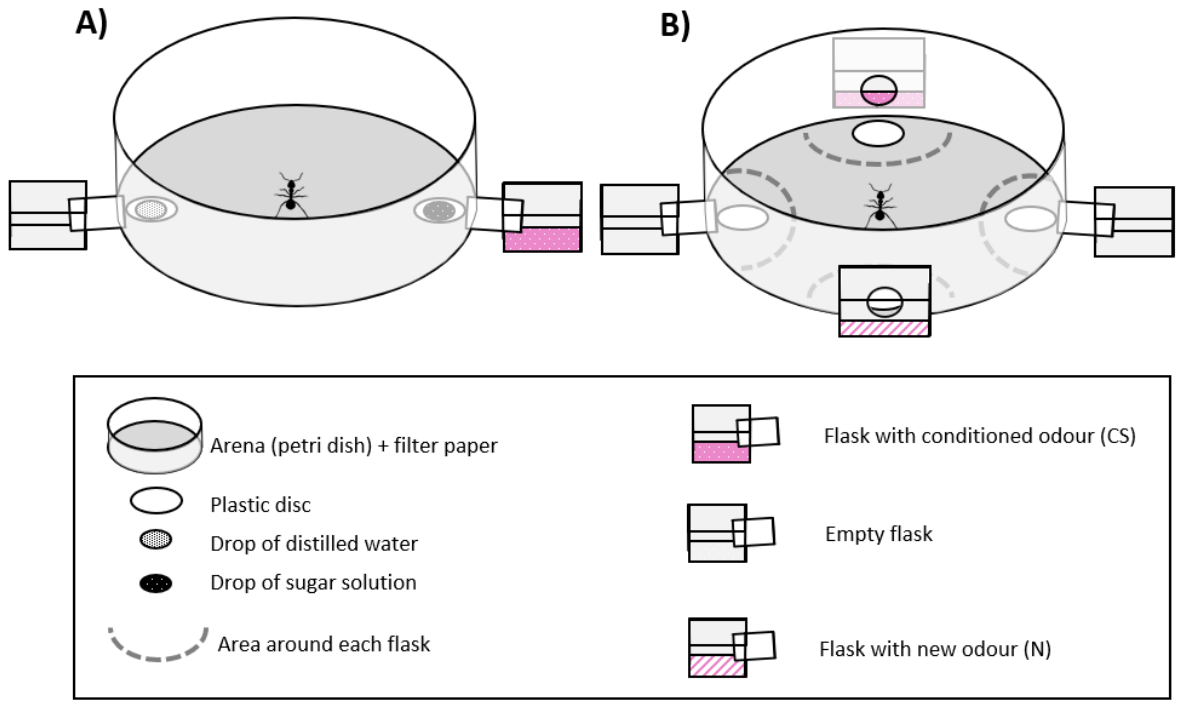
Human ovarian cancer IGROV-1 cell lines were cultivated at the Curie Institute similarly to the other cell lines (MCF-7, MCF-10A and MDA-MD-231). IGROV-1 cells were cultivated in 25 mL culture flasks with vented cap (Fisher Scientific, USA), and placed in an incubator at 37 °C and 5% CO_2. They were propagated in 10 mL of DMEM in each flask. The flasks were used as stimuli one week later. For control, we used DMEM alone prepared in the same flasks and kept in the same conditions.

Conditioning

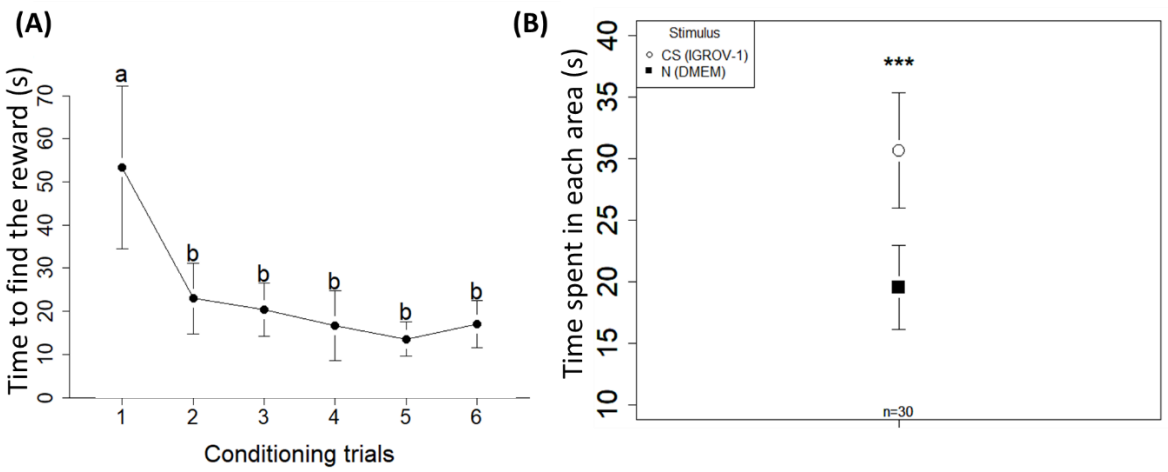
Ants were placed in a circular arena similar to the one used for the frozen cell lines. The major differences were 1) the absence of water-bath, and the use of polystyrene flasks instead of glass vials as stimuli. The arena had two holes in the wall facing each other. Culture flasks were inserted into the holes with their openings towards the centre of the arena. The flask presenting the CS contained IGROV-1 cells in 10 mL of DMEM, while the other flask was empty (used as control for visual and tactile cues). On each side, a metallic grid prevented the ants from entering the flask, while allowing passive diffusion of the odorants in the arena. Small plastic discs (Ø = 6 mm) placed at 1 cm in front of each flask received 1 ml of sugar solution (30% w/w) on the CS side and 1 ml of distilled water on the other side (Supplementary Fig. 1A). Between each trial, we also replaced the flasks with new ones to ensure that cells were still fresh and not dying (which could modify the emitted odours). The orientation of the arena and the position of the experimenter were also modified between trials to limit the possible use of visual or other spatial cues. Ants underwent 6 conditioning trials. In the course of conditioning, the time needed by the ant to find the reward decreased significantly ($F = 9.90$, $d.f. = 5$, $p < 0.001$, $N = 30$, Supplementary Fig. 2A), indicating that ants learned to associate the odour of the fresh cells with the reward.

Memory test

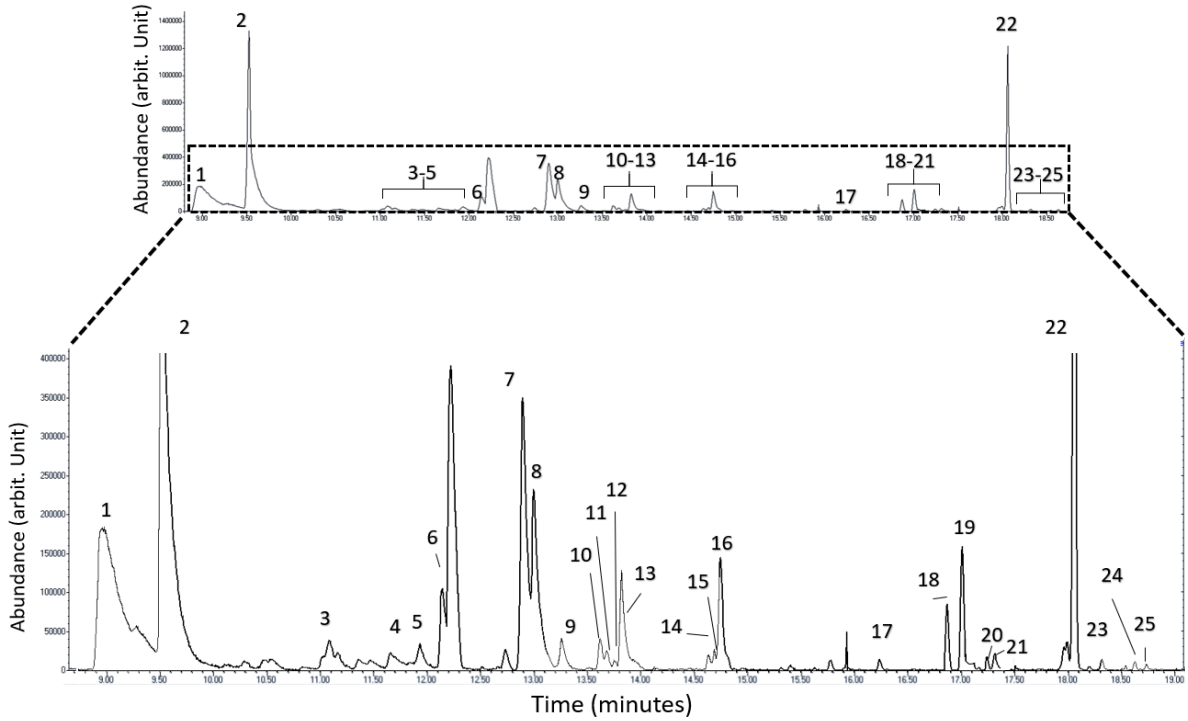
The arena used was similar to the one used for the conditioning phase, but here the arena had four holes in the wall (instead of two), which were connected to four flasks (Supplementary Fig. 1B). Four empty plastic discs were placed in front of them. There were two flasks with odorants: the CS (IGROV-1) and the novel odour, N (DMEM alone), and two flasks without odorants. Each ant underwent only one memory test 15 min after the end of conditioning. In this test, ants spent more time near the flasks with odour than without odour ($F = 22.064$, d.f. = 1, $p < 0.001$, N = 30). They also spent more time near the CS (IGROV-1) than near the N (DMEM alone) ($F = 16.05$, d.f. = 1, $p < 0.001$, N = 30, Supplementary Fig. 2B). This demonstrates that ants can use VOCs to detect the presence of fresh cells in a medium.



Supplementary Figure 1: Schema of the arena used in the experiment with fresh cell samples. During conditioning, the setup in (**A**) was used. We recorded the time needed by the ant to find the reward. During memory tests, the setup in **(B)** was used. The time spent by the ant in the areas in the vicinity of each odour source was recorded for two minutes. Each area measured 35.5 cm^2 (dashed lines). Cells were used directly after being taken from the incubator. As they went from 37°C to room temperature, and to be sure that cells did not start to die and modify their odour, a new stimulus was used at each trial. Metallic grids stopped the passage of ants from the arena itself to the stimuli but allowed passive diffusion of the odours. The orientation of the arena in the experimental room was changed between trials so that ants could not learn spatial cues.

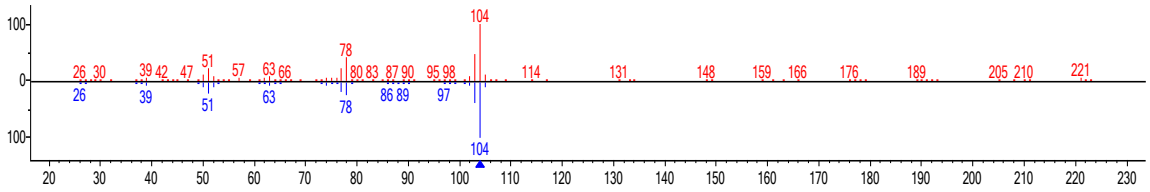


Supplementary Figure 2: Ants' performances during acquisition **(A)** and memory tests **(B)** in an appetitive conditioning experiment with fresh cancer cell samples. (A) Ants were conditioned to associate a reward (sugar solution 30%) with the odour of IGROV-1 cells. The graph shows the time needed by ants to find the reward in the circular arena. Six conditioning trials were applied with an inter-trial interval of three minutes. (B) Time spent by individual ants in each area during the memory tests (white circle: CS area; black square: N area). The memory test was performed 15 minutes after the end of conditioning. Circles and squares represent the mean and error bars show confidence intervals (95%). Significant differences between conditioning trials are noted with different letters. Significant differences between stimuli are noted with asterisks (***: $p \leq 0.001$).



Supplementary figure 3: Some peaks have a high relative abundance (e.g. peak 2 in this IGROV sample) and are hiding the smaller ones (e.g. 17). In order to better see the small peaks, we presented a zoomed chromatograph (lower panel) with cut compounds above 400.000 units of abundance.

1



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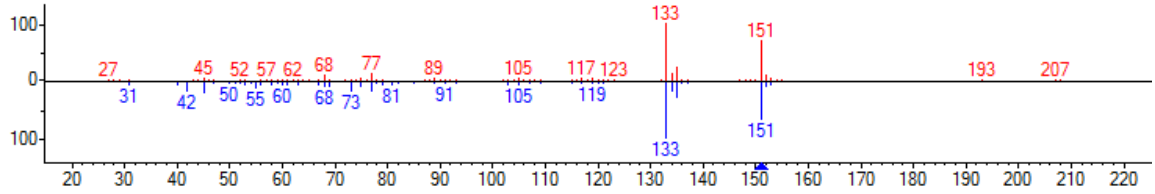

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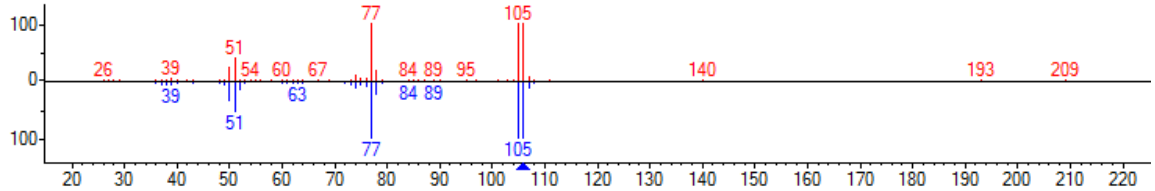

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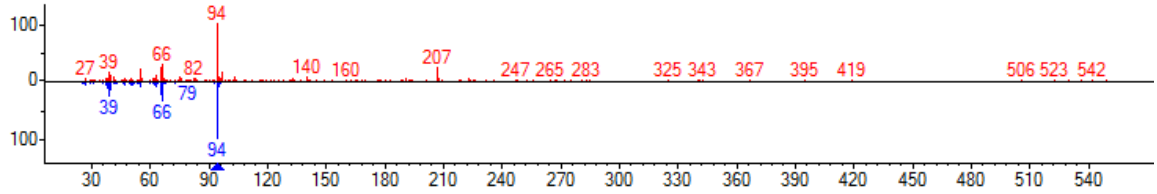

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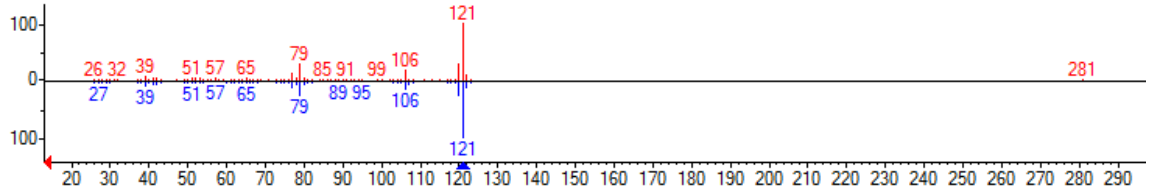

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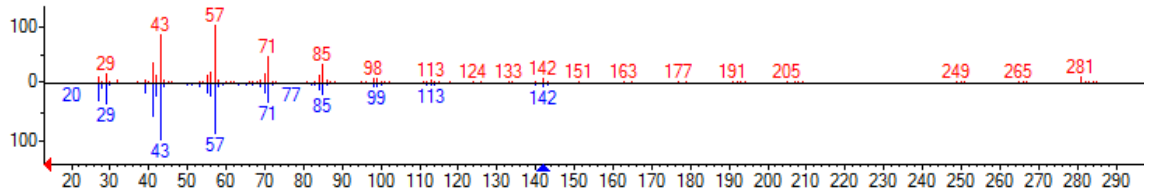

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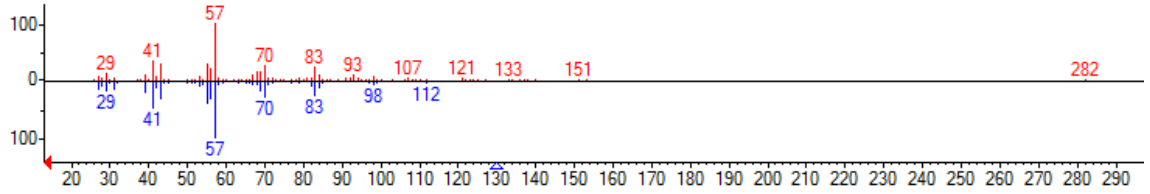

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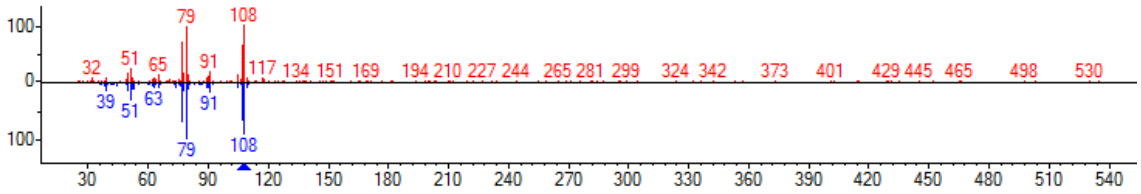

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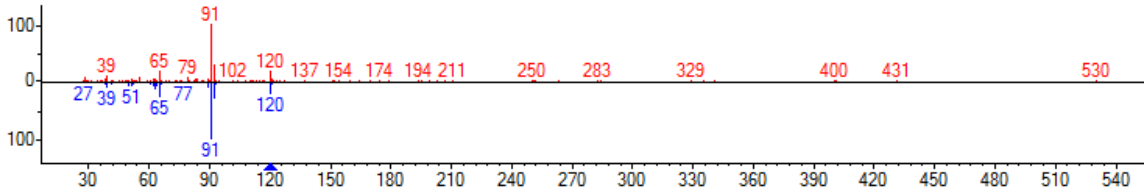

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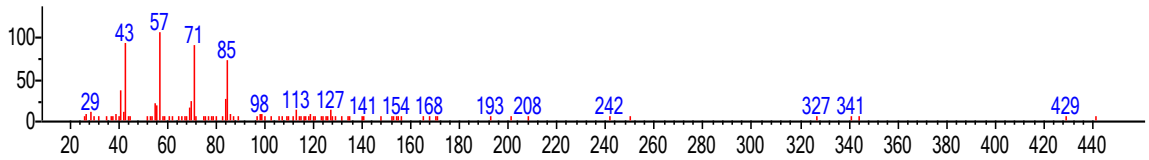

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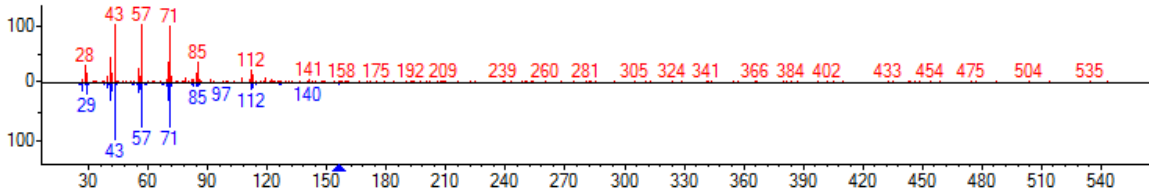

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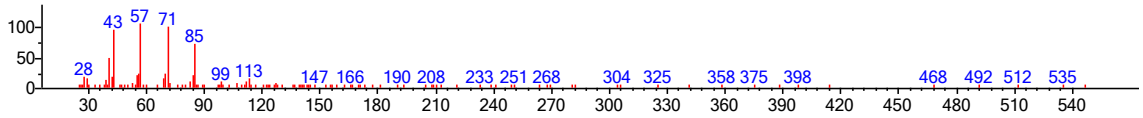

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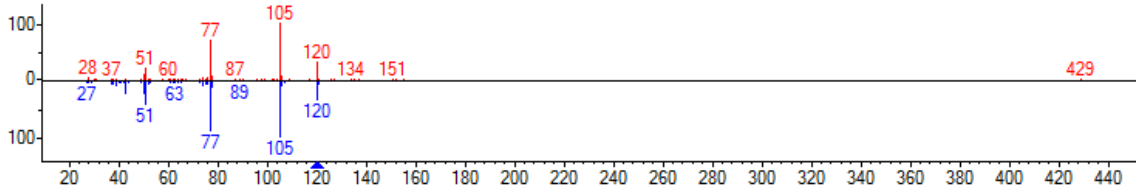

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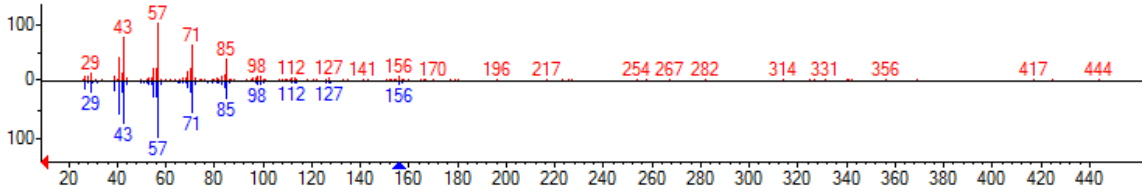

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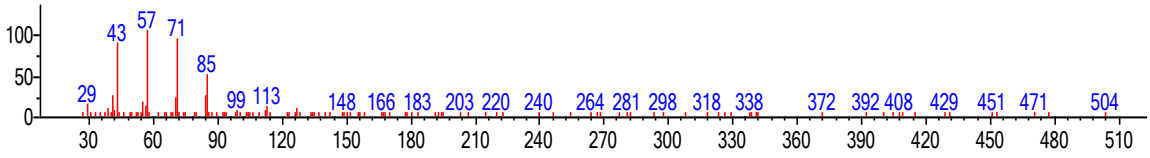

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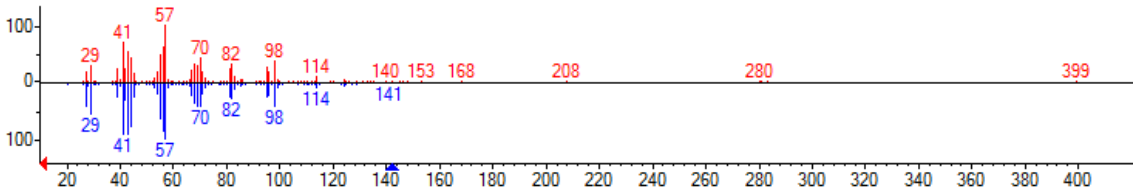

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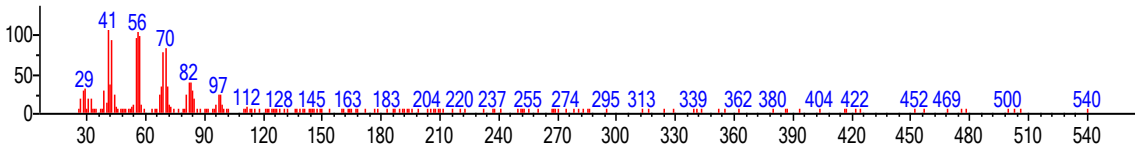

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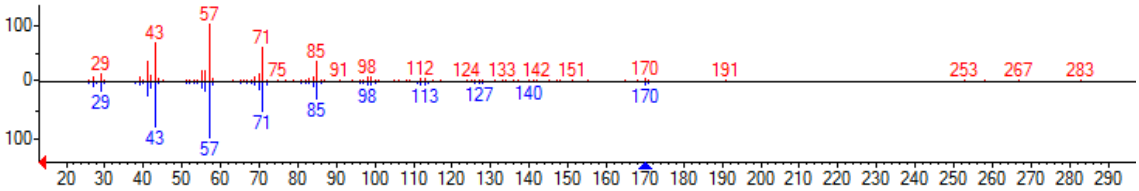

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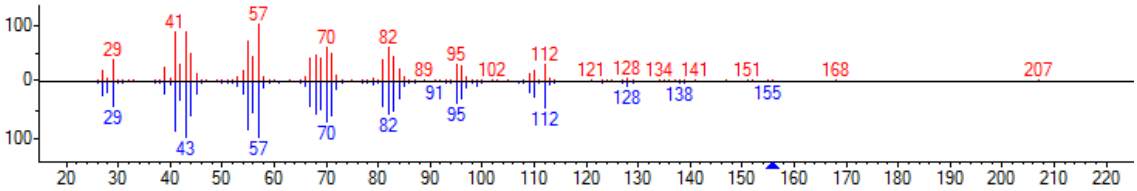

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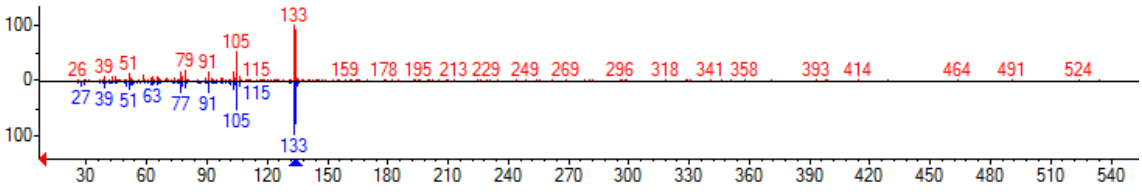

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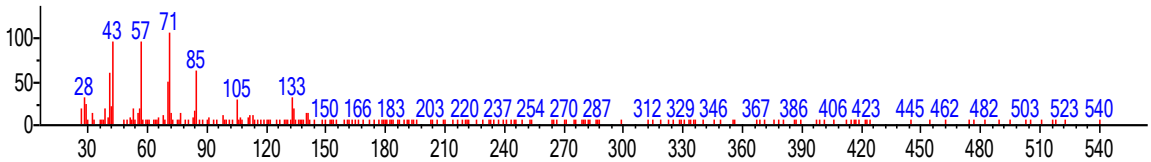

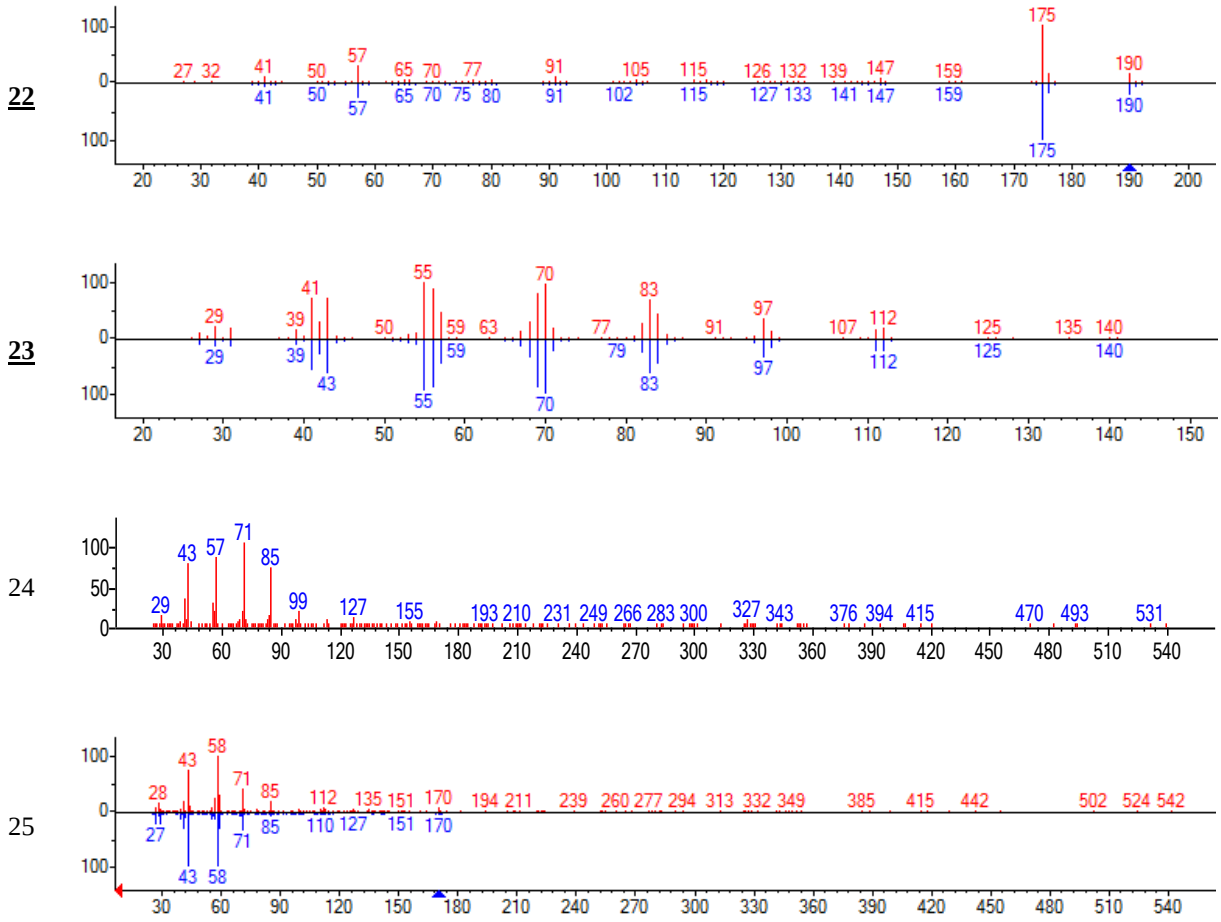

Supplementary figure 4: Spectra of the different compounds found in cell samples (red spectra) compared with spectra from databases (blue spectra). The underlined and bold compounds (#6 ,8, 13, 14, 16, 18, 19, 22, and 23) were also compared to injected standards. Over the 25 compounds, we identified 19 compounds. The spectra of the 6 unidentified compounds are provided for further identifications and comparisons.

