THE ROLE OF INTENSITY, TEMPORAL SYNCHRONY, AND BIOGENIC AMINES FOR UNIMODAL AND MULTIMODAL INTEGRATION DURING LEARNING AND MEMORY OF HONEY BEES AND BUMBLE BEES

By

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A Dissertation Presented in Partial Fulfilment
of the Requirements for the Degree of

DOCTOR EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS

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Doctorado en Ciencias Biomédicas y Biológicas
Universidad del Rosario

Bogotá, Colombia

July, 2023

UNIVERSIDAD DEL ROSARIO, BOGOTÁ COLOMBIA

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This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text or the acknowledgments below. None of the work has been submitted for a degree, diploma, or dissertation at any other university.

Oswaldo Gil Guevara

AGRADECIMIENTOS

Primero que nada, quiero agradecer a mi amada esposa Solimar por su apoyo incondicional su amor infinito y comprensión sin barreras, a lo largo de años de ausencia, viajes, aventuras y sacrificios juntos y en también en soledad. Soli siempre me escuchó, aportó ideas y además me aconsejó. Ella incluso ha sostenido la familia en estos últimos años durante mis estudios doctorales. También quiero recordar a Orión por todo su amor y compañía durante tantas noches.

Quiero agradecer a mi director de tesis, Dr. Andre Riveros por darme la oportunidad de vincularme y trabajar en el campo del aprendizaje asociativo de las abejas. Sobre todo, agradezco su apoyo y confianza que me dieron la libertad de explorar los temas que culminan con mi tesis. No deja de asombrarme la belleza, complejidad y las oportunidades de investigación de las múltiples facultades cognitivas que las abejas poseen. Las discusiones científicas que sostuvimos fueron la base de esta tesis. Muchas gracias por ser un mentor.

Quisiera también agradecer al Dr. Marc Seid de la Universidad de Scranton, PA., US. por su apoyo y supervisión durante mi estancia de pasantía doctoral en su laboratorio durante los difíciles tiempos de la emergencia COVID. Agradezco mucho la colaboración, y el apoyo de Hernan A. Bernal del programa de Ingeniería de Biomédica, Medicina y Ciencias de la Salud de la Universidad del Rosario, durante el desarrollo del dispositivo electromecánico para la ejecución de los experimentos comportamentales. Agradezco mucho las enriquecedoras discusiones y aportes en la biofísica de la visión de insectos por parte de Juan Pablo Hernández. Al profesor Juan Posada, por su ayuda durante la calibración del montaje experimental. También agradezco a Lina García por mostrarme las generalidades del condicionamiento clásico en abejas.

Agradezco al profesor Rodulfo Ospina y a los miembros de su grupo de investigación en la Universidad Nacional de Colombia por permitir el acceso a las colmenas de abejas que ellos mantienen, a la profesora Marisol Amaya en el instituto de Ciencias de la Universidad Nacional de Colombia por abrir las puertas de su laboratorio, donde realicé los experimentos de uno de los capítulos de mi tesis. A Juan Carlos Hernández, por las discusiones sobre el comportamiento de forrajeo de las abejas que sostuvimos en el Instituto de Ciencias. Estoy muy agradecido con el profesor Germán Antonio Gutiérrez, del Departamento de Psicología de la Universidad Nacional de Colombia, por brindarme un espacio en los laboratorios de Neurociencia, durante el desarrollo de uno de los capítulos de mi tesis.

Mi gratitud con la Dra. Diana Galindo de la Pontificia Universidad Javeriana por nuestra amistad legendaria y por enseñarme el poder de los Modelos lineales generalizados mixtos. También agradezco al Dr. Wulfila Gronenberg del Departamento de Neurociencia de la Universidad de Arizona, y al Dr. Christopher Jernigan del Departamento de Neurobiología y comportamiento de la Universidad de Cornell por sus comentarios que mejoraron el manuscrito del primer capítulo. A María Fernanda González por su amistad, sus consejos, por las risas y todo su apoyo. A Olga y Julio Bedoya por su amistad y apoyo incondicional todos estos años. También agradezco el apoyo de mis compañeros y amigos Nicole Rueda, Carlos Vargas y Carolina Álvarez por ser un apoyo en tantos momentos difíciles a lo largo de estos años. Quisiera expresar mi más sincero agradecimiento a los miembros del jurado por su tiempo y valiosos comentarios sobre mi tesis.

Este trabajo de Tesis fue posible gracias a la financiación a través del Ministerio de Ciencia, Tecnología e Innovación de Colombia, mediante la Beca Colfuturo Doctorado Nacional 727. Estoy agradecido con The Animal Behavior Society por otorgarme el premio ABS Developing Nations Award 2020 . Agradezco al Dr. Riveros por brindarme acceso a su laboratorio y a la dirección de Investigación de la Universidad del

Rosario por su apoyo a través del Big Grant de Investigación. También agradezco a los fondos N.S.F. del Laboratorio del Dr. Marc Seid en la Universidad de Scranton, PA, USA, por proporcionarme acceso a sus instalaciones. Estos fondos no solo apoyaron la investigación, sino también la divulgación de resultados a través de la participación en congresos.

A mis profesores y amigos a lo largo años anteriores en la P. Universidad Javeriana, La Universidad de los Andes en Bogotá y en NJIT / Rutgers University NJ USA que han sido parte importante de mi formación, muchas gracias.

A mis padres Sonia y Raúl, por supuesto por su apoyo, eternamente agradecido.

ACKNOWLEDGEMENTS

First and foremost, I want to thank my beloved wife Solimar for her unconditional support, infinite love, and unwavering understanding throughout years of absence, travels, adventures, and sacrifices we have experienced together and in solitude. Soli always listened to me, contributed ideas, and advised me. She has even sustained our family during these past years while I pursued my doctoral studies. I also want to remember Orion for his unconditional love and countless sleepless nights together.

I want to express my gratitude to my thesis advisor, Dr. Andre Riveros, for giving me the opportunity to engage and work in the field of associative learning in bees. Above all, I appreciate his support and trust, which allowed me the freedom to explore the topics culminating in my thesis. The beauty, complexity, and research opportunities presented by the bees' cognitive faculties never cease to amaze me. Our scientific discussions formed the foundation of this thesis. Thank you very much for being a mentor.

I would also like to thank Dr. Marc Seid from the University of Scranton, PA, US, for his support and supervision during my doctoral internship in his laboratory during the challenging times of the COVID emergency. His logistical and academic support were crucial. I greatly appreciate the collaboration and selfless support from Hernan A. Bernal of the Biomedical Engineering, Medicine, and Health Sciences Program at Universidad del Rosario during the development of the electromechanical device for executing behavioural experiments. I am grateful for the enriching discussions and contributions on the biophysics of insect vision by Juan Pablo Hernández. To Professor Juan Posada, thank you for your support and assistance during the calibration of the experimental setup. I also want to thank Lina García for introducing me to the fundamentals of classical conditioning in bees.

I express my gratitude to Professor Rodulfo Ospina and the members of his research group at the Universidad Nacional de Colombia for granting access to the bee colonies they maintain. To Professor Marisol Amaya at the Institute of Sciences, Universidad Nacional de Colombia, thank you for opening the doors of your laboratory, where I conducted experiments for one of the chapters of my thesis. To Juan Carlos Hernández, thank you for the discussions on bee foraging behavior we had at the Institute of Sciences. I am very grateful to Professor Germán Antonio Gutiérrez from the Department of Psychology at Universidad Nacional de Colombia for providing me with a space in the Neuroscience laboratories during the development of one of the chapters of my thesis.

My gratitude goes to Diana Galindo of the Pontificia Universidad Javeriana, for our legendary friendship and for teaching me the power of Generalized Linear Mixed Models. I also want to thank Dr. Wulfila Gronenberg from the Department of Neuroscience at the University of Arizona, and Dr. Christopher Jernigan from the Department of Neurobiology and Behaviour at Cornell University for their comments that improved the manuscript of the first chapter. To Maria Fernanda Gonzalez for her friendship, her advice, the laughs and all her support. To Olga and Julio Bedoya for their friendship and unconditional support all these years I also appreciate the support of my PhD colleagues Nicole Rueda and Carlos Vargas and Carolina Alvarez. I would like to express my sincere gratitude to the members of the jury for their valuable time and insightful feedback on my dissertation.

This thesis work was made possible thanks to funding from the Ministry of Science, Technology, and Innovation of Colombia and through the Colfuturo Doctorado Nacional 727 Scholarship. I am grateful to The Animal Behavior Society for granting me the ABS Developing Nations Award 2020. I am grateful to Dr. Riveros for providing me access to his laboratory, and to the Research Department of Universidad del Rosario for their support through the Big Research Grant. I also appreciate the NSF funds from Dr. Marc Seid's Laboratory at the University of Scranton, PA, USA, for granting me access to their

facilities. These funds not only supported the research but also facilitated the dissemination of results through conference participation.

To my professors and friends throughout the previous years at P. Universidad Javeriana, Universidad de Los Andes in Bogotá and at NJIT/Rutgers University in NJ, USA, who have been a significant part of my education, thank you very much.

And, of course, to my parents, Sonia y Raúl, thank you for your support. I am forever grateful.

“The only true voyage… would be not to visit strange lands but to possess other eyes … to see the hundred universes that each of them sees”.

-Marcel Proust

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GENERAL ABSTRACT

Multisensory integration is a fundamental aspect of learning and memory across animals and is particularly relevant during ecological tasks such as foraging and pollination. This study aimed to investigate the influence of external physical properties of unimodal elements of a composed signal such as intensity and the temporal relationships (synchronicity and order) on the multimodal integration of olfactory and visual signals in honey bees. Furthermore, this study targeted the exploration of the impact of biogenic amines on unimodal and multimodal learning and memory processes in bumble bees, recognizing the crucial role of the inner neuromodulatory environment in the formation of rewarding associations. Through an electromechanical setup, bees were trained using precisely controlled intensity levels and temporal relationships (sync /out of sync /alternate orders presentation) of unimodal and bimodal stimuli. The Proboscis Extension Response (PER) conditioning protocol was employed as a measure of reward learning. To manipulate the neuromodulatory environment of bumble bees, oral administration of biogenic amines, octopamine (OA) and dopamine (DA) agonist 6,7-ADTN was employed. Our findings support the Principle of Inverse Effectiveness (PoIE), indicating that bimodal stimuli are more effectively learned and retained when the individual unisensory responses are relatively weak. The interaction between synchrony and intensity significantly influenced bimodal learning and memory, with maximal enhancement observed at low intensities and synchronous stimuli. Furthermore, our investigation into the role of biogenic amines revealed concentration-dependent and opposing effects OA and DA during unimodal and bimodal appetitive learning. Higher doses of OA improved performance across all modalities, while DA had modality and dose-dependent inhibitory effects. These results provide valuable insights into the complex mechanisms and neural modulation underlying appetitive learning tasks in bees, contributing to our understanding of their behavioural adaptation to complex signals. Ultimately, these findings suggest remarkable parallels between the mechanisms of multisensory integration and rewarding systems in bees and vertebrates. These shared characteristics underscore the significance of bees as a valuable comparative model in neuroscience research.

GENERAL INTRODUCTION AND RESEARCH PROBLEM

Multisensory integration, initially explored in vertebrates, warrants investigation in diverse animal taxa, including insects, to uncover universal patterns in nervous systems (Ghosh et al., 2017; Gnaedinger et al., 2019; Haberkern and Jayaraman, 2016; Hou et al., 2019; Krueger Fister et al., 2016; Rössler, 2023). While there are structural similarities between vertebrate and invertebrate central nervous systems, notable physiological and anatomical differences exist (Barron and Klein, 2016; Donaldson, 2010; Sanes and Zipursky, 2010). However, across taxa, the brain centres responsible for multimodal integration govern animals' abilities to perceive, acquire, utilize, and retain relevant information (Alvarado et al., 2007; Groh and Rössler, 2020; Leo et al., 2008; Menzel, 2014; Otto et al., 2013; Strube-Bloss and Rössler, 2018a). These processes can be influenced by the neuromodulation of rewarding systems that impact learning and memory (Barron, 2022; Barron et al., 2010; Søvik et al., 2015). However, the applicability of vertebrate-based principles of multisensory integration to other animal taxa remains uncertain. Specifically, the effects of varying intensity and synchronicity levels of individual components during multimodal stimulus presentation and their interactions during associative learning are fully understood (Ghosh et al., 2017; Stein and Stanford, 2008; Strube-Bloss and Rössler, 2018a). Moreover, recent findings on the role of octopamine and dopamine in modulating associative appetitive and aversive learning require further investigation, particularly in the context of multimodal learning (Barron et al., 2010; Perry and Barron, 2013; Søvik et al., 2015; Waddell, 2013).

Multisensory integration enables the simultaneous processing of signals from multiple sensory modalities, creating a unified representation of the environment (Ghosh et al., 2017) that is, creating an immersing perception of reality. The applicability of the principles of multisensory integration, originally described in vertebrates, to other taxa, particularly insects, remains unknown. While there are similarities in the basic structure and organization of vertebrate and invertebrate central nervous systems (Spong et al., 2016), some important physiological differences exist that might be related to the smaller size of invertebrates. The presence of myelinated axons in mammals, while found in some invertebrates are absent in insects (Hartline and Colman, 2007). There are also significant anatomical differences between them (e.g., morphology of neuron types) (Katz et al., 2013), as well as biochemical: while in the mammalian brain most of excitatory neurotransmission is mediated by ionotropic glutamate receptors, the main excitatory transmitter in the insect CNS is acetylcholine (Oleskevich, 1999a; Osborne, 1996a; Smarandache-Wellmann, 2016). Examples on the domain of signal processing and perception include recent evidence of the identification of insects as possessing processes thought to be exclusive of vertebrates: *corollary*

discharge circuits (allow distinction between sensory inputs and those result of motor actions) or *forward models* (CNS systems of prediction of motor control that also differentiate self and imposed stimulation) (Crapse and Sommer, 2008; Spong et al., 2016; Webb, 2004). Despite the "back-bone divide" in neuroscience and behaviour, the search for principles of structure and function across nervous systems requires an active comparison.

To gain a comprehensive understanding of multimodal interactions and their impact on memory, learning, and behaviour, it is crucial to compare the effects of parameter variations across different taxa. However, such comparison is still lacking. The exploration of multisensory integration phenomena has predominantly focused on vertebrates, resulting in a noticeable taxonomic bias. While a few exceptions exist with invertebrates, most of the research has been conducted using vertebrate models. Some vertebrate examples include the pioneering studies that described the neurophysiological underpinnings of multisensory integration in the cat *superior colliculus* (SC) (Meredith and Stein, 1983; Meredith and Stein, 1986; Meredith and Stein, 1996; Stein and Stanford, 2008; Stein et al., 2004a), cortical areas of multimodal integration in rats (Wallace et al., 2004), as well as audio-visual topographical maps of sensory space in the SC of ferrets (King et al., 2004). Similarly, studies of audio-visual (AV) integration of speech in humans (Altieri et al., 2015; Bernstein et al., 2004; Krueger Fister et al., 2016), non-human primates (Kaas and Collins, 2004; Kajikawa et al., 2012) have advanced greatly in developing anatomical and neurophysiological models of AV processing and showing physiological effects of AV integration perceptual gains that overcome environmental noise, interactions between intensity and perception of timing. There has been limited research on the multisensory faculties of invertebrates, with only a few classical studies dedicated to this topic. Example of special adaptations that involve merging senses were described for crayfish (Hatt and Bauer, 1980), moths (Olberg and Willis, 1990). In honey bees, there exist considerable work on the physiology of unisensory perception, such as olfactory system (Carcaud et al., 2015; Carcaud et al., 2016; Carcaud et al., 2018; Guerrieri et al., 2005a; Riveros and Gronenberg, 2009a; Sandoz, 2011a), visual system (Dyer et al., 2011; Ehmer and Gronenberg, 2002a; Hempel de Ibarra et al., 2014; Horridge, 2009; Kevan et al., 2001; Riveros and Gronenberg, 2012a), however, most of the research on multimodal phenomena has focused on exploring its adaptive role and evolution (Kulahci et al., 2008a; Leonard and Masek, 2014a; Leonard et al., 2011a; Raguso, 2001; Raguso, 2004) and from the point of view of flower advertisement (Raguso, 2001; Raguso, 2004). A relatively new field of research termed, *cognitive ecology*, focusses on the adaptive intersection of processes by which information is processed, store and retrieved (Giurfa, 2003; Giurfa, 2007a) has received considerable attention employing honey bees (Carcaud et al., 2016; Carcaud et al., 2018; Chittka et al., 2001; Dobrin and Fahrbach, 2012a; Giurfa

and Lehrer, 2001; Giurfa et al., 2001a; Jernigan et al., 2014a; Menzel and Giurfa, 2001a; Mota et al., 2011a; Sandoz, 2013) as well as in bumblebees (Riveros and Gronenberg, 2009a; Riveros and Gronenberg, 2009b; Riveros and Gronenberg, 2012a). However, the exploration of the physiological basis of multimodal integration within the context of cognitive ecology has often been understudied in insects in general and bees in particular (but see Erber, 1978; Homberg and Erber, 1979; Strube-Bloss and Rössler, 2018).

Multimodal integration research has primarily centred around vertebrates, neglecting the exploration of complex natural systems where the integration of multiple sensory sources plays a crucial adaptive role. A clear example is pollination. The variation that flower displays (colour, pattern, shape, and scent, alone or in combination) is mainly the result of coevolution with pollinators (Chittka and Thomson, 2001). In turn, the pollinator perception has been greatly shaped by this relationship (Leonard et al. 2011; Schiestl & Johnson 2013a). Therefore, the olfactory and visual systems of the honeybee are an ideal model to advance our knowledge on multisensory integration (Giurfa and Sandoz, 2012a; Leonard and Masek, 2014b). Indeed, plant – pollinator interactions offer the opportunity of analysing the implications of complex floral stimuli in diverse topics within neuroscience, signal evolution and sensory ecology (Leonard and Masek, 2014b; Leonard et al., 2011a). Indeed, in recent years, researchers investigating plant-pollinator interactions have started to refocus its attention towards production, transmission, and processing of complex signals during the multimodal interactions (Gil-Guevara et al., 2022; Kulahci et al., 2008a; Lawson et al., 2017; Leonard et al., 2011a; Riveros, 2023).

While multisensory integration in plant-animal systems has not been extensively explored, the honey bee has emerged as a reliable model for understanding the underlying mechanisms of learning and memory (Giurfa, 2003; MaBouDi et al., 2017; Matsumoto et al., 2012a; Reichert and Quinn, 2017; Takeda, 1961a). These studies have been performed remarkably on the olfactory system (Bitterman et al., 1983a; Giurfa, 2003; Giurfa, 2007a; Giurfa and Sandoz, 2012a; Giurfa et al., 2001a; Kuwabara, 1957a; Matsumoto et al., 2012a; Mauelshagen, 1993a; Menzel, 2001a; Menzel and Giurfa, 2001a; Sandoz, 2011a; Takeda, 1961a), while visual conditioning has only being recently implemented (Dobrin and Fahrbach, 2012a; Hori et al., 2006a; Jernigan et al., 2014a; Mota et al., 2011a; Riveros and Gronenberg, 2012a). However, the visual and olfactory systems have predominantly been investigated in isolation from each other (Altieri et al., 2015; Dobrin and Fahrbach, 2012a; Giurfa and Sandoz, 2012a; Hori et al., 2006a; Hori et al., 2007a; Leonard and Masek, 2014b; Leonard et al., 2011a; Mota et al., 2011a). Nevertheless, several studies on insects have employed methodologies that integrate associative learning to investigate the interactions between different sensory modalities (Guo and Guo, 2005; Mota et al., 2011a; Srinivasan et al., 1998).

