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Cold vs. CO_2: anaesthetic effects on insect antennal functionality

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Abstract

Anaesthesia methods play a crucial role in ensuring the integrity of the animal during experimental studies. This study investigates the impact of two anaesthesia methods, CO_2 and cold treatment, on an insect antennal response to synthetic alarm pheromone compounds. Adult worker hornets were anesthetised, and their antennae excised and tested using an electroantennography set-up with controlled stimulation of alarm pheromone components. Results showed that CO_2-anesthetised hornets exhibited robust antennal responses, while cold-anesthetised individuals displayed none. This result suggests that freezing may impair the functionality of olfactory receptors. In contrast, CO_2 anaesthesia preserves receptor integrity, offering reliable and interpretable results. This study highlights the importance of selecting appropriate anaesthesia techniques to avoid artefacts in insect sensory physiology research and underscores the ecological relevance of studying *Vespa velutina nigrithorax* alarm signalling.

Introduction

Electroantennography (EAG) is a widely used technique to investigate the olfactory detection of arthropods to volatile organic compounds (Cork *et al.*, 1990; Piersanti *et al.*, 2024). However, the method of anaesthesia prior to antennal dissection can influence the integrity and functionality of sensory receptors, potentially affecting the results of such studies.

Cold anaesthesia, being a very economical and easy method, is commonly employed to temporarily immobilise invertebrates for experimental preparations, typically by placing the individual in a freezer until immobilisation occurs (Frost *et al.*, 2011). However, while cold anaesthesia is convenient for handling single individuals, it is less practical for immobilising groups, like honey bees, as they may cluster to generate and retain heat (Stabentheiner *et al.*, 2010). Moreover, cold anaesthesia has been shown to negatively affect bees' behaviour and physiology, including impacts on short-term memory (Frost *et al.*, 2011), locomotion (Chen *et al.*, 2014), foraging (Poissonnier *et al.*, 2015; Wilson *et al.*, 2006), and defensive behaviour (Groening *et al.*, 2018). On the contrary, CO_2 exposure is a widely used method due to its simplicity and rapid immobilisation, making it ideal for experimental studies (Kohler *et al.*, 1999). However, it is known to alter behaviour due to hypoxia-induced stress, as highlighted in some old studies (Ribbands, 1950). It has also been shown to provoke changes in fecundity and longevity (Tasei, 1994) and in juvenile hormone titres (Bühler *et al.*, 1983). Diethyl ether, though less common, is effective for immobilising insects quickly, but its flammability and potential for adverse effects on both researchers and specimens make it less desirable for regular use (Arora and Gautam, 2025; Cooper, 2001). While more costly, recent studies proposed isoflurane and sevoflurane as alternatives (Gooley and Gooley, 2023). These volatile anaesthetics offer precise, reversible immobilisation with minimal impact on longevity and behaviour, making them particularly advantageous in experiments requiring repeated anaesthesia (Cooper, 2001, 2011; Rayl and Wratten, 2016).

Eusocial insects use chemical communication to coordinate complex behaviours such as foraging, defence, and reproduction (Yew and Chung, 2015). Among these chemical signals, alarm pheromones play a critical role in colony defence, triggering aggressive responses to perceived threats (Bruschini *et al.*, 2008).

In the Yellow-legged hornet, *Vespa velutina nigrithorax*, a species that has become an invasive pest in Europe (Darrouzet, 2024; Robinet *et al.*, 2019) and Asia (Choi *et al.*, 2012; Takeuchi *et al.*, 2017), understanding the function and detection of alarm pheromones is of particular ecological and applied interest (Berville *et al.*, 2023; Cheng *et al.*, 2017). This species has garnered increasing scientific interest as a study model due to its invasive nature and impact on native ecosystems and pollinator populations (Darrouzet, 2024). Its complex social behaviours (e.g., communication, foraging strategies, and colony organisation), along with its ecological and economic significance, make this species a valuable subject for research. The growing concern over its rapid spread and the threats it poses to biodiversity and apiculture

(Carisio *et al.*, 2022; O'Shea-Wheller *et al.*, 2023; Rojas-Nossa *et al.*, 2023) has amplified the need for laboratory and field studies to acclimate, maintain, and manipulate live hornets. For example, this need is important to analyse pheromone production and perception by females (Berville *et al.*, 2023). In laboratory settings, anaesthesia is essential when handling individual hornets, as their venomous stings pose a risk of injury to researchers. Cold and CO_2 narcosis are the most commonly used anaesthesia methods for hornets. While freezing is a common aesthetic in insect studies, its physiological effects on sensory perception are not fully investigated.