Supplementary table 4 | Comparison of relative abundance of each VOCs between cell lines. Grey font is for the culture medium (DMEM), green font for the ovarian cell line (IGROV) and light red for the breast cell lines (MCF-10A, MCF-7, and MDA-MD-231). For each VOCs the relative abundance was calculated (mean ± standard error).

#	Compounds	DMEM	IGROV	MCF-10A	MCF-7	MDA-MD-231
1	Styrene (C_8H_8)	13.8 ± 4.5	15.6 ± 0.2	21.2 ± 3.4	19.0 ± 3.1	12.7 ± 4.9
2	Oxime-, methoxy-phenyl ($C_8H_9NO_2$)	12.8 ± 5.6	31.3 ± 4.1	16.2 ± 3.2	22.0 ± 5.8	40.3 ± 5.1
3	Benzaldehyde (C_7H_6O)	17.5 ± 3.0	6.3 ± 1.2	7.6 ± 1.4	10.3 ± 0.9	9.1 ± 1.0
4	Phenol (C_6H_6O)	1.7 ± 0.7	1.5 ± 0.2	0.9 ± 0.1	2.8 ± 0.6	0.7 ± 0.2
5	Aromatic compound	5.5 ± 2.6	1.9 ± 0.4	2.6 ± 0.4	2.0 ± 0.5	1.8 ± 0.3
6	Decane ($C_{10}H_{22}$)	1.4 ± 0.4	1.5 ± 0.5	1.7 ± 0.2	1.9 ± 0.3	1.3 ± 0.1
7	1-Hexanol, 2-ethyl- ($C_8H_{18}O$)	5.6 ± 4.7	7.6 ± 0.5	11.0 ± 7.1	4.4 ± 0.5	3.7 ± 0.9
8	Benzyl alcohol (C_7H_8O)	2.8 ± 1.8	5.8 ± 2.2	10.3 ± 5.1	7.5 ± 2.6	0.2 ± 0.1
9	Benzeneacetaldehyde (C_8H_8O) (Phenylethanal)	1.1 ± 0.2	1.2 ± 0.2	0.6 ± 0.2	1.2 ± 0.4	0.8 ± 0.3
10	HC	1.4 ± 0.3	0.9 ± 0.3	1.8 ± 0.2	1.1 ± 0.2	1.8 ± 0.8
11	Decane, 4-methyl-	0.5 ± 0.1	0.6 ± 0.3	0.6 ± 0.1	0.9 ± 0.2	0.3 ± 0.1
12	Hydrocarbon 2	0.6 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.2
13	Acetophenone (C_8H_8O)	1.7 ± 0.3	2.3 ± 0.2	1.5 ± 0.7	1.8 ± 0.5	1.5 ± 0.6
14	Undecane	0.8 ± 0.4	0.5 ± 0.3	0.6 ± 0.3	0.7 ± 0.1	0.4 ± 0.0
15	HC	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
16	Nonanal ($C_9H_{18}O$)	3.1 ± 0.8	3.2 ± 0.1	1.4 ± 0.3	2.5 ± 0.8	1.4 ± 0.5
17	Unknown VOCs	0.7 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.5 ± 0.1	0.4 ± 0.2
18	Dodecane ($C_{12}H_{26}$)	1.1 ± 0.2	1.4 ± 0.1	1.6 ± 0.3	1.6 ± 0.2	1.6 ± 0.3
19	Decanal ($C_{10}H_{20}O$)	3.9 ± 1.2	2.7 ± 0.4	1.6 ± 0.5	1.7 ± 0.8	0.9 ± 0.4
20	Benzaldehyde, 3,4-dimethyl- ($C_9H_{10}O$)	0.3 ± 0.1	0.7 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.1
21	Unknown VOCs	0.1 ± 0.0	0.4 ± 0.4	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
22	Benzene, 1,3-bis(1,1-dimethylethyl)- $C_{14}H_{22}$ = 1,3di-tert-butyl benzene ($C_{14}H_{22}$)	8.7 ± 1.7	12.7 ± 0.1	13.7 ± 1.9	14.1 ± 0.8	18.0 ± 2.4
23	Decanol ($C_{10}H_{22}O$)	14.0 ± 9.3	0.2 ± 0.1	2.3 ± 1.8	1.6 ± 0.2	0.6 ± 0.2
24	Unknown VOCS	0.5 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.8 ± 0.4
25	2-Undecanone ($C_{11}H_{22}O$)	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.2 ± 0.1

Chapter 3:

Ants as olfactory bio-detectors of tumour in patient-derived xenograft mice

Ants as olfactory bio-detectors of tumour in patient-derived xenograft mice

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Abstract

Early detection of cancer is critical in medical sciences, as the sooner a cancer is diagnosed, the higher the chances of recovery. Tumour cells are characterized by specific volatile organic compounds (VOCs) that can be used as biomarkers to detect cancer. Through olfaction, animals such as dogs can detect these VOCs and act as an early detection tool. Training dogs is long and expensive, whereas insects, such as ants, have a refined sense of smell and can be easily and quickly trained using olfactory conditioning. Ants are known for their reliable olfactory memory and impressive discrimination abilities. They could therefore represent ideal bio-detectors. Using urine from patient-derived xenograft (PDX) mice as stimulus, we demonstrate that individual ants can be trained to discriminate the odour of healthy mice from that of mice bearing a tumour, and do so after only three conditioning trials. Chemical analyses confirmed that the presence of the tumour changed the urine odour of PDX mice,

supporting the results of the behavioural experiments. Our results demonstrate that ants reliably detect such odour changes and have the potential to act as bio detectors for cancer.

Keywords: *Formica fusca*, conditioning, triple-negative breast cancer, GC-MS, SPME

Introduction

With 10 million deaths, and more than 19 million cases in 2020, cancer is a leading cause of mortality worldwide (International Agency for Research on Cancer, updated on the 14/12/2020). A way to increase cancer survival rate is to improve diagnosis methods. Indeed, the sooner the tumours are detected, the higher are the chances of recovery for the patients. Current early detection methods are often invasive (coloscopy) and/or expensive (MRI), therefore relatively few people profit from available detection techniques. For example, in France, the most prevalent cancer for women is breast cancer. However, despite France is a relatively rich country, less than half (48.6 % in 2019) of the risk population (women between 50 and 74) underwent screening for this cancer (Santé Publique France, 2020).

A promising method to increase early cancer detection rate is the use of animal olfaction. Trained dogs can detect tumours in cell samples or body odour samples (Brooks et al., 2015; Pirrone & Albertini, 2017). This method is based on the detection of volatile organic compounds (VOCs) that are characteristic of tumours and linked to their altered cell metabolism (Hanahan & Weinberg, 2011). Dogs are not the only animal species used as bio-detectors of cancer. Recently, other vertebrates but also invertebrate species were tested. Mice were trained to discriminate tumour-bearing mice from healthy ones (Matsumura et al., 2010). The nematode *Caenorhabditis elegans* showed attractive chemotaxis to some cancer VOCs (Hirotsu et al., 2015). In fruit flies, using in vivo calcium imaging, a cutting edge and expensive technology, it was shown that olfactory neurons formed specific activation patterns when exposed to the volatiles from a given cancer cell line (Strauch et al., 2014). Using a well-known and simple paradigm for olfactory conditioning, the proboscis extension response (Giurfa & Sandoz, 2012), honeybees were conditioned to express an appetitive response when exposed to cancer odour (Schallschmidt et al., 2015). Insects are promising detection tools as they are relatively easy to handle, do not require expensive rearing facilities, are available in large numbers and can be trained to detect an odour in very few trials. Amongst insects, ants, and especially *Formica fusca*, demonstrated remarkable learning abilities using ecologically

relevant odours. In this species, one training trial is enough to form a genuine long-term memory lasting for days. Furthermore, these ants are highly resistant to memory extinction: they can be tested up to 9 times without reward before their responses start to decline (Piqueret et al., 2019, **Chapter 1**). Recently, we tested the olfactory detection abilities of *F. fusca* towards cultured cancer cell lines. Using ovarian (IGROV-1) and breast cell lines (MCF-7, MCF-10A and MDA-MD-231) as stimuli, we showed that ants could correctly discriminate between a cancerous cell line and a healthy one, and between two cancerous cell lines (Piqueret et al., in preparation, See **Chapter 2**). Cell lines have the advantage of being easily available and to provide reproducible odour samples over time, but they do not represent the exact reality of tumours, which are complex tissues composed of different cell types. The use of tumour tissues or whole organisms is therefore a critical step for testing ants as clinical detection tools.

In this study, we used olfactory stimuli (urine samples) from patient-derived xenograft (PDX) mice carrying human tumours. Compared to cell lines that grow on a stable and known environment (culture medium), PDX mice represent a more realistic model, as the cancerous cells composing a tumour are growing in a live organism with all its complexity. Furthermore, human tumours retain their characteristics when grafted on mice (heterogeneity, relative proportion of cells, and genomic architecture). Finally, tumours used in PDX are stable in time and can be duplicated, which allows for a virtually infinite number of drug tests and preclinic investigations, ensuring that the patient at the origin of the grafted tumour receives the optimal treatment (Byrne et al., 2017; Dobrolecki et al., 2016).

Here, PDX were established using a “triple-negative” human breast tumour. Urine was chosen as body-odour stimuli, as it can easily be collected and stored (Becker, 2020). Ant workers of the species *Formica fusca* were used as bio-detector since they are able to learn and detect cancer related odours stemming from cancer cell lines (Piqueret et al., in preparation, **Chapter 2**). Using a simple conditioning paradigm, we tested whether ants could discriminate urine from mice carrying a tumour from that of healthy mice. In a preliminary study, we found that ants were able to discriminate two individual healthy mice based on their odours. To avoid this possible confound, we used as healthy samples the urine odour of the tumour mice before they were grafted. As the time between the collection of urine (before and after the graft) can impact the VOCs composition of the urine, using mice from a control group (not grafted), we

tested whether ants could discriminate the urine collected at two different time points. Lastly, to characterize changes in VOCs composition due to the presence of cancer, urine odour samples were analysed using solid-phase micro-extraction (SPME) and gas chromatography coupled with mass spectrometry (GC-MS)

Material and methods

Insects and origin of colonies

Formica fusca is a relatively common ant species in the Northern hemisphere. Colonies may contain several hundred individuals and are headed by one queen (monogynous) or several queens (polygynous). Seven queenright colonies were collected in the forest of Ermenonville (France, 49°09'51.5" N, 2°36'49.2" E) and kept under laboratory conditions (25 ± 2 °C, 50 ± 10% relative humidity, 12 h/12 h: day/night) at the Laboratory of Experimental and Comparative Ethology (LEEC, University Sorbonne Paris Nord). Tested ants were foragers (ants that leave the nest to search for food) and were individually marked with a dot of oil-based paint (Mitsubishi Pencil) on the abdomen and / or thorax one day prior to the conditioning. For all the conditioning and testing procedures, each ant was used only once.

Mice and odour source

Female immunodeficient mice (Swiss nude mice from Charles River Laboratories) were maintained under specific pathogen-free conditions at the Laboratory of pre-clinical investigation (LIP, Institut Curie, Paris, France). Patient-derived xenografts (PDX) from a triple-negative breast cancer (HBCx-11, mesenchymal subtype (Coussy et al., 2019)) were established with consent of the patient. The study was approved by the local ethics committee (Breast Group of Institut Curie Hospital). Three mice (tumour group) were graft at 9 weeks old and kept for seven weeks in group cage of three. Mice were euthanized at the end of the experiment before the PDX had a critical size of 15/15 mm. Care and housing of animals were in accordance with Institutional Animal Care and French Committee–approved criteria (project authorization no. 02163.02).

Three additional mice (of the same age of the tumour group) composed the control group and were kept in the same conditions as the tumour group, but without receiving the graft procedure.

Urine of mice was used as olfactory stimuli. Urine was collected one day before the graft (T_0 samples) and seven weeks after (T_1 samples), both for the tumour group and the control group. Mice were placed individually in a cage (43 cm x 27 cm, height = 16 cm) without food and water for 3h. The floor of the cage was covered with filter paper, which was collected at the end of the 3h soaked with urine. Samples of 1 cm² of urine imbibed filter paper were inserted individually in an Eppendorf tube and stored at -20 °C until they were used as a stimulus for the behaviour tests, or for the chemistry analysis (Fig. 1). Six mice underwent this procedure (three mice for the control group, three mice for the tumour group). Because one mouse from the control group did not urinate, we had the two series of urine samples from 5 mice. For each mouse, between 18 and 88 urine samples (each of 1 cm² filter paper in Eppendorf tube) were collected.

Experimental protocol

The experimental protocol was similar to the one used by Piqueret et al., (2019). In brief, we trained individual ants to associate an odour (Conditioned Stimulus - CS, urine of mice) with a reward (Unconditioned Stimulus - US, 30% sugar solution) using a circular arena (Fig. 2A). Three conditioning trials were performed by each ant. The time needed by the ant to find the reward was the variable measured in each conditioning trial.

To test if ants had learned the CS, we performed memory tests without reward using an arena similar to the one used for the conditioning (Fig. 2B). In these memory tests, two odorant stimuli were presented at the same time, on each side of the arena. One was the CS, while the other one was a new stimulus (N). The CS and the N odours used for memory tests were urine samples from the same mouse, but at different collection times. When T_0 urine was used as CS, we used T_1 urine as N, and vice versa. Two unscented stimuli were also present, to control for the potential learning of visual cues. Circular areas were drawn around each stimulus, which allowed us to record as variable the time spent by the ant in the vicinity of each odorant. Each memory test lasted 2 min and the behaviour of the ant was scored using a behavioural

transcription tool (software Ethoc v. 1.2, CRCA, Toulouse, France). All experiments were video recorded with a camera (Canon, Legria HFR806) placed above the experimental arena. Ants underwent two consecutive unrewarded memory tests at 15 and 20 min after the end of the last conditioning trial.

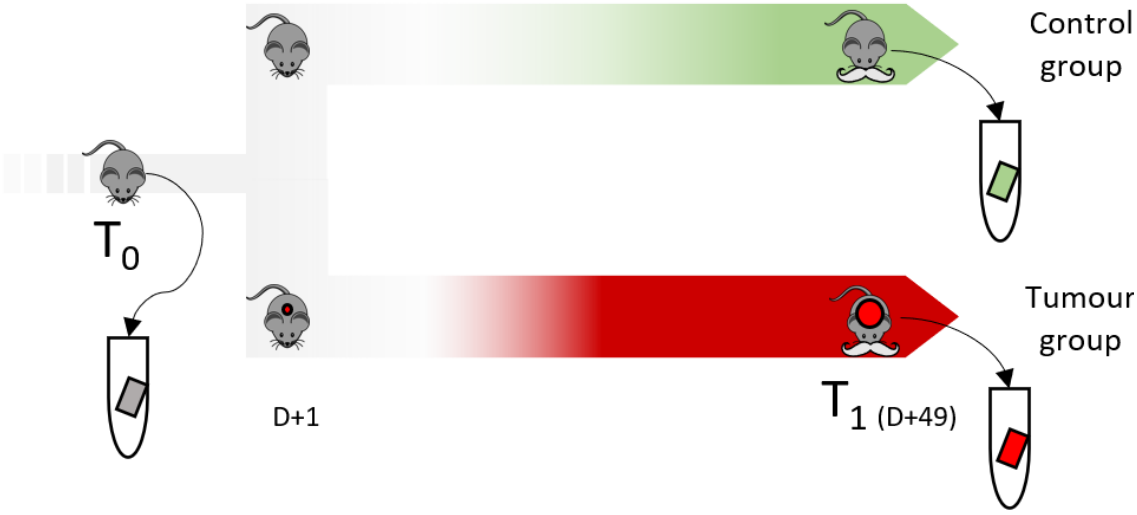

Figure 1: Timeline for the production of urine samples. One group of mice underwent the tumour graft procedure (Tumour group, in red, n = 3), whereas the other did not (Control group, in green, n = 3). Mice belonging to the same group were housed together. To collect urine, a mouse was left alone in an empty cage with filter paper covering the floor of the cage for 3 h. We then cut pieces of 1 cm^2 of filter paper soaked with urine as samples and kept them individually in Eppendorf tubes. The urine was collected twice for each mouse: at $\mathbf{T_0}$ (day 0) and at $\mathbf{T_1}$ (day 49). Red circles inside the mouse indicate the presence of tumour. Whiskers indicate older mice ($\mathbf{T_1}$) at the end of the procedure compared to young mice (no whiskers) at the beginning ($\mathbf{T_0}$).

Two series of experiments were performed. The detailed experimental design is summarized in Fig. 3. First, using the urine of mice from the tumour group, we tested whether ants were able to discriminate the urine of a mouse carrying a tumour from that of a healthy mouse. Ants were conditioned either using the urine of a healthy mouse (T_0) or the urine of a mouse with tumour (T_1) as CS. We therefore had six groups of ants (total N = 59), each conditioned to the urine of one of the 3 mice from the tumour group, either collected at T_0 or at T_1. For the memory tests, we used the urine of different mice than the one used for conditioning, because we wanted to test whether the ants distinguish tumour specific compounds and not individual specific compounds. Indeed, a preliminary experiment showed that ants can discriminate two individual mice based on their individual olfactory identity (see

Supplementary Fig. 1). If the ants are able to detect the presence of the tumour from the urine samples, they should do it independently of the mouse individual identity.

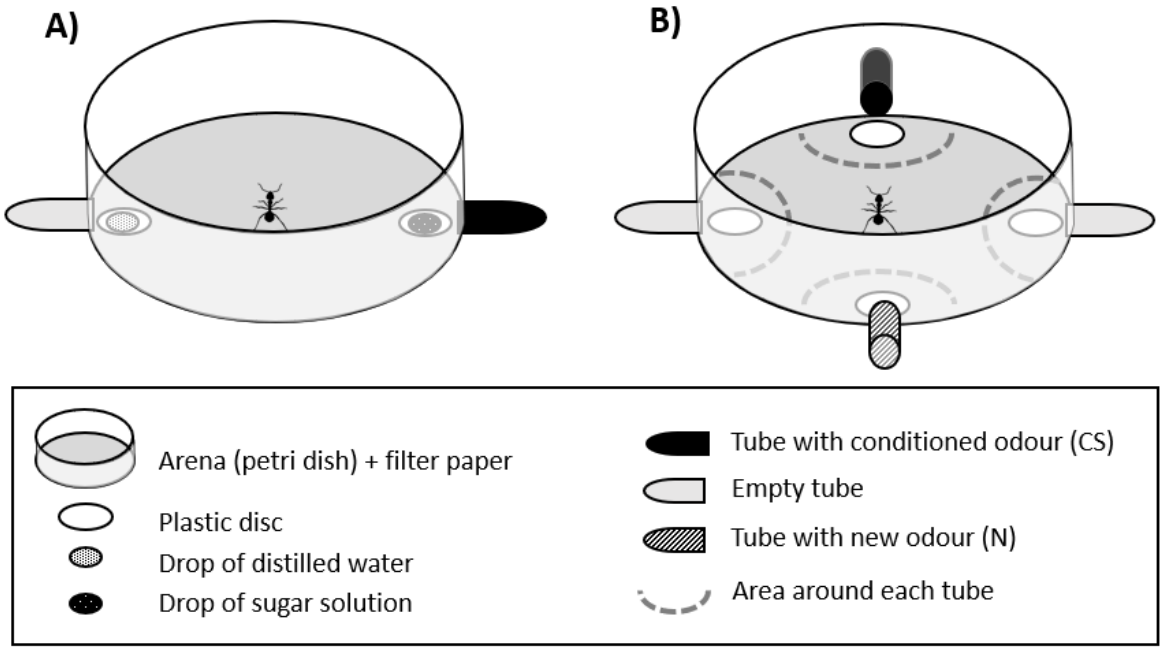

Figure 2: Schema of the experimental device (arenas). During the conditioning, the setup in (**A**) was used. The time to find the reward was noted. During the memory tests, a slightly different setup was used (**B**). The time spent by the ant in the vicinity of the odours (areas, in dashed lines) was recorded during two minutes. Each area measured 35.5 cm². The orientation of the arena in the experimental room was changed between trials so that ants could not learn visual cues.

At each test, ants were presented with a tumour odour (T_1) and a healthy odour (T_0), which were for each ant either the CS or the N odour. During this first experiment, ants encountered the odours from three different mice. One during the conditioning trials (either healthy T_0 or with tumour T_1), the odour of a second mouse for the first memory test (both the T_0 and the T_1 samples), and the odour of a third mice for the second memory test (both the T_0 and the T_1 samples).

In this first experiment, mice have a different age at T_0 and T_1. Ants might be able to perceive this maturation effect in the urine samples independently of the presence of a tumour. The second experiment excluded this possibility, using mice from the control group. We tested whether the time elapsed (49 days) between the collection of the two urine samples might be perceptible by ants. Ants were conditioned either to a urine sample collected at the beginning from young mice (T_0) or at the end from older mice (T_1). Urine samples from a different mouse

were used for the memory tests and, ants were presented with T_1 and T_0 urine odours, which were for each ant either the CS or the N odour. During this second experiment, ants encountered the odours from two mice (and not three, as one mouse did not urinate during urine collection). A sample from one mouse (either T_0 or T_1) was encountered by ants during the conditioning, and both samples from another mouse were encountered during the two memory tests. We therefore had four groups of ants (total N = 58), each conditioned to the urine of one mouse from the control group, either collected at T_0 or at T_1.

Note that for a group of ants (six in the first experiment, four in the second), stimuli were reused between ants, which is one advantage of using urine stimuli (Becker, 2020).

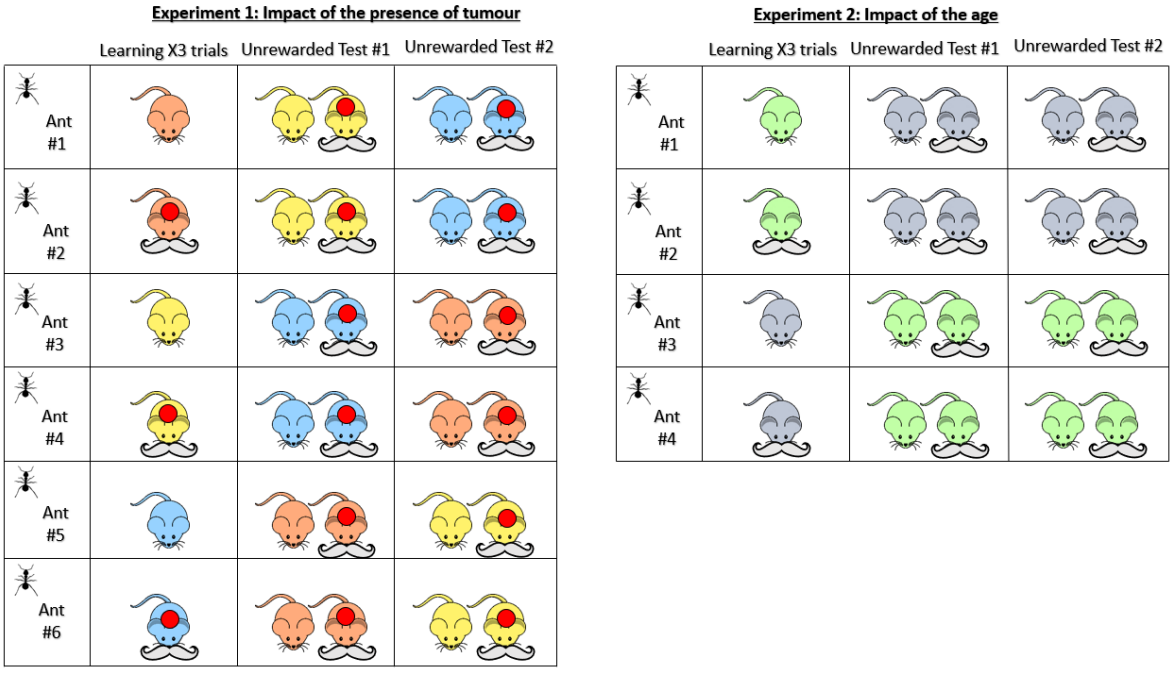

Figure 3: Schema of the experimental procedure. Individual ants were trained to associate the odour (urine) of mice to the reward (sugar solution) three times, and later tested with odours from other mice.
Experiment 1: Using urine of mice from the tumour group, we tested whether ants could discriminate mice based on the absence (collected at T_0) or presence of tumour (collected at T_1). Ants were conditioned either to the odour of a mice with tumour (red circles: ants #2, #4 and #6) or without (#1, #3 and #5). They were later tested with the odours from another mice (indicated by a different colour) at without tumour (T_0) and with tumour (T_1).
Experiment 2: Using mice from the control group, we tested if ants could discriminate mice based on their age. Ants were conditioned either to the odour of a young (T_0, no whiskers: ants #1 and #3) or old (T_1, with whiskers: #2 and #4) mice. They were later tested with the odours from another mouse (indicated by a different colour) at a young age (T_0) and old age (T_1).

Chemical analysis

VOCs emitted by the urine of mice used in the behavioural experiments were characterized using chemical analysis. A filter paper (1 cm^2) soaked with urine was put in a 15 mL glass vial and placed at 37 °C using a water bath. A SPME fibre (50/30 DVB/CAR/PDMS, Supelco) was introduced through the PTFE/silicone 1.5 mm cap of the vial for 50 min (Hanai et al., 2012). After that, the fibre was immediately inserted into an Agilent Technologies 7890A gas-chromatograph, equipped with a HP5MS GC column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, Les Ulis Cedex, France). The carrier gas was helium (1 $mL.min^{-1}$), and the injection was split less (250 °C). The oven temperature was programmed at 40 °C for 5 min, then increased to 220 °C at 7 $°C.min^{-1}$, and to 300 °C at 15 $°C.min^{-1}$ and was held for 3 min. The GC was coupled with a 5975 C mass-spectrometer (Agilent Technologies). Mass spectra were recorded with electron impact ionization at 70 eV. Peak areas were integrated with MSD ChemStation software version E.02.01.1177 (Agilent Technologies). Peaks were identified by comparing their ion spectrum to the NIST library (NIST v2.2, 2014) and to standards injected with the same temperature programme (acetophenone, nonanal, and decanal, all from Sigma Aldricht, Saint-Louis, MO, USA). We found high consistency between the spectra of the standards and those of the compounds extracted from our urine samples (see supplementary material Fig 2).

Statistics

1. *Behaviour analysis*

Data were analysed using R software (v. 4.0.0, R Core Team, 2020). Significance was fixed at $\alpha = 5\%$. All data were analysed using linear mixed models (LMMs, lmer function from the package *lme4*, Bates et al., 2015). Normality of dataset was obtained either with log function (for the conditioning dataset) or with square root transformation (for the memory tests dataset). The identity of the ants and the colony were included as nested random factors.

a. *Acquisition*

We analysed the effect of the number of conditioning trials (factor *trials*) on the dependent variable *time* (continuous variable, the time to find the reward). We analysed the effect of the

conditioning odorant (factor with two levels, odour at the beginning (T_0) or at the end (T_1) of urine collection). We also looked at the interaction *conditioning odorant* × *trials* to detect possible differences in ants' responses depending on the odorant used.

b. *Memory tests*

First, we checked whether ants spent more time near the tubes with odours or near the control unscented tubes by analysing the effect of the independent variable *presence of odour* (factor with two levels, yes or no) on the dependent variable *time* (continuous variable, the time spent near the odorant vials or the unscented ones). Then, in all experiments, we analysed the effect of the independent variable *stimulus* (factor with two levels, CS or N) on the dependent variable *time* (continuous variable, the time spent in the vicinity of a stimulus) during the memory tests. Finally, using data subsets, we analysed the first and the second memory test independently for each experiment.

We also tested whether significant differences in terms of memory abilities were present when ants were tested with fresh or reused urine samples. Ants that were tested with fresh samples did not perform differently from the ones tested with reused samples (*stimulus* × *sequence*: $F = 0.63$, $d.f = 1$, $p \geq 0.1$).

2. **Chemical analysis**

After a first screening where we discarded the contaminants (silicate-derived molecules originating from the GC column, n = 4), the areas of 45 regularly occurring peaks were standardized by calculating the $\ln (P_i / g(P))$ (Aitchison,1986), where P_i is the area of a peak and g(P) is the geometric mean of all the peak areas of the individual. On these variables, we run a Principal Component Analysis (PCA). We compared the scores of the first principal component (PC1, explaining 26.95% of the total variance) between groups (T_0, T_1 control, and T_1 tumour) using Wilcoxon's rank sum test. Using the same 45 variables, we then run a Hierarchical Cluster Analysis with Ward's classification method to classify urine samples. The significance ($p < 0.05$) of each node in the cluster was determined by multiscale bootstrap clustering with 10.000 iterations using the *pvclust* package (Suzuki et al., 2019).

We then performed a second type of analysis where we compared each clustered group (T_0, T_1 tumour and T_1 control) with each other using the relative abundance of each peaks. The relative abundance of a peak was calculated using its raw area divided by the total area of all the peaks in a given sample. For each peak, differences between groups were calculated using Wilcoxon's rank sum test.

Results

1. Behaviour analysis

Can ants discriminate the urine of tumour-bearing mice from that of healthy mice?