Yet, some of those relatively recent approaches using *Drosophila* as a research model for instance differ significantly to honeybees in several ways. For example, Guo & Guo (2005) implemented aversive conditioning to study crossmodal interactions between olfactory and visual learning in *Drosophila*; while studies on fruit flies suggest that multiple sensory systems can facilitate learning in their natural environment, it is important to consider that fruit flies lack the coevolutionary context and significant anatomical and physiological similarities found in plant-pollinator systems. In the honeybee brain, both the olfactory and visual signals are processed and integrated in the mushroom bodies, a relevant structure for learning, memory, and motor control (Ehmer and Gronenberg, 2002a; Erber, 1978a; Homberg and Erber, 1979a; Strube-Bloss and Rössler, 2018a; Strube-Bloss et al., 2011; Strube-Bloss et al., 2016). Quite the reverse in *Drosophila*, where different types of visual memory are stored on different brain regions, independent from the mushroom bodies (Wolf et al., 1998). Both honey bees and bumble bees as research model system provides a valuable opportunity to study coevolved multimodal signals that play a crucial role in learning and memory formation. It also offers an anatomically defined centre for multisensory integration.

Furthermore, these multimodal centres are directly influenced by neuromodulation, highlighting the unanswered question of their connection to reward systems. To achieve a thorough comprehension of multisensory integration and its effects on learning and memory, investigating the involvement of biogenic amines, specifically octopamine and dopamine, in insect nervous systems is essential (Barron et al., 2010; Burke et al., 2012; Owald et al., 2015; Perry and Barron, 2013; Søvik et al., 2015; Waddell, 2013). Biogenic amines have been shown to influence task specialization, motivation states, sensory sensitivity, and learning and memory processes in insects (Huang et al., 2022; Perry and Barron, 2013; Vieira et al., 2018). In the honeybee brain, biogenic amines modulate the sensitivity of sensory receptors and interneurons and are involved in reward pathways (Barron et al., 2002; Barron et al., 2007; Scheiner et al., 2002a). The mushroom bodies, also receive convergent unimodal and multimodal projections, along with the reward pathways mediated by biogenic amines (Perry and Barron, 2013; Søvik et al., 2015). Understanding the convergence of sensory processing and reward systems in the honeybee brain aligns with the reward prediction error signal, which plays a significant role in classical conditioning (Hammer, 1997; Hammer and Menzel, 1995; Rescorla and Wagner, 1972; Terao and Mizunami, 2017; Waddell, 2013).

Therefore, the aim of the present doctoral work is to investigate the principles of multisensory integration with particular emphasis in honey and bumble bees. The study aims to examine the effects of variations in intensity and synchronicity of stimuli on associative learning and memory formation. Additionally, it aims

to elucidate the role of biogenic amines, such as octopamine and dopamine, in modulating learning, memory, and behaviour in bumble bees. This research contributes to the understanding of the generalizability of multisensory integration principles and the role of biogenic amines in cognitive processes in insects.

ARE MULTIMODAL SIGNALS BETTER THAN UNIMODAL SIGNALS?

Despite the previous discussion, recent work on bimodal learning in bees (Riveros et al., 2020) has shown that the performance using a compound bimodal signal does not always exhibit a straightforward positive synergistic effect of signal components. The interaction of components within a multimodal signal can vary among individuals and depends on the nature of the components (Balkenius et al., 2006). In other words, performance following bimodal stimulation is not consistently superior to performance based on unimodal stimulation. This observation suggests that the prevalence of multimodal signals is dependent of specific conditions.

Several hypotheses aim to explain the prevalence of multimodal signals and numerous studies provide experimental evidence supporting the significance of multiple components within a signal (Hebets and Papaj, 2005; Leonard and Francis, 2017; Leonard and Masek, 2014a; Leonard et al., 2011b; Leonard et al., 2011c; Siddall and Marples, 2008). Various conditions might favor the evolution of complex signals from the receiver's perspective (Wilson et al., 2013). One compelling argument is that complex signals, particularly multimodal ones, might be more detectable in noisy environments (Hebets and Papaj, 2005) —a common challenge in natural signaling systems. Another widely accepted hypothesis, known as the multiple messages' hypothesis, suggests that signal components are not truly redundant. Instead, different components may convey distinct aspects of an underlying quality (Hebets and Papaj, 2005; Johnston, 1982; Moller, 2002). Under this framework, complex signals, especially multimodal ones, may be more economically stable. Multicomponent signals may combine reliable and unreliable cues, providing receivers with various ways to interpret and respond to the signal. Several theories propose how secondary signal components can enhance the detectability, discriminability, and memorability of primary components (Rowe, 1999; Rowe, 2005; ten Cate and Rowe, 2007a). This includes the alerting and attention-altering hypotheses, suggesting that a conspicuous but less reliable signal component can draw attention to a more reliable one (Hebets and Papaj, 2005). These mechanisms may contribute to the overall effectiveness and stability of complex signaling systems. The prevalence of multimodal signals suggests that combining various information sources enhances receiver performance. In this context, senders benefit from increased signal conspicuousness and redundancy, while receivers enjoy advantages in learning,

memory, attention, and overall information processing (Akre and Ryan, 2010; Kulahci et al., 2008a; Partan, 2017; Rubi and Stephens, 2016a; Rubi and Stephens, 2016b; Siddall and Marples, 2008; Sutherland and Mackintosh, 1971; ten Cate and Rowe, 2007a).

Hence, together the evidence and hypotheses suggest that multimodal signals may lead to differential performance depending upon factors other than the sole addition of sensory components. Thus, in this thesis, I aimed to investigate the intrinsic properties of unimodal (olfactory and visual) and bimodal signals, including the intensity of unimodal components (**Chapter 1**) and their temporal relationships and possible interactions (**Chapter 2**). Additionally, I examined the bees' inner neuromodulatory state, which can also impact the effectiveness of multimodal signals (**Chapter 3**). Below, I will briefly review the general neuroanatomy of the bee brain, including its primary structures, neurocircuitry, and neuropharmacology as the neural substrate supporting processing of multimodal information. Additionally, I will touch upon the historical significance of the proboscis extension response (PER) protocol, which has played a crucial role in unravelling the mechanisms underlying learning and memory in honey bees and is central to the development of this thesis. The PER protocol, despite its limitations, is a potent tool for comprehending the interaction of multimodal signals in the formation of enduring memories in honey bees.

THE HONEY BEE BRAIN: GENERAL STRUCTURE AND NEUROPHARMACOLOGY

THE HONEY BEE BRAIN

Insects have traditionally been considered simple and small reflex automata; yet, they have proven to be extremely flexible organisms that process information in order to adapt to most environments on earth. Hence, the insect brain must provide intelligent and adapted solutions to a broad range of ecologically relevant problems in order to assure the impressive evolutionary success (Menzel and Giurfa, 2001b). Several general evolutionary trends, common to both invertebrates and vertebrates, such as bilateral symmetry and unidirectional locomotion have both influenced the organization of the nervous system and the distribution of sense organs. Another equally important evolutionary trend, the cephalization, produced the centralization and concentration of the major coordinating elements of the nervous system (Brusca et al., 2016). Indeed, the brain in the insects as well as in the vertebrates, is the main association center in the body. All posterior ganglia send sensory inputs to the brain via ascending interneurons, and naturally, the brain also receives the sensory inputs from all the head sensory organs (Chapman et al., 2013).

Anatomically, the insect brain is organized in three regions: the protocerebrum, the deutocerebrum, and the tritocerebrum. Overall, the protocerebrum is bilobed and contains the optic lobes, a central zone, the pars *intercerebralis* that contains fibers of the ocellar nerves (anterodorsal to the pars), cells that are important for sleep and cells with octopaminergic properties that project to dorsal regions of the protocerebrum . More laterally, cells enter the protocerebral bridge (*pons cerebralis*) that is basically a mass of neuropile that connects other parts of the brain. More importantly, the protocerebrum also gives rise to the mushroom bodies (see below) (Chapman et al., 2013). The deuterocerebrum contains the antennal lobes, as well as mechanosensory and motor support for the antennae. The tritocerebrum is a small part of the brain that contains a pair of lobes, as well as the subesophageal ganglion and the tritocerebral lobes (Chapman et al., 2013).

THE MUSHROOM BODIES: GENERAL FUNCTION

The mushroom bodies (MBs) are regions in the insect brain involved in processing complex multimodal information (Gronenberg, 2001a) (Fig. 1). MBs neuropils are prominent neuropils in the insect brain that are implicated in higher order processing such as sensory integration, learning and memory, and spatial orientation (ref). The large population of its intrinsic cells, the Kenyon Cells (KCs; see below) and associated neuronal microcircuits are highly suggestive for an elaborated computational potential and increased neuronal plasticity as well as associated storage capacities (Rössler and Groh, 2012). This latter rationale suggested the adaptive value having such complex and elaborate neuropil. The MBs has been suggested to be analogous to the vertebrate hippocampus and is implicated in learning and memory, as suggested by anatomical, behavioral, and molecular studies (Oleskevich, 1999b). Other of these studies suggest that the size of the mushroom bodies, more than any other brain region, closely correlates with an animal's behavioral complexity (Gronenberg, 2001a). The high divergence of sensory input to the KCs, their sparse coding properties, and the necessity of coincident activity via PNs makes it likely that KCs code sensory modalities in a highly specific way (Rybak and Menzel, 2010)

MUSHROOM BODIES: GENERAL ANATOMY

The mushroom bodies (*corpora pedunculata*) are structures found in all insect brains and also have been found in some Annelida and Crustacea. The cellular organization of the MBs first described in the honey bee by Kenyon (1896) is similar in all neopteran insects (Gronenberg, 2001a). The MBs are composed of thousands of intrinsic neurons called Keyon cells and all of its dendrites form the most distal neuropil, the

calyx. In the honey bee, the MBs are two bilaterally symmetric neuropils located in the dorsal protocerebrum; they consist of an extremely dense packing with a parallel arrangement of the intrinsic neuron axons (Rybak and Menzel, 1993). Each MB is subdivided into several regions: the pedunculus (PED), the medial (ML) and vertical lobes (VL, the main output regions of the MBs) and the calyces, cup-shaped neuropils that are in turn subdivided in three compartments: the lip, the collar, and the basal ring neuropils (Rössler and Groh, 2012; Rybak and Menzel, 1993) that constitute the main sensory input regions of the MBs. In particular in the honeybee and other Hymenoptera, the MBs form a lateral (LCA) and medial (MCA) calix in each brain hemisphere (Rössler and Groh, 2012). Visual fiber tracts terminate in the collar, whereas the antennae relay neurons projecting into the lip neuropil of the calyces (Mobbs, 1982a; Rybak and Menzel, 1993). A second-order olfactory neuropil, the ventral lateral protocerebral lobe, receives input from both medial and lateral antenna-glomerular tracts (AGT) (Mobbs, 1982a) (Fig 1).

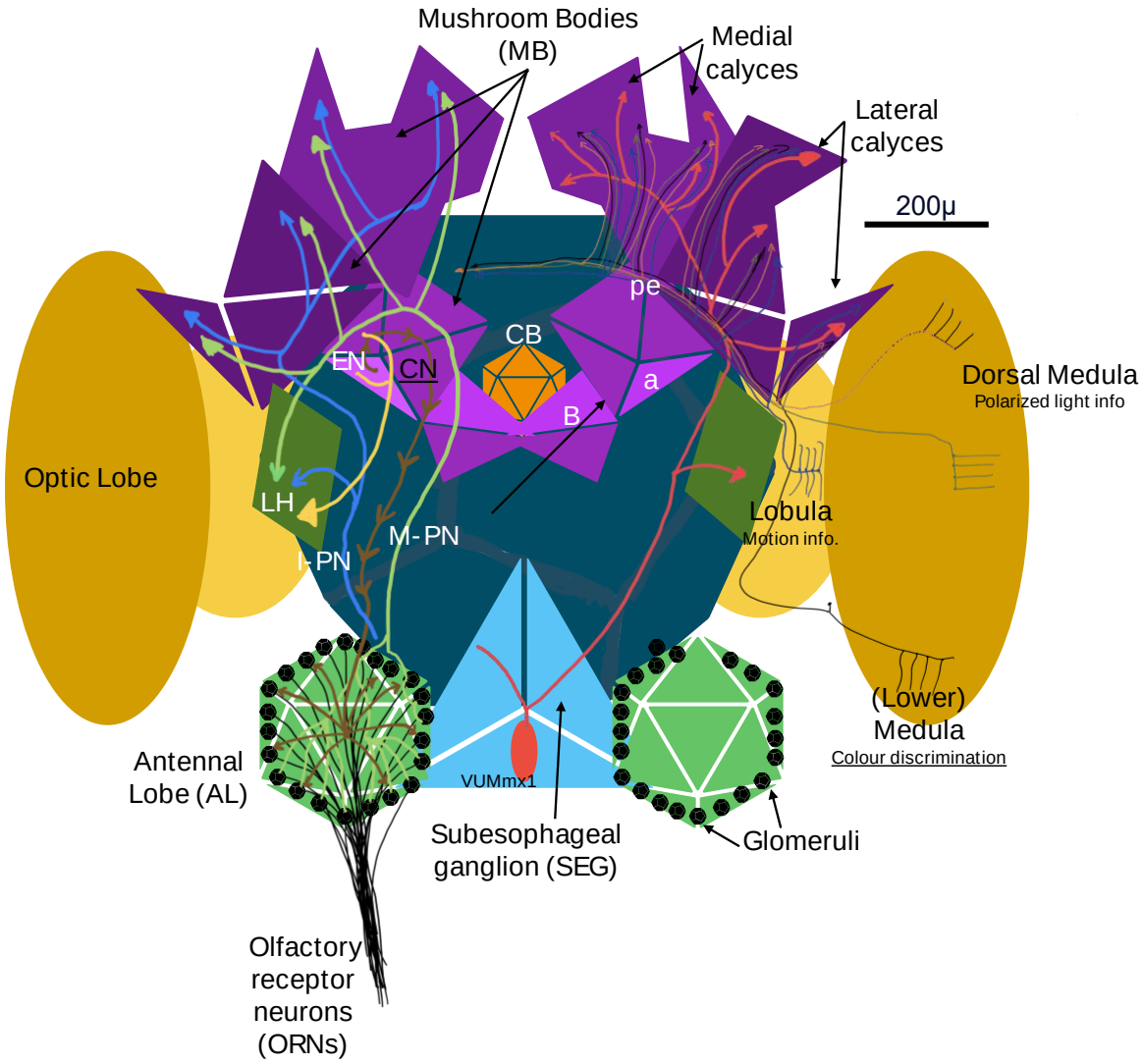

Figure 1. Schematic representation of the bee brain. The main structures are indicated in relation with the neural pathways for gustatory stimulation (unconditioned stimuli or US) as well as olfactory and visual information (conditioned stimuli or CS). The olfactory pathway (CS, left side): After odorant detection on the antennae, approximately 60,000 olfactory receptor neurons (ORNs) project to the Antennal Lobe (AL), a first-order olfactory neuropil. In the AL, ~160 glomeruli (Glo) receive input from ORNs, approximately 4000 inhibitory local neurons (LN) perform local computations, and about 800 projection neurons (PN) transmit processed information to higher brain canters through various tracts. The lateral antenno-protocerebralis tract (I-PN) initially targets the lateral horn (LH) before extending to the mushroom body (MB) calyces (ca), including the lips and basal ring. The medial projection neuron tract (m-PN) projects in the reverse order to the same structures. Kenyon cells (KCs; see text), the MB's intrinsic neurons, have dendrites forming the calyces and axons forming the pedunculus and two output lobes: the vertical (a) lobe and horizontal (b) lobe, each from two collaterals of a KC axon. Feedback neurons within the MBs (not shown) project from the pedunculus and lobes to the calyces, offering inhibitory feedback to the MB input areas. Extrinsic neurons (ENs) convey information from the pedunculus (at the base of the calyces) and lobes to

various regions of the protocerebrum, particularly the LH. Centrifugal neurons (CN) likely play a role in retrograde modulation of antennal lobe circuits. Right side: both the US pathway (gustatory) and the visual inputs tracts (CS). Gustatory sensory neurons in antennae, tarsi, and mouthparts detect sucrose reinforcement and project to the subesophageal ganglion (SEG). A specific octopaminergic neuron, VUM-mx1, represents reinforcement in appetitive conditioning. VUM-mx1 dendrites are in the SEG, where they likely receive input from sucrose receptor neurons and project to three brain regions: the AL, MB calyces, and LH, converging both with the olfactory and visual pathways. In turn, also in the right side a schematic representation of the visual tracts shows the projections to the mushroom body calyces, originating from the lobula lot (blue), lower medulla (dark green), and medulla (orange and green). Note the olfactory, visual, and gustatory convergence in within the calyces. Other anatomical subdivisions of the calyces (Lips, Collar and Basal ring, not shown) are responsible of receiving olfactory, visual, and bimodal sensory integration; among the lips and collar PNs from (SEG) converge information from gustatory and mechanosensory neurons. Figure based and modified from (Ehmer and Gronenberg, 2002a; Galizia and Rössler, 2010; Giurfa and Sandoz, 2012a; Groh and Rössler, 2020; Gronenberg, 2001b; Kropf and Rössler, 2018a; Mobbs, 1982b; Strausfeld, 2002a; Strube-Bloss and Rössler, 2018a).

Starting at the calices, each dendritic tree of the KCs provides an axon that projects from the calix through the peduncle (PED); then these, in turn, branch to reach the vertical and medial lobes, sometimes referred as alpha (α) and beta (β) lobes, respectively (Gronenberg, 2001a). That is, from each of the subdivisions of the calyces (the lip, collar, and basal rings) the dendrites of the KCs are connected with their cell bodies, at the center of the calyces, and their axons, then, are projected towards the pedunculus. At the base of the pedunculus, each fiber bifurcates, sending one branch into the α and β lobes (Rybak and Menzel, 1993) (Fig 1). Electron microscopic studies indicate that the calyces are the main input regions and the α and β lobes are both output and input regions (Schürmann and Elekes, 1987). Intrinsic KCs axons project ventrally with a specific order into the pedunculus. Here, these axons form three U-shaped bands, each derived from a different calycal compartment. The junction of the pedunculus, α-lobe and β-lobe, K-cells subdivide into several strata, continuing into the α and β lobes.

THE CALYX

Each MB hemisphere has a pair of cup-like neuropils called the calyces, each concentrically organized (the lip, collar and basal ring) (Mobbs, 1982a; Rybak and Menzel, 1993; Strausfeld, 2002b) (Fig 1). Each of these regions of the calyces is targeted by a characteristic set of afferents from various peripheral sensory neuropils; The antennal lobes (Fig 1) are represented in the lip and basal ring; the visual system is represented in the calyces by endings of projection neurons that separately originate from the medulla and lobula: the calyx *collar* is innervated by visual afferents from ipsilateral lobula and from both ipsi- and contralateral medullae (Ehmer and Gronenberg, 2002b; Gronenberg, 2001a). An additional region of the *collar* and basal ring, receives terminals from mechanosensory interneurons and gustatory afferents (Strausfeld, 2002b). Axons originating from lateral and medial calyx converge to form a peduncle that then

divides into the α and β lobes (see next section) (Gronenberg, 2001a). The general cytoarchitecture of the calyces consists of an elongated tube with thick walls, forming a cup-like profile; the walls of the tub comprise the neuropil which in turn is composed of the dendrites of the Kenyon cells, and of the axons of afferent neurons terminating on it, while the cell bodies of the KCs fill the tub (Gronenberg, 2001a). The ultrastructure of the calyx neuropil consists in turn of very small sphere-like units (the microglomeruli) composed by the synapses between incoming and intrinsic neurons. The microglomeruli tend to be larger in the basal ring than elsewhere; the borders between lip, collar, and basal ring, coincide with invaginations of the calyx wall. When it comes to sensory input, olfactory projection neurons (PNs) primarily extend into the lip, while visual PNs target the collar region. Mechanosensory PNs branch out within the basal ring, and gustatory PNs are concentrated in a small area between the lip and collar. This distinct organization maintained by the PNs within the Kenyon cells (KCs) continues throughout the mushroom bodies (MB), resulting in a systematic segmentation of the lobes (Gronenberg, 2001a). The notion that the calyces are partitioned in those discrete regions is supported by the shape and distribution of K-cells' dendritic domains.