Consequently, prior to analysing the antennal responses of *V. velutina nigrithorax* to synthetic alarm pheromone components, in this study, we investigated which simple anaesthesia methods could be used, i.e. either CO_2 or freezing. By comparing the electroantennographic responses to key compounds, we aimed to assess the impact of anaesthesia method and to select the least detrimental one for this type of study.

Materials and methods

Insect sampling

Adult workers (foragers) of *V. velutina nigrithorax* were captured with a net at an apiary located within the experimental garden of the CEFE laboratory (Centre d'Écologie Fonctionnelle et Évolutive) in Montpellier (France). The individuals were housed separately in Faclon™ tubes (50 mL, 115 × 30 mm) before being tested. Experimental procedures were carried out at the CEFE from 25 November to 7 December 2024.

Aesthesia treatments

Hornets were anesthetised using either carbon dioxide (CO_2) exposure ($n = 14$) or cold treatment ($n = 11$). Individuals were placed under a CO_2 flow for a few seconds until they stopped moving, or in a freezer (−20°C) for 2 min. Following anaesthesia, hornets were handled with forceps and one antenna (randomised between individuals) of each hornet was excised with microscissors, and prepared for EAG analyses.

Stimulation solutions

We prepared dilutions (0.01, 0.1, and 1 μg/μL) in paraffin of four synthetic compounds present in the alarm pheromone. These included 2-nonanone (Aldrich, CAS: 821-55-6), 2-undecanone (Aldrich, CAS: 112-12-9), and the two enantiomers (R and S) of 4,8-dimethyl-7-nonen-2-one (provided by the Scyll'Agro company) (Berville *et al.*, 2023). Ten microliters of each dilution was applied to filter paper and inserted into a glass pipette connected to an airflow delivery system.

EAG system

One of the two antennae (randomised) of each hornet was excised, and the distal tips were inserted into two glass capillaries filled with Ringer's solution ($NaCl/KCl/CaCl_2/NaHCO_3$, Na^+ 131 mmol/L, K^+ 5 mmol/L, Cl^- 111 mmol/L, $C_3H_5O_3$ 29 mmol/L). These capillaries were subsequently mounted onto silver electrodes of an EAG set-up Kombi Probe PRG-3 (SYNTECH®, Kirchzarten, Germany).

The antenna was placed within a continuous flow of purified and humidified air directed through a tube at a rate of 435 mL/min for stimulation. The tip of an odour cartridge, constructed from a Pasteur pipette, was inserted into a small opening in the airflow tube. Odour stimulation was delivered by a 0.5-s pulse of purified air through the cartridge, with a flow rate of 890 mL/min controlled by a CS-55 Stimulus Controller (Syntech, Kirchzarten, Germany). Electrophysiological responses were recorded using GcEad 2014 v1.2.5 software (Syntech, Kirchzarten, Germany). Each antenna was tested with three stimulus sequences, where each sequence included all four selected compounds at specific doses along with paraffin controls. The doses were presented in ascending order (0.01, 0.1, 1 μg/μL) for each sequence, with compounds applied in a randomised order. Paraffin controls were included at the beginning and end of each sequence. The EAG response amplitude was calculated by subtracting the average response to the paraffin for each sequence.

Analysis

Depolarisation amplitudes were quantified by subtracting the response to the solvent control from the response elicited by each test compound. Statistical analyses were performed using R (version 4.3.1, R Core Team). We performed t-tests to compare our two conditions for each concentration and compounds. Graphical representations were obtained with *ggplot* package.