Using samples from the tumour group, we tested whether ants were able to discriminate healthy from tumour mice based on their urine.

a. Acquisition

During the acquisition phase, the ants learned the two different stimuli (from healthy mice (T_0) and with tumour (T_1)) in a similar way, as shown by the absence of any significant *conditioning odorant* × *trial* interaction (Linear Mixed Models (LMMs): $F = 0.018$, d.f. = 2, $p > 0.1$). The time spent by the ants to find the reward decreased significantly across the three training trials ($F = 19.31$, d.f. = 2, $p < 0.001$, see Supplementary Table 1 for details). This suggests that ants learned that the urine odour CS predicted the sucrose reward (Fig. 4A). Memory tests without reward were then performed to assess if ants could recognize the learned urine type.

b. Memory tests

Ants spent more time near the odorant vials than near the unscented ones ($F = 166.0$, d.f. = 1, $p < 0.001$, Supplementary Table 2), meaning that ants were not conditioned to the presence of a vial, but to the odour. When focusing on the time spent near the vials with odours, the conditioning odour had an impact on the ants' response, as indicated by the significant *stimulus* × *conditioning odorant* interaction ($F = 9.19$ d.f. = 1, $p < 0.01$). When using the odour of mice without tumour as CS (T_0), ants spent more time near the CS than near the novel odour

N ($F = 19.09$, d.f. = 1, $p < 0.001$, Fig. 4C). This is confirmed when analysing each test separately ($6.80 \leq F \leq 13.61$, d.f. = 1, $p < 0.05$). However, when the odour from mice with a tumour (T_1) was used as CS, the ants did not make the difference between the CS and the N odour both in the global analysis ($F = 0.0001$, d.f. = 1, $p > 0.1$) and in the separate analyses of each memory test ($0.0154 \leq F \leq 0.0416$, d.f. = 1, $p > 0.1$). These results suggest that the odour of mice may be changing with the presence of tumour, although ants showed a significant differentiation between the two urine types only when conditioned to the healthy urine odour.

Can age be a confounding factor?

The results of the first experiment could be explained by the presence of the tumour, or by the elapsed time between the two urine collections (49 days). This second hypothesis is tested here.

a. **Acquisition**

During the acquisition phase, the ants learned the two different stimuli (urine from a young or from an aged mouse) in a similar way, as shown by the absence of any significant *conditioning odorant* × *trial* interaction ($F = 0.119$, d.f. = 2, $p > 0.1$,). The time spent by the ants to find the reward decreased significantly across the three training trials ($F = 5.24$, d.f. = 2, $p < 0.01$, see Supplementary Table 1 for details). As in the first experiment, this suggests that ants learned that the CS (urine odour) predicted the US (reward) (Fig. 4B). Memory tests without reward were then performed to evaluate if the ants could recognize the learned urine type.

b. **Memory tests**

Ants spent more time near the odorant tubes than near the unscented ones ($F = 75.2$, d.f. = 1, $p < 0.001$, Supplementary Table 2). In this experiment, the conditioning odour did not impact the ants' response, as indicated by the non-significant *stimulus* × *conditioning odorant* interaction ($F = 0.7708$, d.f. = 1, $p > 0.1$). In both conditions (T_0 or T_1 urine as CS), ants did not spend significantly more time in the CS area than in the N area during the memory tests ($0.1709 < F < 3.3029$, d.f. = 1, $p > 0.05$, Fig. 4D). This result was confirmed when analysing each test separately, as ants never made the difference between the two stimuli ($0.0031 \leq F \leq$

1.8588, d.f. = 1, $p > 0.1$). This indicates that the difference of treatment observed in the first experiment between the urines from healthy and tumour-bearing mice could not be explained by the age difference at the two urine collection stages (49 days). The results of the first experiment are thus rather explained by the presence of tumours.

2. **Chemical analysis**

Principal component analysis (PCA)

The first two PCs accounted for 47.91% of the total variance (Fig. 5A, supplementary table 3 for details) and their plot separates samples collected at T_0 from samples collected at T_1 (control and tumour groups). This suggests that the time between the collection of samples had an impact on the VOCs composition of urine. In addition, at T_1, samples from the tumour group are clearly separated from control samples and further away from T_0 samples, suggesting that the tumour also has an impact on urine odour. This effect is particularly clear on PC1. On this PC, samples at T_0 had an average coordinate of 2.53 ± 3.24, samples from the control group at T_1 an average coordinate of -1.6 ± 0.9, and the group with tumour at T_1 an average coordinate of -3.2 ± 1.2. Using post-hoc comparison, we confirmed that all the three sample types are statistically different from each other (Wilcox tests: T_0 *vs* T_1 control, W = 81.5; T_0 *vs* T_1 tumour W = 132; T_1 control *vs* T_1 tumour, W = 48, $p < 0.05$ in all cases). These observations suggest that, from a chemistry point of view, the presence of a tumour changes the urine odour of mice, and the age (49 days) participates also in the observed changes. To confirm that we have three chemically group patterns, we performed a hierarchical cluster analysis.

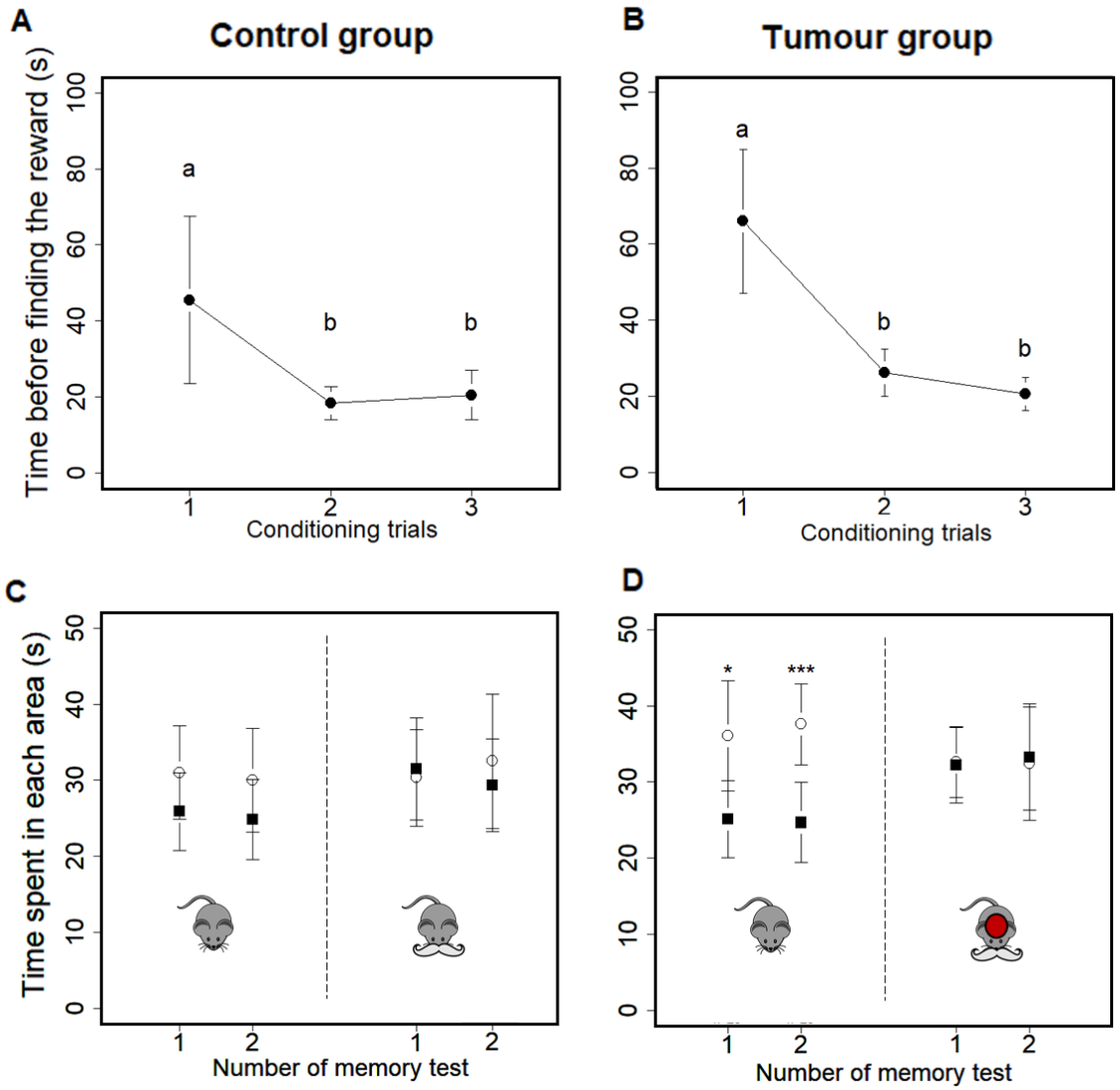

Figure 4: Ants' performances during acquisition (**A**, **B**) and memory tests (**C**, **D**) in an appetitive conditioning experiment with mice urine as odour stimulus. **A**: In the first experiment, ants were conditioned to associate the odour of mice from the tumour group (N = 59) either before (T_0, N = 29) or after the graft of a tumour (T_1, N = 30) with a reward (sugar solution 30%) ; **B:** in the second experiment, ants learned to associate the odour of mice from the control group (N = 58), either at the beginning (T_0, N = 29) or at the end (T_1, N = 29) of urine collection. The graphs show the time needed by the ants to find the reward in the circular arena. Three conditioning trials were done with an inter-trial interval of three minutes. Significant differences between conditioning trials are indicated with different letters.
C - D: time spent by individual ants in the vicinity of each stimulus during the memory tests (circle: CS area; square: N area). On the left panels, odours from mice taken at the beginning of the urine collection (T_0) were used as CS (normal mice; **C**, N = 28; **D**, N = 28). On the right panels, odours from mice at the end of urine collection (T_1) were used as CS (mice with whiskers; **C**, N = 30; **D**, N = 29). In **C**, the red circle indicates the presence of a tumour. Circles and squares represent the mean while error bars show confidence intervals (95%). Significant differences between stimuli are noted with asterisks (*: $p \leq 0.05$, ***: $p \leq 0.001$).

Hierarchical cluster analysis

A hierarchical cluster analysis based on the 45 common peaks showed a clear separation of the three sample types (T_0, T_1 tumour, and T_1 control) from each other (Fig. 5B). All samples are correctly clustered in separate groups, apart from a single mismatched group (red / green striped lines, mismatched between control and tumour individuals). In this analysis, the first

node separates the samples at the beginning of the collection (T0, black lines) from the ones collected at the end (T1, red and green lines). For the T1 samples, the main node for control mice (without tumour) is significant (green lines, Control group, $p < 0.05$) and the main node for the mice with tumour, although not being significant, is well supported too (red lines, Tumour group, $p < 0.1$).

These two exploratory methods suggest that we have three distinct groups of individuals, the T_0 individuals, the T_1 control group individuals, and the T_1 tumour group individuals.

Relative abundance of peaks

We compared the relative abundance of peaks from our three established groups. Amongst the 45 peaks we found that 20 were differently expressed in tumour and/or control group compared to the samples at T0 (Table 1) and 25 were not different (Supplementary table 4).

In the control group, six compounds were less expressed (5-Hepten-2-one, 6-methyl- [compound 2], one hydrocarbon [40] and four unidentified compounds [39, 43, 44, 45]), and four were more expressed (acetophenone [5], Benzaldehyde, 2,5-dimethyl- [11] and three unidentified compounds [4, 31]) compared to the T_0 group.

For the tumour group, we found that six compounds were more expressed (7-exo-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]oct-3-ene [3], Benzyl methyl ketone [8], Benzaldehyde, 2,5-dimethyl- [11], 2-Propanol, 1-(2-butoxy-1-methylethoxy) – [12] and two unidentified compounds [4, 13]), whereas, eleven compounds were less present (nonanal [7], decanal [9], 5 hydrocarbons [34, 36, 37, 40, 42], and 4 unidentified compounds [39, 41, 43,45]) compared to the T_0 samples.

Control and tumour groups expressed some compounds in a similar way. For example, the compounds [4, 11] are up regulated in both groups, and the compounds [39, 40, 43, and 45] are always down regulated. Interestingly, the VOCs of the urine of the tumour group are more often down regulated (eleven compounds) than up regulated (six compounds). This same pattern is found in the control group, but to a lower degree, with four VOCs up regulated, and six down regulated.

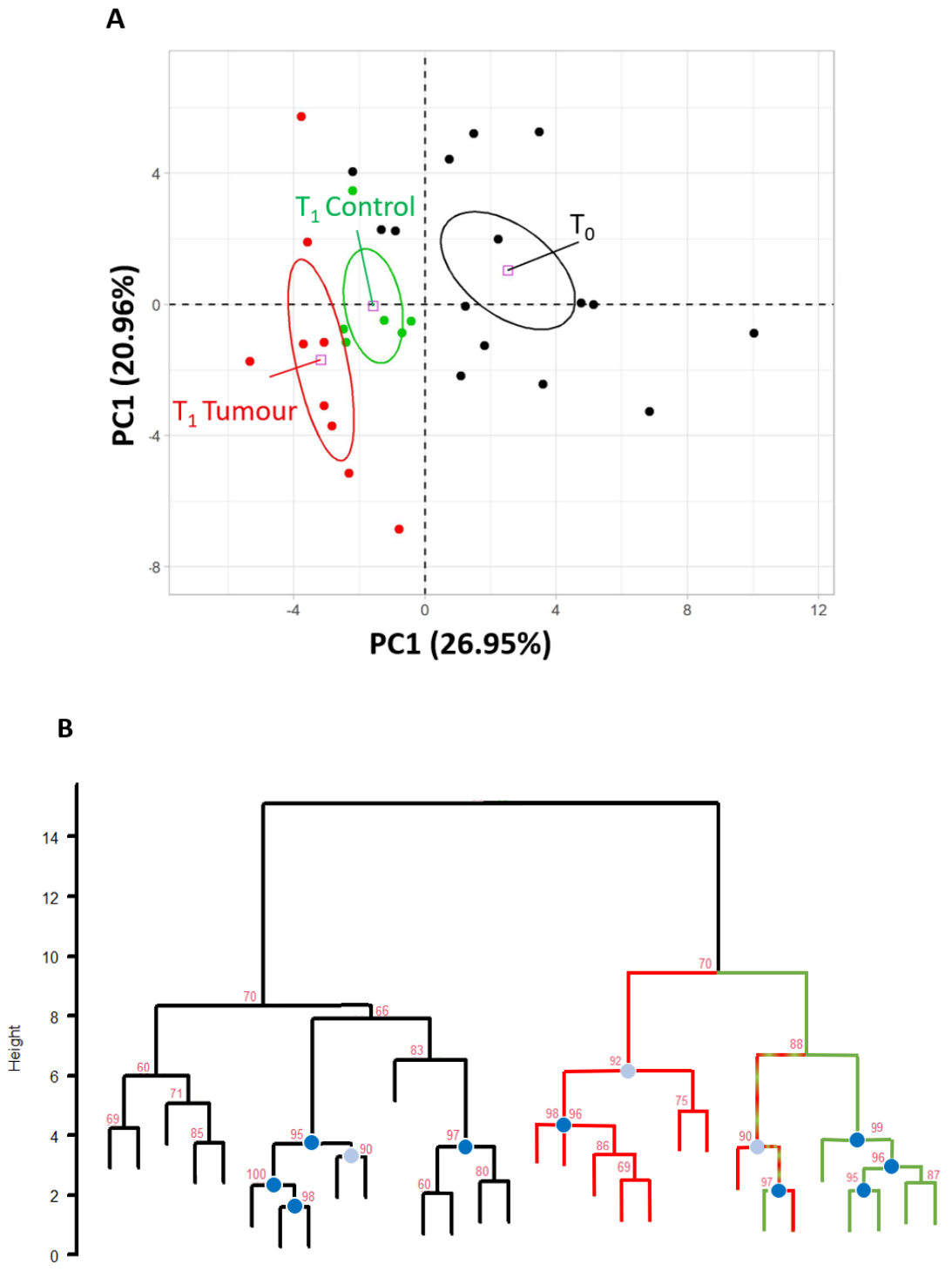

Figure 5: Visualisations of the categorisation of the urine samples. **A**: Plot of the first two Principal Components (PC), explaining 47.91% of the total variance. Urine samples are well separated on the first Principal Component (PC1). Samples at the beginning of the urine collection (T_0) are in black, the samples at the end of the collection (T_1) from the control group are in green and the ones from the tumour group are in red. Ellipses represent the confidence interval (0.95). **B**: Hierarchical Cluster analysis. The significance ($p < 0.05$) of the sample marker clusters was determined by multiscale bootstrap clustering with 10.000 iterations. Dark blue circles represent nodes with $p < 0.05$, light blue circles are for nodes with $p < 0.1$. Black lines are samples at T_0. Red lines are for samples at T_1 from the tumour group. Green lines represent T_1 control samples. Red / green striped lines are for mismatched samples (two T_1 tumour and one T_1 control).

Table 1| List of the 20 VOCs that are significantly different in their relative proportion between groups (Wilcoxon's Rank sum test): healthy mice at T_0, mice with no tumour (control) at T_1, and mice with tumour (T_1). Red arrows indicate that the compound is down regulated compared to the T_0 samples. Green arrow indicates that the compounds is up regulated. Compared to the T_0 mice group, control group mice have 4 up and 6 down regulated compounds. The tumour group have 6 up regulated and 11 down regulated compounds. Compounds in grey were not identified, however some were reduced to hydrocarbons (HC).

#	Compound	Regulation (× fold) compared to the T_0	
		Control group	Tumour group
2	5-Hepten-2-one, 6-methyl-	↓ (× 0.5)	
3	7-Exo-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]oct-3-ene		↑ (x 3.9)
4	Unidentified VOC	↑ (× 2.1)	↑ (x 2.2)
5	Acetophenone	↑ (× 2.2)	
7	Nonanal		↓ (× 0.5)
8	Benzyl methyl ketone		↑ (× 4.4)
9	Decanal		↓ (× 0.6)
11	Benzaldehyde, 2,5-dimethyl-	↑ (× 17.9)	↑ (× 13.9)
12	2-Propanol, 1-(2-butoxy-1-methylethoxy) -		↑ (× 1.7)
13	Unidentified VOC		↑ (× 1.5)
31	Unidentified VOC	↑ (× 1.4)	
34	HC		↓ (× 0.6)
36	HC		↓ (× 0.6)
37	HC		↓ (× 0.6)
39	Unidentified VOC	↓ (× 0.4)	↓ (× 0.7)
40	HC	↓ (× 0.5)	↓ (× 0.5)
41	Unidentified VOC		↓ (× 0.5)
42	HC		↓ (× 0.6)
43	Unidentified VOC	↓ (× 0.5)	↓ (× 0.4)
44	Unidentified VOC	↓ (× 0.5)	
45	Unidentified VOC	↓ (× 0.5)	↓ (× 0.5)

Discussion

In this study, ants were able to learn to associate a complex blend of odours (composed of more than 45 compounds) with a reward, after only three conditioning trials. The simple and fast conditioning protocol we used allowed *Formica fusca* ants to discriminate tumour-free PDX mice from tumour-bearing PDX mice in unrewarded tests. This discrimination was based on the VOCs altered by the tumours. Using SPME and GC-MS analysis, we demonstrated that

samples used for the behaviour tests can be clustered according to the chemical characteristics of each group.

Formica fusca ants are fast learners and show impressive memory abilities after only three associations between an odour and a reward. Such learning abilities were already described in this species (Piqueret et al., 2019), but also in other ant species, like *Lasius niger* (Czaczkes & Kumar, 2020), *Camponotus spp.* (Dupuy et al., 2006; Josens et al., 2009) and *Linepithema humile* (Rossi et al., 2020). Odours used in these studies were pure compounds (e.g. hexanal) or odour blends (e.g. food flavouring). However, they were always ecologically relevant for ants, as they were based on floral or fruit odours. In our previous study focused on the learning of cancer-related odours by ants (Piqueret et al., in preparation, **Chapter 2**), *F. fusca* ants had to associate the odours from cultured human cancer cells (IGROV-1, MCF-7, MCF-10A, or MDA-MD-231) with a reward. Obviously, cancer cells are not ecologically relevant for ants, but many animals are known to be able to learn olfactory stimuli that are not present in their natural environment, such as drugs or explosives, given the right incentive (Frederickx et al., 2011). In that study, ants were able to discriminate a cancerous cell line from a control one, and a cancerous cell line from a different cancerous cell line. The stimuli used in our previous study had the advantage of a relatively simple volatile composition (25 VOCs), emitted by a single cell line in a cultured medium.

PDX mice established with human breast tumour are a step forward in clinical cancer detection and represent an even more complex task for bio-detection as *i)* the odour used as stimulus is not directly extracted from the cancer cells, *ii)* tumours are not composed of a single cell type and are therefore heterogeneous, and *iii)* cancer cell odours might be overshadowed by the body odours of individuals or altered when passing from the local micro-environment of the tumour tissue to the body fluids used as conditioning stimulus (e.g. blood, exhaled breath, faeces, sweat, or urine). Despite all these potential problems, ants could discriminate tumour-free urine samples (T_0) from tumour-carrying ones (T_1) when conditioned to the tumour-free samples ($p < 0.001$ in global effect, Fig. 4C). However, they did not show a successful differentiation between the two samples when the tumour stimuli (T_1) were used as conditioned odour.

How can we explain this finding? A key element for olfactory learning success and generalization responses is the salience of the odour stimuli (e.g. Rescorla et al., 1972; Sutton

& Barto, 1990), *i.e.* how perceptually strong they are for the animal sensory system. Salience is therefore a product of the absolute quantity of odour molecules present in the sample (Pelz et al., 1997), their composition (Schubert et al., 2015), and the particular perceptual sensitivity of the ant olfactory system for these molecules. The asymmetry we observed between tumour-free and tumour carrying samples could be explained by a more salient quality of the tumour-free samples than the tumour carrying samples. When learning the tumour-free sample, a clear and strong stimulus, they had no trouble recognizing this sample compared to the less salient tumour-carrying sample. On the contrary, after learning the weaker stimulus (tumour-carrying sample), they had more trouble finding it again in the tests in the presence of the stronger, more salient, tumour-free stimulus.

The hypothesis of a higher possible salience of tumour-free samples compared to tumour-carrying samples, which could be a by-product of the altered metabolic rate of tumour tissues (see Warburg effect, used by cancer cells for facilitate incorporation of nutrients and to produce new cells rapidly (Vander Heiden et al., 2009)), is supported by our chemical analysis.

We found 45 VOCs shared by all samples (Suppl. Table 4). Using PCA and hierarchical cluster analysis (Fig. 5A and 5B), we observed that the urine odours from mice with a tumour were chemically different from those of healthy mice. When we compared the relative abundance of each peak between our groups, we found that 11 VOCs were more expressed in mice before the graft of tumour (T_0 compared to the tumour ones, T_1), whereas only 6 VOCs were more expressed after the graft. Interestingly, this down regulation of VOCs in cancer-mice was also described in other studies using urine from mice (Matsumura et al., 2010; Woollam et al., 2019). When compared with tumour free mice, Matsumura et al., (2010) remarked that individuals injected with lung cancer cell lines were mostly characterized by patterns of down regulation in VOCs. Woollam et al., (2019), using mice injected with breast cell lines, found that over the 17 VOCs of interest for the classification of cancer-bearing / cancer free mice, 14 VOCs were more present in cancer-free mice, and only 3 were more present in cancer-bearing mice. As they noted, this could be explained by the higher metabolic utilization of compounds from cancer mice compared to healthy ones. Note, however, that this pattern may depend on the type of cancer and conditions, as an opposite pattern of up-regulation in tumour-bearing mice was described elsewhere (Hanai et al., 2012).

Several VOCs detected and identified in our study were also found in other cancer related VOC studies, such as 5-Hepten-2-one, 6-methyl- [2], acetophenone [5], nonanal [7], decanal [9], 2,4-Di-tert-butylphenol [38] (Altomare et al., 2013; Amal et al., 2015; Filipiak et al., 2014; Hanai et al., 2012; Liu et al., 2019; Matsumura et al., 2010; Silva et al., 2017). Over these VOCs, we found that the relative proportion of nonanal and decanal was lower in tumour-bearing mice than in healthy mice. However, both compounds were found in higher concentration in breath of patients with ovarian cancer (Amal et al., 2015) and decanal was found in higher concentration in breath of patient with lung cancer (Filipiak et al., 2014). Here, we used urine of PDX mice that received a breast tumour. To this date, no consensus on which biomarkers one can use for the detection of cancer was established. It is likely that the biomarkers of cancer depend on the type of cancer (lung, breast, ovarian…), and that the biomarkers are altered when passing from the micro-environment of the tumour, to the blood system, and to the final body fluids. Thus, when studying different cancer models and body fluids, this adds difficulties for comparative results.

The goal of our study was to test the olfactory abilities of ants using PDX odours. We found that the ants are indeed able to learn this type of stimuli, but with varying success. For improving conditioning and differentiation success in the tests, it would be useful to investigate how the ants perceive these samples, in particular which VOCs they consider as important when making the discrimination between samples. This could be performed by observing the neural activity of brain using calcium imaging (Galizia et al., 1999; Strauch et al., 2014), or by testing the neural activity of the insect antenna with electro-antennography (d'Ettorre et al., 2004). The number of VOCs of interest could then be reduced to the one detected by the individual.

In this study, we used urine from mice with a well-developed tumour (few days before the ethical sacrifice) as odour stimuli. It would be interesting to test the olfactory abilities of ants using smaller tumours, in order to find the threshold of detection. From a clinical point of view, the optimal conditioning protocol would have the shorter delay possible between the screening and the result. *Formica fusca* ants have the ability to learn a simple stimulus after a single presentation (Piqueret et al., 2019), and we aim at testing this ability using complex cancer-related odours. Finally, would it be possible to train not a single ant at a time, but thousands of individuals at the same time? Social insects (such as ants) live in colonies, and

communication is essential for them (Piqueret & d'Ettorre, 2021, **Annexe of this thesis**). It was demonstrated that one trained honey bee (social insects like ants) could transfer a learned association to a naïve bees when their antennae were in contact (Cholé et al., 2019). Social learning could provide an exponentially faster conditioning methods if it is shown to be successful using complex odours.

In this first study combining olfactory abilities of ants and the use of body fluids from human tumour in PDX mice, we found that ants can be used as bio-detectors, with encouraging results. Compared to single cancer cell lines, body odours are more complex, and as we have shown, individual variability, including age, has an effect on the emitted VOCs. However, ants managed to discriminate healthy individuals from tumour-bearing ones. With this proof of concept, ants are a step closer to becoming a fast, efficient, inexpensive, and non-invasive tool for detection of human tumours.

Data availability

The data used in this publication are available in the supplementary information.

Code availability

R code is available in the supplementary information.

Authors' contributions

B.P proposed the original idea. B.P., P.d.E. and J.-C.S. conceived the project and designed the experiments. The conditioning and memory test arenas were built by P.D and B.P. E.Ma & E.Mo proposed the different PDX and E.Mo oversaw the mice cares during the study. B.P. performed the behavioural experiments and analysed the data with the help of P.d.E. and J.-C.S. C.L oversaw the chemistry data acquisition. Chemistry data were analysed by B.P with help of C.L and P.d.E. The manuscript was written by B.P. and revised by P.d.E., J.-C.S., E.Ma and E.Mo. Final manuscript was approved by all authors.

Competing interests

We declare we have no competing interests.

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Supplementary Information:

Ants as olfactory bio-detectors of tumour in patient-derived xenograft mice

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Supplementary Table 1| Result of the different models used for analysing ants' conditioning performances (Fig. 3A and 3B, main text): Time was always the dependent variable. Significant effects ($p < 0.05$) are indicated in bold.

Focus on the factor(s)	Df	F value	p value
Tumour factor experiment			
Conditioning odorant × trials	2	0.018	0.982
Conditioning odorant	1	0.108	0.744
Trials	2	19.305	**<0.001**
Trials (1 *vs* 2)	1		**<0.001**
Trials (1 *vs* 3)	1		**<0.001**
Trials (2 *vs* 3)	1		1.000
Age factor experiment			
Conditioning odorant × trials	2	0.119	0.888
Conditioning odorant	1	0.303	0.584
Trials	2	5.239	**0.007**
Trials (1 *vs* 2)	1		**0.029**
Trials (1 *vs* 3)	1		**0.014**
Trials (2 *vs* 3)	1		1.000

Supplementary Table 2 | Results of the model used for analysing ant's performances in the memory tests (Fig 2D-F, main text): Time was the dependent variable. Significant effects ($p < 0.05$) are indicated in bold. Tendency effects ($p < 0.1$) are underlined.

Focus on the factor(s)	Df	F value	p value
Tumour factor experiment			
Global effects			
Presence of odour	1	165.99	**< 0.001**
Stimulus × Trial × conditioning odorant	1	0.1696	0.6809
Trial × Conditioning odorant	1	0.2991	0.5849
Stimulus × Trial	1	0.0075	0.9309
Trial	1	0.0021	0.9638
Stimulus × Conditioning odorant	1	9.1928	**0.0027**
No tumour (T_0) odour as CS			
Stimulus × Trial	1	0.1361	0.7129
Stimulus	1	19.0945	**<0.001**
Stimulus Test 1	1	6.797	**0.0118**
Stimulus Test 2	1	13.612	**<0.001**
Tumour (T_1) odour as CS			
Stimulus × Trial	1	0.0471	0.8286
Stimulus	1	0.0001	0.9906
Stimulus Test 1	1	0.0416	0.8391
Stimulus Test 2	1	0.0154	0.9018
Age factor experiment			
Global effects			
Presence of odour	1	75.22	**< 0.001**
Stimulus × Trial × conditioning odorant	1	0.0799	0.7777
Trial × Conditioning odorant	1	0.0410	0.8398
Stimulus × Conditioning odorant	1	0.7708	0.3809
Stimulus × Trial	1	0.0524	0.8191
Conditioning odorant	1	1.164	0.2817
Trial	1	0.2017	0.6538
Stimulus	1	2.2213	0.1375
Young (T_0) odour as CS			
Stimulus × Trial	1	0.0020	0.9641
Stimulus	1	3.3029	0.0719
Stimulus Test 1	1	1.8588	0.1784
Stimulus Test 2	1	1.4469	0.2343

Old (T_1) odour as CS			
Stimulus × Trial	1	0.1133	0.7370
Stimulus	1	0.1709	0.6801
Stimulus Test 1	1	0.0031	0.956
Stimulus Test 2	1	0.2586	0.6131

Supplementary Table 3| Results of a Principal Components analysis (PCA) based on the normalized peak area of 45 peaks extracted from the chemical analysis of the urine samples. We selected 12 principal components (PCs), with an eigenvalue > 0.80, which together explain more than 90% of the total variance.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
Eigen value	12.13	9.43	4.55	3.24	2.79	2.32	1.48	1.37	1.16	0.89	0.87	0.80
Variance	26.95	20.96	10.12	7.19	6.21	5.15	3.28	3.05	2.58	1.99	1.94	1.78
Cumulative Variance	26.95	47.91	58.03	65.22	71.43	76.58	79.86	82.91	85.49	87.48	89.42	91.20

Supplementary Table 4| List of the 45 VOCs found in all the urine samples. Compounds in bold were identified by comparison with standards analysed with the same temperature programme (see supplementary figure 2). The spectra of the other VOCs were identified using a reference database (NIST v2.2. 2014). Compounds in grey had a match probability too low for correct identification. However, we reduced the identification to hydrocarbons (HC) for most of the unknown peaks. We compared the relative area of each peak present in the tumour group or the control group to the T_0 group (Wilcoxon's Rank sum test). We provided the direction of the regulation: compounds are either up regulated (in green), or down regulated (in red).