The relationship between sensory input and anatomic distribution is represented in the calyx (Fig 1). The olfactory information reaches the calyx from the collaterals from antennal projection neurons (axons that run in the medial and lateral antenno-cerebral tracts (Gronenberg, 2001a). These projections are ipsilateral and connect the antennal glomeruli with the lip and basal ring. The projections from the optic lobes to the collar and the basal ring are provided by the anterior superior optic tract, carrying axons from the medulla and lobula (Mobbs, 1982a). Specifically, each of the calyx regions (lips, collar, basal ring) exhibit some differences with regard to the distribution of sensory afferents that they receive: **The lip:** terminals of antennal lobe relay neurons are larger or more numerous in the lip's outer layer; although among hymenopterans, honey bees exhibit the least noticeable differences or subdivisions in the lip region. **The Collar**: here visual inputs terminate as three long and broad fibers that in preparations are visible because they appear as separated by a thin unstained layer. **The basal ring:** the calyx receives afferent input from the antennal lobes, the medulla, and probably from the lobula; neurons that supply either the lip or the collar have short blebbed collaterals in the basal ring; however, visual and antennal lobe inputs are segregated in the basal ring.

As mentioned earlier, the significance of the architecture of the calyx is that matches the layered architecture of the α and β lobes. The calycal regions comprise one or more Kenyon cell type that is in

turn, represented by a group of corresponding axons in the lobes (Mobbs, 1982a). Since the projection neurons originating in different sensory neuropils innervate the calyx in an orderly fashion, where olfactory projection neurons (PN) are targeting the lip, visual PNs the collar, mechanosensory PNs the basal ring, and gustatory PNs in a small region between the lip and collar (Rybak and Menzel, 2010). The microstructure of the junction between PNs and Kenyon cells within the MBs leads to KCs are divided into two classes: the ventral part of the lobe is composed of Kenyon cells type 2 (KII-type) homogeneously distributed throughout all calycal zones, while the dorsal α lobe is layered by the orderly projections of the Kenyon cells type 1 (KI type) from different calycal sub-compartments (Rybak and Menzel, 2010).

THE NEURONAL CONNECTIONS AND THE α AND β LOBES

By means of focal cobalt chloride injections and Golgi staining methods, Rybak and Menzel (1993) studied the neuronal connections between the MB and the cells of the α-lobe. The α-lobe is a neuropil of the MB that forms seven clusters of somata in both protocerebral and deuterocerebral regions. Rybak and Menzel (1993) identified the morphology of 15 cell types that summarize the arborizations of ~400 cell bodies that reach the α-lobe within each hemisphere. These 15 cell types are localized and compensated in seven somata clusters (A1-A7). The α-lobe extrinsic neurons form mainly intra-protocerebral neuronal circuits between the mushroom body and the ipsilateral protocerebral lobe, particularly the lateral proto-cerebral lobe (LPL). Rybak and Menzel stated that all neurons examined exhibit structural polarity, which might indicate input and output sites. Most extrinsic neurons were found to form a dense banding pattern in confined zones in the α-lobe; the banding pattern is probably corresponding to input sites. These α-lobe neurons project to the calix, pedunculus and to the β - lobe and are presumed to be an indication of presynaptic structures. Hence, the arborizations in the α-lobe are described as dendritic and those in other projection areas as axonal or terminals.

Rybak and Menzel (1993) described three major morphological groups of neurons, distinguishable according to their projection patterns and cell body location in the proto- and deutocerebrum: **1)** *Unilateral neurons* (clusters A1, A2, A4 and A5) **2)** *Recurrent neurons* (clusters A3, -d and -v) and **3)** *Bilateral neurons* (clusters A6 and A7). *The* ***Unilateral neurons***, are restricted to one protocerebral hemisphere, invade the α-lobe ventrally forming dense horizontal bands in the ventral (A4, A5), ventral and medial (A5), and medial and dorsal (A1, A2) α-lobe; therefore, these regions are connected with the neuropil (MBs) around the a-lobe and the LPL (lateral protocerebral lobe). *The* ***Recurrent neurons*** are neurons that

connect α or β lobes with the calices. These have been characterized by means of intracellular electrophysiological recordings in the honeybee, where many presented multisensorial properties (Homberg and Erber, 1979b). This characterization concluded that the spontaneous discharge frequency of these neurons ranged between 5 and 95 impulses per second. When stimulated, about 80 percent of the neurons responded to at least one of five different sensory modalities: scent, light, air current to the antennae, sugar water applied to the antennae and to the proboscis. Importantly, 45 percent of the neurons responded to two or more modalities (Homberg and Erber, 1979b). On the other hand, anatomical studies (Mobbs, 1982a) showed that feedback neurons of the protocerebral -calycal-tract create many branches in the α and β lobes and pedunculus and connect these neuropils with the calyces. The ***Bilateral neurons*** are a small number of extrinsic neurons originating in the A6 and A7 cluster and project within the ipsilateral protocerebrum towards the lobe and (partially) to the β lobe (Rybak & Menzel 1993). In the MBs, the spatial segregation of sensory inputs into the calyces is maintained in the pedunculus and lobes (Mobbs, 1982a; Rybak and Menzel, 1993). For example, olfactory information spatially segregated in the MBs is transferred to other brain regions by subsets of α-lobe output neurons (unilateral A4 neurons). Both Recurrent neurons (A3) and Bilateral neurons (A7) have shown evidence of multisensory activity in the MBs (Erber, 1978b), explained by different mechanisms (e.g., multimodal inputs to one region of the calyces; feedback neurons connecting zones between calyx and α lobe; synaptic connections between KCs; intrinsic network properties of the MBs mediated by KCs) (Rybak and Menzel, 1993). Finally, it can be concluded that in general each of the MB lobes (α and β-lobes) comprises three parallel divisions representing one of the three concentric zones of the calyces (the lip, collar and basal ring) (Mobbs, 1982a; Rybak and Menzel, 1993; Strausfeld, 2002b). With more precision it was demonstrated that the lip of the calyx is not represented in the lower third of the α-lobe, but in the lower half of a layer previously mentioned by Mobbs (1982) as recipient of projections from the collar; that evidence indicates that the lower third of the α-lobe receives axons from (clawed) K-cells that together represent the entire calyx (Strausfeld, 2002b).

THE KENYON CELLS

The honeybee olfactory pathway comprises an intriguing pattern of convergence and divergence: ~60.000 olfactory sensory neurons (OSN) convey olfactory information on ~900 projection neurons (PN) in the antennal lobe (AL). To transmit this information reliably, PNs employ relatively high spiking frequencies with complex patterns. PNs project via a dual olfactory pathway to the mushroom bodies (MB). This

pathway comprises the medial (m- ALT) and the lateral antennal lobe tract (l-ALT). PNs from both tracts transmit information from a wide range of similar odors, but with distinct differences in coding properties. In the MBs, PNs form synapses with many KCs that encode odors in a spatially and temporally sparse way (Kropf and Rössler, 2018b). Each MB is formed from many thousands of small intrinsic interneurons, the K Cells; interestingly, the shape of the mushroom bodies reflect the geometry of the individual KCs each of which has its cell body and dendritic arborization in the calyx; then , from here each KC sends a long projection into de pedunculus that divides into two branches which enters the α and β lobes (Mobbs, 1982a). With respect to the calyces and its sub compartments (lip, collar and basal ring), the KC dendrites forming the lips arise from the population of larger cell bodies within the cup and around the lip. The collar contains projections of large KC bodies within the cup and those small KC covering the undersurface of the collar area. The basal ring, gets its volume from the population of small cell bodies that occupy the central regions of the cup and also from small KCs located underside the calyx at around the basal ring (Mobbs, 1982a). Regarding the Kenyon cell morphologies themselves, they have an approximate total path length of 900 μm within the pedunculus and lobes; they can be divided into subclasses on morphological basis: 1) directly attached to primary dendritic segments of the calycal arborization (Ka cells) or 2) isolated from it by a neurite connected to the peduncular projection in the immediate vicinity of the calycal dendritic field (Kb cells). There are several other subdivisions based on their dendritic specialization within the calycal regions (Mobbs, 1982a). However, based on staining techniques (Golgi-stained) two main categories are described: cell types KI and KII. The KI neurons are exclusive of the lip zone, its cell bodies are distributed around the lip. The KII neurons are Kb cells found only within the collar zone and their cell bodies occupy positions in the other group of K cell somata. Other types of K cells (KIII, KIV and KV) are also present. KIII are found only in the basal ring zone. KIV neurons can be either Ka or Kb cells and are also found only within the basal ring; the dendrites of these cells do not extend along the basal ring but are restricted to the contour by two tracts (inner and outer ring tracts). Finally, the KV neurons (that can be either Ka or Kb) are found within both the collar and the basal ring (Mobbs, 1982a).

NEURONAL CORRELATES OF COLOR CONTEXT AND OLFACTORY LEARNING

When animals are conditioned to respond to a cue (e.g., an odor) in presence of a reinforcer (e.g., sugar) they also learn the contexts and conditions (time of the day, temperature, visual stimuli). Then two alternative options arise. The first option suggest that all stimuli present at the occurrence of the reinforcer are associated separately with the reinforcer (Rescorla and Wagner, 1972). Alternatively, during

conditioning, all co-occurring stimuli represent unique combinations or configurations, that result distinct from the elements that compose them, an idea favored by Pearce (Pearce, 1994). Hussaini and Menzel (Hussaini and Menzel, 2013a) attended this unresolved question of how the different elements of a cue or context compounds are integrated neurally as either separate elements developing each their specific associative strengths (Rescorla and Wagner, 1972) or as unique configurations that produce a combined associative strength (Pearce, 1994). Hussaini and Menzel assumed that bees do not show conditioned responses to visual stimuli, supposing that these might only enhance the response to the learned olfactory cue. This is perhaps similar to the occasion setting, a sophisticated form of non-elemental learning where animals learn to disambiguate an uncertain conditioned stimulus using an alternative stimulus that do not enter into direct association with the unconditioned stimulus (Mota et al., 2011a). However, visual stimuli indeed can form direct conditioned responses (Avarguès-Weber and Mota, 2016; Dobrin and Fahrbach, 2012a; Hori et al., 2006a; Jernigan et al., 2014a; Lichtenstein et al., 2018; Lichtenstein et al., 2019; Riveros and Gronenberg, 2012a).

Interestingly, the neuronal data of Hussaini and Menzel (2013) support the Rescorla-Wagner model, contrary to other interpretations of behavioral data in bees about learning odor configurations (Mota et al. 2011). Hussaini and Menzel (2013) focused on the MB extrinsic neurons exiting the alpha lobe (αL) in its ventral aspect (β exit). These neurons are known to respond to multiple sensory modalities changing their properties during associative learning (Strube-Bloss et al., 2011). In short, Hussaini and Menzel observed general properties of αL extrinsic neurons such as firing rate (spikes per seconds) during both spontaneous activity and in response to contexts (light or no light) and odors. Here, the firing rate of these neurons increased with the onset of contexts (light/no light) and odors. They used two inverse related metrics: the change in spike firing rate (ΔSFR) and the inter spike interval (ISI: the interval between two spiking events); when the SFR increases naturally, the ISI is lowered. After simple differential conditioning experiments the ISI during spontaneous activity reduced (i.e. the ΔSFR increased) and the firing rate became more stable. Then, when the experiments switched to discriminant context-dependent learning (odor + light / odor alone) in which only one of two odors in one of the two contexts was rewarded. Bees learned this simple rule quickly. The change in the neuronal response of MB β neurons specifically was negative toward rewarded odors and positive toward rewarded contexts. This suggest that firing rates of MB β neurons are different for the cue (odor) and context (color). This seems to be a general pattern in animals: cues and contexts appears to be processed differently.

In mammals, cue learning and context-dependent learning are related to different neural mechanisms (see (Phillips and LeDoux, 1992), where context-dependent learning requires an intact hippocampus, whereas cued learning does not require the hippocampus (Hussaini and Menzel, 2013a)(Hussaini and Menzel, 2013b). It seems that in insects, visual and olfactory learning may involve the MBs differently. While simple forms of visual learning are independent of the MBs, simple forms of olfactory learning require the MBs (Heisenberg, 2003); similarly, more complex forms of both visual and olfactory learning also require the MBs. On the other hand, the complexity elementary olfactory task is increased as a function of the number of stimuli.

Therefore, the MBs are required for configural (context) forms of learning (Hussaini and Menzel, 2013a)(Hussaini and Menzel, 2013b). The MBs integrates across multiple sensory modalities, organizes associative plasticity of its intrinsic neurons according to the value of the stimulus combinations. The results of Hussaini and Menzel support the idea that the elements of stimulus combinations are processed separately, and that the neural trace can be tracked experimentally. More importantly, these results might indicate that since during learning of colors and odors, neurons are processing the two modalities differently (and perhaps within different sparsening frames) and not as a configuration, there is room for phenomena such as those implicated during multisensory integration (i.e. temporal, spatial rule and principle of inverse effectiveness PoEI) (Meredith and Stein, 1983; Meredith and Stein, 1996; Meredith et al., 1987; Stein and Stanford, 2008; Stein et al., 1988; Stein et al., 2004b). The no-unification of multimodal stimuli as a unique configuration during processing might indicate that MB neurons can include independent parameters of each modality such as receptive fields (for spatial integration) temporal windows, and intensity (PoEI) to reach a conclusion during multimodal integration about whether or not these two inputs constitute a single event, a crucial function of brains during the prediction of reality (Llinas and Roy, 2009).

BRAIN MICROCIRCUITS AND NEUROPHARMACOLOGY

Although electrophysiological studies have helped to understand the honeybee reward system (Hammer 1993), most studies have used neuropharmacological methods to explore circuit functions (Scheiner et al., 2002b). The calyx constitutes a matrix of connectivity: on average, one KCs receives input from 10-15 olfactory PNs, and each of these PNs provides input to 10-100 KCs (Rybak and Menzel, 2010). Each PN button is composed of a microcircuit made of multiple outputs to KCs, input and outputs from a to GABA profiles representing the A3 neurons (Recurrent), the output from these profiles onto KC spines and

modulatory neurons (Rybak and Menzel, 2010). Olfactory PN boutons respond to odor stimulation with excitation and/or inhibition in an odor identity–specific way. Odors are coded in KCs (type KII) in a sparse way both in the temporal and the population domain (Szyszka et al., 2005). Interestingly, pairing an odor as a conditioned stimulus with sucrose reward in an associative learning situation induces prolonged KCs responses; after the conditioning, KCs responses recover (repetition-induced decrease -habituation?) while nonrewarded odor responses decrease even more. Therefore, KCs responses are subject to non-associative plasticity during odor repetition and undergo associative plasticity after appetitive odor learning (Rybak and Menzel, 2010).

Of the three types of ionotropic glutamate receptors, AMPA, kainite and NMDA (N-methyl-D-aspartate), the latter is localized throughout the bee brain, including the MBs. Silencing the expression of the NR1 subunit of the NMDA receptor (NMDAR) in the MBs by RNAi impairs selectively the acquisition phase and the formation of middle-term memory leaving long-term memory intact. It is concluded that NMDARs are not coincidence detectors in the MBs but are rather involved in the formation of particular memories (Rybak and Menzel, 2010). Outside the MBs, the neuron VUMmx1 is thought to be an octopaminergic neuron, and represents the reinforcement function during olfactory conditioning of the bee; this neuron receives input from sucrose receptors in the sub-esophageal ganglion; its axons innervate bilaterally both antennal lobes, lateral horns and the lip and basal regions of the calyces (Hammer, 1993).

Biogenic amines are important neuromodulators, neurohormones, and neurotransmitters in the honey bee. The biogenic amines are an important class of compounds, including adrenaline, ACh, dopamine (DA), noradrenaline (NA), adrenaline (AD), tyramine, octopamine (OA), synephrine, 5-hydroxytryptamine (5-HT, serotonin) and histamine (HA), which influence many aspects of insect physiology (Osborne, 1996b). In particular, octopamine and dopamine are found in high amounts in the nervous system of adult honey bees; they modulate the sensitivity of sensory receptors and interneurons (Scheiner et al., 2002b). These biogenic amines can modify the intrinsic properties of neurons, the efficacy of synaptic connections and the properties of motor systems (Scheiner et al., 2006). Therefore, these amines might affect sensory sensitivity and behaviors ranging from perceptual responses to learning and memory formation (Scheiner et al., 2002b). One of those interesting behaviors is the sucrose responsiveness measured using PER. Scheiner et al (2002) measured different concentrations of sucrose finding the sucrose response threshold. Then, the authors tested for changes in the responsiveness on sucrose as a function of treatments of different biogenic amines (octopamine, tyramine, dopamine, and the dopamine receptor agonist 6,7-ADNT). They either injected the biogenic amines into the thorax or fed into sucrose solution. They found

that Injection and feeding of tyramine or octopamine significantly increased sucrose responsiveness. Dopamine decreased sucrose responsiveness when injected into the thorax. Feeding of dopamine had no effect. Injection of 6,7-ADTN into the thorax and feeding of 6,7-ADTN reduced sucrose responsiveness significantly. As a consequence, the responsiveness of honey bees can be modulated by biogenic amines (Scheiner et al., 2002b).

The ventral unpaired medial (VUMmx1) cell mediates the interaction between the sucrose sensitive taste receptors in the unconditioned stimulus (US) pathway and olfactory sensory afferents in the conditioned stimulus (CS) pathway of the antennal lobe (AL). The way that VUMmx1 mediates these relationships is by means of the release of octopamine (OA) into the glomeruli of the ALs, being responsive to odor changes in a correlated way with associative changes during conditioning (Hammer, 1993). By means of pharmacological (microinjections of OA receptor antagonist at the AL) and molecular (RNA-mediated interference) techniques, Farooqui et al (2003) blocked and disrupted the octopamine receptor of the honeybee *Apis mellifera.* They founded that octopamine mediates consolidation of a component of olfactory memory at this early processing stage in the antennal lobe. And, after consolidation, octopamine release becomes essential for recall, which suggests that the modulatory circuits become incorporated as essential components of neural representations that activate odor memory (Farooqui et al., 2003).