Results and discussion

A significant difference was observed in antennal responses of *V. velutina nigrithorax* to synthetic alarm pheromone compounds tested depending on the method of anaesthesia. Hornets anesthetised with CO_2 exhibited robust electroantennographic responses to all tested compounds, while those anesthetised by cold showed no detectable response in any of the concentrations (fig. 1, table 1).

These results suggest that cold anaesthesia, achieved by placing the individual hornet in a freezer, may probably impair the functionality of antennal olfactory receptors. Freezing and thawing are known to cause cellular damage, potentially affecting the delicate structures of the antennal sensilla and their associated neurons. Additionally, rapid cooling may disrupt ion channels and synaptic transmission, leading to a loss of olfactory signal transduction (McGann *et al.*, 1988).

In contrast, CO_2 anaesthesia gives interpretable results and appears to probably preserve the integrity of antennal receptors, allowing reliable detection of volatile compounds. This aligns with previous studies indicating that CO_2 has transient and reversible effects on insect physiology, minimising long-term impacts on sensory systems (Barie *et al.*, 2022; MacMillan *et al.*, 2017). For instance, exposure to CO_2 anaesthesia has been shown to increase chill coma recovery time in *Drosophila melanogaster*, but this effect diminishes after a recovery period in air, suggesting minimal long-term physiological disruption (Nilson *et al.*, 2006). Poissonnier *et al.* (2015) showed the bees were more active after a CO_2 treatment compared to control and cold-treated bees, which could be beneficial for behavioural experiments.

The absence of response in cold-anesthetised hornets raises concerns about the reliability of freezing as a method of immobilisation in studies involving sensory physiology. While freezing is a convenient and commonly used technique, our results suggest it may introduce significant artefacts, particularly in chemical communication and behavioural ecological studies. A study by Wilson *et al.* (2006) on honeybees suggested that the

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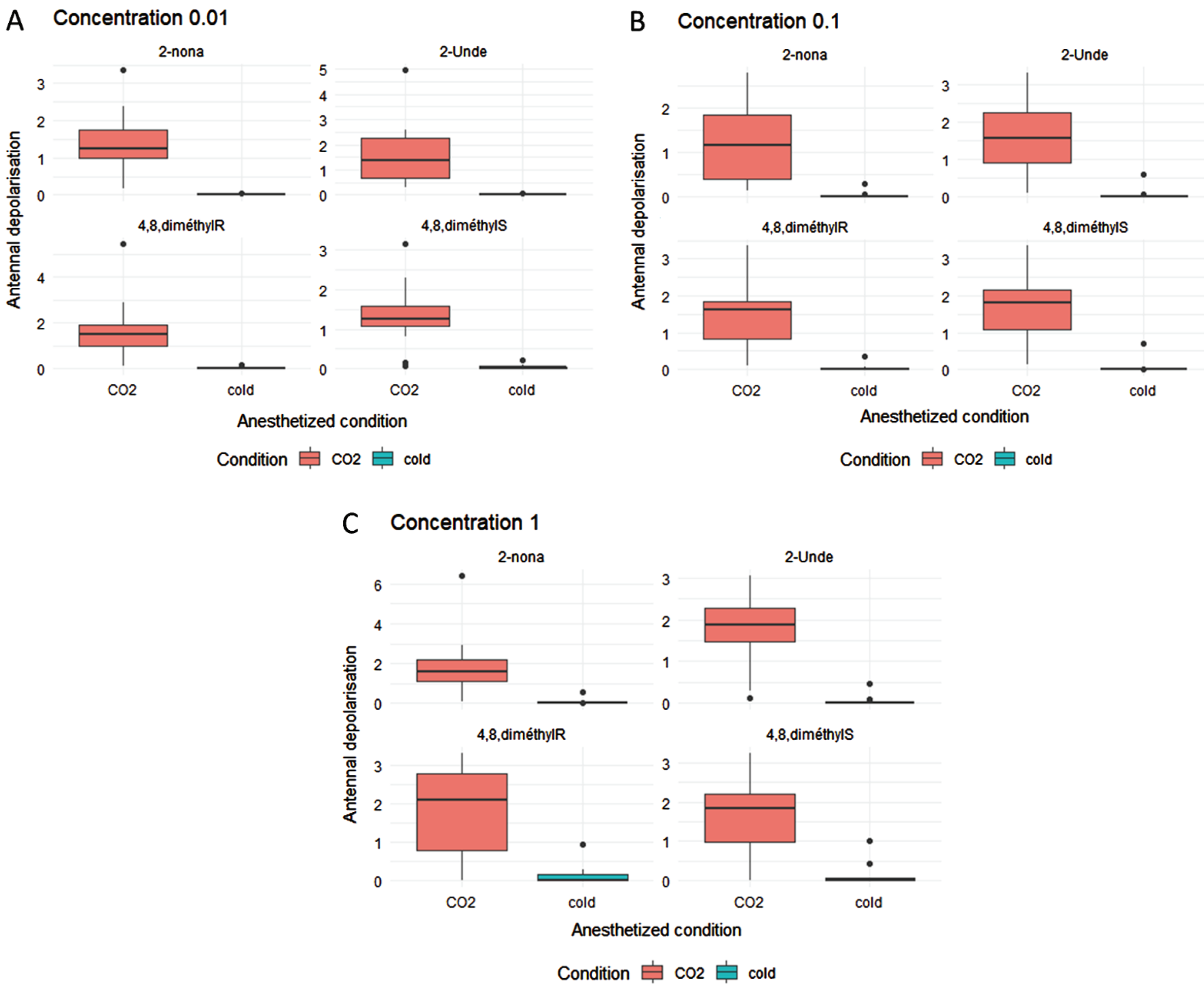