Peak	Compounds	T0 samples	T1 samples					
			Tumour group			Control group		
		% area	% area	P value	regulation	% area	P value	regulation
1	Dimethyl sulfone	16.3 ± 16.5	15.8 ± 13.5	0.815	---	21.2 ± 11.3	0.205	---
2	5-Hepten-2-one, 6-methyl-	3.0 ± 1.9	1.9 ± 1.0	0.109	---	1.5 ± 1.5	0.014	down
3	7-Exo-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]oct-3-ene	1.0 ± 1.0	3.9 ± 3.0	0.004	up	1.7 ± 1.2	0.112	---
4	Unidentified VOC	0.8 ± 0.5	1.8 ± 0.7	<0.001	up	1.7 ± 0.6	0.003	up
5	**Acetophenone**	1.0 ± 0.4	1.3 ± 0.7	0.215	---	2.1 ± 0.7	<0.001	up
6	1,7-Octanediol, 3,7-dimethyl-	1.0 ± 0.4	1.4 ± 0.8	0.2631	---	1.2 ± 0.7	1	---
7	**Nonanal**	4.8 ± 1.9	2.2 ± 0.8	<0.001	down	4.6 ± 1.2	0.970	---
8	Benzyl methyl ketone	1.4 ± 1.3	6.3 ± 5.3	0.018	up	1.3 ± 0.8	0.970	---
9	**Decanal**	2.7 ± 1.3	1.5 ± 0.7	0.018	down	1.9 ± 1.0	0.112	---

10	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-(Synonyme:2-Pinen-4-one)	3.5 ± 1.9	4.3 ± 2.1	0.238	---	2.9 ± 0.9	0.733	---
11	Benzaldehyde, 2,5-dimethyl-	0.3 ± 0.1	4.3 ± 2.4	<0.001	up	5.6 ± 1.6	<0.001	up
12	2-Propanol, 1-(2-butoxy-1-methylethoxy) -	1.0 ± 0.3	1.7 ± 0.8	0.025	up	0.9 ± 0.3	0.622	---
13	Unidentified VOC	1.8 ± 0.7	2.8 ± 0.9	0.015	up	2.4 ± 0.5	0.080	---
14	HC	3.2 ± 1.6	3.9 ± 2.3	0.558	---	4.0 ± 1.1	0.154	---
15	HC	1.8 ± 0.6	2.2 ± 0.8	0.263	---	1.7 ± 0.4	0.677	---
16	HC	1.3 ± 0.5	2.1 ± 1.3	0.055	---	1.6 ± 0.5	0.235	---
17	HC	1.6 ± 0.6	2.0 ± 0.5	0.096	---	1.9 ± 0.4	0.178	---
18	HC	0.9 ± 0.5	0.9 ± 0.6	0.861	---	1.0 ± 0.3	0.340	---
19	Unidentified VOC	0.8 ± 0.3	0.7 ± 0.4	0.482	---	0.6 ± 0.1	0.570	---
20	HC	0.7 ± 0.3	0.6 ± 0.4	0.446	---	0.5 ± 0.1	0.235	---
21	HC	1.2 ± 0.7	1.3 ± 0.6	0.599	---	1.0 ± 0.3	0.677	---
22	HC	1.8 ± 0.6	2.5 ± 1.5	0.238	---	1.6 ± 0.3	0.235	---
23	HC	0.6 ± 0.3	0.8 ± 0.2	0.155	---	0.7 ± 0.3	0.470	---
24	HC	2.1 ± 0.9	2.3 ± 0.9	0.599	---	2.0 ± 0.5	0.791	---
25	HC	0.9 ± 0.4	1.0 ± 0.6	0.953	---	0.8 ± 0.2	0.850	---
26	Unidentified VOC	1.3 ± 0.6	1.1 ± 0.4	0.599	---	0.9 ± 0.4	0.267	---
27	Unidentified VOC	2.3 ± 1.0	1.7 ± 0.7	0.238	---	1.8 ± 0.5	0.302	---
28	Unidentified VOC	0.8 ± 0.4	0.8 ± 0.3	0.907	---	0.8 ± 0.3	0.791	---
29	Unidentified VOC	2.5 ± 1.2	1.8 ± 0.8	0.123	---	2.3 ± 0.6	0.470	---
30	HC	1.3 ± 0.5	1.0 ± 0.4	0.178	---	1.1 ± 0.3	0.381	---
31	Unidentified VOC	0.8 ± 0.4	1.1 ± 0.4	0.290	---	1.2 ± 0.3	0.049	up
32	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl) -	1.3 ± 0.6	1.3 ± 0.4	0.682	---	1.3 ± 0.3	0.970	---
33	Unidentified VOC	1.7 ± 1.5	1.3 ± 0.3	0.599	---	1.2 ± 0.4	0.235	---
34	HC	2.0 ± 0.8	1.2 ± 0.5	0.021	down	1.6 ± 0.4	0.424	---
35	HC	2.8 ± 0.9	2.1 ± 0.7	0.084	---	2.5 ± 0.5	0.424	---
36	HC	6.7 ± 1.8	3.9 ± 1.3	<0.001	down	5.2 ± 0.7	0.066	---
37	HC	2.7 ± 1.1	1.6 ± 0.4	0.001	down	1.9 ± 0.3	0.112	---
38	2,4-Di-tert-butylphenol	3.8 ± 1.7	3.4 ± 1.1	0.558	---	3.0 ± 0.7	0.302	---
39	Unidentified VOC	1.3 ± 0.8	0.9 ± 1.0	0.048	down	0.6 ± 0.2	0.006	down
40	HC	1.9 ± 1.1	1.0 ± 0.5	0.012	down	1.0 ± 0.2	0.011	down
41	Unidentified VOC	1.5 ± 0.7	0.8 ± 0.3	0.018	down	1.0 ± 0.2	0.156	---
42	HC	4.4 ± 1.7	2.8 ± 0.9	0.008	down	3.4 ± 0.3	0.131	---

43	Unidentified VOC	2.1 ± 1.4	0.9 ± 0.4	0.001	down	1.0 ± 0.1	0.014	down
44	Unidentified VOC	1.8 ± 1.5	1.0 ± 0.6	0.055	---	0.9 ± 0.2	0.045	down
45	Unidentified VOC	1.6 ± 1.1	0.8 ± 0.4	0.008	down	0.8 ± 0.3	0.045	down

Can ants discriminate two mice based on their urine?

Preliminary experiments were performed in July and August 2019 with six mice that come from wild house mice caught around Lyon (France) and bred for at least 10 generations at the laboratory (LEEC). Animals were kept under laboratory condition with a 14h/10h light/dark cycle (light off at 09:00 am), in a room at 20 ± 2.0 °C with a 50% humidity level. At the time of the collect of urine, mice were between 4 - 6 months old. Urine of 6 wild-type males was collected the same way as indicated in the main paper for the PDX mice.

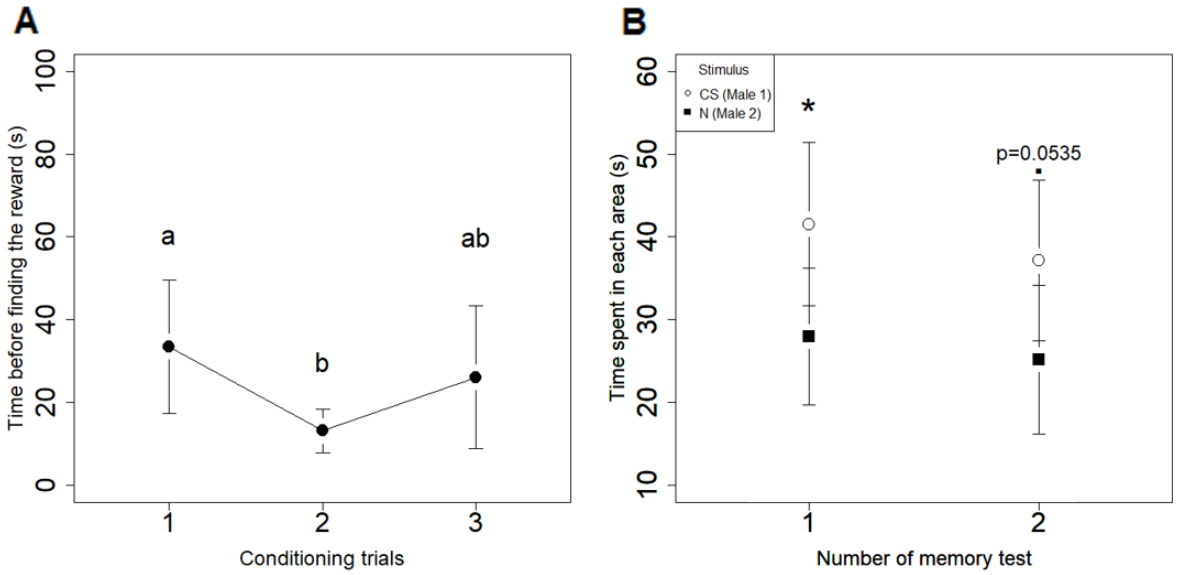
The aim of this preliminary experiment was to test whether ants could discriminate two individuals based on the mice's odours. Using the same protocol and setup described in the main text (see Fig. 2, main text), we conditioned ants to associate the odour of a given male to a reward with three conditioning trials. Then we tested the memory with two unrewarded consecutive memory tests, by keeping the same stimulus used during the learning (CS) and by adding another new stimulus (N), the urine from a different male.

Conditioning results

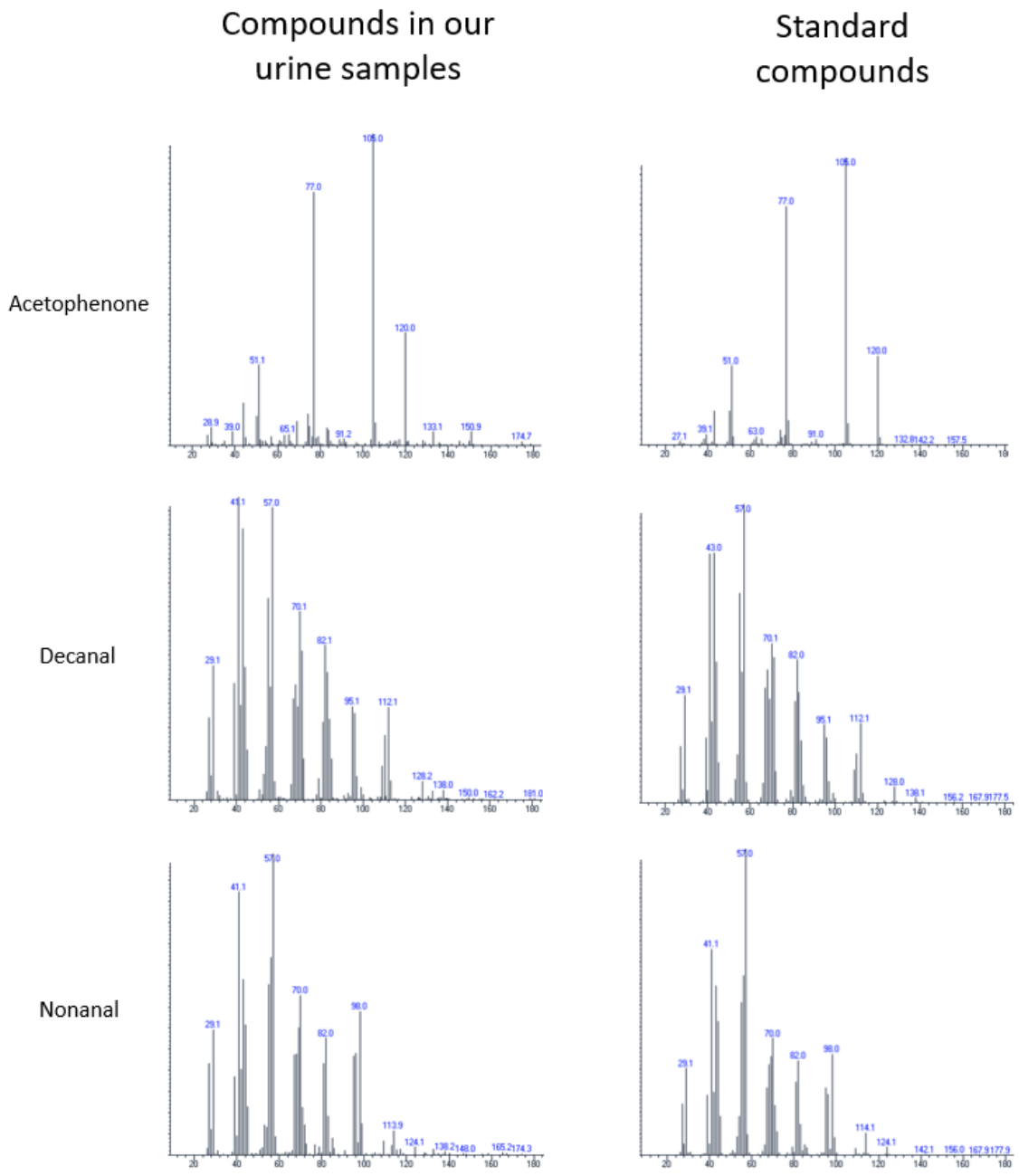
During the conditioning, the time to find the reward decreased significantly for the second conditioning trial ($F = 4.0513$, d.f. = 2, $p < 0.05$, Supplementary Fig. 1A). This could indicate that ants have learned to associate the odour of the mice with the reward. We tested this hypothesis using unrewarded memory tests.

Memory tests results

Ants spent more time near the area with the individual learned odour than the area with the new odour ($F = 8.5166$, d.f. = 1, $p < 0.01$, Supplementary fig. 1B). This is confirmed when using subsets, with the first memory test being significant ($F = 4.3221$, d.f. = 1, $p < 0.05$), and the second memory test being on the edge of significant difference ($F = 4.0194$, d.f. = 1, $p = 0.0535$). This indicates that ants can make the difference between two individual mice. For the main experiments, we choose to not use the same individuals between the conditioning trials and the memory tests, to control for this effect. Instead, ants had to focus on other factors, such as the presence of a tumour or the age of individuals.



Supplementary figure 1: Ants' performances during acquisition **(A)** and memory tests **(B)** in an appetitive conditioning experiment with mice's urine as odour, where the individual discrimination was tested.
A: Ants (N = 17) were conditioned to associate a reward (sugar solution 30%) with the odour of a given mouse. The graph shows the time needed by ants to find the reward in the circular arena. Three conditioning trials were done with an inter-trial interval of three minutes. Significant differences between conditioning trials are indicated with different letters.
B: Time spent by individual ants in the vicinity of each stimulus during the memory tests (circle: CS area; square: N area). Odour from the same mice used for the conditioning was used as CS, whereas the new odour was from another individual. Circles and squares represent the mean while error bars show confidence intervals (95%). Differences between stimuli are noted with asterisks (*: $p \leq 0.05$, ●: $p \leq 0.1$).



Supplementary figure 2: Spectra of the compounds found in urine samples (left column) identified with injected standard (right column) show high similarities. Up-panels: Acetophenone. Middle panels: decanal. Bottom panels: nonanal. On the Y-axis, the abundance of each ions. On the X-axis, the mass divided by charge number (m/z).

Discussion:

General discussion

The studies presented in this thesis allowed us to quantify the olfactory learning abilities of *Formica fusca* ants, in term of acquisition, memory retention, and resistance to extinction, in the light of using individuals of this species as a bio-detector tool for screening of human cancer, by first testing them with human cancer cell lines, and then with body-odour from cancerous organisms.

The first objective of this thesis was to investigate the learning abilities of *Formica fusca* ants. Despite the important number of ant species (more than 14.000), relatively few species were tested in an olfactory conditioning. Notably, before this thesis project was started, only *Camponotus* ant (Bos et al., 2012; di Mauro et al., 2015; Dupuy et al., 2006; Guerrieri & d'Ettorre, 2010; Perez et al., 2015b, 2016) had been used in olfactory conditioning experiments. During the course of our experiments, *Lasius niger* (Oberhauser et al., 2019), and *Linepithema humile* (Rossi et al., 2020) had their olfactory learning abilities tested, and both these species demonstrated the ability to learn quickly, in less than 3 trials. Furthermore, they also showed good memory retention when tested in unrewarded choice test.

The formation of a long-term memory after a single conditioning trial

In the first Chapter (Piqueret et al., 2019), we investigated the learning abilities of *Formica fusca* ants using a simple free-walking protocol. Our experiments highlighted the unconventional abilities of these ants amongst other invertebrates. Notably, this species is able to form a genuine long-term memory (LTM) after a single CS/US association. Classically, the formation of LTM requires several conditioning trials, and leads to a robust and stable memory over time, after consolidation involving *de novo* protein synthesis (Menzel, 2001) (Figure 8A). In our study, a single association (that can be performed in a few minutes) leads to a strong memory up to three-days after the end of the conditioning. When ants were fed with cycloheximide (a protein synthesis inhibitor), no trace of the memory was observable

after three-days, supporting the hypothesis that these performances relied on a genuine long-term memory form.

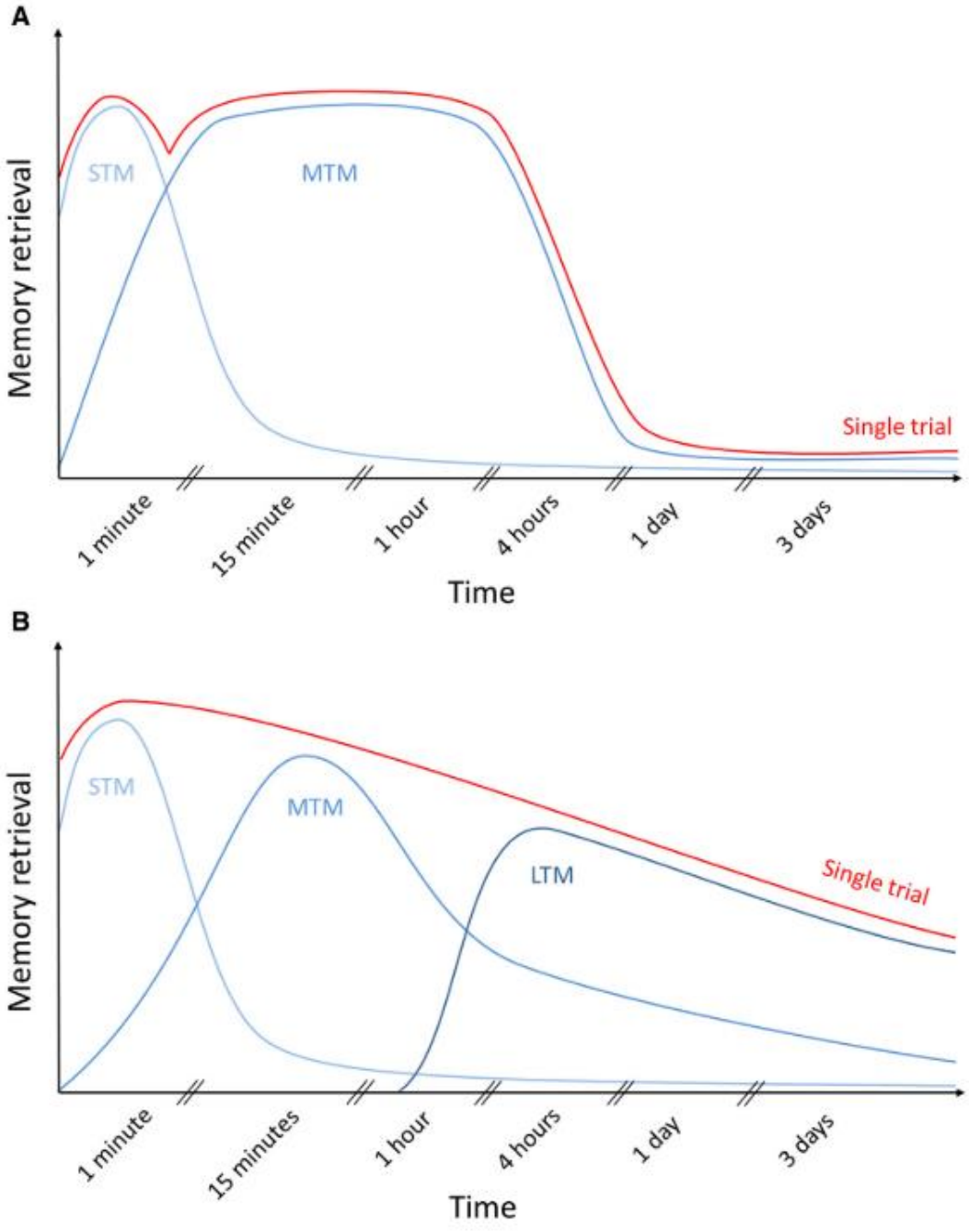


Figure 8 : Commonly admitted model of memories in insects, compared to a novel model

(A) The commonly admitted model of memories induced by olfactory PER conditioning in bees; a single conditioning trial leads to short-term memory (STM) and mid-term memory (MTM) (the latter has not been characterized in term of molecular substrates so far) and eventually, at longer delays, to a decaying memory that does not depend on protein synthesis.
(B) A novel model: a single conditioning trial can lead to protein-synthesis-dependent long-term memory (LTM). The 4-h memory depends on translation but not on transcription so that it has the characteristics of an early-LTM. The 24-h and the 72-h memories depend on both translation and transcription and should thus be considered as late-LTM. This model still mentions MTM for consistency with the previous model shown in (A), but note that MTM after one conditioning trial is still hypothetical and awaits characterization. From Villar et al., 2020.

This finding was surprising compared to the other insect species. This ability was only described in *Drosophila melanogaster* (Colomb et al., 2009; Krashes & Waddell, 2008). Fruit flies were trained using a differential conditioning, where one odour was positively reinforced (CS +), whereas the second was not reinforced (CS -). However, the researchers conducting these studies did not use the same type of conditioning as we did, as we used absolute conditioning, where only the CS was rewarded. *Formica fusca* was then the first insect species where a LTM was described after a single conditioning trial using absolute conditioning. It seems that the duration of insects' memory abilities were underestimated for decades, as this formation of LTM after a single trial was supposed not to be possible (Menzel, 2001). However, honeybees displayed memory trace of a single conditioning trial after 4 days (Sandoz et al., 1995). Recently, the molecular basis of this memory was investigated (Villar et al., 2020), and was characterized as a true LTM using absolute conditioning. As in our study, the colleagues tested the type of memory formed after a single conditioning trial using protein synthesis inhibitors 1h and three-days after the conditioning, and obtained similar results to ours in ants. The memory of bees depended on protein synthesis after three days but not after 1 h. This led to a novel model of memory formation in honeybees (Figure 8B) that is consistent with our finding in *F. fusca* ants.

To our knowledge, genuine long-term memory in ants was studied after several conditioning trials (*Camponotus fellah*, Guerrieri et al., 2011), but no other study tested long-term memory after a single trial. It is likely that other species share comparable memory abilities. For example, *Camponotus aethiops* (Bos et al., 2012) and *Linepithema humile* (Rossi et al., 2020) were tested after respectively six and three conditioning trials in a circular free-walking arena similar to the one used in our experiments. Although their long-term memories were not tested, they showed similar performances for short-term memory (tested a couples of minutes after the conditioning).

Furthermore, when comparing the learning curve (the latency to find the reward) of these two species with our own data, we observe remarkable similarities between these two species and *F. fusca*. In all the cases, ants greatly improved their latency between the first and the second conditioning trial, and then seem to reach a limit that cannot be improved during the third and the next conditioning trials. This could be seen as a hint that they are able to learn fast, after a single conditioning trial. These similarities between *F. fusca* and the others ant species

should be further investigated, to state whether the learning and memory abilities of *F. fusca* are specific to this species, or shared across ant species.

However, for now, as no other ant species were documented (or even tested) for this LTM formation after a single conditioning, we can only be surprised by the abilities of *Formica fusca* ants.

Ants are surprisingly resistant to extinction

Another objective of the first chapter was to test the resistance of the CS-US association to extinction. Usually, when the CS is presented repeatedly without reinforcement, the animal's responses starts fading (a process called extinction) (Sandoz & Pham-Delègue, 2004). In our experiments, ants were extremely resistant to extinction, and we did not observe any decrease of their performances after 6 extinction trials, although they had received only one conditioning trial. We had to prolong our extinction protocol up to 9 trials to observe the beginning of extinction. We propose that this undescribed resistance to extinction, and the ability to form a LTM after a single trial are linked to the ecology of *F. fusca*.

As we noted in **Chapter 1**, individuals of this species do not have a clear tasks specialization when they work outside of the nest. In some species (e.g. *Formica polyctena*), ants have a clear task specialization, with guards (low exploration, low food collection, but important time dedicated to standing still near the nest entrance and be ready to attack), scouts (high exploration, low food collection, and low aggression), or food collector (low exploration, low aggression, but important time dedicated to the food collection) (Novgorodova, 2015). However, in other *Formica* species, such as *F. fusca*, workers are unspecialized and they share their time outside the nest between exploration, food collection, patrolling and guarding. In the case of specialized workers, the need for forming a strong memory fast is not important, as the ant will do the same job for the rest of its life. However, for unspecialized workers, as they perform multiple tasks in parallel, it is beneficial to learn fast a given task, otherwise they will never be efficient in their job.

F. fusca ant species was also described as being able to colonize new environments, especially in early stages of forest succession (Vepsalainen et al., 2000). However, when others ant species (competitors) colonize the environment, *F. fusca* are less present. In this context,

being able to learn with only one trial could help *F. fusca* to be quickly efficient in a new environment. However, the resistance to extinction is more difficult to interpret. When ants are in a new environment without competitors, a learnt behaviour can be optimal as long as the environment is not changing. However, when competitors are colonizing the environment, not being able to rapidly modify a learnt behaviour is a clear disadvantage, as a learnt association can become useless, and the associated behaviour will be sub-optimal, which could partly explain why *F. fusca* are less present when competitors are colonizing their environment.

Unfortunately, as for the formation of LTM after a single trial, data on extinction in ants are extremely scarce; therefore, it is difficult to make comparisons. We can only relate our results with few research on *Camponotus* ants. In Dupuy et al., (2006), the researchers tested *Camponotus mus* and *Camponotus fellah* in a Y-maze using differential conditioning and, after 24 conditioning trials, they tested the ants' memory twice and found that ants spent the same time in the correct Y-maze arm (the one that was rewarded during the conditioning). Thus, no extinction was observed between the two memory tests. As the conditioning was extremely intense (24 trials, compared to our strong conditioning of 6 trials) and the number of extinction trials reduced, it would be difficult to state whether or not *Camponotus fellah* ants are resistant to extinction. Similarly, *Camponotus aethiops* ants underwent two memory tests after a six-trial conditioning, and ants chose the same stimulus during the first and the second memory test (Bos et al., 2012). However, in both studies, ants were rewarded between the two memory tests, thus, they cannot be considered as genuine extinction trials, as no CS/US association should be present. To state if resistance to extinction is common in ants, true extinction procedures with several extinction trials are needed.

Honeybees were studied for their resistance to extinction (Figure 9), and their behaviour was impacted by extinction phenomenon. After few unrewarded trials, the learnt behaviour started fading. What cause bees to be different from *F. fusca* ants on this particular memory feature? In ants, we hypothesized that it was due to their ecology characteristics, where individuals from this ant species are unspecialized workers. By contrast, bees have a relatively strict division of labour (Johnson, 2010). In experimental learning protocols, summer bees are the standard, whereas winter bees are less tested, as they are less sensitive to sugar solution, and perform worse than summer bees in PER conditioning (Behrends & Scheiner, 2010).

Summer bees follow a strict age polyethism, with bees between 4-12 days being nurses, and bees older than 21 days being foragers (with a middle-aged bees category in between). When in a trip looking for food, foragers usually specialize on a single food source (e.g. nectar or pollen) (Johnson, 2010). As bees are more specialised than *F. fusca*, being resistant to extinction is not important, as they will encounter the same stimuli across several days.