While it is often assumed that memories are stored and formed in areas where input from disparate regions converges (e.g., after several steps of information processing), information does not flow in one direction in the *Drosophila* or honey bee nervous systems. In fact, neurons from the MBs also give feedback to target PNs in the AL in *Drosophila* (Hu et al., 2010). In honey bees, histochemical and immunocytochemical labeling studies show that the neurotransmitter acetylcholine (ACh) and receptors of acetylcholine (AChR) are present in the MBs (Oleskevich, 1999b). Calcium imaging studies demonstrate that cultured MBs cells respond to ACh via nicotinic AChR; Glutamate immunoreactivity and excitatory responses to glutamate have been reported in the MBs of different insects (Bicker and Kreissl, 1994). Biogenic amines such as dopamine (DA), serotonin, norepinephrine, and octopamine (OA) are also present in the MB. Oleskevich (1999) investigated the synaptic transmission in the MBs of several biogenic amines. Acetylcholine receptor antagonists, D-tubocurarine and alpha-bungarotoxin attenuated the postsynaptic response by 61 and 62% of control, respectively. On the other hand, the glutamate receptor antagonists, (+)-2-amino-5-phosphonopentanoic acid and 6-cyano-7-nitroqui- noxaline-2,3-dione, had no effect. However, the invertebrate monoamine and neuromodulator, octopamine, transiently increased the postsynaptic response by 130% of control (Oleskevich, 1999b). Therefore, it was concluded that the

synaptic transmission of the olfactory input pathway in the MB is mediated by acetylcholine and modulated by octopamine (Oleskevich, 1999b).

LEARNING AND MEMORY: ASSOCIATIVE CONDITIONING IN HONEYBEES BY MEANS OF PER

Throughout evolution, animals express sensory biases that results from phylogenetic, developmental and physiological constraints within their sensory and central nervous systems; these, in turn, are shaped and fined-tuned by natural selection to particular ecological functions (Endler and Basolo, 1998). The coevolution between plants and pollinators results in the modification of preexisting sensory preferences by means of learning (Gegear and Laverty, 2001; Leonard and Masek, 2014b). Receivers have a key role in shaping the communication systems by means of perception and learning (learning-based biases) with adaptive consequences (ten Cate and Rowe, 2007b). Indeed, learning is expected to be adaptive, since is probably a universal property of all or most animals with a nervous system (Dukas and Ratcliffe, 2009). Consequently, a critical function of the brain is to generate predictions of future events, anticipating likely outcomes based on inherited instincts, sensory stimuli and remarkably, in previously learned experiences (Llinás and Roy, 2009). Learning, can be defined as the acquisition of neuronal representations of new information; such information may be kept for a short period (short-term memory) and also for longer durations (long-term memory) and has consequences in behavior and decision making (Dukas and Ratcliffe, 2009). Among the types of learning, elicited behaviors (i.e. habituation and sensitization) constitute the simplest form (i.e. non- associative learning) and are simultaneously the basis for a simple form of long-term memory (implicit, procedural memory); these types of responses constitute reflex reactions to events in the environment and occur in any situation that involves repeated exposure to a stimulus (Domjan, 2015; Kandel et al., 2013). A more complex and versatile form of acquisition of new information involving more than one stimulus is associative learning. Here, the animal learns about the relationship between two stimuli or between a stimulus and a behavior (Kandel et al., 2013). Associative learning entails the capacity to extract the relationships between sequences of orderly events in the environment to perform proper actions in anticipation of future occurrences (Domjan, 2015). Associative learning is a fundamental property of the nervous systems with conserved rules across species and sensory modalities (Matsumoto et al., 2012b).

The simplest form of associative learning is called *classical conditioning.* Here, organisms adjusts to events in their environment that they do not directly control (Domjan, 2015). Animals can learn that a stimulus that is biologically neutral (the conditioned stimulus or CS), which does not originally elicit a behavioral response, act as a predictor of a biologically relevant stimulus which innately evokes a response (the unconditioned stimulus or US) (Matsumoto et al., 2012b; Mota et al., 2011b; Pavlov, 1927). Through this association, the originally neutral stimulus acquires the capacity to elicit a conditioned response (Giurfa and Sandoz, 2012b). In this way, while the CS initially lacks any connection with the response, following a pairing between the CS and the US the animal learn that CS predicts US delivery and starts to respond to CS; the new response elicited by the CS is called conditioned response (CR) (Matsumoto et al., 2012b; Pavlov, 1927).

During classical conditioning, the interactions of two conditioned stimuli (CS) typically are explained around the two related phenomena of blocking and overshadowing (Vandbakk et al., 2020). The former occurs when one previously conditioned stimulus (CS1) is followed by another stimuli (CS2) during the conditioning trials, and as a result C2 fails to become associated on its own. That is, CS1 effectively blocked CS2. To explain this phenomenon, it is thought that the organism has already learned the association between the US and CS1 and adding CS2 do not provide any additional predictive information about the US (Cassaday, 2010; Vandbakk et al., 2020). The related phenomenon of overshadowing occurs when two or more stimuli are presented together during conditioning, and one of the stimuli (usually the more salient or intense one) becomes a much stronger conditioned stimulus (CS) than the others. In this case, the more salient stimulus "overshadows" the conditioning of the less salient one. The overshadowing stimulus captures more of the organism's attention and is a better predictor of the US, leading to stronger conditioning for that stimulus (Cassaday, 2010; Schmajuk, 2010).

The experiments of the present thesis does not directly attempt to answer the occurrence of these phenomena, but instead, I am interested in exploring how the multimodal integration rules (external factors of the stimuli) and the inner neuromodulatory environment of the bees affect such integration. However, these phenomena might occur simultaneously or independently depending on specific circumstances (Schmajuk, 2010). Despite pointing towards different levels of explanation, multimodal integration and phenomena like blocking and overshadowing are not mutually exclusive. In fact, depending on the framework used for analysing levels of explanation (such as Tinbergen’s four questions, Mayr’s two questions, or Marr’s levels of analysis), these phenomena may occupy several levels (Kaplan, 2015; Marr, 1982; Mayr, 1961; Taborsky, 2014). The process of multimodal integration in the brain can influence behavioural phenomena like blocking and overshadowing, and vice versa. Understanding these

interactions can provide a more comprehensive picture of how organisms perceive and learn from their environment.

THE PROBOSCIS EXTENSION RESPONSE (PER)

Classical conditioning in invertebrates such as Crustacea and Cephalopoda was reported from the beginning of the 20th century and during the following fifty years the Pavlovian conditioning protocol was implemented in the honey bee as well (Kuwabara, 1957b; Takeda, 1961b). These pioneering conditioning studies in honeybees discovered that bees restrained in tubes form an association between an olfactory stimulus and a sucrose reward (Menzel, 2001b). This association is the basis of the proboscis extension response (PER) protocol: During PER restrained bees exhibit an unconditioned response (UR) after the contact with a sucrose solution or unconditioned stimulus (US) on the antennae, tarsus or mouthparts; then during the actual conditioning, an odor (the conditioned stimulus, CS) is presented in close temporal proximity with the US. At the end of the training, the CS alone evokes the PER, an indication that the bee has learnt the association between odor and the sucrose solution (Bitterman et al., 1983b; Giurfa, 2003; Giurfa, 2007b; Giurfa and Sandoz, 2012b; Giurfa et al., 2001b; Kuwabara, 1957a; Matsumoto et al., 2012b; Mauelshagen, 1993b; Menzel, 2001b; Menzel and Giurfa, 2001b; Sandoz, 2011b; Takeda, 1961b)

By means of the implementation of the PER protocol several aspects learning has been investigated. First, because of the bees ability to associate natural (e.g. pheromones) and arbitrary odors with sucrose reinforcement, the behavioral and neural basis of olfactory learning have been well studied (Sandoz, 2011b). In terms of olfactory perception, honey bees possess remarkable discriminatory abilities (out of 1816 pairs of odors, bees discriminate more than 95%) and generalize odors (i.e., transfer of information from the learned instructional setting into a different one) useful to measure the perceptual relationships among odors (Giurfa and Sandoz, 2012b; Guerrieri et al., 2005b; Sandoz, 2011b; Sandoz and Menzel, 2001). In general, the associative (reward) memory formation and learning in honey bees follows the general dynamics of memory processing described in other animals (*Aplysia*, *Drosophila*, bumblebee, mouse, chicken, man) with respect to short-term memory, mid-term memory and long-term memory (memory lasting minutes, hours, 1d and longer, respectively) (Menzel 2001a; Riveros & Gronenberg 2009a, 2012). On the contrary, PER conditioning using color stimuli to study visual learning has poorer performance under this study model: it is estimated that only a 40% of bees are capable of learn an association between a color stimulus and a sucrose solution after ~20 training seasons, that is learning is

very slow (Hori et al., 2006b; Jernigan et al., 2014b; Mota et al., 2011c). Indeed, for reasons that still remain unknown, European honey bees can achieve this level of visual learning only after the amputation of the antennae, which in turn eliminates the possibility of studying multimodal learning (Balamurali et al., 2015; Dobrin and Fahrbach, 2012b; Hori et al., 2006b; Jernigan et al., 2014b; Kulahci et al., 2008b; Mota et al., 2011c; Strube-Bloss and Rössler, 2018b).

Certainly, only a few studies have attempted to study visual learning using Africanized honey bees (AHB; feral bees near *Apis mellifera scutellata*) instead of European honey bees (EHB, *Apis mellifera*). Most notably, Jernigan et al (2014) highlight an important difference between EHBs and AHBs: these latter do not require antennal amputation in order to learn visual tasks, which opens the possibility to study bimodal (olfactory and visual) learning and memory. This result also suggests important cognitive differences between these two groups of bees; the mechanisms responsible for such differences remain unknow. More importantly, Jernigan et al (2014) suggest that Africanized *A. mellifera* can be successfully trained to learn colors using the PER protocol and that the learning performance will vary depending on the colors used. Another relevant study validating the possibility of achieving successful PER visual conditioning using antennae-intact foragers of EHB (Dobrin and Fahrbach, 2012b). They attribute their success in achieving visual PER to changes in the timing of the training protocol (reduction in the time interval between trials, and methods of bee restraint) and the detection of a negative relationship between age and visual performance.

Other studies have shown that bees learn visual cues from moving patterns (Hori et al., 2007b) and that motion can improve the performance of visual learning (Balamurali et al., 2015). In addition, recent studies have shown that visual PER conditioning is also possible in bumblebees (*Bombus impatiens*) with intact antennae as well (Riveros and Gronenberg, 2012b). Despite the limitations of the PER protocol, which immobilizes bees and restricts their natural flower approach behaviour, it has been standardized for three decades (Giurfa and Sandoz, 2012a; Matsumoto et al., 2012a; Menzel and Bitterman, 1983). Over time, deviations from the original procedures have emerged, encompassing not only olfactory conditioning but also visual aspects. These adaptations have extended to neuropharmacological studies involving PER conditioning and drug interventions in the bee nervous system. Specifically, the ability to randomly assign different foraging bees to various experimental treatments ensures a homogeneous distribution among treatments, encompassing bees of similar brain developmental states, ages, hormonal conditions, hive roles, and foraging functions.

Due the difficulties to perform visual PER in honeybees, multimodal learning remained mostly out of reach given the traditional training protocol (time interval of visual stimuli), or honeybee strain (EHB instead of

AHB) (Dobrin and Fahrbach, 2012b; Jernigan et al., 2014b). Despite this, some interactions between modalities have been detected. In free flying bumble bees foraging on artificial flowers, multimodal signals were associated with higher accuracy, taking more effective decision when flowers signaled in more than one modality (Kulahci et al., 2008b). Similarly, in another study in bumblebees, a positive relation was found between different levels of multimodal complexity and learning performance (Katzenberger et al., 2013). Moreover, although Mota et al (2011) found that honey bees (EHB) could not form a direct association between color and sucrose (using a traditional PER training protocol), they found that bimodal conditioning of PER is possible using colors as occasion setters (i.e., as a contextual signal) that modulated the conditioned odor; the color served as an occasion setter, for responding or not to the odor (disambiguation). Classical studies employing single cell recordings in the mushroom body and the α-lobes (see below) of the honey bee brain, revealed that although is not possible to classify these cells as responding to a particular modality, most cells within this area responds to more than one modality (Erber, 1978b; Homberg and Erber, 1979b). Finally, in a recent neurophysiological study of the olfactory-visual integration at the neuronal level, a sensory modality convergence was found on mushroom body neurons (see below), by means of extracellular recordings (Strube-Bloss and Rössler, 2018b). The significance of this result is that a neuronal amplification (enhancement) of olfactory and visual inputs when occurring as compounds, is detectable in such neurons.

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THESIS OUTLINE

This thesis comprises three chapters:

First, I aimed to investigate the interactions between sensory modalities and intensities in a learning task. Using Africanized honey bees (*Apis mellifera*), which can be trained to associate olfactory and visual stimuli with a reward. For this, I compared the learning and memory performance of bees under restrained conditions (PER) when exposed to bimodal and unimodal stimuli with varying intensity components. My hypothesis was that bimodal stimulation would enhance learning and memory performance compared to unimodal signals, and we also examined the effects on response latency. Additionally, I explored the effect of intensity on learning performance comparing both unimodal and bimodal stimuli. This allowed me to test the "principle of inverse effectiveness," where lower relative effectiveness of individual components in a multimodal stimulus leads to higher performance when combined.

Second, based on the results from chapter 1, I examined how intensity and temporal factors shape the conditioned responses in honey bees during bimodal learning and memory tasks. Specifically, I investigated the impact of different types of bimodal stimulation on performance and response latency. Stimuli were presented synchronously or asynchronously at varying temporal orders and intensities. My goal was to determine the existence of an optimal temporal window of integration and whether it interact with stimulus intensity. I hypothesized that synchronous bimodal stimuli would result in higher performance compared to asynchronous stimuli, based on predictions of the temporal window of integration. I further investigated how stimulus intensity modulates the effect of temporal synchrony on integration probability. I also analysed the impact of temporal order in asynchronous stimulation and assessed memory retention effects. The study aimed to uncover crossmodal interactions influenced by temporality and intensity during memory recall.

Third, multimodal learning and memory are influenced by both external physical properties of sensory stimuli and the inner neuromodulatory state. While biogenic amines' role in olfactory learning is well-studied, their involvement in visual and multimodal learning is not fully understood. Therefore, my objective was to compare the effects of octopamine (OA) and dopamine (DA) on appetitive olfactory, visual, and bimodal learning in bumble bees (*Bombus impatiens*). I hypothesized that OA would enhance learning and memory across all modalities, based on its role in mediating sucrose reinforcement. On the

other hand, the role of DA in appetitive learning is still being explored, and its impact on associative strength in olfactory, visual, and bimodal learning remains uncertain. A lack of effect would suggest that dopamine does not influence learning and memory formation, while a reduction in associative strength may occur due to its involvement in appetitive learning and decreased sucrose responsiveness. My findings suggest that the dopaminergic system plays a role in modulating appetitive learning, not only in olfactory tasks but also in visual and potentially multimodal learning.

CONTRIBUTIONS OF THE AUTHOR

Oswaldo Gil-Guevara, Hernán A. Bernal and Andre J. Riveros 2022. **Honey bees respond to multimodal stimuli following the principle of inverse effectiveness.** Journal of Experimental Biology (2022) 225, jeb243832. doi: https://doi.org/10.1242/jeb.243832

Oswaldo Gil-Guevara & Andre J. Riveros. 2023 **Stimulus intensity modulates the temporal window of integration during multimodal learning and memory in honey bees**. *Under review*

Oswaldo Gil-Guevara, Andre J. Riveros and Marc A. Seid. 2023. **Complementary roles of octopamine and dopamine during olfactory, visual, and bimodal learning and memory in bumble bees (*bombus impatiens*).** *Under review*.

In addition, all chapters were presented on the following international congresses:

Oswaldo Gil-Guevara and Andre J. Riveros 2020. **The effect of stimulus intensity during multimodal learning in Africanized honey bees. Oral presentation** at the 2020 Virtual Animal Behavior Society Conference July 28-31, 2020.

Oswaldo Gil-Guevara and Andre J. Riveros 2022. **The role of temporal congruency and intensity during multimodal learning in honey bees. Oral presentation** at the 59th annual conference of the Animal Behavior society June 20-23, 2022.

Oswaldo Gil-Guevara, Andre J. Riveros and Marc Seid 2022. Multimodal learning modulation by biogenic amines in bumble bees (*Bombus impatiens*). Poster presentation. **The International Congress of Neuroethology 2022, held in Lisbon, Portugal from 24 to 29 July 2022.**

CHAPTER 1

HONEY BEES RESPOND TO MULTIMODAL STIMULI FOLLOWING THE PRINCIPLE OF INVERSE EFFECTIVENESS

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Keywords: Multimodal integration, Bimodal signals, PER, Associative learning, Cross modal integration, *Apis mellifera*, Absolute conditioning.

Summary statement: Multisensory integration produces a coherent depiction of the environment. We explore the role of stimulus intensity on integration during associative learning through a behavioural approach in honey bees.

ABSTRACT

Multisensory integration is assumed to entail benefits for receivers across multiple ecological contexts. However, signal integration effectiveness is constrained by features of the spatiotemporal and intensity domains. How sensory modalities are integrated during tasks facilitated by learning and memory, like pollination, remains unsolved. Honey bees use olfactory and visual cues during foraging, making them a model to study the use of multimodal signals. Here, we examined the effect of stimulus intensity on both learning and memory performance of bees trained using unimodal or bimodal stimuli. We measured the performance and the latency response across planned discrete levels of stimulus intensity. We employed the conditioning of the Proboscis Extension Response protocol in honey bees using an electromechanical setup allowing us to control simultaneously and precisely olfactory and visual stimuli at different intensities. Our results show that the bimodal enhancement during learning and memory was higher as the intensity decreased when the separate individual components were least effective. Still, this effect was not detectable for the latency of response. Remarkably, these results support the Principle of Inverse Effectiveness, traditionally studied in vertebrates, predicting that multisensory stimuli are more effectively integrated when the best unisensory response is relatively weak. Thus, we argue that the performance of the bees while using a bimodal stimulus depends on the interaction and intensity of its individual components. We further hold that the inclusion of findings across all levels of analysis enriches the traditional understanding of the mechanics and reliance of complex signals in honey bees.

INTRODUCTION

The integration of information from multiple sensory modalities enables a more precise representation of the environment and often increases behavioural performance (Ghosh et al., 2017; Hartline et al., 1978; Meredith and Stein, 1983; Shams and Seitz, 2008; Stein, 1998; Zahar et al., 2008). While multimodal integration occurs across diverse animal taxons and behavioural contexts, the extent of the behavioural enhancements elicited by multimodal signals depends on key physical factors of the compound stimuli (Cappe et al., 2010; Chandrasekaran, 2017; Otto et al., 2013; Stein and Stanford, 2008). Signal intensity is a conspicuous feature of signals that have been rarely evaluated in behavioural contexts where learning a multimodal signal possesses a clear adaptive value. Multisensory integration is especially relevant in tasks requiring learning and memory of signals such as those used in the interactions between pollinators and plants (Kulahci et al., 2008; Leonard and Masek, 2014a). At an adaptive level, the multi-component flower traits (colour, pattern, shape, and scent) may reduce pollinator uncertainty by increasing the amount of information (Chittka and Thomson, 2001; Leonard and Francis, 2017; Leonard et al., 2011b; Leonard et al., 2011c). At the proximate level (a non-mutually exclusive perspective), the physical properties such as spatiotemporal and salience aspects of signals may affect the effectiveness of multimodal signals (Meredith and Stein, 1983; Otto et al., 2013; Riveros et al., 2020; Rubi and Stephens, 2016a; Stein et al., 1988). In particular, deciphering the effects of intensity variation of the multicomponent signal elements and their possible interactions is needed. Despite this, the effect of varying intensity level during multimodal learning is still unclear.