Figure 1. Comparison of the antennal depolarisation between cold- and CO_2-anesthetised hornets for different concentrations of the selected pheromone compounds. (A) Concentration of 0.01 μg/μL. (B) Concentration of 0.1 μg/μL. (C) Concentration of 1 μg/μL.

Table 1 *T*-test results of the antennal response between cold- and CO_2-anasthetised hornets for the different synthetic alarm pheromone compounds at different concentrations (0.01, 0.1, and 1 μg/μL)

μg/μL	4,8-Dimethyl-7-nonen-2-one (R)	4,8-Dimethyl-7-nonen-2-one (S)	2-Nonanone	2-Undecanone
0.01	$t = 4.98$, $p = 0.0002511$	$t = 6.02$, $p = 4.026e\text{-}05$	$t = 5.99$, $p = 4.525e\text{-}05$	$t = 4.8$, $p = 0.0003468$
0.1	$t = 5.78$, $p = 5.561e\text{-}05$	$t = 6.48$, $p = 1.158e\text{-}05$	$t = 5.07$, $p = 0.0001987$	$t = 5.86$, $p = 4.045e\text{-}05$
1	$t = 5.33$, $p = 8.36e\text{-}05$	$t = 5.08$, $p = 0.0001156$	$t = 4.54$, $p = 0.0005084$	$t = 6.72$, $p = 1.052e\text{-}05$

cold-induced decrease in foraging could be due to impaired cognitive or sensory receptor abilities and therefore the lack of detection and response to recruitment stimuli. With our results, we could imagine that antennal receptors could be damaged, leading to the non-detection of pheromones and other molecules needed in this kind of behaviours.

Future research should explore the cellular and molecular impacts of freezing on insect sensory organs and evaluate alternative methods of immobilisation. In addition, further work is needed to determine whether the observed effects are consistent across other insect species and sensory modalities. By optimising experimental methodologies, we can improve the accuracy and reproducibility of studies in neurophysiology and chemical communication in arthropods.

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Author contributions. Dissections, data collection, and statistical analyses were carried out by C.M. EAG analyses were performed by C.M. and implemented in the analytic instrument by B.L. Writing and editing were carried out by all co-authors. D.E. managed the project and obtained the funds. All authors read and approved the final manuscript.

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Competing interests. The authors declare no competing or financial interests.

Data availability. All relevant data and resources can be found within the article and its supplementary information.

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