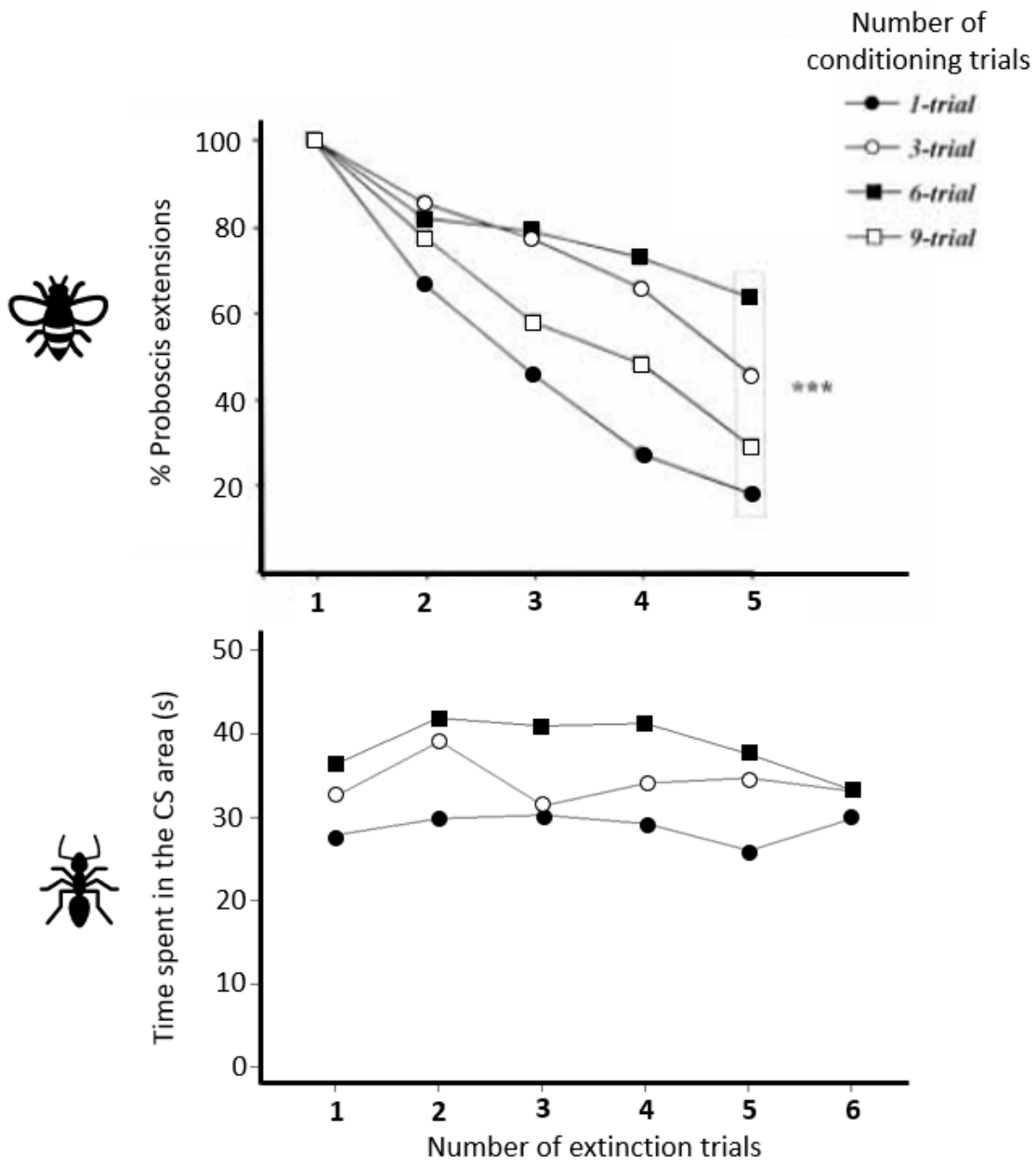


Figure 9: Resistance to extinction of bees and ants.
Honeybees (upper panel) and ants, *Formica fusca* (lower panel), were tested for extinction after one (black circles), three (blank circles), six (black squares) or nine (blank squares) conditioning trials. The performance of bees (% Proboscis extensions) starts decreasing after the first extinction trial, whereas ants keep a stable response (time spent in the CS area) over time. For the bees, N = 33 for 1 conditioning trial, N = 35 for 3 trials, N = 33 for 6 trials, & N = 31 for 9 trials. For the ants, N = 37 for 1 trial, N = 22 for 3 trials, & N = 39 for 6 trials. Ants were not tested for 9 conditioning trials. Data on bees from Sandoz & Pham-Delègue (2004), data on ants from **Chapter 1** - Piqueret et al., (2019).

It is still unclear how being resistant to extinction is advantageous in a natural stochastic environment. However, **the ability of *Formica fusca* to learn olfactory associations rapidly and to form strong and resistant memories suggests that they have a high potential as a bio-detector tool. Indeed, concerning the possibility of using ants as bio-detectors of cancer, testing conditioned individuals multiple times without a decrease in their performances would be an important advantage.**

The detection of cancer cells by ants

In **Chapter 2**, we tested ants' ability to learn the odour from human cancer cell lines. In our first approach, we addressed a simple question: can ants detect the presence of cells in a sample? To maximize our chances of obtaining good performances, we applied a long conditioning protocol with 6 trials and used new stimuli source at each trial. This intensive protocol allowed ants to learn the odour from cancer cell lines and to discriminate it from the culture cell medium alone during the memory test. However, this protocol was particularly time consuming, as seven culture flasks were needed for a single ant, and involved complex safety protocols (bio-safety level 2 laboratory, BSL 2), as we were manipulating live human cancer cells. Our next objective was to simplify and optimize our protocol. As found in **Chapter 1**, ants can be conditioned in a single trial, but as the performances of ants rapidly reached a threshold after few trials, we decided to train ants with only three conditioning trials instead of six. Furthermore, instead of using live cells, we used the culture medium that was in contact with the cells as stimulus after centrifugation. This allowed us to freeze the samples without killing any remaining cells (that could alter the VOCs composition), and to transport the samples out of the cancer-cell facility (BSL 2), as they were cell-free. Given that no living cells were present in the samples, we could use just one sample for the conditioning of one ant, instead of replacing the sample with a fresh one at each conditioning trial. Under these simplified conditions, ants were still able to differentiate the cancer samples from the culture medium alone. Later, we trained ants to discriminate cancer cell samples from non-cancerous cell samples, and to discriminate different types of cancer samples. In all the conditions, ants performed well and chose the conditioned odours in the tests.

Dogs were previously used to detect the smell of cells cancer cells, including ovarian (Murarka et al., 2019), breast (Yoel et al., 2015), and lung cancer cells (Schallschmidt et al., 2015). Dogs were first asked to detect the cancer samples compared to culture medium, and gradually, they were asked to discriminate a cancer sample from other ones. The results of these studies are very variable. For example, in Yoel et al., (2015), the two trained dogs succeeded in the tasks, in Murarka et al., (2019) only one dog out of three reached the end of the test procedure, and in Schallschmidt et al., (2015) none of the two dogs was able to differentiate the cell samples from the culture medium. During the testing procedures, the results were also extremely variable, ranging from 10% of correct identification (Schallschmidt et al., 2015), to 100% (Yoel et al., 2015). However, in this last error-free study, the two dogs underwent a six-month extensive training procedure, and each dog was tested with cell-samples only 15 times, so dogs need an important time investment for few screening tests. Long training and few tests characterize other studies as well, with 8 months of conditioning and 10 tests per dog in Schallschmidt et al., (2015), and several months of training with 9 tests for the dog in Murarka et al., (2019). The exact number of conditioning trials that dogs were subjected to was never indicated in these studies. Only one study gives clear indications. Thuleau et al., (2019) trained dogs with skin secretions of women breast cancer during a 5-month period and more than 750 conditioning trials. Here, the time to train dogs was similar as in the error-free study using cells (5-month vs 6-month, Yoel et al., 2015), we can therefore hypothesize that the number of conditioning trials was also similar in both studies, which means that dogs needed 750 trials to performed 15 tests, which leads to a conditioning / test ratio of *50*. Therefore, experiments with dogs seem to be extremely costly in term of conditioning effort. In comparison, our last experiments involving the discrimination of two cell lines (either both cancerous, or one cancerous and one non-cancerous) required 3 conditioning trials and produced 2 tests per ant. However, we do not consider the individual response of an ant as meaningful as the response of a single dog. That is why we conditioned dozens of ants to obtain a similar result in term of reliability. As our groups of ants were always composed of less than 50 individuals (maximum 49 ants, figure 2F, **Chapter 2**), we needed 150 trials to obtain 2 tests where ants will be collectively equivalent to dogs. Using these numbers, ants have a conditioning / test ratio of *75*, which is higher than the dogs. However, knowing the high resistance to extinction expressed by ants **(Chapter 1),** we could probably test them more than twice. If the resistance to extinction after three conditioning trials is as resistant as the

one after a single conditioning trial, we could test ants up to 9 times (ratio = *17*). We are confident that ants can be tested more than 9 times after three conditioning trials, as we did preliminary experiment using 6 conditioning trials and 12 extinction trials. Here, ants kept the same high performances, and no clear extinction was present along the 12 extinction trials (unpublished data), which leads to a ratio of *12.5*. This is just an assumption, as we did not test the resistance to extinction after three conditioning trials more than 6 times (**Chapter 1**, Figure 4B). Here, the ratio would be *25* (150 conditioning trials / 6 memory tests), which is lower than the ratio of dogs. Furthermore, using 50 ants is not necessary since fewer ants are required to obtain a reliable response (see *Are ants efficient as bio-detectors?),* which further reduces the conditioning effort when using ants.

The dog is not the only species that was tested using cancer-cell line odours for conditioning. Notably, another insect species, the honeybee, was also tested albeit in a very cursory experiment (Schallschmidt et al., 2015). Using lung-cell line odours and differential conditioning protocol, bees were not able to discriminate the cell samples from the control after five rewarded conditioning trials using cells (CS +), and five unrewarded trials using culture medium (CS -). In the same study, dogs were also tested and failed to perform the task. There might be some concerns about this study, especially since only 14 bees from a single colony were tested, whereas in other conditioning studies, the sample size is at least 30-40 bees for a given condition (Giurfa & Sandoz, 2012; Matsumoto et al., 2012; Villar et al., 2020). Further studies are needed before excluding the possibility of using bees as bio-detectors, indeed, they were successfully conditioned for the detection of landmines (Bromenshenk et al., 2003). Knowing that ants can be conditioned using cancer cell odours, and in the light of the similarities between ants and bees, we suggest redoing these experiments with bees.

To conclude using cancer cell line odours seems a good approach for evaluating the potential interest of a particular animal as a bio-detector for cancer. However, tumours are not composed of a single cell lines, but are complex tissues, which are known to modify the body odours of the tumour bearer.

How do ants perform in a realistic screening protocol?

In **chapter 3**, we aimed to test ants in a more realistic screening scenario, by using real tumours in a whole organism as odour sources for conditioning. In studies employing sniffing animals, several body-products from patients have been tested, such as skin secretions (Thuleau et al., 2019) or blood plasma (Murarka et al., 2019), but almost all studies focused either on breath (Buszewski et al., 2012) or urine (Mazzola et al., 2020) as a source of odour (reviewed in Brooks et al., 2015, and Pirrone & Albertini, 2017). These two body fluids were chosen because they are easily accessible (no invasive methods such as the collection of blood) and can be produced by the body in high quantities. We chose to use urine, as it has advantages over breath. Notably, urine can be frozen and stored for a nearly unlimited duration after collection, high quantities can thus be stored in a contained volume, and initial samples can later be divided as needed in sub-samples without alteration (Becker, 2020). In our experiments, we used the urine of patient-derived xenograft (PDX) mice, and not directly from human patients. The collection and utilisation of bio-samples from humans are tightly controlled, and includes many parameters such as the sex of patients, the age, the type of cancer, the stage of cancer, or the diet that could modify the body odours. By contrast, PDX mice are a great model for a first approach of a realistic screening scenario, as mice are from the same lineage, have the same diet, are reared in similar conditions, and are grafted with the same tumour (Byrne et al., 2017; Dobrolecki et al., 2016), thus displaying similar body odours between individuals.

In our experiments, ants were able to discriminate a tumour-bearing sample from a non-tumourous one only when conditioned to the non-tumourous one. Our hypothesis for this learning asymmetry is linked to the relative salience of the samples for the ants. In this case, the tumour samples would be less salient than the tumour-free samples. This hypothesis was supported by SPME and GC-MS analysis. We found that, over the 45 volatile organic compounds (VOCs) common between the odour of mice before and after the graft of the tumour, 6 VOCs were more present in tumour mice, whereas twice (11 VOCs) were higher in abundance in tumour-free mice, which could make the odour of tumour-free mice more salient.

Tumour cells have a different metabolism compared to normal cells, and as tumours multiply very fast, they tend to incorporate as many nutrients as possible to grow in body mass. This is

called the Warburg effect, and here, cancer cells use the anaerobic metabolism, which is faster (but less efficient) instead of the aerobic one (slow but efficient)(more details about the Warburg effect action on cell proliferation in (Vander Heiden et al., 2009), and in the introduction, 10th The hallmarks of cancer). Therefore, from a chemistry point of view, more VOCs would be consumed than released when cells are in an exponential growth phase, thus decreasing the amount of VOCs emitted and therefore the odour salience for the ants.

To determine which candidate biomarker VOCs are the most salient for ants, one might use electro-antennography (EAG) or GC-MS coupled with electroantennographic detection (GC-EAD) (d'Ettorre et al., 2004). The EAG technique allows to measure global neural activity at the level of the insect antenna, in response to olfactory stimuli (Schneider, 1957). When coupled with a GC-MS, it allows to evaluate insects' detection ability for each compound present in a sample, at the concentration present in this particular sample. This method would allow us to test our *salience* hypothesis. Similarly, calcium imaging could also be used to directly observe neural activity patterns in the brain of individual ants (Brandstaetter & Kleineidam, 2011; Dupuy et al., 2010; Galizia et al., 1999). This method was successfully implemented using human cancer cell odours (Strauch et al., 2014), which produce specific brain patterns in the fruit fly *Drosophila melanogaster*.

Using these EAG and calcium imaging approaches, we could reduce the number of VOCs of interest by discarding the ones that are not detected by ants. Note however, that the fact that an odour is detected by an individual, does not necessarily implicate that it is used for discrimination. Therefore, when the putative biomarkers are highlighted, a possible approach would be to condition ants to individual biomarkers, or to reduced blends of several biomarkers, and to test if they can recognize the real body odours (e.g. urine) in memory tests. Such approaches could help to determine behaviourally relevant biomarkers for cancer detection by ants, which could then be used as a standard conditioning sample for clinical implementation. Note however, that mixture perception is a complex phenomenon in general and also in ants, where some components in a mixture are known to mask perceptually the presence of others, a phenomenon termed overshadowing (Perez et al., 2015a; Schubert et al., 2015). The search for the optimal biomarker blend will thus require intensive work, and ants may represent a tractable insect model for such endeavour. In this thesis, we used cell-line odours and PDX mice odour for the conditioning of ants. To obtain these stimuli, one

needs appropriate research facilities including a cell culture laboratory with specific safety measures (as human cancer cells are involved), free-germ animal facilities, and an access to tumour from patients. All of these facilities are expensive, require full time researchers, and are time consuming. However, if a tumour-matching blend is found, it could be easily produced, could potentially be kept in glass vials for weeks or months, or even be frozen for years before the conditioning trials of individuals. The search for cancer biomarkers is limited by the lack of standardisation such as the origin of the odours, the cancer type, the origin of cancer, the different GC-MS or SPME fibers tested, or the GC oven temperature programme. Some biomarkers (e.g. hexanal or nonanal) were found in different studies (review by Krilaviciute et al., 2015), although huge variability was observed.

Are ants efficient as bio-detectors?

Ants are able to detect cancer cells and to discriminate a cell line from another after an extremely short conditioning, and to discriminate tumour free mice from tumour bearing ones, but are they as efficient as other available sniffing animals?

Dog responses are categorised using a binomial response (good identification or bad identification of a sample), which simplifies the calculation of the rate of positive responses. For ants, we chose to measure a continuous response (the time spent near the CS and N odours), which leads to more subtle *performance scores*, but does not allow a direct comparison with dog's performances, currently the most used animal species for cancer detection. To compare the performances of both species, we used bootstrap calculations.

We are asking two questions. The first is: *are ants efficient?* The efficiency is measured by the success rate in discrimination. Above 90% of success, we consider that ants are efficient. The second question is: *how many ants do we need to train to obtain an accurate response*?

Our bootstraps consist in randomly choosing a number of ***N*** ants, over our initial sample of ***S*** ants, *a total of* ***i*** times and see how many times ants choose the right odour ($p < 0.05$) over the ***i*** iterations. We used the same statistics as for the memory tests used in this thesis, the linear-mixed-models (LMMs). For bootstrapping, resampling was allowed. This can only cause problems when the initial sample size is very low, as the probability to randomly sample the same individual ***N*** times is $(\frac{1}{S})^N$. For $S = 25$ (the average number of ants we tested for each

condition), and a resampling of *N* = 10, the probability of having the same individual sampled 10 times is close to 1.10^{-14}, so statistically very unlikely. Furthermore, as we put the individual identity of ants in our LMMs, we were notified when only a single individual was presented in our bootstrapped samples, and this never happened over the thousand iterations we performed.

The first parameter to determine is the optimal number of ***i*** iterations for our bootstraps. We performed this first bootstrapping on the simplest task we asked ants: after a 6-trial conditioning, they had to choose the fresh IGROV-1 cell line (ovarian cancer) over the culture medium alone (**Chapter 2**). Thirty ants were conditioned to IGROV-1 (*S* = 30) and resampling of 30 individuals (*N* = 30) was performed ***i*** times (50, 100, 1 000 or 10 000 iterations were done (Table 1)).

Table 1 : Optimal number of iterations for bootstrapping

***(i)* Iteration**	**% of correct iteration**
1 (original dataset)	**100**
50	**94**
100	**97**
1 000	**96.4**
10 000	**96.69**

We can observe that the threshold of correct iteration was rapidly reached. After 100 iterations, 97% of the times the 30 randomly selected ants performed well and spent statistically more time near the CS than the N odour. We chose to use ***i*** = 1 000 iterations as a trade-off between accuracy and calculation time. Now that we know the optimal value of ***i***, we tested different values of ***N***. We tested values ranging from 2 ants to 50 ants (Figure 10).

As we noted before, we tested 30 ants randomly resampled, and they chose the right odour 96.4% of the time. With N = 40, they are correct 99.2 % of the time, and with N = 50, this number goes up to 99.99%. When decreasing this ***N*** number to 20, ants choose 88.8 % of the time the correct answer. We recommend not to go below this value, as the number of correct responses was rapidly decreasing. The threshold for having > 90 % of correct responses is 22

ants, whereas for 95 %, the minimal number of ants is 27. The number of ants (N = 30) we tested in our behaviour experiments is very close to the maximum value and adding up to 50 individuals does not seem to be optimal for gaining only ~3 % of performances.

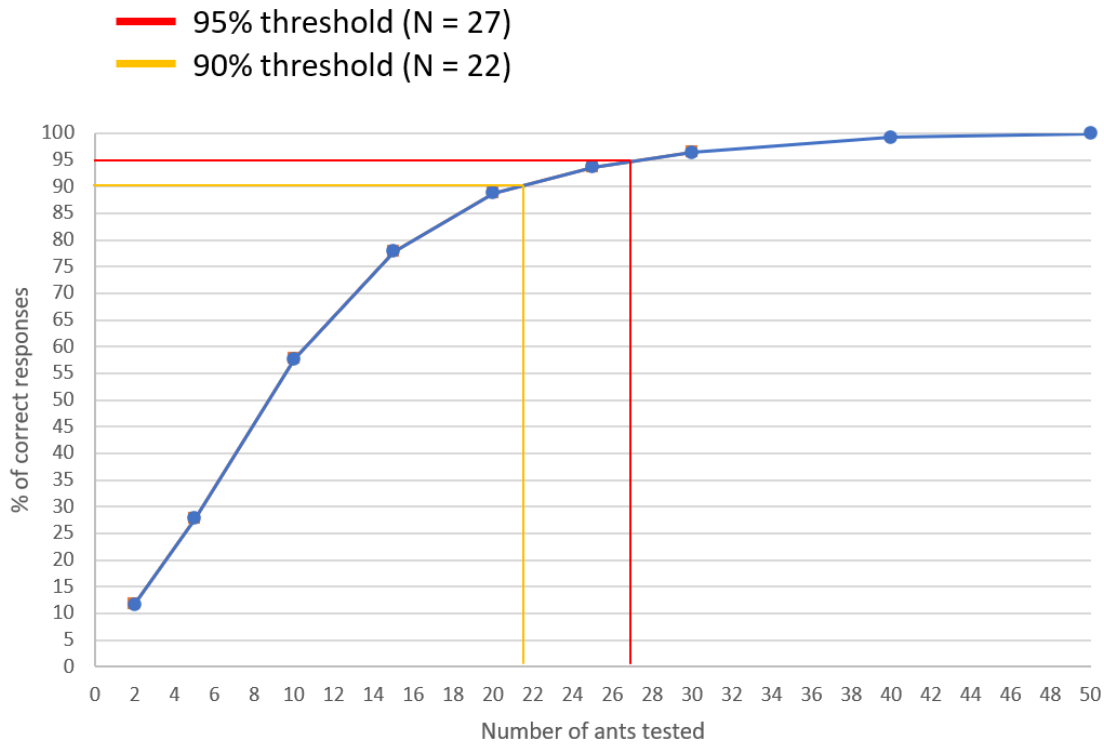


Figure 10 : Optimal number of ants in a cancer-cell odours conditioning

Performances of ants using cell line odours as stimuli were tested with bootstraps. Amongst the 30 ants that were conditioned using fresh IGROV-1 (ovarian cancer) cell lines, and later tested to discriminate IGROV-1 from culture medium, ***N*** were ramdomly sampled a thousand times, and the proportion of times ants chose the right odour is expressed (% of correct responses). Here, the thresholds for 90% and 95% of correct responses are highlighted in yellow (N = 22) and red (N = 27) respectively. Ants underwent 6 conditioning trials and 1 memory test. Data from **Chapter 2,** supplementary figure 2.

This situation (cell lines *vs* culture medium) is far from a real screening protocol, where ants should be conditioned to real body odours of patients. In **Chapter 3**, we used urine of mice with human tumour. As ants managed to make the discrimination between the tumour odour and the non-tumorous ones only when conditioned to the non-tumorous ones, we used this dataset for bootstrapping (*S* = 28) (Figure 11).

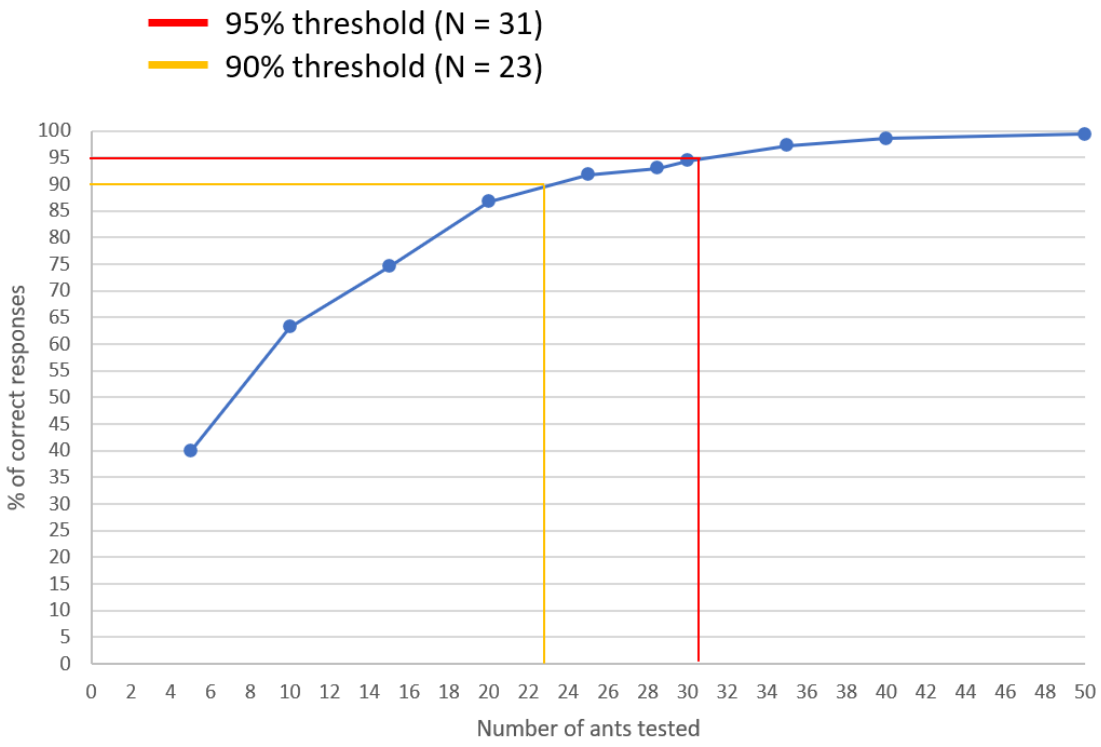


Figure 11 : Optimal number of ants in a real screening conditioning (urine from tumour mice)

Performances of ants using body odours as stimuli were tested with bootstraps. Amongst the 28 ants that were conditioned using tumour-free mice, and later tested to discriminate tumour-free from tumour bearing mice, ***N*** were ramdomly sampled a thousand times, and the proportion of times ants chose the right odour is expressed (% of correct responses). Here, the thresholds for 90% and 95% of correct responses are highlighted in yellow (N = 23) and red (N = 31) respectively. Ants underwent 3 conditioning trials and 2 memory test. Data from **Chapter 3,** figure 4C.

In a real screening protocol (urine from whole organisms), 23 ants are needed for reaching a score of more than 90 %, and 31 ants are needed for 95 % of correct identification. These results are highly similar to those observed using cancer cells (Figure 10). However, a major parameter was different between the two experiments. In the mice tumour experiment, ants performed not one, but two consecutive memory tests, which means that, for 25 ants, 50 memory tests were performed, whereas 25 memory tests were performed for the fresh cell experiment. It would be interesting to investigate this further, in order to find the minimal experimental investment (the minimal number of conditioned ants, and the maximum number of memory tests one ant can perform) and the optimal statistical power. For example, are 40 ants performing a single memory test as efficient as 10 ants performing 4 memory tests (40 memory tests in total)? In the light of our results from **Chapter 1**, we are confident that

ants can be tested more than twice without losing performances. As it is more rapid to train one ant performing two memory tests than to train two ants performing one memory test, if this hypothesis is validated, then the time for conditioning and screening would be even shorter.

Here, we demonstrated that ants can be efficient bio-detectors with a success rate superior at 90%. But are they as efficient as the current standard, the dog olfaction?

Comparison of dogs vs ants performances

First, in term of time and effort needed to train individuals, ants have a clear advantage. Dogs need months of proper cancer training (after having followed a basic training of months for obedience). In recent studies using human samples, dogs needed between 2 months (Junqueira et al., 2019), 5 months (Thuleau et al., 2019) or 1 year of cancer-related training (Mazzola et al., 2020). Ants need half an hour. However, as we demonstrated, several ants (~25-30 ants) are needed to have a reliable response. Still, ants are clearly faster in learning the given task compared to dogs. Another advantage of ants is that almost all the individuals are passing the criteria for the test (e.g. 467 ants over 496, so 94% in Chapter 1), whereas around half of dogs did pass the conditioning: 3 over 6 in Mazzola et al., (2020), 1 over 3 in Murarka et al., (2019), 3 over 4 in Junqueira et al., (2019), and 2 over 2 in Thuleau et al., (2019). Note that dogs were not randomly chosen but demonstrated good olfactory abilities before the start of the studies. In the case of ants, the sole criteria were that tested ants have to be foragers and outside the nest. Compared to ants, dogs need longer training time, and the probability of not successfully conditioning a dog to cancer samples is not negligible. The number of individuals tested in ants is also profitable, as, if one ant is not efficient, the global result will barely be impacted, as the dozens of other ants will be performing well. However, if one dog over two dogs (mean number in previous cited dog studies) is not performing well, the outcome will be catastrophic.

In term of costs, despite the exact cost of training a dog is not clear (estimation are starting at 20.000€, including the initial cost of the dogs, the food, the veterinary costs…), we can be sure than ants are less expensive, as one can buy a small ant colony for a dozen of euros, with no veterinary costs, and a minimum cost of maintenance (honey and frozen insects).

Finally, in term of efficiency, dogs have an advantage, as they were tested using real samples from patients, whereas in these first studies investigating ant cancer detection abilities, we did not tested samples from patients, but from PDX mice which are less variable between individuals (no diet, age, sex, medication confounders). Still, ants managed to make the difference from healthy *patient* to sick ones, with an efficiency (success rate) of 93% (calculated after 1.000 bootstrapping iterations, and 28 ants). In the latest studies using dogs, the success rate was 57.57% when using urine as source of odours (Mazzola et al., 2020), 90.3% when using skin secretion (Thuleau et al., 2019), and between 33% (Murarka et al., 2019) and 97% (Junqueira et al., 2019) when using blood. These important success rate differences between studies were also noted in a recent review (Pirrone & Albertini, 2017), where the success rate was oscillating from 13% to 100% between studies. The differences between dogs and ants in terms of training time, number of trained individuals, of performed tests, and success score are listed in Table 2.