Signal detection and discrimination in noisy backgrounds is a universal problem in sensory processing (Babineau et al., 2007) and different species rely on different strategies to tackle the challenge; the use of multiple modalities is one of such tactics (Wiley, 2006). Multimodal information occurs across a wide range of ecologically relevant tasks (perception, locomotion, communication) (Cappe et al., 2012; Cowan and Fortune, 2007; de Luna et al., 2010; Narins et al., 2003). The omnipresence of multimodal signals fosters the idea that an enhancement of the receiver's performance is the main benefit of integrating multiple sources of information (Kulahci et al., 2008; Partan, 2017; Siddall and Marples, 2008). Here, the senders gain from higher signal conspicuousness and redundancy, and the receivers benefit from enhanced learning and memory, attention, and overall information processing (Akre and Ryan, 2010; Arak and Enquist, 1993; Balkenius and Balkenius, 2016a; Hebets and Papaj, 2005a; Redhead, 2007; Rubi and Stephens, 2016a; Sutherland and Mackintosh, 1971; ten Cate and Rowe, 2007). The cognitive basis of the perception of the diverse floral displays offered by plants is also dependent on the innate and learned

behavioural responses of pollinators (Schiestl and Johnson, 2013b). As a consequence, the increased amount of information derived from multiple floral traits should facilitate discrimination and learning, enhancing foraging efficiency (Kulahci et al., 2008; Leonard et al., 2011b; Leonard et al., 2011c). For a floral diurnal visitor, visual and olfactory elements are the most conspicuous components of the signal and determine the initial contact (Raguso, 2004). It has been suggested that even some nocturnal bees rely on both olfaction and vision to explore flower resources (Liporoni et al., 2020). In short, it has been hypothesized that multimodal signals might boost pollinators' attention towards a particular floral display, due to increased conspicuousness (Leonard et al., 2011c). Therefore, a potential enhancement during learning and memory is also predicted (Leonard and Francis, 2017; Leonard et al., 2011c). Importantly, previous studies have detected the modulation of one modality by the learning of another, suggesting the existence of interactions during the acquisition of bimodal elements, at the neural level (Giurfa, 2003b; Mota et al., 2011; Sandoz, 2011).

Multimodal signals might be more beneficial than unimodal ones, either because they provide a higher amount of information or because they facilitate receiver perception a pair of non-mutually exclusive hypotheses (Rubi and Stephens, 2016b). From the information theory point of view, however, multimodal signals might not necessarily be better than unimodal ones; that is, in some instances, receivers perform equally well facing unimodal or multimodal signals (Rubi and Stephens, 2016a; Wilson et al., 2013). This reinforces the idea in which multimodal signals enable performance enhancements mainly at the signal processing level (Rubi and Stephens, 2016a; Rubi and Stephens, 2016b). Also, recent studies have not found the expected differences in the performance between bimodal and unimodal stimuli (Riveros et al., 2020; Rubi and Stephens, 2016b). To understand how multimodal signals might benefit receivers, a direct comparison between the effects of unimodal and multimodal signals is required. Such comparison should avoid confounding the effects of multiple components with multiple modalities or the inappropriate distinctions between innate and learned responses (Rubi and Stephens, 2016b). It is also necessary to consider the physical properties of the stimuli (i.e., synchrony and intensity level). In addition, bumble bees do not necessarily enhance their performance when trained using bimodal versus unimodal stimuli under restrained conditions (Riveros et al., 2020), which contrast with performance of bees in free flight (Kulahci et al., 2008). However, it is not clear whether the discrepancy derives from differences intrinsic to the methods, such as a/synchrony in presentation of components or perceived variation in olfactory and visual stimuli intensities (Riveros et al., 2020).

The honey bee has been used to study the functional mechanisms of learning and memory (Giurfa, 2003b; MaBouDi et al., 2017; Matsumoto et al., 2012; Takeda, 1961). Most of the attention has historically focused on anatomical, neuronal, and behavioral aspects of unisensory olfaction (Carcaud et al., 2018; MaBouDi et al., 2017; Mauelshagen, 1993; Riveros and Gronenberg, 2012; Sandoz, 2011) or vision (Ehmer and Gronenberg, 2002; Horridge, 2009; Jernigan et al., 2014; Riveros and Gronenberg, 2012). Multisensory integration of bees during learning has received less attention, with some significant accounts (Gerber and Smith, 1998; Kulahci et al., 2008; Leonard et al., 2011b; Leonard et al., 2011c; Leonard et al., 2011a; Mota et al., 2011; Riveros et al., 2020; Strube-Bloss and Rössler, 2018). Although the role of intensity thresholds has been extensively examined both in vision (Avarguès-Weber and Giurfa, 2014; Backhaus, 1991; Chittka, 1992; Hempel De Ibarra et al., 2000; Katzenberger et al., 2013; Neumeyer, 1981; Nouvian and Galizia, 2020) and olfaction (Bhagavan and Smith, 1997; Wright and Smith, 2004; Wright et al., 2002; Wright et al., 2005), the intensity variation during multimodal learning and memory tasks has rarely been directly explored (but see Katzenberger et al., 2013).

Typically, and across contexts, an animal response increases together with the intensity of the stimulus (Bhagavan and Smith, 1997; Gil-Guevara and Amézquita, 2020; Hempel De Ibarra et al., 2000; Mackintosh, 1974; Wright et al., 2005). Similarly, the higher the intensity, the stronger the association between a stimulus and a reward, following associative learning model predictions (Rescorla and Wagner, 1972). In nature, several factors influence the intensity of individual floral components, mainly during signal production and transmission (Bradbury and Vehrencamp, 1998). Importantly, at the perceptual level, the so-called principles of multisensory integration compare the effectiveness of bimodal stimulus relative to its unimodal components (Meredith and Stein, 1983; Otto et al., 2013; Stein and Stanford, 2008; Stein et al., 1988). Unimodal components are effectively integrated (thus, increasing the strength of the multisensory response) when they originate from the same place, occur synchronously and, when the individual unisensory responses are weak due to signal intensity variation (Meredith and Stein, 1986a; Stanford and Stein, 2007; Stein et al., 1988). In field conditions, these physical properties of stimuli (location, timing, and intensity) determine the extent of the integration, may interact with other components of the signal, affecting pollinator performance (Hebets and Papaj, 2005a; Leonard and Masek, 2014a).

To study the effect of intensity levels during multimodal learning, precise control of the stimuli presented to individuals is therefore required, a difficult task when using free-flight protocols where salience and synchrony vary depending on the particular flight pattern (speed, angle, etc.) (Leonard and Masek, 2014a; Riveros et al., 2020; Wright et al., 2009). Alternatively, the conditioning of the proboscis extension

response (PER) protocol (Giurfa and Sandoz, 2012; Matsumoto et al., 2012), where bees are tested under restrained conditions, enables a more precise stimuli delivery (Leonard and Masek, 2014a). The PER is a natural appetitive response where bees extend the proboscis upon sensory stimulation (antennae, tarsi) with a sweet substance (floral nectar) (Bitterman et al., 1983; Takeda, 1961). During a training experiment, the PER is conditioned by pairing the presentation of the unconditioned stimulus (US; sucrose solution) with a conditioned stimulus (CS; here, odour/colour). After several repeated pairings, a response to the CS eliciting a PER in the absence of the US (the CS now serves as a predictor of the US) is considered as a proxy of learning. This protocol has been historically used to examine learning and memory capabilities (Giurfa and Sandoz, 2012).

In this study, we aimed to explore the possible interactions between sensory modalities and intensities during a learning task. We evaluated the learning performance of honey bees under restrained conditions (PER) by comparing the effect of variations in the intensity components between bimodal and unimodal stimuli. We relied on Africanized honey bees (*Apis mellifera scutellata*) as they can be readily trained to both olfactory and visual stimuli (Jernigan et al., 2014). We tested whether an enhancement in learning and memory performance results from bimodal stimulation relative to unimodal signals and whether latency of response is affected. We constructed this hypothesis based on the idea that a compound signal should provide redundant information, eliciting better learning (Mackintosh, 1974). Here, since redundant signals possess multiple components that improve the accuracy of information transmission (Hebets and Papaj, 2005a; Leonard et al., 2011a), and hence, from the receptor point of view, might lead to a stronger association between the compound signal and its message is possible than in the case of unimodal signals (Mackintosh, 1974; Rowe, 1999). We also tested whether increasing intensity enhances learning performance when trained using unimodal (olfactory/visual) and bimodal stimuli. This latter hypothesis allowed us to test a cross-modal phenomenon, known as the "principle of inverse effectiveness" previously detected on some mammals, in which the lower the effectiveness of the individual components of a multimodal stimulus, the higher the relative performance when combined (Holmes, 2009; Otto et al., 2013; Stein and Stanford, 2008). That is, we tested whether a bimodal stimulus comprised of low-intensity units, results in a higher performance relative to those produced by the unimodal stimuli of the same intensity. The principle of inverse effectiveness (PoIE) is part of a set of conceptually simple rules that predict when multisensory integration is physiologically more strong, efficient, or prevalent. These rules suggest that several unimodal stimuli are more likely to be integrated as a single compound when presented from the same location (spatial rule), temporal window (temporal rule) or when the unisensory components are relatively weak (PoIE) (Chandrasekaran, 2017; Guo and Guo, 2005b; Stein and Stanford, 2008). Under

this framework, the PoIE would make behavioural sense if unimodal sensory stimuli are sufficient to solve learning tasks when presented at high intensities, while unimodally, would be insufficient at low intensities, being surpassed by the learning induced by bimodal stimulation at the same intensities (Guo and Guo, 2005b; Stein and Stanford, 2008; Stein et al., 1988). Finally, we test the effect of intensity levels within each modality (olfactory, visual, and bimodal) across intensities. Using the PER conditioning protocol, we examined how the learning ability of bees is affected by intensity within different modalities.

We found that bees trained using a bimodal stimulus did not necessarily exhibit the highest performances. During bimodal learning and memory tasks, the highest enhancement in performance was achieved when the signal components consisted of low-intensity stimuli. However, this relative bimodal enhancement was not observed in bees trained with stimuli of mid and high intensities. Similar trends were followed when evaluating memory retention. Also, we found that the latency of response during bimodal learning was not affected by the variation in stimuli intensity and was only affected by the modality type. Our results suggest that the complex interactions between modalities during multimodal learning can be modulated by the intensity levels.

MATERIALS AND METHODS

Bee collection and maintenance

Africanized honey bees were obtained from hives maintained at the Universidad Nacional de Colombia in Bogota (4.642419 N, -74.081839 W; ≈2600m elevation; annual average climatic conditions: relative humidity: 80-85 %; temperature:14.2°C -19.7°C ±8.4°C). Worker bees leaving the hive were collected (13:00h-16:00h) using a pyramidal translucent acrylic trap (Matsumoto et al., 2012). Then, honeybees were ice anesthetized (Jernigan et al., 2014) and harnessed into custom 3D printed plastic tubes. After recovery, bees were fed *ad libitum* with sucrose-water (50% w/w) and maintained overnight in a polypropylene box with a window that enabled natural illumination (aiming to maintain photoperiod) and humidity stability (58% RH). The next morning, the bees were tested for motivation using the proboscis extension response (PER) elicited by the antennal stimulation with the sucrose solution (50% w/w). Only those individuals responding were included for experiments. At the end of both the training and memory retention tests, all surviving bees were released. To avoid using a bee more than once, we labelled them with a small drop of enamel paint on the dorsal surface of the thorax before release.

Training apparatus

We adapted a training apparatus that allows both precise and automatic delivery of olfactory and visual stimuli (Fig. 1A**)** (Jernigan et al., 2014; Riveros and Gronenberg, 2009a; Riveros et al., 2020). The setup included 12 individual chambers coated with aluminium foil tape to homogenize the reflectance of light emitted from a LED located at the bottom of the compartment (see Fig. 1A). Each chamber was attached to an acrylic rotatory platform (diam.: 0.52 m) and had two openings: one in the front and in one the back, enabling a stream of pumped air to flow through the chamber and access for the experimenter to provide the reward. Since each chamber contained an individual harnessed bee, we trained 12 bees at a time.

For stimuli delivery, a sequence of instructions with different concentrations and intensities was programmed in advance. An *Arduino Uno* microcontroller (v. REV 3 SMD) using custom code implemented in *Arduino* (v. 1.8.7) on a PC running *Processing* software (v. 3.5.3) (Reas and Fry, 2014), read and executed the stimuli sequence. This system controlled the air flow provided by a pipe system connecting: an air pumping device, a set of parallel electronic valves that allowed the air flowing into a set of three parallel glass tubing containing filter paper with a scent. The air flow reached the chamber at a volume of 1.08 L/min (Fluke VT Plus HF gas flow analyser) after mixing with a parallel constant airflow of clean air (0.33 L/min) aimed to reduce the possibility that bees learned the mechanical stimulation by air. Finally, the odour airflow was effectively cleaned out by an air extractor in the back of the chamber before and after each odour stimulation (0.30 L/min) (Fig. 1A). Simultaneously, our system controlled light intensity by automatically varying the electric current reaching the LED. In addition to controlling the stimuli delivery, our software code also allowed recording the time of behavioural events (latency of response, see below) with a built-in synchronized chronometer. Due to the restraining method, the lower portion of the bee eye may have received direct light, while other regions receive diffused light; however, we did not measure light distribution inside the chamber (Jernigan et al., 2014). We applied 10 μL of the molecule solution at the corresponding concentration (see training stimuli, below) to a piece of filter paper (~10 x 4mm) and place it in the respective glass tube of the training device (see Fig. 1A). We replaced the filter paper with the solution concentration after 3 consecutive puffs during the training trials of each training session.

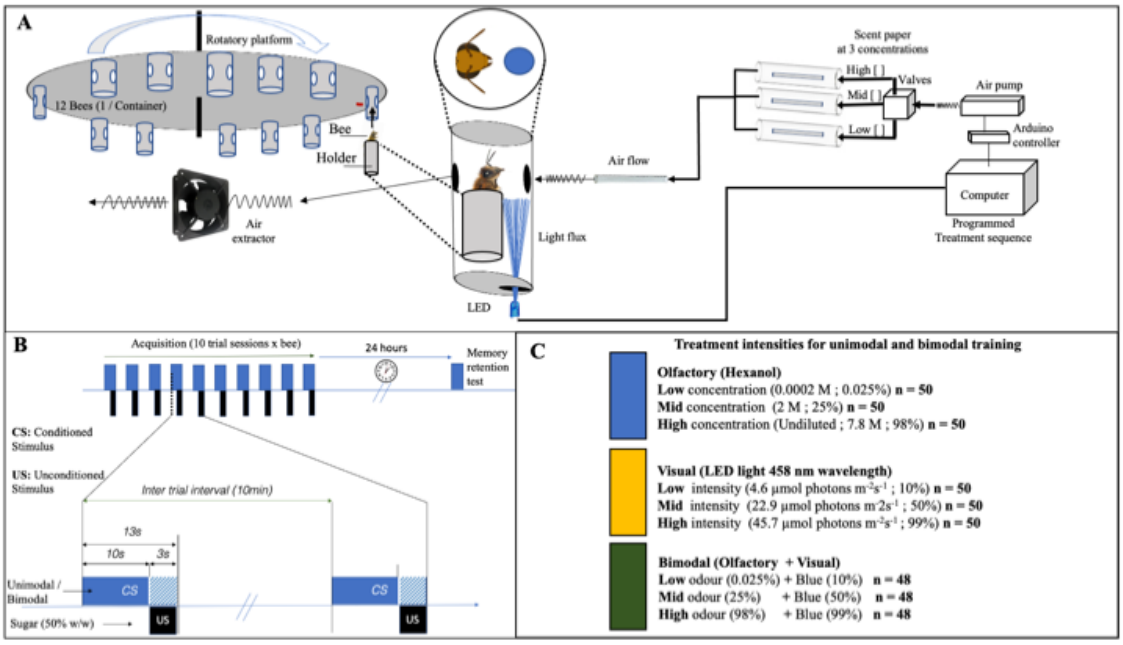

Fig. 1. Conditioning of restrained bees to olfactory, visual, and bimodal stimuli. (A). Scheme of the electromechanical set up to achieve precise control of the light intensity, odour concentration and timing delivery of unimodal and bimodal stimuli. A pre-programmed sequence of Visual and Olfactory stimuli loaded on a PC, implemented custom software to direct an Arduino Uno microcontroller. The instructions triggered a set of electro-valves allowing different concentrations of 1-Hexanol and different intensities of light intensity (LED) to be delivered on individual honeybees. B. A classical conditioning protocol under the Proboscis Extension Reflex (PER) paradigm, allowed the training of 12 bees harnessed on a rotatory platform per session. The experimental procedure for absolute conditioning (Conditioned Stimulus (CS) = unimodal: olfactory or visual; bimodal: olfactory and visual). Bees were conditioned using 10 trials. The conditioning sequence consisted of 10 seconds of stimulation followed by 3s of paired (shaded areas) CS and US (unconditioned stimulus= sucrose solution 50%w/w). Then, an inter-trial interval of ten minutes without stimulation followed for each bee before receiving the following trial. This procedure was repeated until 10 trials were completed for all 12 bees. A memory retention test on the particular stimuli (unimodal or bimodal) was performed 24 h later without the US. Binary behavioural responses (PER) and latency time (s) to PER were registered. C. Schematic depiction of the experimental treatments and the final sample sizes achieved per level of treatment during the experiments.

Training stimuli

To define the minimum low-intensity level, near the threshold for visual and olfactory stimulation in our experimental setup, we trained bees (see training protocol below) using a range of intensity levels (Fig. 2). We defined the minimum level for visual and olfactory learning as the lowest possible magnitude of stimulation that induced a learning performance significantly different from a negative control and from that induced by other higher intensity levels of stimulation. To establish the minimum intensity level for unimodal visual learning, we trained bees to associate a reward (see training protocol below) using light from a monochromatic blue LED (peak λ = 458 nm) with intensities that ranged from 0 to 45.7 µmol photons $m^{-2}s^{-1}$ measured with an LI-COR portable spectroradiometer (model Li-1400, Lincoln, NE, USA). We programmed the automatized system (see above: *Experimental setup*) to deliver five intensities (in µmol photons $m^{-2}s^{-1}$): control (no light), 4.6 (10% of max intensity), 18.3 (40%), 22.9 (50%), 45.7(100%) (Fig. 2).

The median learning score of bees (MLS; see *Calculation of response variables,* below) differed across intensity levels (Kruskal-Wallis $H_{(df = 4)}$ = 33.018 p<0.0001). Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction. The post hoc analysis (Fig. 2, top) showed that the MLS elicited by the lowest light intensity level (4.6 µmol photons $m^{-2}s^{-1}$, 10%, mean rank = 87.85) differed from the medium level (2.9 µmol photons $m^{-2}s^{-1}$; 50%; mean rank = 118.62) (p= 0.035) and from the control (0%; mean rank = 42.19) (p=0.007).

Similarly, to delimit the lower end of olfactory stimulation, we used 1-Hexanol 98% (Sigma-Aldrich #H13303) and obtained five different concentrations that varied between 0 and 98% diluting the pure molecule in mineral oil: control (0%, no odour), 0.0002M (0.025%), 0.02M (2.5%), 2M (25%), 7.8M (98%; undiluted) (Fig. 2, bottom). We found differences in the induced MLS responses across odour concentrations (KW $H_{(4)}$ = 51.825; p<0.0001). In particular, the MSL differed between the low concentration (0.025%; 0.0002M; mean rank = 78.91) and the undiluted condition (98%;7.8M; mean rank = 130.79) (Dunn's, p<0.0001), while the MSL elicited at all concentrations differed from the control concentration (Dunn's, p= 0.0001, Fig. 2, bottom).

After defining minimum intensity levels of visual and olfactory stimulation, we use these intensity levels in subsequent experiments using unimodal (visual or olfactory) stimuli as well as bimodal stimulation (visual + olfactory) that included middle and higher levels of intensity, aimed to represent a range of saliences. The range for unimodal visual stimulation was as follow: *Low* intensity (4.6 µmol photons $m^{-2}s^{-}$

[1],10% of max intensity), *Mid* intensity (22.9 µmol photons $m^{-2}s^{-1}$, 50% of max intensity) and *High* (45.7 µmol photons $m^{-2}s^{-1}$, 100% max intensity). The range for unimodal olfactory stimulation was determined in this fashion: *low concentration* (2x10-4M; 0.025%), *Mid* (2 M; 25%) and *High* (7.8 M; 98%; undiluted). Finally, the bimodal stimulation was provided by the execution of the programmed electronic setup sequence that delivered combinations of simultaneous stimuli of olfactory and visual at *Low* (odor 0.025% + blue light 10%), *Mid* (odor 25% + blue light 50%) and *High* intensities (odor 98% + blue light 100%).

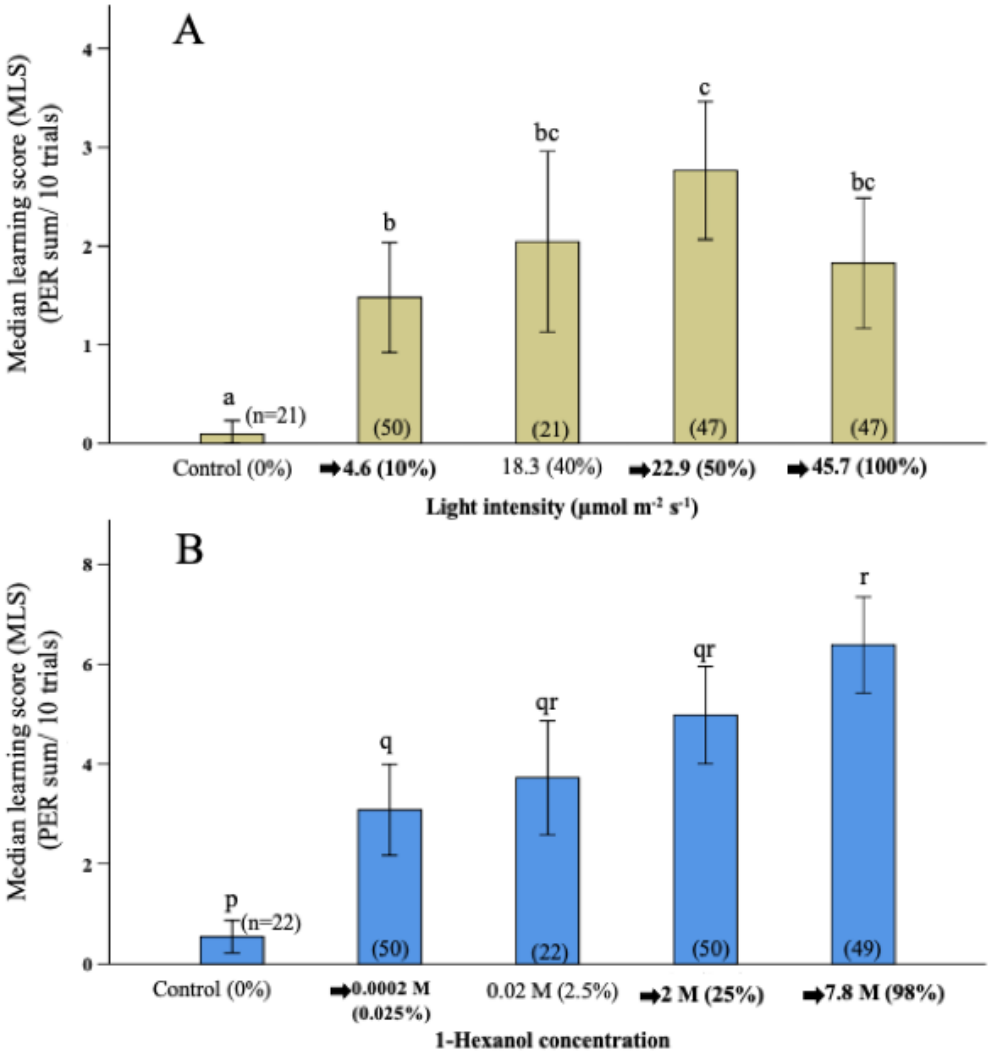

Fig. 2. Minimum thresholds for visual and olfactory learning. (A) Five light intensities were tested (from 0 to 45.7 µmol photos $m^{-2}s^{-1}$, the maximum current supported by the LED). The MLS in response to the lowest intensity tested (4.6 µmol photos $m^{-2}s^{-1}$; 10% of maximum intensity) was significantly different from the intensity medium intensity (22.9 µmol photos $m^{-2}s^{-1}$; 50%) and from the intensity at 0% (see methods). (B) Five concentrations of 1-Hexanol (from 0 to 7.8M, 98%). The MLS elicited in response to 0.025% and 98% were significantly different. The MSL at all concentrations differed significantly from the control concentration (see methods). The number in parenthesis refers to the number of bees examined. Error Bars: 95% CI. The arrows indicate the intensity levels used for subsequent experiments.

Experimental settings

We compared the performance of bees trained to unimodal and bimodal stimuli within each of the three intensity levels. Each experimental bee received a single type of stimulation either unimodal (visual or olfactory) or bimodal at a single intensity level: low, mid, or high. We delivered all combinations of modalities and intensities in a pseudorandom order within each experimental cycle. We applied absolute conditioning where a specific conditioned stimulus (CS+) is associated with a reward. The treatments in our experiment consisted of the application of CS+ using the distinct unimodal (visual/olfactory) or one bimodal (visual + olfactory) stimulus at one of the three possible combinations of intensity levels that we defined.

Training protocol

We used classical conditioning of the PER (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Hori et al., 2006; Matsumoto et al., 2012; Takeda, 1961). We adapted the original protocol as described by (Jernigan et al., 2014). For the acquisition phase, we allowed the bees to acclimate during 15s before starting the training procedure. A pipette holding a small drop of sugar-water (≈1µl; 50% w/w) was sustained within 1 cm near the chamber entrance during the first 10s in which the stimuli (CS+) was delivered. During the following 3s of CS+ stimulation, we paired both the CS and the sucrose reward (unconditioned stimulus, US), by gently touching the tip of the antennae to elicit the PER. We allowed the bee to drink the reward for 3s. Thus, we trained the individuals to associate the CS+ presented for 13s with the US that overlapped for 3s. We waited 15s before turning the rotatory platform to relocate the following bee. Each training trial (15s of acclimation, 13s of stimulation and the final 15s period of post-stimulation) was repeated ten times at intervals of 10 min for each bee (see Fig. 1B). Finally, a memory retention test was conducted after 24 h by exposing the bees to the CS without providing the reward (see Fig. 1B). Individuals were tested for motivation and if a PER was not observed it was discarded from the subsequent experiments. For both acquisition and memory retention, we recorded the PER response and latency time to exhibit PER.

Calculation of response variables

We employed the proboscis extension reflex (PER) directly as a binary dependent variable (1/0) into both the Generalized linear and mixed models (see Statistical analyses, below). In addition, the learning

performance of bees at the group level was measured as the percentage of PERs over ten trials. The latency to eliciting a PER response was measured as the time in seconds between the start of the CS presentation and the beginning of a PER. We computed a median learning score (MLS) of bees of each individual bee as the sum of PERs across trials to summarize the dynamics over trials. Following previous work (Riveros and Gronenberg, 2012; Riveros et al., 2020) we computed the average latency response for bees responding in at least three trials.

Statistical analyses

To explore how the bees' response was affected by the different stimuli modalities and by the manipulation of the intensity, we divided our analyses in two phases: acquisition and memory retention. First, we employed a Generalized Linear Mixed Model (GLMM) to study the effect of Modality (levels in the model = olfactory, visual, and bimodal), Intensity (levels in the model = low, mid, and high), and the interaction between these factors on the PER response (binary) across 10 training Trials during acquisition (10 levels in the model). Then, to study the bees' conditioned PER response during the memory retention test, we used a Generalized linear model (GLM). We carried out both GLMMs and GLMs in R v.4.0.3.(R Core Team, 2020) with binomial error distribution using the glmer() function (Bates et al., 2015). These models permit analyzing binary PER data and, in the case of acquisition, allows to incorporate the training trials as within-subjects factors (repeated measures) as well as between-group comparisons (Harrison et al., 2018; Pirk et al., 2013). We used PER response as dependent factors for the GLMMs and GLMs. In both cases, we introduced Modality (3 levels), Intensity (3 levels) and Trial (10 levels; the repeated measurement component, during acquisition) as independent factors; individual honey bees were included as random factors. We checked on adequate models based on the Akaike Information Criterion (AIC). To test the effect of individual factors, we used χ^2 analysis for both GLMMs and GLMs, using the function "Anova" of the *car*() package (Fox and Weisberg, 2019). Then, to determine where significant effects lay across the different levels of factors, we used the package *emmeans*() to obtain pairwise comparisons (Tukey HSD method with Bonferroni correction), estimated marginal means (EMMS), odds ratios, and predicted probabilities (Lenth, 2017). We also employed GLMMs to test for intensity effects within modalities.

Finally, to study the reaction times of bees during both acquisition and memory retention tests, we employed Two-way ANOVAs. To analyze the reaction time (s) of conditioned PER during acquisition, we obtained the mean latency across the 10 Trials for each individual bee. Therefore, mean latency time was included as dependent variable. Modality, Intensity, and the interaction term *Modality X Intensity* were considered as independent factors, respectively on both analyses.

RESULTS

We collected and prepared for training 680 bees. We excluded individual bees before the onset of the experiment if they failed a motivation test (a PER after approaching the reward to the bee without touching the antennae and preventing it from drinking) or if they exhibited spontaneous responses to the conditioned stimuli. After the exclusion of bees failing these two criteria (total = 232 bees; due to lack of motivation = 223; by a spontaneous response = 9 bees), we conducted the experiments employing 448 individuals. The experiments consisted of nine treatments in a fully factorial design with three modalities: olfactory, visual, and bimodal at three levels of intensity each: low, mid, and high (see methods and Fig 1). We studied the conditioned PER responses of bees across ten trials during the acquisition phase and 24 hours later during the memory retention test.

Effects of intensity and sensory modality on learning acquisition

Bees learned three intensities levels of unimodal (olfactory/visual) and bimodal stimulation, associating them with the reward while showing increasing and differential PER responses across trials (GLMM: *Trial* effect: $\chi^2_{1,444} = 29.506$; $p < 0.0001$; Table S1A, Fig 3). Such differential conditioned associations, measured as changes in the probability of PER responses, significantly depended upon the specific *modality* (GLMM: Modality effect: $\chi^2_{2,444} = 129.508$; $p < 0.0001$; Table S1A) and *intensity* level (GLMM: *intensity* effect: $\chi^2_{2,444} = 6.891$; $p = 0.0305$; Table S1A). In consequence, the learning acquisition of bees was affected by the modality and the intensity interaction (GLMM: *modality*intensity* interaction effect: $\chi^2_{4,444} = 19.263$; $p < 0.0001$; Table S1A).

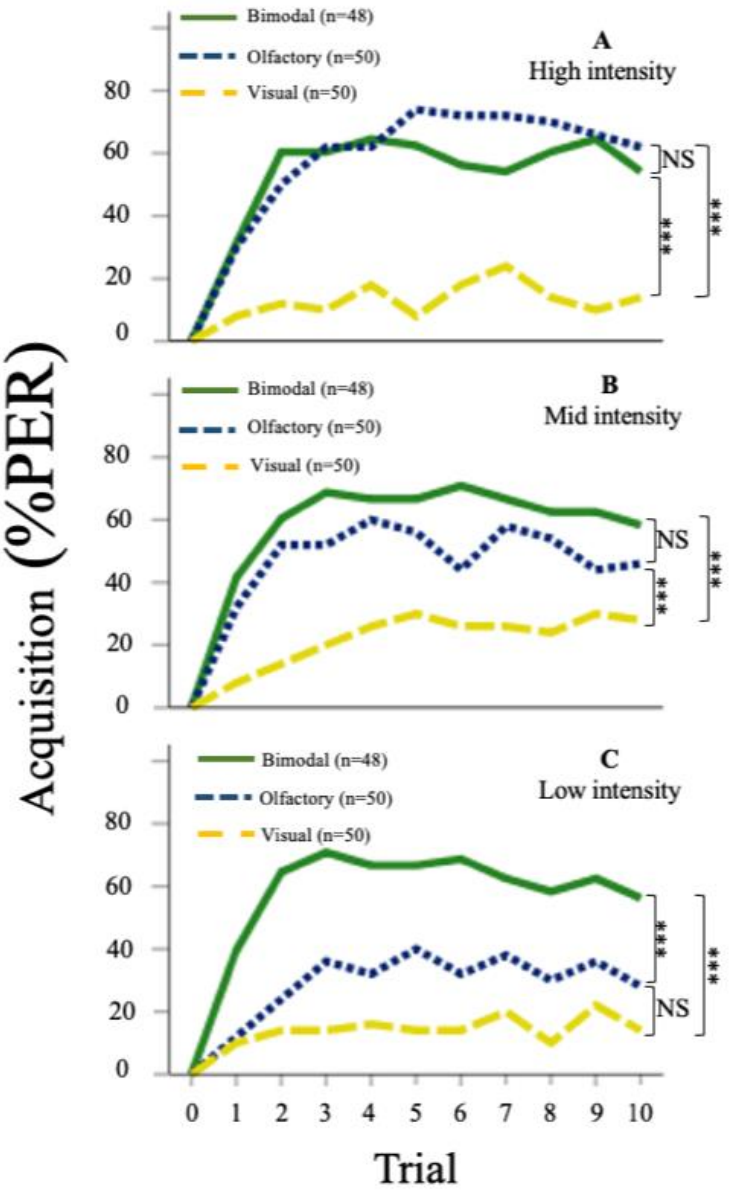

Fig. 3. Learning curves comparing the percentage of proboscis extension response (PER) of honey bees during acquisition. During 10 trials of absolute conditioning, honey bees were trained with one of three different stimuli, either unimodal (olfactory or visual) or a bimodal compound (O+V), at different intensities (A) high, (B) mid, and (C) low, (see methods). Each bee was trained with a single combination of stimuli. The range of visual intensities: low (4.6 µmol photons m-2s-1,10% of max intensity), Mid (22.9 µmol photons m-2s-1, 50% of max intensity) and High (45.7 µmol photons m-2s-1, 100% max intensity). Olfactory stimulation: low concentration (2x10-4M; 0.025%), Mid (2 M; 25%) and High (7.8 M; 98%; undiluted). Bimodal stimulation consisted of combinations of simultaneous stimuli of olfactory and visual at Low (odor 0.025% + blue light 10%), Mid (odor 25% + blue light 50%) and High intensities (odor 98% + blue light 100%). Significance levels are indicated by $p<0.0001$, NS= no significant differences (see results).

At low intensities bees had significantly higher conditioned PER responses when trained using bimodal stimuli compared to unimodal stimuli (post hoc, *low intensity*: olfactory – bimodal, Tukey: z ratio=4.654, $p<0.0001$; visual – bimodal, Tukey: z ratio=6.953, $p<0.0001$; Fig. 3C; Table S2A). There was no difference between unimodal stimuli at low intensities (Tukey: z ratio=2.423, $p=0.0407$; Fig. 3C; Table S2A). At mid and high intensities there was no difference in acquisition performance between bees trained using the unimodal olfactory stimulus conditioning and the bimodal stimulus conditioning (*mid intensity:* olfactory – bimodal, Tukey: z ratio=1.735, $p=1.1923$; Fig. 3B; *high intensity*: olfactory – bimodal, Tukey: z ratio=0.667, $p=0.7824$, Fig. 3A; Table S2A). At mid and high intensities unimodal visual conditioning performance was significantly lower than the other modalities (*mid intensity*: olfactory – visual, Tukey: z ratio=4.073, $p=0.0001$, Fig. 3C, *high intensity:* olfactory – visual, Tukey: z ratio=7.376, $p=0.0001$, Fig. 3A; Table S2A). We display these post hoc contrasts for the GLMM model (Table S2a) as predicted probabilities of PER responses shown in Figure 4A.

We examined the effects of *modality* and *intensity* on the reaction time of bees. The reaction time of bees during acquisition was affected by the type of modality (Two-way ANOVA, $F_{2, 334} = 14.580$, $p<0.0001$) but not by the intensity level (Two-way ANOVA, $F_{2, 334} = 0.538$, $p=0.584$). The reaction times elicited by olfactory and bimodal stimulation were the same (Tukey=0.165, $p=0.766$). However, the visual stimulation produced significantly longer latency times compared to olfactory and bimodal stimulation (Tukey= 1.149, $p<0.0001$; Tukey=1.314; $p<0.0001$, respectively).

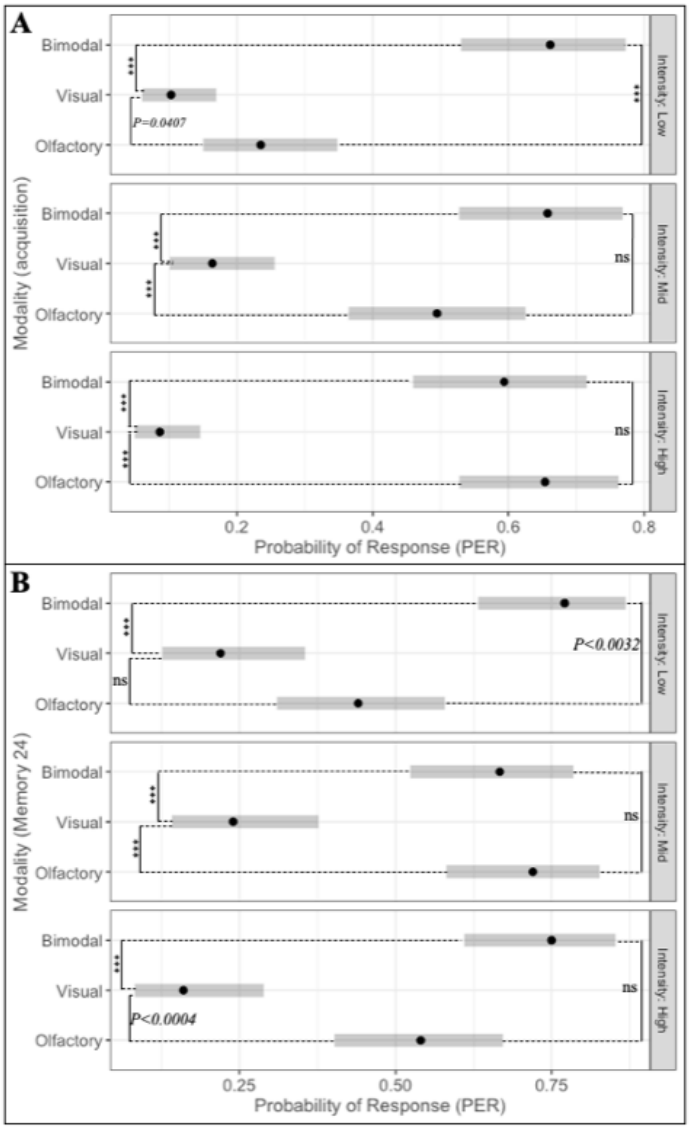

Fig. 4. **Predicted probabilities of PER response for each modality and intensity level during acquisition and memory retention.** (A) During acquisition at low intensities, bimodal stimulation is predicted to produce elevated PER responses, much higher than those induced both by olfaction and visual stimulation. However, at higher intensities, the predicted probabilities induced by olfactory stimulation increase and become indistinguishable from those resulting from bimodal stimulation; at high intensities, the predicted probabilities of a PER response remain low for visual stimulation. (B) During memory retention, the same pattern of predicted probabilities for PER response shown during acquisition remains: the highest enhancements of the PER response in the bimodal relative to unimodal stimulation are produced at low intensities, while at higher intensities such advantage attenuates. The predicted probabilities of PER responses are derived from the post hoc analyses for the GLMM and GLM models for acquisition and memory retention respectively (see results; Table S1 and Table S2) and were obtained after correcting the number of contrasts by the Tukey method (based on EMM pairwise comparisons see Table S2); p values are directly indicated or as *** ($p<0.0001$), ns (non-significant); bars represent the 95% confidence intervals.