Table 2 : List of advantages of using ants

Study	Training time	Number of tested individuals	Number of tests performed	Success score
Mazzola et al., 2020	1 year	3	11 per dog	57.57%
Thuleau et al., 2019	5 months	2	4 for dog 1 27 for dog 2	90.3%
Murarka et al., 2019	Months (no clear details)	1	6 per dog	33%
Junqueira et al., 2019	2 months	3	10 per dog	97%
Study with ants	30 min	162 in Chapter 2 58 in Chapter 3 (+ 59 for control)	2 per ant, with the possibility to test them at least 6 times (Chap. 1, Figure 4B), and probably 9 times (Chap. 1. Fig. 4C) before reconditioning ants	93%

In the end, what species should we use for cancer screening? *It depends*. As long as ants will not be tested using human body odour samples, we cannot state that they can be used for screening cancer. However, they demonstrated impressive abilities, when tested with cells (**Chapter 2**), or urine from human tumour-bearing mice (**Chapter 3**). Furthermore, even if

several ants are needed for having a reliable response, as they can be trained very rapidly, and at a negligeable cost, they are clearly advantageous compared to dogs. Additionally, they are resistant to extinction, and can thus be tested several times in unrewarded tests, where, in a real screening procedure, the investigator will have no idea of which odour should be rewarded. Once the abilities of ants in a *true* screening-controlled experiment will be known, one last parameter should be taken in account: the willingness and trustiness of patient to be tested using ants. In term of approval, dogs are mostly accepted, as they are used for centuries for their olfactory abilities for hunts, drug, or explosive detection, and more recently for prospective cancer detection. On the other hand, ants seem to be a novel and potential model for cancer screening, but without proper communication, patients might refuse to be screened using the olfaction of ants. For example, insect phobia or misconception of the conditioning and tests (ants are not in contact with patients) could be problematics.

How can we improve our protocol

Our cancer protocol was based on three conditioning trials, with an ITI of three minutes. This time allows the ant to perform trophallaxis with nestmates to empty its social crop, and to ensure a full motivation for sugar solution at each trial. After the training, two consecutive memory tests (ITI of three minutes) were performed. This simple protocol produced good performances and could easily be mastered by anyone after three days of training (personal observation). However, this protocol could still be improved. Notably, we would suggest adding more memory tests / extinction trials as this species is resistant to extinction. This would add statistical power to the analysis, and potentially lower the minimal number of trained ants needed for an accurate diagnosis.

In the course of our experiments, ants did not manage to master one of the tasks. When conditioned to associate the odour from tumour-bearing mice with the reward, they did not discriminate it from the odour of tumour-free mice in the tests. We have two suggestions to improve ants' performances in this case. First, one could subject ants to more conditioning trials (up to six trials for instance, see **chapter 1**), in order to improve their learning of the less salient odour. This would not be very time consuming, as the protocol is already established. However, as the performances of ants in **chapter 1** were not so different when training them with one, three or six conditioning trials, we cannot be sure that adding more trials here would

solve the problem. Our second suggestion would be to perform differential conditioning instead of absolute one. In fact, we started to test such differential conditioning. During a COVID-19 national lockdown, I brought some ant colonies from the lab (LEEC) to my home and performed sugar/quinine differential conditioning. However, results were not reproducible from one day to the other. This result can be explained by several factors. First, hygrometry and temperature were not stable over the day and between days. Secondly, despite the fact that ants are biologically resistant species, the colonies showed signs of decay (loss of brood) after some weeks of minimal maintenance before the conditioning (due to lockdown). Lastly, they endured car transportation with all the related stress, including vibrations. Even absolute conditioning did not produce reproducible results during this period. Therefore, such experiments should be performed again in a stable lab setting. It is also interesting to note that in a screening procedure, ant colonies should probably be reared in steady conditions, in proximity to the screening centres, to prevent any stress that could negatively impact the ant discrimination abilities. It would also be interesting to investigate the performance of ants along the time of day. Although we did not observed any difference in learning performance between ants trained on the morning or the afternoon, the circadian rythme can play a role in response to odour (Gadenne, Barrozo, and Anton, 2016)

Finally, now that individual learning is well documented in this ant species, it would be interesting to investigate social learning. Through social learning (where at least two individuals are involved), ants could reinforce nestmates, and condition naïve ants to a given stimulus. One possibility would be to condition the whole colony (Cammaerts, 2004). Here, the training was passive, as food was placed inside the colony near visual stimuli. Another option would be to use *active* training, by conditioning a certain percentage of ants using an individual learning protocol (such as the one we used), allowing then conditioned individuals to spread the learnt information inside their colony. This was done by Provecho & Josens (2009) using *Camponotus mus* ants. One ant was trained to associate an odour with a reward and was later placed near a naïve one. When they performed trophallaxis, the naïve ants received part of the sugar solution (used as reward), but also olfactory cues, and was able to choose the learnt odour from a new one. Preliminary tests were performed, during a summer student internship, on *Formica fusca*, however, the protocol was still in a tuning-phase, so no conclusion could be extracted from this experiment.

Conclusion

In this thesis, we investigated the possibility of using *Formica fusca* ants as bio-detectors of cancer, and ants proved that they have the potential to fulfil this role. They can be trained in less than three conditioning trials (which can be achieved in couples of minutes), are highly resistant to extinction and can be tested several times after training. Ants of the species *F. fusca* are also easily available, form nests of thousands of individuals that are not particularly aggressive (they neither bite nor sting), and, in memory tests, they show high and stable performances (correct responses can easily be superior to 95 %) as dog, which is the main sniffer animal used for cancer detection. Our protocol needs some adjustments for ants to master all the discrimination tasks, but this first investigation of the potential of using ants for detecting cancer (and by extension, any diseases that alter the VOCs composition of the body) was successful and proved that ants (and insects in general) should not be underestimated for their cognitive abilities.

In 2020, almost 10 million people died from various forms of cancer worldwide. Any detection tool (animal olfaction, e-noses, molecular biomarkers…) that can prevent deaths, burden, or any loss of life quality due to this disease should be heavily investigated. This thesis aimed to make a contribution to this objective.

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Annexes – Published articles

Communication in Ant Societies

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Small insects like ants have evolved many different ways to communicate, some very sophisticated. Before describing the different communication strategies in ants, we shall clarify what an ant is, and what we mean by communication.

Ants

Ants are insects that live in societies. They appeared in the Jurassic and diversified during the Cretaceous, around 100 million years ago. Ants are closely related to bees and wasps (Figure 1), but while we can find species of bees and wasps that are solitary, there are no solitary ant species. Ants can be easily observed in our gardens. Many of us spent hours as children watching these tiny insects transporting pellets of dirt to build their nest, carrying food along trails, or fighting against intruders. In each of these examples, ants communicate among each other.

Ants are present all around the world (except at the poles) – they colonized the Amazonian forest, the Sahara Desert, the Australian Bush, the Iberian Peninsula, the European countryside, and they live in our cities as well. They represent on average 15–20 percent of the terrestrial animal biomass, except in the tropical forest, where they are abundant and represent 25 percent of the biomass (Schultz, 2000). With such a quasi-ubiquitous presence in areas occupied by humans, it is not surprising that ants have had an important impact on our culture. In Greek mythology, Zeus created the myrmidons from an ant colony, which were extremely strong and obedient soldiers that fought during the Trojan War under the command of Achilles. Ants are used in human initiation rites, to show bravery, where people let the so-called bullet ants (*Paraponera clavata*) sting them (Bosmia, Griessenauer, Haddad, & Shane Tubbs, 2015). As ants can have a strong bite, ancient populations in various

continents used ants for stitching wounds (Schiappa & Van Hee, 2012). More recently, in comics and movies released in the last decades, a superhero “Ant-Man” saved our (fictional) world from destruction with help of ants!

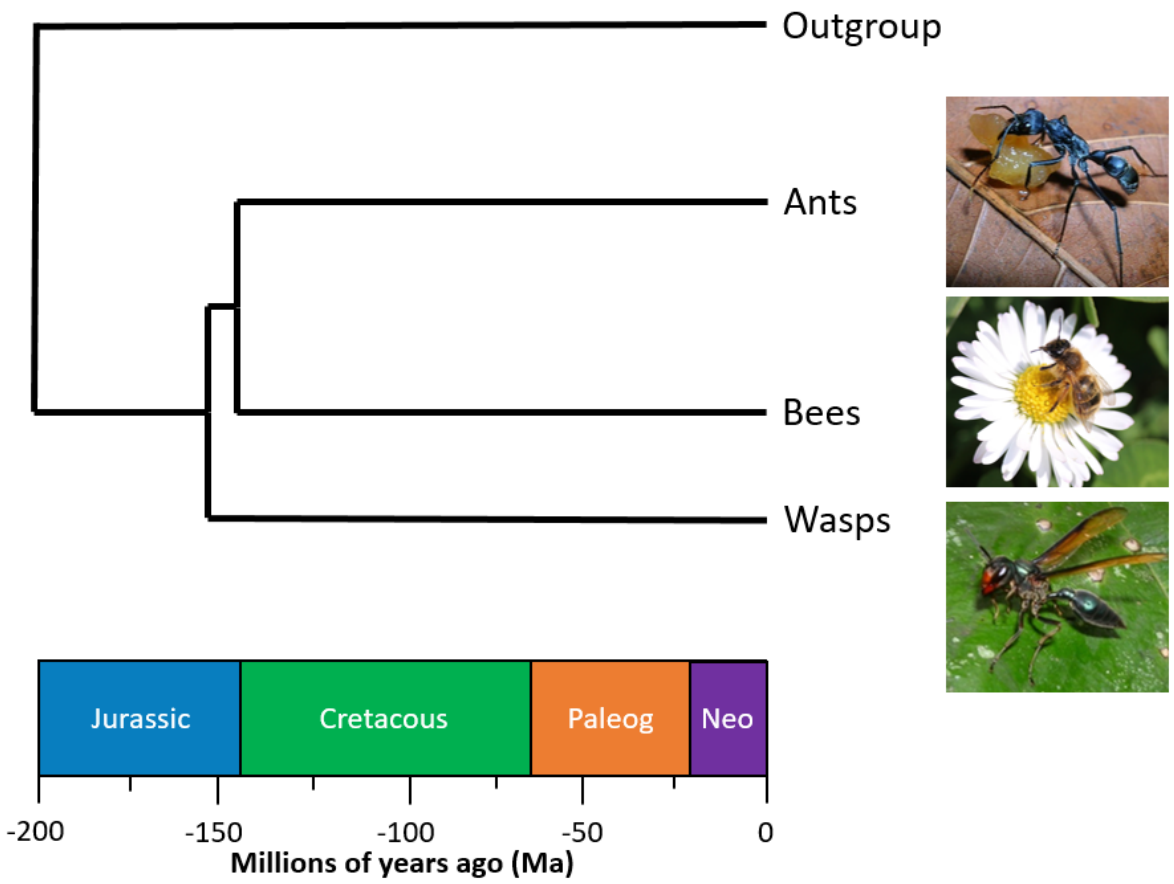

Figure 1: Simplified phylogenetic tree of the insect order Hymenoptera. At the end of the Jurassic era, wasps differentiated from the common ancestor. The differentiation between ants and bees followed several million years later. Bees and wasps include solitary and social species. Ants are always social. Adapted from Branstetter et al., 2017. ©Paul Devienne (ants & wasps) & Véronique Sallé (bees), both used with permission of the author.

With such a global distribution, more than 14,000 ant species described (Agosti & Johnson, 2005), and probably thousands still unknown, it can be expected that interspecific differences are extreme. It is very difficult to define a typical ant species. If we consider the morphology, for instance by focusing on the size of individuals, some ants can be just a few millimeters long, such as the pharaoh ants (*Monomorium pharaonis*), while others are 3–4 cm long, such as *Dinoponera gigantea*. Even within the same species, morphological traits may significantly differ between individuals, with the presence of morphologically differentiated worker castes.

For example, in species of the genus *Camponotus*, where we find adult workers of different sizes, major workers can be twice as large as minor workers. Other traits, such as the form of the head and the mandibles, or the orientation of the legs (Khalife et al., 2018) can differ within and between species. The form of the mandibles can give us information about the diet of ants. Some species have long mandibles, which can be closed in few milliseconds, such as *Odontomachus* ants – these are excellent for hunting insects. Workers of an ant species living in tropical Africa, Australia, and Southeast Asia, *Mystrium camillae*, can snap their mandibles and strike in only 23 µsec, with a peak velocity of 90 meters per second, one of the fastest movements in the animal kingdom. They use this ability for hunting, but also to feed on the hemolymph of their own larva, thus earning the nickname "Dracula ants" (Larabee, Smith, & Suarez, 2018). More than 50 million years before humans discovered agriculture, ants were already farming fungi. Attine ants (*Atta* and *Acromyrmex*, for instance) grow a symbiotic fungus inside their nest and feed it with leaves collected by ant foragers. Ants practice husbandry as well, keeping root aphids in their nests or tending aphids on nearby plants to eat honeydew (*Lasius*, *Formica*). Others are harvester ants, collecting and gathering seeds, which they can consume using their strong mandibles (*Messor*). Apart from morphology and dietary habits, ant species differ in the number of queens heading the colony. There are monogynous colonies with one queen and polygynous colonies with multiple queens. They can also differ in the number of nests that a colony inhabits: one nest (monodomy) or more nests (polydomy). However, the most notable characteristic of ants is that they always live in societies, which can vary in size from dozens to thousands of individuals. These societies are very well organized and characterized by efficient division of labor. Typically, only the queen lays eggs, while the workers are in most cases sterile (in some species, workers can lay unfertilized eggs, which develop into males, given the haplodiploid sex determination in social Hymenoptera). The various tasks are carried out by different groups of workers (such as nurses, foragers, guards), and a given worker can change tasks depending on age or the needs of the colony (Hölldobler & Wilson, 1990). For these societies to be functional and ecologically successful, individuals must have a way to communicate between themselves. Otherwise, there would be chaos.

Communication

Animal communication is difficult to define and can take different forms depending on the specificity of the definition. It is generally accepted that the process of communication involves the provision of information by a sender to a receiver (Bradbury & Vehrencamp, 1998). But what, then, is the nature of this information? When a pigeon is moving, it may send information about its position to a hawk, thus allowing the predator to localize its prey. Is the pigeon communicating with the hawk? When the stimulus produced by an animal is perceived by another animal, and possibly used to the detriment of the sender, this is not considered a case of communication but of eavesdropping – and the stimulus is a cue.

In "true communication," the sender does not produce inadvertent cues but voluntarily emits a signal that contains information aimed at obtaining a specific response from the receiver. When a male bird is singing, it intentionally sends information about its quality and/or social status to females. Females hear the song and react by approaching the male for mating, for example. This is a case of true communication. However, a predator can hear the song and locate the bird to capture it. The same stimulus can thus act as both a signal and as a cue. It is crucial to consider who will benefit from the information provided. We agree with the definition of Zahavi (2008), who considers signals those traits that evolve in a sender in order to provide information to a receiver, aiming to change the behavior of the receiver to the benefit of the sender. However, signals evolve and persist over time when both senders and receivers gain from their interaction. Therefore, communication is a cooperative endeavor.

This definition of communication can be applied to extremely different communication systems, from Silbo (a whistled language used by inhabitants on the Spanish island of La Gomera), to insect trail pheromones, to binary systems between computers, to bird songs, to bees' waggle dances, and to human languages. Through communication, individuals obtain food, sexual partners, avoid danger, establish hierarchies, and so on. Organisms have found multiple ways to communicate. Some species use the magnetic field, electric fields, vision, sounds, olfaction, taste, or tactile sensation. Ants use mainly the tactile, acoustic, visual, and especially the chemical channel to communicate, and in some cases more than one channel is involved in multimodal communication. We provide some representative examples for each of the main communication channels.

Visual Communication

If you ask yourself what is the most important sense for humans – sight, hearing, smell, taste, or touch – your answer would probably be that vision is the most important. If you were asked to go from point A to point B, to drive, or to eat, you could easily perform these tasks without audition, olfaction, taste, or the sense of touch, but without vision, even if it were feasible, it would be very difficult.

Visual communication is used by many different species for orientation, aggression, or communication during mating, for instance. In case of a conflict over territory or access to the opposite sex, one possible outcome is to fight directly with the opponent, regardless of the chance of success. A smarter way is to evaluate the chance of winning and act upon it. To determine the chance of success, an individual must know its own strength and estimate the strength of the opponent, for instance by using visual cues. In fallow deer, males compete for access to females. The strongest males begin with a vocal display (which is costly in terms of energy) and if two males remain, they will walk side by side, allowing each to compare the opponent with themselves. If they seem to be of similar strength, they will fight. However, if one seems stronger (e.g., larger body size) than the other, the opponent will leave (McElligott et al., 2001). Cichlid fishes also use visual communication to detect the opponent's strength. In this case, body size is not a valuable criterion, so fish compare their color patterns (Ziegelbecker et al., 2018). In ants, none of this has been recorded. Ants rarely use the visual channel alone to communicate. If visual communication is used, it is usually paired with the chemical or acoustic channel. When disturbed, *Crematogaster scutellaris* ants raise their abdomens. This behavior can be perceived as a visual communication to enemies or to increase the aggression of nestmates, but ants produce a drop of venom to repel opponents, thus using the chemical channel as well. Reproductive individuals (queens and males) also use visual communication paired with either the chemical (pheromones) or the acoustic channel (stridulation) when seeking sexual partners.

Ant workers possess two composed eyes, and reproductive individuals have also three ocelli on the top of the head that help them to navigate when flying (virgin queens and males typically have wings – the males die after mating, while mated queens shed their wings). Although ants can have up to five different eyes, the majority spend most of their life in the

dark, inside the nest, and when outside can only detect vague forms. However, workers of some species, such as *Gigantiops destructor*, which forage in complex environments, possess large eyes that occupy almost half of their head. With the help of these gigantic eyes, ants are capable of foraging on the ground and in the tree canopy. When they fall from a tree, they still manage to come back to the nest. During foraging trips, *Gigantiops destructor* ants use visual cues, not the magnetic field or odorant cues, to locate their nest (Macquart et al., 2006). Workers of the desert ant, *Cataglyphis*, use a sun compass and an internal odometer to count the number of steps they take between the nest and the food source and then integrate the path to find their way back (Andel & Wehner, 2004). However, they also use visual landmarks to navigate. So, few ant species have large eyes and use visual cues, but the majority rely on other channels. For example, leaf-cutting ants use landmarks, pheromones, the sun's position, and also the earth's magnetic field during foraging (Banks & Srygley, 2003).

Acoustic Communication

Producing sound is costly for individuals, but as sound travels fast (330m/s in the air, 1,500m/s in water) and far (cetaceans can communicate with conspecifics that are hundreds of kilometers away), it is a suitable option to reach conspecifics quickly. Another interesting feature of acoustic communication is that sounds vanish rapidly. In that way, when information is no longer reliable, individuals simply stop emitting the sound and others will quickly react and change their behavior. Contrary to most mammals and birds, insects (including ants) do not have structures similar to vocal cords to produce sound. However, they have other means of acoustic communication. Ants can produce sound by knocking walls or the ground with their bodies (drumming) or by rubbing two parts of their bodies (stridulation).

When ants drum, they usually beat the substrate using their gaster (abdomen). Drumming behavior has been recorded in several ant species that nest inside trees. When nests are built into the soil, the substrate does not act as a good vibratory conductor, unlike wood, which is an efficient vibratory conductor. Workers of *Camponotus senex* use the silk produced by their larvae to build an arboreal nest. When the nest is disturbed, for example by an experimenter who drops a little ball on the top of the nest, vibrations coming from ants' drumming can be recorded. Drumming is likely a signal to recruit nestmates against a potential predator, or it

could mimic the sound produced by aggressive sympatric wasps, which usually scares predators (Santos, Korndörfer, & Del-Claro, 2005).

To stridulate, ants (and many arthropods) have developed a specialized stridulatory organ. Stridulation consists of rubbing two body parts (Figure 2).

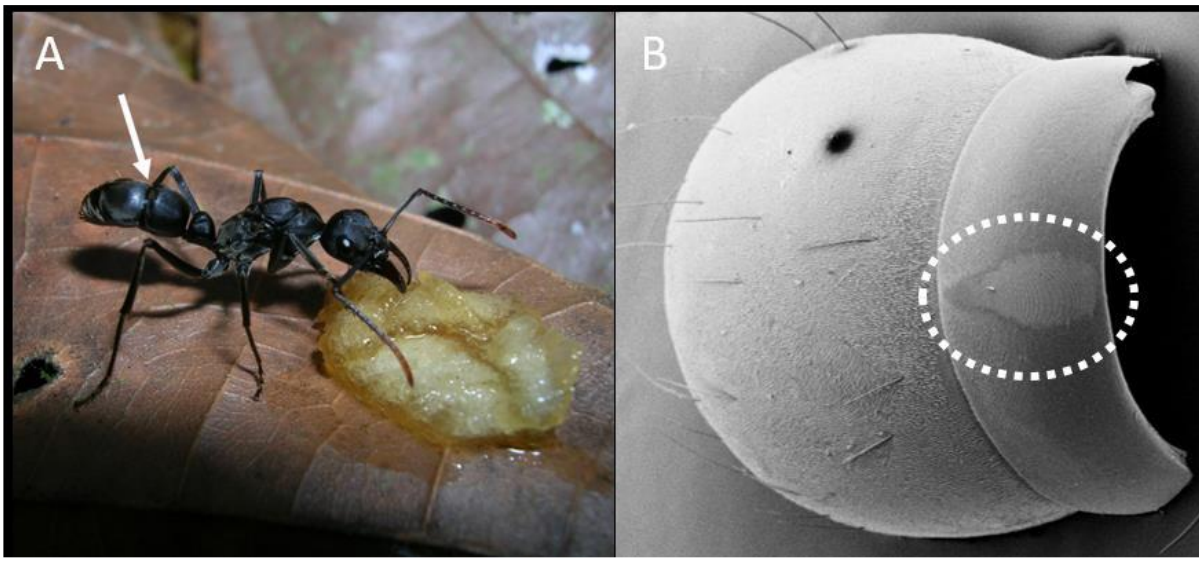

Figure 2: The stridulatory organ of the ant *Neoponera apicalis*. Stridulatory organs are composed of two parts: the scrapper and the file. In *N. apicalis* (A), the file is located on the second segment of the abdomen, and the scrapper is on the first one. When both parts are moving, the ants are stridulating. The arrow represents the position of the stridulatory organ. Scanning Electron Microscopy (x91) of the file (B). The file is made up of parallel and rectilinear ridges (here vertical lines) © Paul Devienne, used with permission of the author.

For a long time, it was thought that the original function of the stridulatory organ was linked to the call for help (Markl, 1973). When ants are covered by soil, they stridulate to alert nearby nestmates that can dig and rescue them. This is especially useful for monogynous terrestrial species where, if the queen dies, the colony dies. Based on this hypothesis, scientists expected to find a stridulatory organ in nearly all terrestrial or subterrestrial ants where the selective pressure is strong for this protective system to evolve. Few arboreal ants should have this stridulatory organ, because of the impossibility of being buried. However, a recent study found exactly the opposite pattern. More arboreal ant genera possess a stridulatory organ, compared to genera that nest in the ground, and none of the subterranean genera studied has one (Golden & Hill, 2016). The stridulatory organ probably evolved independently several times with potentially different uses. Ants stridulate during aggression, disturbance of the nest, food recruitment, trophallaxis, and mating.

In *Neoponera* ants, laboratory colonies left undisturbed do not stridulate, but when experimentally disturbed (by shaking the nest or opening the nest box), ants start stridulating (Ferreira, Cros, Fresneau, & Rybak, 2014). This indicates that stridulation can be used to communicate alarm within the colony. It is also possible that stridulation acts as an aposematic signal used as a warning for other species (that caused the disturbance) because these ants sting and are potentially dangerous (Ferreira et al., 2014).

Stridulation was recorded during mating in *Cardiocondyla* ants, where stridulation and antennation of the male toward the female was observed. Males that stridulate more before mating were more likely to succeed (Mercier et al., 2007). However, in *Neoponera* (formerly called *Pachycondyla*), queens stridulate after mating, which could indicate their mating status and the fact that they will not accept any further mates (Ferreira et al., 2014).

Stridulation can be used during foraging recruitment. *Atta sexdens* ants, which forage for leaves, can stridulate when they find a good (tender) leaf. Stridulation goes from the body of the ant through the substrate (the leaf) so that nestmates will detect the vibrations and will help to forage. When ants are experimentally confronted with two paper bridges that vibrate with a short delay (left bridge vibrates first), ants choose to go to the left. However, this preference can be modulated by the presence and concentration of a component (citral) of the alarm pheromone. A high concentration of citral will disrupt the preference for the bridge vibrating first (Hager, Kirchner, & Kirchner, 2017). This result indicates that one signal overrides the other, or that a modulation of the response occurs when both signals are present at the same time, a case of multimodal communication.

Most communication systems might be decoded (or hacked) by an individual that was not supposed to receive this message or to understand the content. An example of “hacking” was recently described in a parasitic butterfly that mimics the sound produced by ants (Sala, Casacci, Balletto, Bonelli, & Barbero, 2014). Maculinea butterflies lay eggs on host plants, and when larvae reach a certain developmental stage, they fall on the ground. *Myrmica* ant workers find the larvae and bring them to the nest. Depending on the butterfly species, adopted larvae can either be fed by the ants or directly devour ant eggs, larvae, or cocoons. The adopting mechanism relies on two communication channels, the olfactory and the acoustic. Chemical analyses showed that butterfly larvae mimic the odor of ant larvae (e.g. Nash, Als, Maile, Jones, & Boomsma, 2008). Playback experiments revealed that vibrations

produced by butterfly larvae mimic vibrations produced by the ant queen (Sala et al., 2014). Workers of this ant species can also produce vibrations, but they are different from those of the queen. The butterfly larvae are thus very precise in their mimicry to be adopted and cared for in the ant nest.

Tactile Communication

In some species, the offspring are totally dependent on parents for food and protection. Assuming all young are equally related to the parents, to maximize their fitness the parents will share food equally among offspring. But for a particular young one, the best strategy is to obtain more food than its brothers and sisters. To obtain more food from the parents, offspring can beg for food. Begging behavior has been described in many species and can rely on visual and acoustic communication, as shown in birds. Chicks that beg more or sooner than others will receive more food (Dearborn, 1998). Acoustic begging has also been observed in mammals, for example in meerkats where hungry pups begged more than well-fed pups (Manser, Madden, Kunc, English, & Clutton-Brock, 2008). Besides acoustic and visual communication, tactile communication linked to begging has also been described: for example, in vampire bats starving individuals beg by licking the mouth or grooming a potential donor, thus inducing food sharing (Carter & Wilkinson, 2013).

Ant brood is totally dependent on adult workers. Ants are holometabolous insects with metamorphosis (once adult, they keep the same size and stop growing). During metamorphosis, at the pupal stage ants are unable to eat. Metamorphosis is a costly phenomenon that can use up to 75 percent of stored sugar, and 40–45 percent of lipids and soluble proteins (Wheeler & Buck, 1992). Therefore, the larval stage is a critical period to accumulate enough energy to allow metamorphosis. Ant larvae hardly move and cannot forage by themselves. They rely on adult workers to bring them the necessary nutrients for their growth and survival. The food intake during the larval stage can also determine the caste of an ant. Queens and sterile workers are both diploid females and possess the same genes, and caste determination is usually the result of differential food provision (Wheeler, 1986; Penick, Prager, & Liebig, 2012;). From the larval point of view, the way to maximize fitness is to become a queen and produce offspring. Therefore, a larva must eat more than other larvae to have a chance to become a queen. On the other hand, from the colony point of view

producing a queen is more costly than producing a sterile worker and for efficient functioning of the colony more workers must be produced than queens. Workers will try to feed each larva equally whereas larvae will try to obtain more food to maximize their chance to become a queen.

Ant larvae use tactile communication to incite workers to give them more food. Typically, nurse workers use their antennae to probe larvae, and hungry larvae move their body while being antennated, to indicate that they need food. In the ant *Gnamptogenys stritula*, begging of larvae has been labeled as swaying behavior: larvae raise their heads and necks, and gently reach and wave toward workers or food items. Laboratory experiments show that larvae performing more swaying behavior are better fed by tending workers (Kaptein, Billen, & Gobin, 2005).

In *Mesoponera caffraria* ants, similar begging behavior has been described. Ants antennate the larvae and if the larva reacts by moving the anterior part of its body, workers take it and set it apart to form a group of "hungry larvae." When all larvae are inspected by antennation, workers feed the hungry larvae by placing a piece of food near them, which the larvae will immediately start to eat. To test if these ant larvae may use another means of communication to indicate their hungriness beside moving their body, researchers added larvae that did not move when antennated by workers to the hungry larvae group selected by the workers. When these two kinds of larvae were together, workers were unable to detect if one larva was hungry or not, and just fed all the larvae of the group. Non-hungry larvae waited a few hours before eating (Agbogba, 1991). This indicates that body movements are the only cue used by the workers to detect hungry larvae. *Myrmica rubra* larvae also display a spontaneous begging behavior by keeping their heads bent upwards in a stretched position; this is not necessarily induced by previous contacts (antennation) with workers (Creemers, Billen, & Gobin, 2003).

As food is essential for the development of larvae and for the maintenance of adult workers and the queen, finding and bringing food rapidly (in a context of competition among neighboring colonies) is essential for the colony. To bring food rapidly to the nest, social insects recruit nestmates to guide them to the food location. One well-known example is the waggle dance displayed by honeybees. When a forager finds food, she comes back to the hive and executes the dance. The bee waggles back and forth in a straight line, then circling and forming an "8" shape (von Frisch, 1974). Naïve bees antennate the forager and get information

to find the food source, which is correlated with the number of waggles (distance) and the orientation of the dance.

Ants are also capable of recruiting nestmates using tactile communication, one example being a behavior called tandem running. Two ants are involved in the tandem running – the leader, which knows the exact location of the food source, and the follower, which is naïve. When the leader finds the food source, she comes back to the nest and recruits a nestmate by doing "back and forth" movements toward the naïve ant ("jerking") (Lenoir & Jaisson, 1982). The leader will then go to the food source with the follower behind. During the way, the follower regularly taps the abdomen of the leader with its antennae. If the leader detects no tapping, she stops and waits for the follower until the contact occurs again, thus reinitiating the running. The leader therefore adapts its speed and acceleration to the behavior of the follower. The fact that a demonstrator modifies its behavior (in a costly way, by slowing down) in the presence of a naïve observer so that the naïve individual learns more quickly, indicates that tandem running is a form of teaching (Franks & Richardson, 2006). In tandem running, only one ant can be recruited by each leader, which is a suitable strategy for ants with small colony size, like *Temnothorax* ants. In species with a large colony size, the chemical channel of communication is preferred in the form of trail pheromones.