Effects of sensory modality and intensity during the memory retention test

During memory retention, the pattern of conditioned response to unrewarded stimuli was very similar to those elicited during the previous acquisition phase. The type of modality stimulus produced a lasting and differential effect on the conditioned response of bees during the memory test (GLM: modality effect: $\chi^2_{2, 444} = 72.2226$; $p<0.00001$; Table S1B). At low intensities, bees showed the greatest relative enhancements in the response. That is, at low intensities, the conditioned PER response to the bimodal compound, was significantly higher compared to both unimodal stimuli (olfactory – bimodal, Tukey: z ratio=3.259, P=0.003; visual – bimodal, Tukey: z ratio=5.119, $p<0.0001$, Table S2B). At low intensities, there was no difference between unimodal stimuli (olfactory – visual, Tukey: z ratio=2.304, $p=0.055$; Table S2B). There was no difference in the bee's response to olfactory and bimodal stimuli at mid and high intensities (*mid*, Tukey: z ratio= 0.572, P=0.835; *high*, Tukey: z ratio=2.143, $p=0.081$; Table S2B). At mid and high intensities, the response to visual stimulation was different to both, unimodal olfactory (olfactory –visual: *mid intensity*, Tukey: z ratio=4.589, $p<0.0001$; *high*, Tukey: z ratio=3.798, $p=0.0004$, Table S2B) and bimodal stimulation (*Mid*, Tukey: z ratio=4.093, $p<0.0001$; *High*, Tukey: z ratio=5.408, $p<0.0001$, Table S2B). The predicted probabilities derived from these post hoc procedures for the GLM model for memory retention (Table S2b) are shown in figure 4B.

The modality explained the general differences in the latency time of bees during the memory retention memory test (Two-way ANOVA, $F_{2, 212} = 5.207$, $p = 0.006$). The response of bees to visual stimuli was slower, compared to olfactory (Tukey=1.92, $p= 0.039$) and bimodal stimuli (Tukey= 1.92, $p= 0.004$). Lastly, the reaction time to olfactory and bimodal stimulation was roughly the same (Tukey=0.44, $p= 0.552$).

Contrasts within modalities across intensities

We also investigated how learning was affected within each modality (visual, olfactory, and bimodal) across the levels of intensities during acquisition (Table S3). Olfactory learning was the only modality significantly affected across levels of intensity (GLMM: Olfactory intensity levels effect: $\chi^2_{2,150} = 21.468$; $p < 0.001$; Trial effect: $\chi^2_{9,150} = 16.527$; $p < 0.0001$; Table S3b). The responses induced by olfactory stimuli of low intensity were different from the learning achieved with olfactory stimuli at both mid and high intensities (Post hoc: low – mid, Tukey: z ratio=-2.923, $p=0.0097$; low – high, Tukey: z ratio=-4.611,

p<0.001; Table S3b), while mid and high intensities was quite similar (Post hoc: mid – high, Tukey: z ratio= -1.698, p=0.2059). The visual learning was not significantly impacted across different levels of intensity (GLMM: Visual intensity levels effect: $\chi^2_{2,150} = 3.7962$; $p = 0.15$; Trial effect: $\chi^2_{9,150} = 10.3532$; $p< 0.001$; Table S3a). Finally, the learning achieved employing bimodal stimulation was unaffected by the different intensity levels (GLMM: Bimodal intensity levels effect: $\chi^2_{2,144} = 0.6618$; $p = 0.718$; Trial effect: $\chi^2_{9,144} = 4.6291$; $p < 0.05$Table S3c).

During memory retention, olfactory learning was again, the only modality impacted by intensity levels (GLMM: olfactory intensity levels effect: $\chi^2_{2,150} = 1.0464$; $p = 0.0189$; Table S4b); Olfactory learning for low and mid intensities were the only significantly different contrasts (Post hoc: low – mid, Tukey: z ratio=-2.792, p=0.0145). Visual learning was unaffected across intensities (GLMM: visual intensity levels effect: $\chi^2_{2,150} = 1.0464$; $p = 0.593$; Table S4a). Lastly, bimodal learning during memory retention test was not impacted differentially across intensities (GLMM: Bimodal intensity levels effect: $\chi^2_{2,150} = 1.4648$; $p = 0.4807$; Table S4c).

DISCUSSION

Our goal was to examine potential interactions between vision and olfaction within bimodal stimuli while inquiring about the role of stimulus intensity during a learning task in harnessed honey bees. We found that relative to its unimodal constituent elements, a multimodal stimulus does not necessarily lead to the highest performance; such difference in the magnitude of learning depends on the intensity of its constituent unimodal elements. Our results suggest that during bimodal learning and memory, the highest relative enhancement in performance is achieved employing unimodal components of low intensity. When employing unisensory stimuli at relatively minimum intensities, and then, after combining them for bimodal conditioning, the bees achieved significantly higher learning performances. However, at higher intensities, the relative advantage of the bimodal condition in learning performance diminished, while simultaneously, the olfactory component underlined a higher performance. This might not be surprising, since, from a pure informational perspective, multimodal signals may not necessarily be more advantageous than unimodal signals (Rubi and Stephens, 2016b; Wilson et al., 2013). At the perceptual level, physical properties of the unimodal components within a bimodal signal interact, thus enhancing or reducing the response and resulting in processing benefits (Stein and Stanford, 2008). Signal intensity may determine those benefits and, hence, it may be important during unimodal and bimodal learning and

memory. The synergistic effects of the unimodal components during bimodal stimulation are relevant, especially at low intensities.

Similar near-threshold situations have been reported in humans and other animals. For instance, the so-called “cocktail party problem” describes a noisy context where visual input may aid in understanding the voice of an interlocutor (Bee, 2015; Kayser et al., 2011). Despite being initially approached as a unimodal phenomenon (i.e., auditory scenes analysis) (Bee, 2015), the cocktail party problem is a well-known scenario that illustrates multisensory integration (Ross et al., 2007a). In essence, the visual information about lip movements enhances the perception of the auditory signal (Kayser et al., 2011). Previous work described a neuronal substrate for an analogous "flower party effect" in honey bees (Strube-Bloss and Rössler, 2018). Likewise, our results support the idea of an interaction between visual and olfactory information that enhances learning and memory at near-threshold intensities, the pattern behind a "flower party effect". On the other hand, our results also agree with a derived prediction of the cocktail party effect: when a single modality is strong enough to surpass the threshold for masking interference (noise), no other additional modality should be required for effective communication. That is, at higher intensities, when unisensory stimuli elicit stronger responses, the processing of two unisensory inputs is more likely to be redundant, thereby reducing the need for multisensory integration (Ross et al., 2007a).

We interpret the results of our experiment with restrained bees, alike to a “flower party effect”, where the multisensory benefits are dependent on the salience of the unimodal components. In real-world situations, under what circumstances a "flower party effect" might be encountered by bees? Typically, flowers emit complex signals (Hebets and Papaj, 2005a), and pollinators tend to find specific plant hosts more efficiently through multimodal signals (Burger et al., 2010; Dötterl et al., 2014). However, during transmission, flowers’ visual and olfactory signals are degraded by several environmental factors (e.g., cloud cover, temperature, humidity, wind, etc.), affecting signal transmission parameters (visual: medium absorption, scattering and filtering; olfactory: distance, wind turbulence) (Bradbury and Vehrencamp, 1998). Therefore, during foraging, bees might experience environmental conditions that influence the conspicuousness of flower multimodal signals. This degradation is also the basis of competing flowering signals, concealing floral displays (Leonard et al., 2011b; Leonard et al., 2011c; Leonard et al., 2011a). Here, the associative learning abilities of bees allows floral constancy – the short-term specialization of pollinators on flower type – depending on the relationship between floral rewards and signals (Schiestl and Johnson, 2013b). Once such floral constancy is established, bees should integrate floral displays, including those near-threshold multimodal signals, to access rewards.

Even when assuming interpretations from information theory where multimodal signals do not offer additional information *per se* (Rubi and Stephens, 2016b; Wilson et al., 2013), the interactions between the intensities of the elements of a composed signal might enhance its detection and / or processing (Hebets and Papaj, 2005a; Leonard et al., 2011a; Solvi et al., 2020) resulting in an improved learning (Katzenberger et al., 2013; Mackintosh, 1974; Rescorla and Wagner, 1972). We suggest that during multimodal learning of harnessed bees, a few combinations of functional interactions (Leonard et al., 2011a; Raguso, 2004) might occur, depending on the intensity of its components. Our finding of a bimodal enhancement in learning performance after combining near-threshold unimodal visual and olfactory stimuli might be considered "synergistic" (Raguso, 2004; Raguso and Willis, 2002), because the combined signal allowed a high bimodal associative learning, while its elements resulted in a low learning performance on their own. On the other hand, although when employing mid and high-intensity levels the relative bimodal advantage decreased, the combined bimodal stimuli still elicited a high learning performance. In addition, at these higher intensities, olfactory stimulation-induced even higher learning performances than the elicited by the bimodal stimuli. Here, the effectiveness of a bimodal signal might be dominated by olfaction alone, while visual stimulation might just be of secondary influence. Therefore, we propose that, at near-threshold intensities, compound signals eliciting a behavioural response deploy synergistic interactions, while only at relatively high intensities, the elements of a multimodal signal might be considered either "complementary" or "redundant" (Leonard et al., 2011a; Raguso, 2004; Raguso and Willis, 2002). Our data, therefore, seems to support a set of explanations within the "efficacy-based hypothesis" (multimodal components increase effective transmission, detection, or signal processing by the receptor) for the establishment of multimodal signals in communication systems in general and in pollination systems in particular (Hebets and Papaj, 2005a; Leonard et al., 2011a). Thus, at low intensities, not only does the idea of a flower party effect fit nicely into the framework where the floral complexity of multimodal signals facilitate detection against background noise (detection-based hypothesis) (Chittka and Spaethe, 2007a; Leonard et al., 2011a) but also a multicomponent signal is beneficial because it might allow a parallel rather than a serial processing (signal-processing hypothesis) (Hebets and Papaj, 2005a; Leonard et al., 2011a).

Indeed, at the neural level, a parallel architecture is characteristic of the mushroom bodies (MB), a region in the insect brain involved in the processing and integration of multimodal information, learning and memory (Ehmer and Gronenberg, 2002; Erber, 1978; Gronenberg, 2001; Homberg and Erber, 1979; Menzel and Giurfa, 2001). In honey bees, MB output neurons exhibit cross-modal integration after

unimodal and bimodal stimulation (Strube-Bloss and Rössler, 2018). These output neurons categorize its responses to visual, olfactory, and bimodal stimuli. Remarkably, a neuronal enhancement of olfactory and visual input was detected when presented as a compound (Strube-Bloss and Rössler, 2018). Our behavioural results might expand this neural circuit perspective towards the modulation of associative learning and memory of bimodal compounds.

Together, these results of cross-modal interactions are related and agree with the narrative of the principles of multimodal integration, postulated after recordings of unimodal and multimodal cells of the superior colliculus of cats (Meredith and Stein, 1983; Meredith and Stein, 1996; Stein and Stanford, 2008). To be effectively integrated by the brain as a multimodal signal, the unimodal elements require some correspondence in the temporal and spatial domains, the first and second "rules", respectively (Otto et al., 2013; Stein and Stanford, 2008). The third principle, termed inverse effectiveness, states that two or more sensory stimuli produce a maximal multisensory response enhancement when the unisensory stimuli are minimally effective in evoking responses (Alvarado et al., 2007; Chandrasekaran, 2017; Holmes, 2009; Stanford and Stein, 2007; Stein and Stanford, 2008). Importantly, when the unisensory stimuli are emitted at high intensities, they evoke stronger responses by themselves, providing redundant information, and reducing the need or the importance of integrating different modalities (Otto et al., 2013; Ross et al., 2007a; Stein and Stanford, 2008). We argue that our results are analogous to this principle. Here, we report that the principle of inverse effectiveness may act during multisensory tasks that involve learning and memory in honey bees.

Our main goal was to compare the learning performance of unimodal (olfactory, visual) and bimodal stimulation within each level of intensity presented (low, mid, and high). Our results shows that the efficacy of bimodal learning performance is relative to its unimodal components' intensity. A secondary comparison of natural interest was to contrast learning within modalities across intensities. Interestingly, only olfactory learning was significantly impacted by intensity when comparing within modalities across intensities, during acquisition and memory retention (see results, Fig 4; Table S1 and S2). Hence, we found that bimodal stimuli are capable of inducing high learning performances across intensity levels (low, mid, or high). Our results indicate that bimodal stimuli retain their associative strength across a wide spectrum of signal-to-noise ratios, from acquisition to memory. Nonetheless, this occurrence with unimodal constituent elements of low salience reflects the modulating effect of intensity during bimodal stimulation, similar to the findings of research in vertebrate systems (Meredith and Stein, 1983; Meredith and Stein, 1996; Stein et al., 1988).

Our results also confirm that the efficacy of olfactory stimulation is significantly impacted by intensity level. This is consistent with previous work showing that odorant intensity correlates with improved performance during learning and discrimination tasks, due to the codification strategy of the olfactory system (Leonard and Masek, 2014a; Wright and Smith, 2004; Wright et al., 2009). In contrast to olfactory learning, the influence of visual stimulation remained both low and mostly unaffected across intensity (see results, Fig 4). The reaction pattern of bees shows that visual stimulation at low and high intensities tends to induce an even lower response, relative to visual stimulation at mid intensities. In general, this pattern appears consistent with the threshold test for visual learning (Fig 2) and also during acquisition and memory retention (Figs 3 and 4). Such response patterns were presented at a higher variance during memory (Fig 4B), which might be interpreted as a consequence of an overall weakening of the association strength. As in many other visual systems, the visual performance in honey bees declines at low light intensity (Warrant et al., 1996); despite this, the responses of trained bees also decline at high intensities (Menzel, 1981; Rose and Menzel, 1981). This bright light effect is explained as the result of the specific response function of the lamina monopolar cells (Menzel, 1981). Our results might be, therefore, consistent with these accounts where the visual perception of bees is less acute at both, low and high-intensity levels (the dim and bright light effects, respectively). We argue that the synergistic interaction between visual and olfactory stimulation is modulated by its intensity and turns out to be critical in shaping the relative associative strength of the bimodal stimulus. Our results highlight this when both visual and olfactory low-intensity stimuli are combined, merging into bimodal stimuli of high associative strength despite their low intensity (Figs 3 and 4). When unimodal components of higher intensities are combined, olfaction takes a leading role in the bimodal condition.

Assessing the effect of the intensity of the elements that compose stimuli during multimodal learning might explain some discrepancies regarding the beneficial nature of multimodal signals. Our results suggest that the benefits of multimodal stimuli might depend on the intensity of the unimodal components. Therefore, multimodal signals are not necessarily better than unimodal ones. Consequently, the intensity of the individual elements should be considered when examining the effectiveness of a multimodal signal in producing synergistic effects during processing even after recognizing intrinsic differences between free-flight and restrained methods (Jernigan et al., 2014; Kulahci et al., 2008; Leonard and Masek, 2014a; Leonard et al., 2011c; Leonard et al., 2011a; Riveros et al., 2020).

Finally, a speed up of responses is predicted to be one the advantages provided be multimodal stimuli (Hebets and Papaj, 2005a; Leonard and Masek, 2014a; Leonard et al., 2011a). Moreover, physiological evidence in mammals and computational models point to benefits in reaction times as a multimodal effect (Chandrasekaran et al., 2011; Colonius and Diederich, 2017). Despite this, we found that reaction times did not conform with the principle of inverse effectiveness, nor did we found a consistent reduction in latency times after employing bimodal stimuli, as shown previously (Riveros et al., 2020). However, the acceleration in response time is predicted to occur only when the elements of a multimodal stimulus elicit similar performance levels; that is, the speedup of the latency should be largest after controlling the salience of unimodal stimuli, ensuring the induction of similar effectiveness (Otto et al., 2013). Further research may address the speedup of PER reactions during the multimodal learning task, controlling for unisensory stimuli leading to equivalent performance.

Conclusion

In conclusion, our data suggest that the performance benefits associated with the use of a bimodal signal during learning and memory tasks are dependent on the interaction of its components with their intensity. Specifically, visual, and olfactory stimuli that independently elicit low performances, when bimodally combined, produce a significant enhancement during both acquisition and memory. This, together with the finding that such magnitude of bimodal enhancements was not present at mid and high intensities, suggest that honey bees integrate bimodal information following the principle of inverse effectiveness during learning and memory. Such integration relies upon neuronal computations occurring when visual and olfactory inputs appear as a compound. The intensity modulation of the components of a bimodal signal would enable honey bees to acquire, retain and respond effectively to changing environmental conditions where bimodal processing is not always the most efficient way to gather useful information. Therefore, the benefits for receivers derived from the integration of multimodal signals result from a fine-tuned relationship between perception mechanisms, cognitive bias and changing physical conditions across environmental contexts.

Acknowledgments

We deeply thank Rodulfo Ospina and all members of LABUN at the Universidad Nacional de Colombia for honey bee colony management and for granting us access to them. We thank Marisol Amaya-Marquez for facilitating research space at her laboratory of the ICN of the Universidad Nacional de Colombia. We also thank all members of the CANNON research group at the Universidad del Rosario; we especially appreciate the contributions of Juan Pablo Hernandez and Juan Manuel Posada during the light intensity

calibration for the experimental setup. We also thank Wulfila Gronenberg, Christopher Jernigan and two anonymous reviewers whose comments substantially improved the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: O.G.G., A.J.R.; Methodology: O.G.G., H.A.B., A.J.R.; Software: O.G.G.; H.A.B.; Statistical Analyses: O.G.G., A.J.R.; Formal analysis: O.G.G; Data curation: O.G.G.; Investigation: O.G.G.; Resources: O.G.G., H.A.B., A.J.R.; Writing-original draft: O.G.G.; Writing-review and editing: O.G.G., A.J.R.; Visualization: O.G.G.; Supervision: O.G.G., A.J.R.; Funding Acquisition: O.G.G., A.J.R.

Funding

This project was funded in part by the National Grant (Colciencias-Colfuturo No 727-2015) of Ministerio de Ciencia, Tecnología e Innovación (Minciencias) de la República de Colombia, granted to O.G.G. It was also supported by the Animal Behavior Society through the Student Research Grant funding (ABS Developing Nations Award 2020), awarded to O.G.G. Additional support was provided by the Big Grant from Dirección de Investigación e Innovación (Universidad del Rosario) to A.J.R.