Tactile communication via antennal movements is also used to resolve intra-colony conflicts. A major source of conflict in social species is to decide who has the right to reproduce. In social insects, the queen produces the majority of the colony offspring and usually inhibits the ovarian development of workers with a queen pheromone (Holman, Jørgensen, Nielsen, & d'Ettorre, 2010). However, in polydomous species for instance, the queen pheromone is less present in secondary nests than in the primary nest, leaving the door open for worker reproduction (via the production of male eggs, which are haploid). Conflicts among workers can emerge to achieve a dominant status leading to egg-laying. The dominance hierarchy among workers is maintained via ritualized agonistic behaviors, such as antennal boxing (Figure 3a), observed for example in *Neoponera goeldii* (Denis et al., 2008). Antennal boxing is also used as a form of worker policing to stop workers that try to reproduce in the presence of a healthy queen (Brunner & Heinze, 2009)

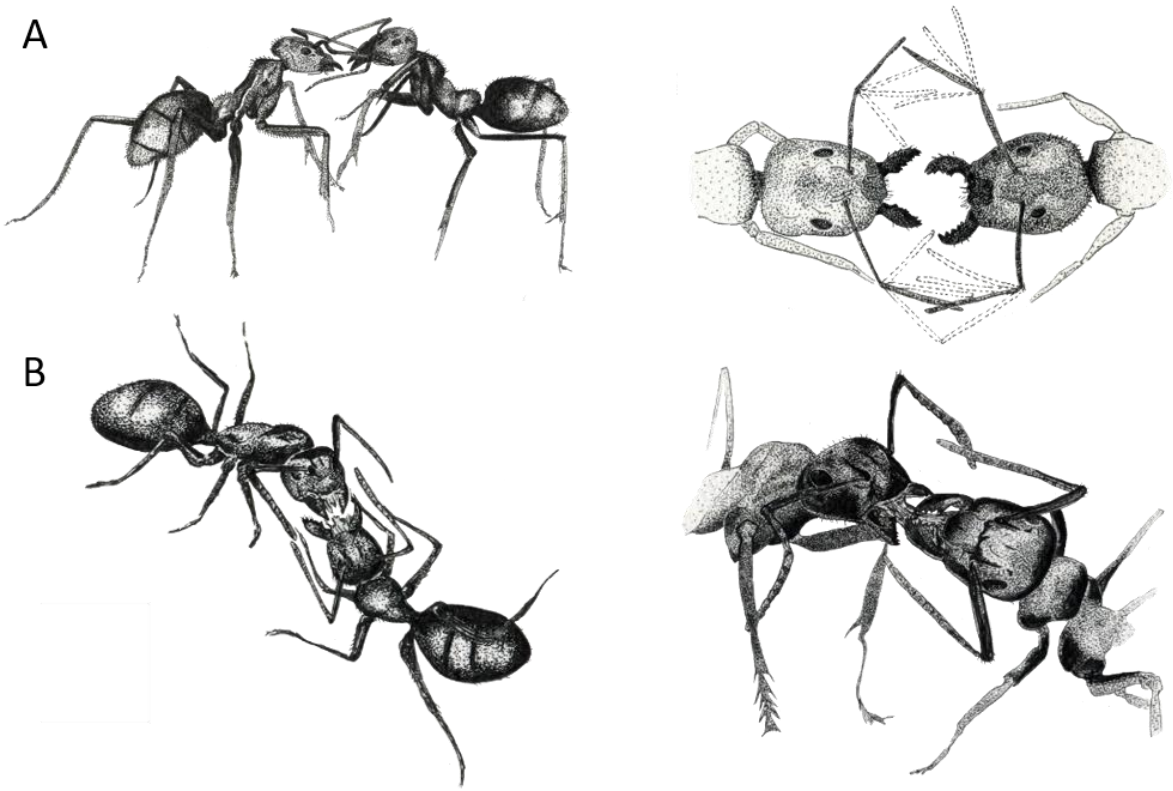

Figure 3: Trophallaxis and antennal boxing between two ants. A) Antennal boxing: when conflicts arise between individuals, they can resolve them with antennal boxing. Two ants are facing each other with open mandibles, then start boxing by doing rapid antennations. If one ant dominates, the other, dominated ant will flee. B) Trophallaxis: During trophallaxis, ants are facing and antennating each other. Fluids are exchanged in both directions, mainly composed of regurgitated foods and cuticular hydrocarbons. ©Louise C. Prévot, used with permission of the author.

Tactile communication is involved in trophallaxis, in which two ants exchange fluid through their mouths (Figure 3b). Trophallaxis occurs in many ant species between adult workers or between workers and larvae (see larval begging). When an ant finds a source of liquid food, she fills her crop and comes back to the colony, where she starts trophallaxis with nestmates that antennate her.

During trophallaxis, ants will principally exchange food but also different chemical products such as cuticular hydrocarbons (Boulay, Hefetz, Soroker, & Lenoir, 2000), micro-RNA, proteins (Leboeuf et al., 2016) and odorant food cues (Provecho & Josens, 2009).

Chemical Communication

Chemoreception is the most common way to map the world and is found from bacteria to mammals, including humans (Ache & Young, 2005; Wyatt, 2014). Ants are particularly efficient in detecting chemicals, for example via olfaction. They have evolved a remarkably large clade

of odorant receptor genes (d'Ettorre, 2016) expressed in specialized sensilla located in their antennae. These sensory receptors relay the information to up to 500 glomeruli, dedicated olfactory units in the brain, and the processed information is then passed to the higher brain centers. Although not as well studied as the olfactory system of the honeybee, *Apis mellifera* (review in Sandoz, 2011), the olfactory system of ants is well developed and allows discrimination between different single odorants and mixtures of odorants. With the recent development of conditioning learning paradigms (e.g., Guerrieri & d'Ettorre, 2010), similar to those used to study olfactory abilities in honeybees, (review in Giurfa & Sandoz, 2012), olfactory detection and discrimination can be explored in ants. Harnessed ants extend their tongue (maxilla-labium) in response to the presentation of a food reward (e.g., sucrose solution). This response can be conditioned using odorants (maxilla-labium extension response) (Guerrieri & d'Ettorre, 2010). This protocol has shown that, for instance, ants can discriminate between two concentrations of the same odorant, even when the difference between the two stimuli is very low (0.005mg/ml vs. 0.05mg/ml) (di Mauro et al., 2015), and that the perception of odorants depends on their chemical characteristics, such as chain length and functional group (Perez et al., 2015). During their lives, ant workers change the way they perceive the world. Foragers are significantly more efficient at detecting chemical cues characterizing the presence of an enemy than younger, intranidal workers (Larsen, Nehring, d'Ettorre, & Bos, 2016). Foragers are also better at detecting low concentrations of a sucrose solution and at olfactory appetitive learning than intranidal workers (Perez, Rolland, Giurfa, & d'Ettorre, 2013). Ants' learning abilities are astonishing, workers of *Formica fusca* are able to learn an olfactory stimulus after only a single presentation when it is paired with food and they retain this memory for days (Piqueret, Sandoz & d'Ettorre, 2019).

In ants, chemical communication is used in many different contexts, such as recognition of group members (van Zweden & d'Ettorre, 2010), foraging (David, 2009), reproduction (Walter et al., 1993), alarm and enemy defense (Blum, 1985), worker policing (Monnin, Ratnieks, Jones, & Beard, 2002). In many of these contexts, communication relies on pheromones. Pheromones are signals that induce a specific and adaptive response in conspecifics. Typically, pheromones are species-wide signals and do not necessitate learning. The first pheromone was discovered in the female silkworm moth, *Bombyx mori*, and acts as a sex attractant for males (Butenandt, Beckmann, Stamm, & Hecker, 1959). Females that are sexually mature

release this pheromone and males follow the increasing pheromone concentration in the air to find their partner. In ants, sexual pheromones can be released by virgin queens to attract males, or by males that gather in specific mating sites to attract females (Hölldobler, 1976; Bourke & Franks, 1995; Ayasse, Paxton, & Tengö, 2001). Sex pheromones have been characterized in few ant species (Hölldobler, 1971; Walter et al. 1993; Greenberg et al. 2007). The attraction power of female sex pheromones has been tested in the lab by recording the activity of males (*i.e.* flying, running in circles) in the presence or absence of pheromones. When no pheromones are present, only 2 percent of males show activity, whereas in the presence of pheromones, more than 80 percent of individuals are active and go toward the pheromone source (Topoff & Greenberg, 1988).

Queens use pheromones not only for finding their partner, but to keep order in the colony throughout their life. Despite the queen being the only individual who is mated and reproduces in an ant colony, in many species, workers retain the ability to lay unfertilized eggs that can develop as males. When this happens, there is a cost at the colony level because workers that lay eggs do not work efficiently (Monnin & Peeters, 1999; Wenseleers, Helanterä, Hart, & Ratnieks, 2004). Therefore, some ant species have evolved a "policing worker force" that reacts aggressively to workers with developed ovaries and eats worker-laid eggs (van Zweden, Fürst, Heinze, & d'Ettorre, 2007). In most cases however, the action of the police is not needed because the queen produces a pheromone that prevents workers from developing ovaries. The first ant queen pheromone was identified in the black garden ant, *Lasius niger*, this is an hydrocarbon found on the cuticle of the queen (Figure 4, 3-MeC31, (Holman et al., 2010)). Chemical profiles of workers, queens and queen laid eggs have been analyzed and the relative amount of queen pheromone was found to be six times higher in the cuticular profile of the queen than in the profile of the workers, and nine times higher than in eggs. In the laboratory, the synthetic version of the pheromone has been used to coat glass queen dummies to show that the mere presence of the chemical compound reduced significantly the ovarian development of workers compared to control treatments. Thus, a pheromone composed by a single substance helps keeping order in the colony.

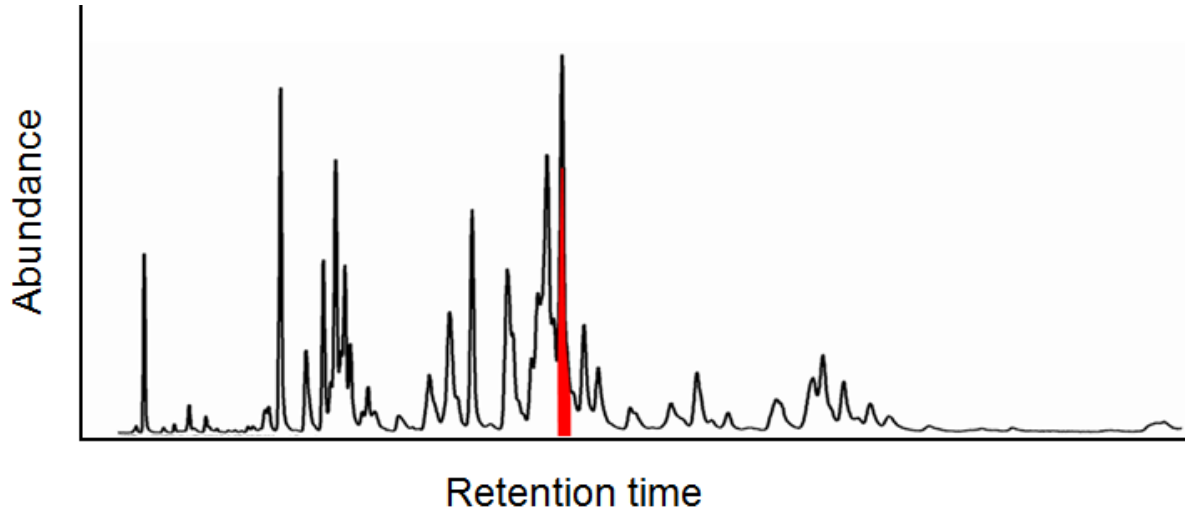

Figure 4 Gas-chromatogram showing the cuticular hydrocarbon profile of a *Lasius niger* queen. The queen pheromone regulating worker reproduction (3-MeC31) is highlighted (for details about the identification of this pheromone see: Holman et al., 2010).

The cuticle of ants, as well as that of insects in general, is covered by a layer of hydrocarbons, but these do not all act as pheromones. The original function of cuticular hydrocarbons was to limit the evaporation of water, since they are hydrophobic (Gibbs, 1998). With the evolution of social life, cuticular hydrocarbons have assumed the function of carrying information about the identity of individuals. Here, the identity of individuals does not depend on a single odorant but rather on a mixture, the so-called "signature mixture" (Wyatt 2014). Although in some particular cases, such as in associations of founding queens, ants can recognize each other individually (d'Ettorre & Heinze, 2005), with up to several thousands of individuals in a colony, it is impossible for each ant to keep track of the individual identity of each nestmate. In fact, in large colonies all individuals share the so-called colony odor, which is a mix of the odors of all individuals. When an ant encounters another ant, a process of odor matching occurs. The behavioral response depends on the similarity between her own odor and the encountered odor: if the two odors match (as for a nestmate), the ant is accepted; if they do not match it is aggressively rejected (van Zweden & d'Ettorre, 2010). In ants, cuticular hydrocarbons are stored in a gland present in the head (postpharyngeal gland) and are spread all over the body cuticle. To obtain a homogenous colony odor, ants exchange hydrocarbons continuously via trophallaxis and allogrooming (Soroker, Vienne, Hefetz, & Nowbahari, 1994; Lenoir, Fresneau, Errard, & Hefetz, 1999). If workers are experimentally isolated from their colony, their body odor changes with time. Isolated ants reintroduced to their colony after

five days received some aggression and many occurrences of trophallaxis. When isolation was longer (forty days), the cuticular hydrocarbon pattern reached a point where nestmates did not recognize them and attacked them as intruders (Boulay et al., 2000). To maintain the colony odor, ants must regularly exchange their hydrocarbons with nestmates. Different colonies have different odors, and the aggressive reaction of workers is typically related to the chemical distance between these odors: the higher the chemical dissimilarity between the two profiles, the higher the aggression (Guerrieri & d'Ettorre, 2008). This recognition system is highly efficient at stoppping intruders coming into the nest. There are worker guards permanently monitoring the nest entrances. These guards investigate every individual approaching the nest, including nestmate foragers returning from foraging trips.

In many ant species, foraging efficiency is enhanced by the use of pheromones (Tumlinson, Silverstein, Moser, Brownlee, & Ruth, 1971), especially when the colony size is large. Indeed, when colonies are composed of few individuals, they can rely on one-to-one communication to inform each other about the location of food, as in the example of tandem running. When colonies are composed of thousands of individuals, personal communication is too costly and time consuming; therefore, ants rely on trail pheromones. When an ant finds a suitable food source, she deposits a trail pheromone on her way back to the colony. Other ants are attracted to the pheromone, follow it, and find the food source. These ants will also release trail pheromone on the way back, thus creating a positive feedback loop that attracts more and more individuals. In the laboratory, the dynamics of this phenomenon can be studied when ants are given the possibility to reach a food source via a short path and a long path. Because of the positive feedback mechanism, the shortest path is quickly selected by the ants (Goss, Aron, Deneubourg, & Pasteels, 1989). Analyzing how ants can solve this kind of problem has helped resolving mathematical puzzles, such as the traveling salesman problem, who has to find the shortest way to visit all the cities he has to go to (Dorigo & Gambardella, 1997; Pintea, Pop, & Chira, 2017). Ant algorithms are used to solve practical problems, such as to improve urban design or public transport (Shokouhi, Shadab Mehr, Mafi, & Rahnama, 2016).

Ants that forage outside the nest are the most exposed to predators. As the strength of ants is in numbers; if a scout ant encounters a competitor ant or a predator near the nest, she will flee and release alarm pheromones (Blum, 1985). This pheromone induces fleeing behavior in other ants. However, if the alarm pheromone is released near or inside the nest, fleeing is not

the best option, as brood is exposed to predators. In this case, ants fight against intruders. Formic acid acts as an alarm pheromone and as a defensive mechanism in many ant species (Löfqvist, 1976). When formic acid is present around the nest, ants become more aggressive and active than normal. More aggressive ants are faster to attack and repel intruders, but increased general aggression might result in recognition errors in which nestmates are also attacked. This, however, is not the case. Indeed, formic acid does not only act as an alarm pheromone in *Camponotus* ants, but enhances discrimination of nestmates from non-nestmates. In an experimental setting, formic acid induced both more non-nestmate rejection and more nestmate acceptance than a control treatment, thus increasing discrimination accuracy (Rossi, Baracchi, Giurfa, & d'Ettore, 2018). Pheromones typically elicit a stereotyped response but their action might be more complex than was previously thought, as they can also act as behavioral modulators.

Concluding remarks: Ant societies are comparable in many ways to human societies. They build complex nests (cities), cemeteries, highways (pheromone trails), they cultivate food, store extra food in a granary, practice animal husbandry, and they take care of the young in nurseries. Work is divided between individuals: some are shepherds, others are nurses, foragers, or guards. In both humans and ants, communication is essential to guarantee efficient and organized societies. Human societies rely mostly on visual (books, newspapers, websites) and acoustic communication (oral languages), whereas ants use principally the chemical channel. According to Billen & Šobotník (2015), eighty-four different glands have been described in ants, whereas there are only fifty-three in bees, forty-nine in wasps and twenty in termites. Each gland can release different molecules (e.g., formic acid) associated with one specific behavior (e.g., alarm pheromone) or that function to modify other behaviors (e.g. recognition of nestmates).

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Ants learn fast and do not forget: associative olfactory learning, memory and extinction in *Formica fusca*

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Learning is a widespread phenomenon that allows behavioural flexibility when individuals face new situations. However, learned information may lose its value over time. If such a memory endures, it can be deleterious to individuals. The process of extinction allows memory updating when the initial information is not relevant anymore. Extinction is widespread among animals, including humans. We investigated associative appetitive learning in an ant species that is widely distributed in the Northern Hemisphere, *Formica fusca*. We studied acquisition and memory between 1 h and one week after conditioning, as well as the extinction process. Ants learn very rapidly, their memory lasts up to 3 days, decreases slowly over time and is highly resistant to extinction, even after a single conditioning trial. Using a pharmacological approach, we show that this single-trial memory critically depends on protein synthesis (long-term memory). These results indicate that individual ant workers of *F. fusca* show remarkable learning and memory performances. Intriguingly, they also show a strong resistance to updating learned associations. Resistance to extinction may be advantageous when the environment is stochastic and individuals need to switch often from one learned task to another.

1. Background

Behavioural flexibility offers significant fitness advantages, especially in environments where resource distribution or threats are characterized by stochasticity. One way to achieve

this flexibility is via learning, defined as a change in behaviour occurring as a result of experience. Many learned behaviours can be further modified to suit changing environmental conditions. The ability to learn and memorize allows animals to respond to environmental stimuli in an adaptive way, for instance, by either ignoring them or by giving them a specific value, positive or negative. This helps in predicting the environment when facing new but similar situations [1].

Storing information is costly; therefore, only essential pieces of information should remain available for the individual. For instance, with time, a stimulus which used to predict a certain resource in the environment (e.g. the presence of food) might lose its significance and be no longer associated with the resource. It is beneficial to learn rapidly that such a stimulus is not reliable anymore. Extinction is the process in which a conditioned response gradually decreases through repeated experience with the stimulus in the absence of its outcome. Extinction generally involves the formation of a new inhibitory memory rather than the destruction of the previous memory [2]. Knowledge about the extinction process has important clinical applications, for instance, for the treatment of drugs addiction and abnormal fear of a past event (e.g. war trauma) in humans [3].

The extinction phenomenon was first described by Pavlov in 1927 in experiments with dogs using classical conditioning, the association of an unconditioned stimulus (US, for example a reward) with an initially neutral stimulus that becomes a conditioned stimulus (CS) producing the response in the absence of the US. After a successful conditioning (CS–US association), Pavlov observed the conditioned responses stopped after a few unrewarded CS presentations, leading to the extinction of the conditioned behaviour. Extinction does not erase the old memory. It is rather a new learning (creation of a CS–no US association). Therefore, two memories coexist. When time passes after successful extinction, the original behaviour may reappear (called spontaneous recovery or relapse), through a decay of the extinction memory [2]. Associative learning and extinction are widespread in the animal kingdom and have been intensively studied in several vertebrate species such as mice [4] or zebrafish [5] and also in invertebrates species such as snails [6], crabs [7] or nematodes [8]. Among invertebrates, insects like fruit flies became key model species for learning and memory [9–11]. Insects are well suited for laboratory studies because they are relatively easy to keep, they have short reproductive cycles and offer easy access to brain structures (e.g. crickets, [12]). Learning and extinction have also been investigated in social insects, including bumblebees [13,14] and honeybees [2,15]. Among social insects, ants are the most diverse group with more than 14 000 described species, which represent up to 25% of the total animal biomass on Earth [16]. Visual learning in ants has been intensively studied, also at the individual level, in the context of spatial orientation and navigation [17–19]. Individual olfactory learning has been less investigated [20–27]. Carpenter ants are very efficient in discriminating between different odorants [24,26] and even between different concentrations of the same compound [27]. Recently, workers of *Lasius niger* were shown to be able to learn odour–reward associations after only one training trial, while more trials were required when using spatial cues instead of odours [23]. However, in this study, the dynamics of memory formation was not investigated. We know that individual ants can form long-term olfactory memories after six CS–US presentations [22], but whether fewer conditioning trials lead to long-term memory (LTM) is unclear. Furthermore, data about extinction of olfactory learned associations are very scarce in ants [28].

In the present work, we present the results of a laboratory study on individual associative olfactory learning, memory and extinction in the ant *Formica fusca*. Among ants, the genus *Formica* was described as one of the most advanced from a cognitive point of view (especially concerning communication and learning) [29]. *Formica fusca* is widely distributed and lives in a variety of environments with a large range of temperatures, resources, predators and competitors. Colonies are populous (hundreds of individuals) and grow well in laboratory conditions. We investigated the acquisition performance of individual ants by changing the number of conditioning trials (from one to six). We tested ants' memory abilities by subjecting them to a memory test between 1 h and one week after training. We then categorized the memory using a pharmacological approach by administrating a protein synthesis inhibitor. Finally, we studied the extinction phenomenon in individual ants, by measuring their behaviour after unrewarded presentations of the CS.

2. Material and methods

2.1. Insects and origin of colonies

Formica fusca is a relatively common ant species found in the Northern Hemisphere. This species can be monogynous or polygynous and colonies may contain several hundred individuals [30]. Nine queenright

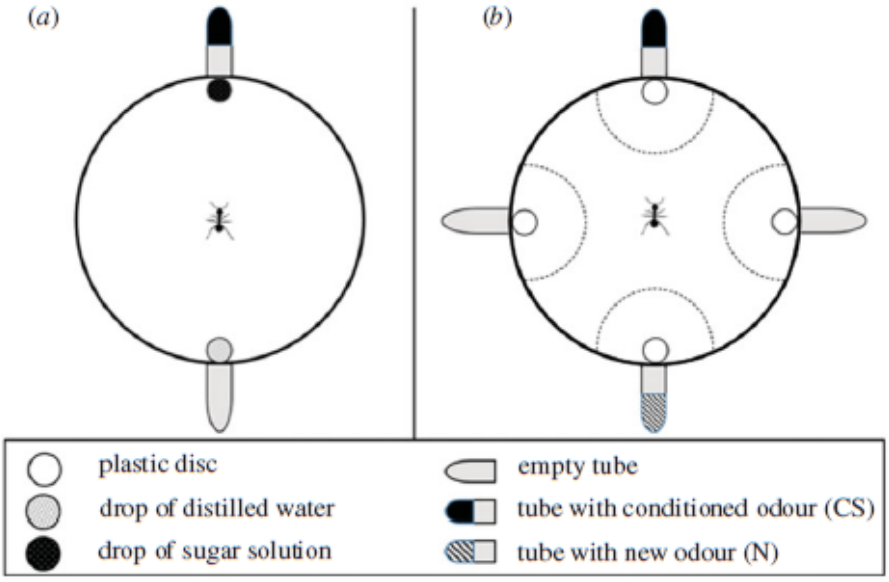

Figure 1. Schema of the experimental device (the arena). During conditioning, the set-up on the left was used (*a*). During memory test and extinction trials, the set-up on the right was used (*b*). The time spent by the ant in the areas around the discs was recorded during 2 min. Each area measured 35.5 cm^2 (dashed lines). The orientation of the arena in the experimental room was changed between trials, so that ants could not learn spatial cues.

colonies were collected in the forest of Ermenonville (France, 49°09′51.5″ N, 2°36′49.2″ E) in September 2013 ($n = 5$) and 2017 ($n = 4$) and kept under laboratory conditions (25 ± 2°C, 50 ± 10% relative humidity, 12 h/12 h: day/night). Tested ants were foragers and were coloured on the abdomen or thorax using oil-based paint. Each ant was used only once in the conditioning and testing procedure.

2.2. Odorant stimuli

Hexanal and 1-octanol (Sigma Aldrich, respectively, USA and Germany, purity greater than 99%) were used as conditioned stimuli. These compounds are found in floral emissions [31] and therefore may be ecologically relevant for *F. fusca* ants who feed on extra-floral nectar. Ants did not show a spontaneous preference for any odorant (details in electronic supplementary material).

2.3. Experimental protocol

2.3.1. Acquisition

Our protocol is a modified version of that used by Bos *et al.* [21] to study perceptual similarity among cuticular hydrocarbons. In absolute olfactory conditioning, a single initially neutral odorant (CS) is associated with a reward (US). For the conditioning trials, the ant was placed in the centre of a circular arena (Ø = 12 cm, height = 3.5 cm) with clean filter paper at the bottom. The arena had two holes in the wall facing each other. Eppendorf tubes were inserted into the holes with their openings towards the centre of the arena. The tube presenting the CS contained a piece of filter paper (1 cm^2) soaked with 1 µl of hexanal or 1-octanol, while the other tube contained a clean piece of filter paper (this tube without odour represents the control for visual and tactile cues). On each side, a cotton plug allowed passive diffusion of the odorant when present but prevented the ants from entering in direct contact with the filter papers. Small plastic discs (Ø = 6 mm) placed at 1 cm in front of each tube received 1 µl of sugar solution (30% w/w) on the CS side and 1 µl of distilled water on the other side (figure 1*a*). Due to the presence of these two drops of liquid, the two stimulus sides were visually indistinguishable. The time needed by the ant to find the sugar solution (US) was recorded during each conditioning trial. The ant was allowed to drink the drop of sugar solution and was then returned to the colony, where the ant could perform trophallaxis. During the trophallaxis, ants exchange the sugar solution drunk during the conditioning trial with nest-mates. Without trophallaxis, the crop of tested ants would be full in only a few conditioning trials and the ants would not be motivated to find more food. Trophallaxis thus ensures high and stable motivation for the tested ants. Tested ants were left for about 3 min in the colony (inter-trial interval, 186 ± 18 s, mean ±

standard deviation), during which they terminated trophallaxis and came back to the foraging arena. During this interval, the filter paper at the bottom of the arena and the plastic discs were replaced with clean ones to avoid the use of any possible chemical cue left by the ant. The orientation of the arena and the position of the experimenter were also modified between trials to limit the possible use of visual or other spatial cues. Three independent groups of ants were subjected to one, three or six conditioning trials (electronic supplementary material, figure S1).

2.3.2 Memory tests

For the unrewarded memory tests, we used an arena with four holes in the wall (instead of two), which were connected to four Eppendorf tubes. Four empty plastic discs were placed in front of them. There were two tubes with odorants (the CS and the novel odour, N, never encountered during conditioning) and two tubes without odorants (figure 1*b*). For ants conditioned with hexanal, 1-octanol was used as N, and conversely, for ants conditioned to 1-octanol, hexanal was N. A circular area was drawn around each tube which allowed us to record the time spent by the ant in the vicinity of each odorant for 2 min with the software Ethoc (v. 1.2, CRCA, Toulouse, France). Independent groups of ants were subjected to the memory test 1, 24, 72 or 168 h after conditioning. Each ant underwent only one memory test.

2.3.3. Extinction

The extinction protocol was performed with the same arena as the memory test and started 1 h after the end of the conditioning. Each extinction trial was similar to the unrewarded memory test: the ant had the choice between CS and N for 2 min. All ants underwent at least six extinction trials. In the case where we tested the resistance to extinction after one conditioning trial, a subgroup of ants underwent six additional extinction trials, for a total of 12 extinction trials. Between two trials, each ant was returned to the colony for 3 min (inter-trial interval). During this time, the filter paper and plastics discs of the arena were replaced with new ones. Spontaneous recovery was tested 24 h after the end of the extinction protocol, by performing a last unrewarded test [32]. After this test, we offered each ant a drop of sugar solution to check whether they were motivated to feed. All ants ($N = 88$) but one drank the sugar solution. The one that did not was discarded from the analyses.

2.3.4. Pharmacological treatment

Memory is divided in different categories depending on its duration and the molecular cascades it involves. For example, a long-lasting memory involving de novo protein synthesis will be qualified as LTM [11,33]. To test if ants' olfactory memory depends on protein synthesis, additional groups of ants were given cycloheximide, a protein synthesis (translation) inhibitor (CHX, Sigma Aldrich, USA, purity greater than 99%). To prevent the drug from spreading in the colony, we created subcolonies consisting of groups of 40 ants in a nest-box with five or six larvae. A maximum of 10 of these 40 ants were tested. Each experimental ant was individually confined in a small cylinder placed inside the subcolony and received either 1 µl of sugar solution (30% w/w) containing 1 µg of CHX (treatment) or 1 µl of sugar solution (control), similarly to Guerrieri *et al.* [22]. After 2 h, the ant was released, allowing interaction with nest-mates. One hour later, therefore 3 h after receiving the treatment, the experimental ant was subjected to one conditioning trial and was then placed back into its subcolony until the memory test. This memory test was performed either 1 or 72 h after the end of conditioning. We verified that CHX did not affect ants' health (electronic supplementary material, table S4).

In total, for all experiments, 496 individual ants were conditioned, of which 467 (94%) underwent a memory test or an extinction protocol. Twenty-nine ants were excluded because they took too long to find the sugar solution during conditioning (more than 10 min for the first trial or more than 2 min for the following ones), they did not drink the CHX or control solution, or they died between conditioning and test procedures.

2.4. Statistics

Data were analysed using R software (v. 3.5.2, R Core Team, 2018 [34]). Significance was fixed at $\alpha = 5\%$. All data were analysed using linear mixed models (LMM, package 'lme4', [35]), details in electronic

supplementary material. Post hoc differences were observed by using LMMs with reduced dataset and the α-level was adjusted using the Holm–Bonferroni correction [36].

2.4.1. Acquisition

We analysed the effects of two independent (predictor) variables: *conditioning odorant* (factor with two levels, hexanal or 1-octanol) and the number of conditioning trials (continuous variable up to 6, named *trials*) on the dependent variable *time* (continuous variable, the time before finding the reward). We looked at the interaction *conditioning odorant* × *trials* to detect possible differences in ants' responses depending on the odorant used.

2.4.2. Memory tests

We analysed the effects of four independent variables: *stimulus* (factor with two levels, CS and N), the time elapsed since conditioning (factor with four levels, 1, 24, 72 and 168 h, called *elapsed time*), the number of conditioning trials (factor with two levels, one or six conditioning trials, called *conditioning groups*) and the *conditioning odorant* on the dependent variable *time* (continuous variable, the time spent in the vicinity of a stimulus). We used post hoc tests to see whether the time spent in the CS or N area varies according to the time elapsed since conditioning. We tested whether ants spent more time in the CS or N area in function of the elapsed time since the conditioning.

For the memory tests performed in the pharmacological experiment (CHX), we tested if ants spent more time in the CS or N area 1 or 72 h after the conditioning trial as a function of treatment (CHX or control).

2.4.3. Extinction

We analysed the effects of four independent variables: *stimulus*, the number of conditioning trials (factor with 3 levels, 1, 3 or 6 conditioning trials, called *conditioning groups*), the number of extinction trials (continuous variable from 1 to 12, called *extinction trials*) and the *conditioning odorant* on the dependent variable *time* (continuous variable, the time spent in the vicinity of a stimulus). At each extinction (or spontaneous recovery) trial, we tested if ants spent more time in the CS or in the N area.

3. Results

3.1. Acquisition

During the acquisition phase, the time spent by ants to find the reward decreased significantly over trials (LMM: $F = 72.45$, d.f. $= 5$, $p < 0.001$) (electronic supplementary material, figure S2). The observed decrease in the time to find the reward suggests that ants have associated the odorant (CS) with the reward (US). To validate that learning occurred, ants underwent memory tests without reward.

3.2. Memory tests

The number of conditioning trials (one or six) did not influence the performance (time spent near the CS) of the ants in the memory tests, as indicated by the non-significant interaction *stimulus* × *conditioning groups* ($F = 0.52$, d.f. $= 1$, $p > 0.05$). However, the time elapsed since conditioning had a significant effect on ants' behaviour (figure 2), as indicated by the significant interaction *stimulus* × *elapsed time* ($F = 7.89$, d.f. $= 3$, $p < 0.001$). Post hoc tests showed that the time spent in the stimuli areas was not significantly different when memory tests were performed 1 or 24 h after conditioning (*stimulus* × *elapsed time*, $p > 0.1$). A tendency was observed between 72 and 168 h ($p = 0.069$): ants performed better, i.e. spent more time in the CS area, at 72 h than at 168 h.

Instead, there was a significant difference in the ants' performance between 1 and 72 h, with ants performing better at 1 h than at 72 h (*stimulus* × *elapsed time*, $p < 0.05$), between 24 h and 72 h (better performance at 24 h, $p < 0.05$), between 1 and 168 h (better performance at 1 h, $p < 0.001$) and between 24 and 168 h (better performance at 24 h, $p < 0.001$).

Finally, we observed that ants spent significantly more time in the CS than in the N area for the memory tests performed at 1, 24 and 72 h (for the three cases, $65.6 > F > 13.1$, d.f. $= 1$, $p < 0.001$), while no significant difference was found at 168 h ($F = 0.49$, d.f. $= 1$, $p > 0.1$, figure 2), indicating that

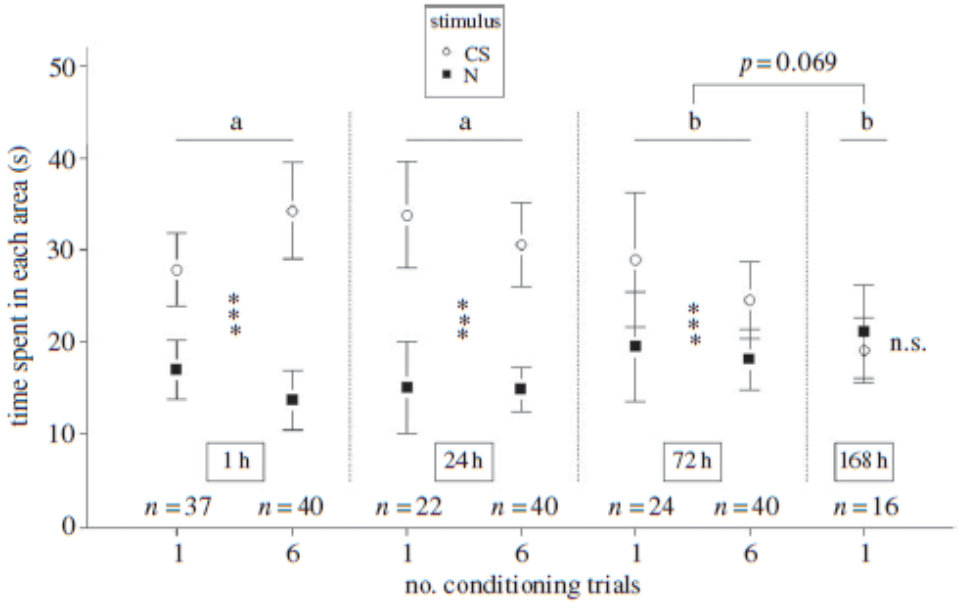

Figure 2. Time spent by individual ants in each area during the memory tests (white circle: CS area; black square: N area). Memory tests were performed 1, 24, 72 or 168 h after the end of the conditioning. Ants underwent one or six conditioning trials. Circles and squares represent the mean and error bars are confidence intervals (95%). Significant differences within a group are noted with asterisks (***$p < 0.001$; n.s.: $p \geq 0.05$). Significant differences between groups (1, 24, 72 and 168 h) are noted with letters.

the ants do not prefer the CS to the N anymore. These results show that ants are capable of forming an appetitive associative memory that lasts for at least 3 days even after a single conditioning trial.

3.3. Pharmacological treatment

After a single conditioning trial, the olfactory memory of *F. fusca* appears to be very strong. We hypothesized that, even with such short training, ants built a LTM. To investigate this, we tested the susceptibility of this single-trial memory to a protein synthesis inhibitor (CHX). During these memory tests, control ants (sham-treated) spent more time in the CS area than in the N area, no matter whether the test was performed 1 h ($F = 4.16$, d.f. $= 1$, $p < 0.05$) or 72 h after conditioning ($F = 6.03$, d.f. $= 1$, $p < 0.05$), thus showing intact memory retention after 3 days. CHX-treated ants, however, displayed a preference for the CS area at 1 h ($F = 8.62$, d.f. $= 1$, $p < 0.01$) but not at 72 h after conditioning ($F = 0.02$, d.f. $= 1$, $p > 0.05$) (figure 3). This show that ants establish an appetitive olfactory LTM [37] after a single conditioning trial.

3.4. Extinction

3.4.1. After six conditioning trials

Ants did not show any significant extinction in the course of the six-test procedure (figure 4*a*). Indeed, the ants' performance was stable over time, as indicated by the non-significant *stimulus* × *extinction trials* interaction ($F = 1.40$, d.f. $= 5$, $p > 0.05$). When comparing the ant performance in the last extinction trial and in the spontaneous recovery test, no significant difference was found ($F = 0.10$, d.f. $= 1$, $p > 0.05$) (figure 4*a*). In all the extinction trials and in the spontaneous recovery test, ants spent more time in the CS than in the N area ($142.7 > F > 50.54$, d.f. $= 1$, $p < 0.001$). Therefore, with six conditioning trials, ants showed high resistance to extinction.

3.4.2. After three conditioning trials

Given that extinction did not occur after six conditioning trials, we decided to subject ants to only three conditioning trials before the six extinction trials. Again, we found that the *stimulus* × *extinction trials* interaction was non-significant, indicating that ants' performance was stable during the extinction procedure ($F = 2.14$, d.f. $= 5$, $p > 0.05$) (figure 4*b*). When comparing the last extinction trial with the spontaneous recovery test, we did not find any significant difference ($F = 0.01$, d.f. $= 1$, $p > 0.05$). In all the extinction trials and in the spontaneous recovery test, ants spent more time in the CS than in the N area ($71.7 > F > 15.15$, d.f. $= 1$, $p < 0.001$). Here, again, ants show very high resistance to extinction.

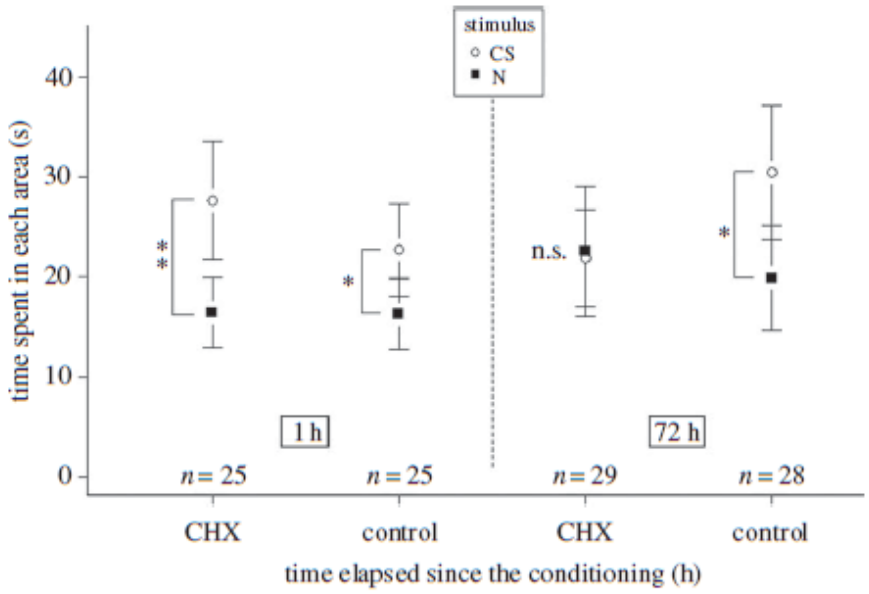

Figure 3. Time spent by individual ants in each area during memory tests. Ants received a drop of sugar solution 3 h before a single conditioning trial (control). In the experimental group (CHX), ants received 1 µg of CHX with the sugar solution. Memory tests were performed 1 h (left) or 72 h (right) after conditioning. Significant differences within a group are noted with asterisks (**$p < 0.01$; *$p < 0.05$; n.s.: $p \geq 0.05$). Circles and squares represent the mean and error bars are confidence intervals (95%).

3.4.3. After one conditioning trial and up to 12 extinction trials

Even with three conditioning trials, no extinction was found after six extinction trials. We then further reduced training strength and subjected the ants to a single conditioning trial before the extinction procedure. If we look at the first six extinction trials, the ants' performance did not decrease along the trials (figure 4*c*) as indicated by the non-significant interaction *stimulus* × *extinction trials* ($F = 0.67$, d.f. $= 5$, $p > 0.1$). For these ants, which underwent one conditioning trial and six extinction trials, there is no significant difference between the sixth extinction trial and the test of spontaneous recovery ($F = 0.17$, d.f. $= 1$, $p > 0.1$). For the first six trials and the test of spontaneous recovery, ants spent significantly more time in the CS than in the N area ($28.38 > F > 12.62$, d.f. $= 1$, $p < 0.001$) (figure 4*c*). Some of the ants that received only one conditioning trial underwent six additional extinction trials. Overall, the ants' performances (i.e. the time spent near the CS) decreased along the 12 tests, indicating extinction (figure 4*c*), as shown by the significant *stimulus* × *extinction trials* interaction ($F = 1.83$, d.f. $= 11$, $p < 0.05$). To detect at which trial extinction started, we tested if ants spent more time in the CS or N area during the last six extinction trials. For the seventh trial, a tendency is observed ($F = 3.69$, d.f. $= 1$, $p = 0.063$), for the eighth trial, ants spent more time in the CS area ($F = 8.74$, d.f. $= 1$, $p < 0.01$) but at the 9th, 10th and 11th trials, ants did not show any significant preference for one of the stimuli ($0.88 > F > 0.001$, d.f. $= 1$, $p > 0.1$). For the last extinction trial, a tendency was observed ($F = 4.14$, d.f. $= 1$, $p = 0.051$). Finally, no significant difference was found between ants' performances in the 12th extinction trial and in the spontaneous recovery test ($F = 0.75$, d.f. $= 1$, $p > 0.05$). During this spontaneous recovery test, ants did not spend more time in the CS area compared to the N area ($F = 0.26$, d.f. $= 1$, $p > 0.1$), indicating that spontaneous recovery did not occur within 24 h.

4. Discussion

By using a simple conditioning paradigm, our study shows that *F. fusca* ants are very efficient in individual olfactory learning, and reveals unconventional characteristics in their learning ability. These ants learn an odour-reward association very quickly (within a single trial) and thereby build a highly stable memory form (genuine LTM, dependent on protein synthesis), which was undescribed in ants. Moreover, the established odour-reward association is highly resistant to contradictory information, being subject to extinction only after many unrewarded trials.

4.1. Formation of a long-term memory after one conditioning trial

During conditioning with three and six conditioning trials, ants showed fast acquisition: the time to find the reward rapidly decreased after the first conditioning trial, suggesting that memory is already formed after a

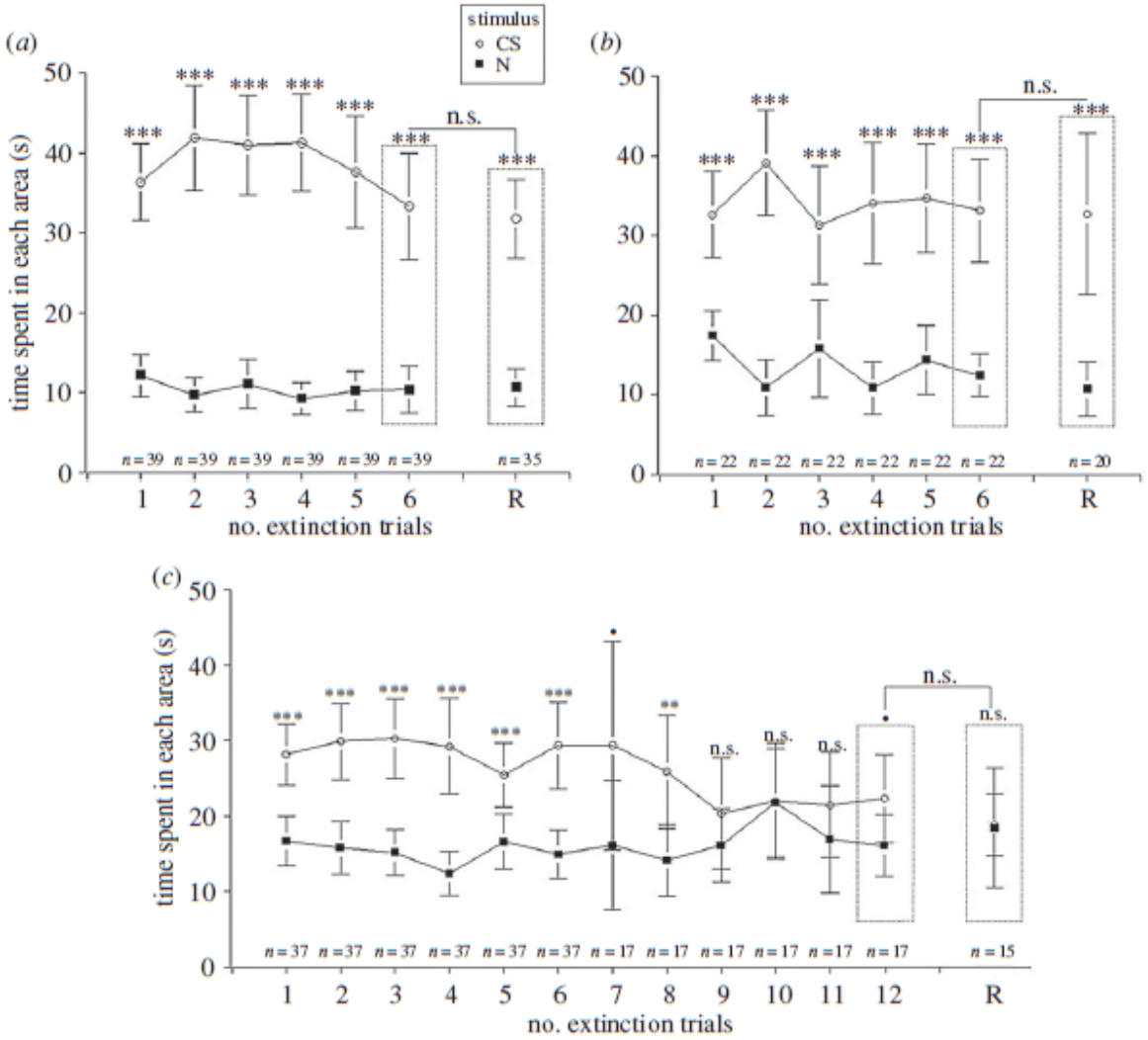

Figure 4. Time spent by ants in the CS and the N area during an extinction protocol that began 1 h after the last conditioning trial. *Y*-axis: time spent in the CS (white circles) and the N area (black squares). *X*-axis: number of extinction trials (6–12). The test for spontaneous recovery (24 h after the last extinction trial) is represented by the letter 'R'. Different panels represent different groups of ants: (*a*) ants that underwent six conditioning trials and six extinction trials ($n = 39$), (*b*) ants with three conditioning trials and six extinction trials ($n = 22$) (*c*) and ants with one conditioning trial and six ($n = 20$) and 12 extinction trials ($n = 17$). Significant differences within extinction trials or between the last extinction trial and the test of spontaneous recovery are noted with asterisks (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n.s.: $p \geq 0.05$). Circles and squares represent the mean and error bars are confidence intervals (95%).

single conditioning trial. When ants that underwent one or six conditioning trials were tested with an unrewarded memory test (1, 24 or 72 h after conditioning), no significant differences in ants' performances were found between the one conditioning trial group and the six conditioning trials group. Typically, in insects as diverse as fruit flies, crickets and honeybees, a single conditioning trial results in the formation of a short-term memory (lasting 1–24 h), whereas several conditioning trials are needed to form an LTM (lasting more than 24 h) [33,38,39]. In ants, a recent study showed that one training trial is sufficient for olfactory learning, but if this leads to LTM was not known [23]. To test whether the memory formed after a single conditioning trial is genuine LTM, we treated ants with a protein synthesis inhibitor (cycloheximide) before conditioning and we observed that, 72 h later, treated ants could not retrieve any memory. This confirms that the memory formed by *F. fusca* ants after a single conditioning trial is true LTM [11,22,40]. The formation of an LTM after one single appetitive conditioning trial is not common in insects, and has been found until now only in the fruit fly, *Drosophila melanogaster* [11,41].

To investigate the limit of this memory, we tested ants one week after this one single conditioning trial and no trace of memory was found. We did not perform this test at one week for ants that underwent six conditioning trials, but we assume that they would behave similarly since their performances were not different from ants that underwent one conditioning trial when tested after 1, 24 and 72 h.

Our study is original in that it shows both single-trial olfactory learning and the formation of a highly stable memory form after this single learning. Single-trial visual learning has been shown in individual foragers of desert ants, for example, *Melophorus bagoti*, but it is unclear whether this short training leads

to LTM [17]. In the case of olfactory learning, previous studies found that ants can learn rapidly or retain memories for a long time, but both abilities were rarely found together. Moreover, these studies were performed at the colony level (not at the individual level) and/or involved very young individuals in an imprinting context. Workers of the desert ant *Cataglyphis fortis* can collectively learn to associate one odorant with food after one trial, and about half of the ants remember this association for up to 26 days afterwards [42]. Leaf-cutting ants, *Atta colombica*, feed their symbiotic fungus with freshly collected leaves. Field colonies learn to avoid plants that are dangerous for their fungus (e.g. experimentally treated with fungicide) and show robust memory for plant unsuitability lasting up to 18 weeks [43].

Young workers of *Formica polyctena* that were reared just after emergence with cocoons of an alien species for 15 days will take care of cocoons of this species when encountering them six months later, while they will eat conspecific cocoons that they never encountered [44]. This is an imprinting-like phenomenon occurring during a critical period after emergence and that has been shown in ants several times (review in [45]). Imprinting-like phenomena also occur with environmental odorants. If young ants are reared from eclosion in a nest with a specific plant odorant (i.e. thyme), and are then kept in a nest without odorant, they will prefer a nest with the odour experienced during their young age when given the choice [46]. Young individuals can possibly form long-term olfactory memories that persist for weeks or even months. Indeed, high retention abilities were found when training cricket nymphs to discriminate an odorant associated with water or saline solution [47], and in very young mice trained to associate an odorant with milk [48], especially after a period of deprivation from these resources. In the present case, we documented ants' adult learning abilities at the individual level and show that they build long-lasting olfactory memories within a single rewarded trial.

4.2. *Formica fusca* ants are particularly resistant to extinction

The second part of our study involved testing resistance to extinction. Based on the social insect literature, we expected to observe a rapid decrease in performances along extinction trials. In honeybees, using the proboscis extension response conditioning paradigm, 80% of the bees show a conditioned response after six conditioning trials. After two extinction trials, 70% of these bees display the conditioned response, and after five extinction trials, 60% still respond. However, with a single conditioning trial, only 10.7% of bees show the conditioned response after five extinction trials [15]. In *Myrmica* ants tested at the colony level, two extinction trials are needed to extinguish an olfactory association established with a 12 conditioning trials procedure [49]. In our first extinction protocol, with ants that underwent six conditioning trials and six unrewarded extinction trials, no extinction was observed. This result in itself is surprising and was replicated with ants that underwent three or only one single conditioning trial. We could observe extinction only in ants that underwent a single conditioning trial and more than six extinction trials, demonstrating an exceptionally high resistance to extinction in these ants. During extinction, the memory formed after conditioning (CS–US association) is not erased, but a new learning usually takes place (CS–no US association) [32]. The behaviour of the individual will reflect the relative strength of each memory. With more extinction trials, the CS–no US association will become stronger than the CS–US memory and individuals will stop responding to the CS. As the CS–US memory is not erased, if we test the behaviour of an individual at a later time after the end of the extinction protocol, positive response to the CS may increase again. This phenomenon, called spontaneous recovery [50], usually corresponds to a decay of the CS–no US memory. Here, we did not observe any spontaneous recovery when extinction took place (i.e. one conditioning trial and 12 extinction trials). This absence of spontaneous recovery could be due to the time elapsed between the end of the extinction trials and spontaneous recovery test, which may have been too short for the decay of the CS–no US association. In honeybees, spontaneous recovery usually appears within a few hours (1 h for [18], 35 min for [51]). In any case, the lack of spontaneous recovery in these ants confirms the stability of the associations they form: just like their CS–US association is highly resistant to time and extinction, their CS–no US association also appears to be highly resistant to time.

Why do ants show such fast learning and high resistance to extinction? In ants, as in other social insects, individuals are usually specialized in a particular task according to their age. Young workers will avoid threats, stay in the nest and take care of the brood, whereas old workers will go out foraging, and therefore be exposed to biotic and abiotic threats [52]. In undisturbed natural colonies, as workers keep the same job for weeks or even months, it is not relevant to learn rapidly and to be resistant to extinction. However, if a category of workers suddenly decreases in number (e.g. due to predation or raids from slave-making ants, of which *F. fusca* is a common host species [53]), task

switching may occur and workers with the ability to learn quickly will be very advantageous for the colony. Being resistant to extinction is an advantage when the environment is extremely stochastic and workers need to switch often from a current task to another, previously learned, task. In *F. fusca*, there is no clear specialization of individuals working outside the nest, which may engage in different tasks such as foraging, scouting and guarding [54]. An ant could act as forager one day, guard another day and then forager again. In this scenario, learning quickly and building strong memories of a previously learned task (i.e. being resistant to extinction) is advantageous because it allows ants to exploit optimally their environment without spending time to learn again.

After documenting the unconventional olfactory learning and memory abilities of *F. fusca*, we are left wondering if this species is really exceptional among ants, and more generally among social insects. While visual learning is well documented in ants, especially in the context of navigation [18], data on individual olfactory learning and memory abilities in ants are relatively scarce and future comparative studies taking into account the ant phylogeny may be useful to provide answers to this question. Furthermore, given that the insect brain shares many similarities in its architecture with the vertebrate brain [55], a better understanding of the neural mechanisms underlying such a stabilized memory and resistance to extinction might help improving treatment of maladaptive behaviours.

Data accessibility. Original data are accessible in electronic supplementary material, together with supplementary methods and results, tables S1–S5 and figures S1 and S2.

Authors' contributions. B.P., P.d.E. and J.-C.S. conceived the project and designed the experiments. B.P. performed the experiments and analysed the data with the help of P.d.E. and J.-C.S. The manuscript was written by B.P. and revised by P.d.E. and J.-C.S. Final manuscript was approved by all authors.

Competing interests. We declare we have no competing interests.

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Title: Olfactory detection of human cancers by ants

Abstract

Cancers are the second most common death cause in Humans, with up to 10 million deaths in 2020. The sooner is it diagnosed, the better the survival is for patients. Currently, the classical methods of diagnostics are either invasive (e.g. mammography) or costly (e.g. RMI), but alternatives methods, based on the learning of characteristics odours of cancers are potential, as non-invasive, rapid and inexpensive. In this context, this thesis investigated the possibility of using the discriminative olfactory abilities of an ant species, Formica fusca, to detect cancers. We first characterized the olfactory associative learning in this species and demonstrated that these ants were able to learn fast, and that the related memory was robust. Then, by using human cancer cell lines, we observed that ants were able to differentiate healthy cells from cancerous ones, as to differentiate a cancer cell line from another. Finally, we used urine from mice grafted with human tumours as a source of odours for the learning. Ants were able to differentiate the urine of healthy mice from cancer mice, but not the other way, which we attributed to the cancer samples being less salient than the healthy ones. The utilization of chemistry analytic tools (GC-MS and SPME) confirmed this hypothesis and helps us established a list of potential cancer biomarkers. Compared to the canine olfaction, the gold standard in this domain, ants are faster, cheaper, and as efficient as dogs. Improvement can be proposed, but this first study highlighted the promise of using ants for early human cancer detection.

Discipline: Ethologie

Keywords: Early detection, human cancers, olfaction, conditioning, GC-MS, SPME, *Formica fusca*

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Résumé

Les cancers sont la seconde cause de décès chez l'Homme avec presque 10 millions de morts en 2020. Plus tôt cette maladie est détectée et diagnostiquée, meilleures sont les chances de survie du patient. Actuellement, les méthodes de dépistage classiques sont invasives (ex : mammographie) et/ou coûteuses (ex : IRM), mais des méthodes alternatives, basées sur l'apprentissage d'odeurs caractéristiques des cancers, sont prometteuses car non-invasives, rapides et peu onéreuses. Dans ce contexte, cette thèse s'est intéressée à la possibilité d'utiliser les capacités olfactives de discrimination d'une espèce de fourmi, *Formica fusca,* pour détecter les cancers. Nous avons tout d'abord caractérisé l'apprentissage olfactif associatif chez cette espèce, et démontré que ces fourmis apprenaient rapidement et que la mémoire formée était robuste. Par la suite, en utilisant des lignées cellulaires cancéreuses humaines, nous avons observé que les fourmis pouvaient différencier des cellules saines de cellules cancéreuses, ainsi que deux lignées cancéreuses entre elles. Finalement, nous avons utilisé de l'urine de souris greffées avec des tumeurs humaines comme source d'odeur pour l'apprentissage. Les fourmis se sont montrées capables de différencier l'urine des souris saines de celle des souris malades, mais pas l'inverse, ce que nous attribuons à la saillance plus faible des échantillons cancéreux. L'utilisation d'outils d'analyse chimique (SPME et GC-MS) ont permis de confirmer cette hypothèse et d'établir une liste de biomarqueurs potentiels des cancers. En comparaison de l'odorat canin, qui est le maitre étalon dans ce domaine, les fourmis sont beaucoup plus rapides, moins chères, et tout aussi efficaces. Même si des améliorations peuvent être proposées, cette première étude a mis en lumière le potentiel de l'utilisation des fourmis pour la détection précoce des cancers.

Discipline : Éthologie

Mots-clefs : Détection précoce, cancers humains, olfaction, conditionnement, GC-MS, SPME, *Formica fusca*

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