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Supplementary information for Chapter 1

Honey bees respond to multimodal stimuli following the Principle of Inverse Effectiveness (Journal of Experimental Biology (2022) 225, jeb243832. doi:10.1242/jeb.243832)

a) GLMM Acquisition Model

=PER response ~ Modality X Intensity + Trial + (1 | individual honeybee)

Coefficients	**Estimate**	**SE**	**z val.**	***p***	
Intercept	-1.591	0.292	-5.453	<0.0001	
Visual	-0.989	0.408	-2.423	0.015	
Bimodal	1.850	0.398	4.654	<0.0001	
Mid intensity	1.157	0.391	2.957	<0.001	
High intensity	1.816	0.389	4.667	<0.0001	
Trial	0.075	0.014	5.432	<0.0001	
Visual *X* Mid intensity	-0.618	0.568	-1.089	0.276	
Bimodal *X* Mid intensity	-1.175	0.556	-2.113	0.035	
Visual *X* High intensity	-2.010	0.576	-3.490	<0.0001	
Bimodal *X* High intensity	-2.107	0.554	-3.804	<0.0001	
b) GLM Memory retention Model =					
=PER response ~ Modality X Intensity					
Intercept	-0.2412	0.2849	-0.846	0.3973	
Visual	-1.0245	0.4447	-2.304	0.0212	
Bimodal	1.4542	0.4462	3.259	0.0011	
Mid intensity	1.1856	0.4247	2.792	0.0052	
High intensity	0.4015	0.4021	0.999	0.3180	
Visual *X* Mid intensity	-1.0726	0.6376	-1.682	0.0925	.
Bimodal *X* Mid intensity	-1.7055	0.6261	-2.724	0.0065	
Visual *X* High intensity	-0.7941	0.6535	-1.215	0.2243	
Bimodal *X* High intensity	-0.5159	0.6251	-0.825	0.4092	

Table S1. A) Summary of the binomial GLMM model for the PER in response to unimodal (olfactory / visual) and bimodal stimuli that varied in intensity (low, mid high) across 10 trials during learning (Model fit: link function (logit), conditional R^2= 0.58, AIC = 4446.34, ICC = 0.48, individual honeybees denoted as random effects); **B)** summarizes the binomial GLM model during the a single memory retention test, 24h later (Model fit: χ^2 (8) = 100.09, p = 0.00, link function (logit); conditional R^2= 0.27, AIC =533.41). For both models, N = 444. Significance values are indicated.

A)		PER Acquisition			
Intensity	**Modality contrast**	**Odds ratio (SE)**	**Estimate (SE)**	**Z. ratio**	***P***
Low	Olfactory - Visual	2.69 (1.10)	0.989 (0.408)	2.423	0.0407
	Bimodal - Olfactory	**6.36** (2.53)	1.85 (0.398)	4.654	<0.0001
	Bimodal - Visual	**17.1** (6.98)	2.839 (0.408)	6.953	<0.0001
Mid	Olfactory - Visual	**4.99** (1.97)	1.607 (0.395)	4.073	<0.0001
	Bimodal - Olfactory	1.96 (0.76)	0.675 (0.389)	1.735	0.1923
	Bimodal - Visual	**9.8** (3.90)	2.282 (0.398)	5.729	<0.0001
High	Olfactory - Visual	**20.07** (8.16)	2.999 (0.407)	7.376	<0.0001
	Olfactory - Bimodal	1.29 (0.50)	0.257 (0.386)	0.667	0.7824
	Bimodal - Visual	**15.52** (6.38)	2.742 (0.411)	6.664	<0.0001
B)		**PER 24h Retention**			
Low	Olfactory - Visual	2.79 (1.24)	1.025 (0.445)	2.304	0.055
	Bimodal - Olfactory	**4.28** (1.91)	1.454 (0.446)	3.259	0.003
	Bimodal - Visual	**11.93** (5.77)	2.479 (0.484)	5.119	<0.0001
Mid	Olfactory - Visual	**8.14** (3.72)	2.097 (0.457)	4.589	<0.0001
	Olfactory -Bimodal	1.29 (0.56)	0.251 (0.439)	0.572	0.835
	Bimodal - Visual	**6.33** (2.86)	1.846 (0.451)	4.093	0.0001
High	Olfactory - Visual	**6.16** (2.95)	1.819 (0.479)	3.798	0.0004
	Bimodal - Olfactory	2.56 (1.12)	0.938 (0.438)	2.143	0.081
	Bimodal - Visual	**15.75** (8.03)	2.757 (0.51)	5.408	<0.0001

Table S2. Summary of pairwise comparisons contrasting the Modalities (Olfactory, Visual and Bimodal) across Intensities (Low, Mid and High) for **A)** the GLMM model for the bee's PER response during acquisition and **B)** after the GLM model for the bee's PER response during the memory retention phase. For both models, interaction contrasts of model fixed factors. Results are given on the logit (not the response) scale. Confidence level used: 0.95. Results given on the log odds ratio scale; *p* values obtained using the Tukey HSD method.

a) GLMM Model comparing Visual intensities during acquisition

=PER response ~ Visual low + Visual mid+ Visual high + Trial + (1 | individual honeybee)

Coefficients	**Estimate**	**SE**	**z val.**	***p***
Intercept (Visual low)	-2.626	0.323	-8.136	<0.0001
Visual mid	0.543	0.386	1.407	0.160
Visual high	-0.183	0.399	-0.459	0.646
Trial	0.088	0.027	3.218	<0.001
b) GLMM Model comparing Olfactory intensities during acquisition				
=PER response ~ Olfactory low + Olfactory mid + Olfactory high + Trial + (1 | individual honeybee)				
Intercept (Olfactory low)	-1.695	0.315	-5.382	<0.0001
Olfactory mid	1.170	0.400	2.923	<0.01
Olfactory high	1.835	0.398	4.611	<0.0001
Trial	0.091	0.022	4.065	<0.0001
c) GLMM Model comparing Bimodal intensities during acquisition				
=PER response ~ Bimodal low + Bimodal mid + Bimodal high +Trial +(1|individual honeybee)				
Intercept (Bimodal low)	0.405	0.313	1.291	0.197
Bimodal mid	-0.020	0.405	-0.050	0.960
Bimodal high	-0.293	0.404	-0.725	0.469
Trial	0.049	0.023	2.152	0.031

Table S3. Summary of the GLMMs models comparing the effect on the PER performance between intensities (low, mid, and high) within each modality of stimulation (olfactory, visual, and bimodal) during the acquisition phase of the experiments.

a) GLMM Model comparing Visual intensities during memory retention

=PER response ~ Visual low + Visual mid+ Visual high + (1 | individual honeybee)

Coefficients	**Estimate**	**SE**	**z val.**	***p***
Intercept (Visual low)	-1.266	0.341	-3.708	<0.001
Visual mid	0.113	0.476	0.238	0.812
Visual high	-0.393	0.515	-0.762	0.446
b) GLMM Model comparing Olfactory intensities during memory retention =PER response ~ Olfactory low + Olfactory mid + Olfactory high + (1 | individual honeybee)				
Intercept (Olfactory low)	-0.241	0.285	-0.846	0.397
Olfactory mid	1.186	0.425	2.792	0.005
Olfactory high	0.402	0.402	0.999	0.318
c) GLMM Model comparing Bimodal intensities during memory retention =PER response ~ Bimodal low + Bimodal mid + Bimodal high + (1|individual honeybee)				
Intercept (Bimodal low)	1.213	0.343	3.532	<0.001
Bimodal mid	-0.520	0.460	-1.130	0.258
Bimodal high	-0.114	0.479	-0.239	0.811

Table S4. GLMM model comparing within modalities and across intensities during the memory retention test.

CHAPTER 2

STIMULUS INTENSITY MODULATES THE TEMPORAL WINDOW OF INTEGRATION DURING MULTIMODAL LEARNING AND MEMORY IN HONEY BEES

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Keywords: Multimodal integration, PER, synchrony, asynchrony, stimulus intensity, temporal order, PoIE.

ABSTRACT

Multimodal integration is a core neural process with a keen relevance during ecological tasks requiring learning and memory, such as foraging. The benefits of learning multimodal signals demand solving whether they come from a single event. This challenge presumably depends on the timing and intensity of the stimuli. Here, we aimed to analyse how variations in temporal synchrony and contrasting intensities interacted to affect bimodal learning. We were also interested in the acquisition of the separate components within the stimuli. We used an electromechanical adaptation of the conditioning of the proboscis extension response protocol (PER) to train honey bees to an appetitive learning task with bimodal stimuli precisely controlled. Thus, bees were trained to different synchronicity and intensity levels. We found that synchronicity, temporal order, and intensity significantly impacted the probability of exhibiting conditioned PER responses and the latency of the conditioned responses. At low intensities, synchronous bimodal inputs produced maximal multisensory enhancement, while asynchronous temporal orders led to differential PER reactions. At high intensities, the relative advantage of the synchronous stimulation diminished, and asynchronous stimuli produced comparable performances. Also, memory retention of the olfactory component and bimodal stimuli was higher than that of the visual component, regardless of the temporal configuration used during training. Our results highlight the importance of the interaction between synchrony and intensity during a learning task involving a bimodal stimulus, particularly at low intensities. Also, these results lead to questions regarding the impact of these interaction in more realistic scenarios during foraging.

INTRODUCTION

Multisensory integration is critical for adaptive sensory processing in all animals. An optimal timing of information flow is crucial for multimodal associative learning and memory tasks (Alais and Cass, 2010; Ghosh et al., 2017; Goyret et al., 2007; Kim et al., 2008; Meredith and Stein, 1983; Meredith and Stein, 1986b; Meredith and Stein, 1986a; Paton and Buonomano, 2018; Shams and Seitz, 2008). Multisensory integration may enhance the accuracy and speed of reactions and more informed decision-making across ecological contexts (Buchholz et al., 2012; Cappe et al., 2012; Chandrasekaran, 2017; Chandrasekaran et al., 2019; Chittka and Spaethe, 2007b; de Luna et al., 2010; Harrap et al., 2019; Kulahci et al., 2008; Leonard and Masek, 2014a; Narins et al., 2003; Siddall and Marples, 2008). Such effects might be due to higher information content and/or facilitated signal processing and integration (Chandrasekaran, 2017; Ma and Pouget, 2008; Otto et al., 2013; Rubi and Stephens, 2016b). Such benefits have been shown during learning tasks across diverse taxa from invertebrates to humans (Gil-Guevara et al., 2022; Shams and Seitz, 2008; Siddall and Marples, 2008; Zhu et al., 2021).

The effectiveness of multisensory signals might be influenced by the fulfilment of three principles: spatial rule, temporal rule, and inverse effectiveness (principle of inverse effectiveness; PoIE), resulting in maximal enhancements in response to multimodal signals arriving from similar locations, timing, and low-intensity stimuli, respectively (Meredith and Stein, 1983; Meredith and Stein, 1986a; Stein and Stanford, 2008). For instance, the PoIE predicts that when the signal strength of a unimodal stimulus is low, it will only elicit a weak unisensory response. Conversely, when these unisensory stimuli are presented together, the lowest intensity of the multimodal stimulus leads to an increase, rather than a decrease in multisensory integration (Chandrasekaran, 2017; Holmes, 2009; Ma and Pouget, 2008; Meredith and Stein, 1983; Otto et al., 2013; Stein et al., 1988). Furthermore, the temporal rule suggests that animal brains are relatively insensitive to lags between unimodal signals presented within a “window of temporal integration”. This ability would allow for maintaining temporal coherence despite delays generated due to different physical properties across sensory modalities. Therefore, all information falling within this hypothetical window might be assigned to the same event (Colonius and Diederich, 2004; Colonius and Diederich, 2017; Keetels and Vroomen, 2012).

Despite the extensive study of the neuronal (Balkenius and Balkenius, 2016b; Chandrasekaran, 2017; Meredith and Stein, 1983; Meredith and Stein, 1986b; Rowland and Stein, 2008; Stein and Stanford, 2008;

Strube-Bloss and Rössler, 2018) and behavioural processes involved (de Luna et al., 2010; Gil-Guevara et al., 2022; Ma and Pouget, 2008; Moller, 2002; Otto et al., 2013; Riveros et al., 2020; Stein et al., 1988), the interaction between time, space, and intensity level in multisensory integration has been given less consideration, especially within the context of learning and memory. Indeed, the stimulus-related factors of the multisensory process have traditionally treated the influences of time, space, and intensity independently (Krueger Fister et al., 2016) despite the high potential for interactions among intensity and synchronicity. It is still mostly unclear how the interaction between intensity and timing affects learning and recall of multicomponent signals despite disparities in arrival times of different sensory modalities (Sarko et al., 2012; Spence and Squire, 2003). The study of audio-visual integration during perceptual tasks in vertebrates showcases the importance of the intensity and temporal dynamics involving behavioural, electrophysiological, and computational approaches (Briscoe, 2016; Calvert, 2001; Chandrasekaran, 2017; Colonius and Diederich, 2004; Colonius and Diederich, 2017; Gingras et al., 2009; Ma and Pouget, 2008; Meredith and Stein, 1983; Ohki et al., 2016; Otto et al., 2013; Stein and Meredith, 1993; Stein and Stanford, 2008). A typical example is the study of the role of audio-visual interactions during the recognition (Chandrasekaran et al., 2019; Doesburg et al., 2008; Kajikawa et al., 2012) and speech (Ohki et al., 2016; Ross et al., 2007b). These include the analysis of crossmodal illusions like the McGurk effect, where visual input affects auditory perception (Briscoe, 2016; Doesburg et al., 2008; Mcgurk and Macdonald, 1976). Another focus of research is the 'cocktail party problem', consisting in understanding speech in noisy settings with multiple people talking and highlighting the unimodal challenge of isolating speech from other sounds (Bee, 2015; Bronkhorst, 2015; Kayser et al., 2011). Simultaneously, this problem has been approached via multisensory integration, where visual information about lip motion can improve the perception of vocal signals (Kayser et al., 2011; Ross et al., 2007b).

Most studies on multimodal integration have focused on vertebrate models, but a few exceptions exist among invertebrates (Hatt and Bauer, 1980; Olberg and Willis, 1990b). The interactions between flowering plants and pollinators provide a clear example of how multisensory benefits can occur (Giurfa, 2003b; Giurfa, 2022; Leonard and Masek, 2014a; Leonard et al., 2011b; Raguso, 2001; Raguso, 2004). These offer a relevant context to investigate the multimodal phenomena in a comprehensive way from an adaptive, behavioural, cognitive, and neurophysiological point of view (Kropf and Rössler, 2018; Raguso, 2001; Raguso, 2004; Rubi and Stephens, 2016b; Rubi and Stephens, 2016a; Strube-Bloss and Rössler, 2018; Strube-Bloss et al., 2011; Szyszka et al., 2008). Despite some exceptions, floral displays tend to be primarily multimodal. Flower trait variation in colour, pattern, shape, and scent can act alone or together to signal and attract animal pollinators (Gegear and Laverty, 2001; Leonard and Masek, 2014a; Leonard

et al., 2011b; Leonard et al., 2011a). The cognitive basis of pollinator attraction to floral signals depends on both innate behavioural responses and on learning, as well as on interactions between these factors (Schiestl and Johnson, 2013c). Traditionally, bees learning and recalling abilities have been studied from the point of view of unisensory olfaction (Carcaud et al., 2018; MaBouDi et al., 2017; Mauelshagen, 1993; Riveros and Gronenberg, 2012; Sandoz, 2011) or visual (Ehmer and Gronenberg, 2002; Horridge, 2009; Jernigan et al., 2014; Riveros and Gronenberg, 2012). Despite the unimodal emphasis, the question of how bees integrate multimodal floral stimuli has also received attention. In real environments, several factors (e.g., light and wind conditions; flower approach pattern) might influence saliency, sequence, and degree of overlap between visual and olfactory stimuli, making the timing and ordering of scent and colour a basic feature of bees' foraging routines (Gerber and Smith, 1998; Kulahci et al., 2008; Leonard and Masek, 2014a; Leonard et al., 2011a; Leonard et al., 2011c; Leonard et al., 2011b; Mota et al., 2011; Raguso, 2001; Raguso, 2004; Riveros et al., 2020; Strube-Bloss and Rössler, 2018; Wright et al., 2009).

In multimodal experiments employing retrained honey bees, we recently obtained behavioural evidence consistent with the PoIE for learning and memory. When comparing unimodal and bimodal stimulation, we found the greatest response enhancements when the multimodal components consisted of low-intensity stimulation (Gil-Guevara et al., 2022). Moreover, when contrasting the effect of synchrony and structure of bimodal signals on bumble bees it was found that a visual component followed by an overlapping, but asynchronous olfactory stimuli was the most effective bimodal structure (Riveros, 2023). This latter supports the relevance of the level of synchrony for foraging bees, further evidencing that the effect of synchrony is dependent on the order in which the components are presented (Riveros, 2023; Riveros et al., 2020). Despite this, several aspects of the temporal dynamics of the processing and perceptual properties of multimodal signals remain to be addressed (Chittka and Spaethe, 2007a; Leonard and Masek, 2014a). One of such areas of relevance is the understanding of the possible interactions between the temporal and the inverse effectiveness principles of multimodal integration during learning and memory tasks.

It is conceivable an interaction between the effects of inverse effectiveness and a temporal window for multimodal integration in insects, inferred from both olfactory and visual neuronal pathways (Kropf et al., 2014; Paulk et al., 2008; Strube-Bloss et al., 2016; Szyszka et al., 2008). The various modalities need to interact following unimodal processing to create a unified perception of the surroundings, occurring at high-order multimodal integration centres, the insect mushroom bodies (MBs) (Gronenberg, 2001; Rybak and Menzel, 1993; Strausfeld, 2002). In honey bees as in other insects (Balkenius and Balkenius, 2016c;

Guo and Guo, 2005b), the MBs are a brain region implicated in the processing and integration of multimodal information and in learning and memory (Ehmer and Gronenberg, 2002; Erber, 1978; Gronenberg, 2001; Homberg and Erber, 1979; Menzel and Giurfa, 2001). Moreover, electrophysiological evidence in honey bees indicates convergence of visual and olfactory pathways where MB output neurons exhibiting cross-modal integration after unimodal and bimodal stimulation (Strube-Bloss and Rössler, 2018). Notably, previous work on multimodal stimulation showed neuronal and behavioural enhancements consistent with the PoIE, even underling a "flower party effect" in honey bees (Chandrasekaran, 2017; Gil-Guevara et al., 2022; Stein and Stanford, 2008; Strube-Bloss and Rössler, 2018). Besides, some temporal effects have been uncovered both via electrophysiological recordings in the antennal lobe and MB of the moth *Manduca sexta* (Balkenius and Balkenius, 2016b) and more recently, at the behavioural level in bumble bees (Riveros, 2023). Nonetheless, an inquiry into the possible interaction effects between intensity and temporal window principles during multimodal learning tasks is still lacking.

Here, we explore whether inverse and temporal principles of multimodal integration interact to shape a conditioned response during learning and memory tasks. To investigate this, we require an alternative method to free-flight protocols that can precisely control the timing and intensity of synchronous and asynchronous bimodal stimuli (Leonard and Masek, 2014a; Riveros et al., 2020; Wright et al., 2009). The conditioning of the proboscis extension response (PER) protocol (Giurfa and Sandoz, 2012; Matsumoto et al., 2012), offers such an alternative since bees are tested under restrained conditions allowing a precise stimuli delivery. The PER protocol exploits the natural appetitive response of bees towards floral nectar (Bitterman et al., 1983; Takeda, 1961) which constitutes the unconditioned stimulus (US; sucrose solution). After successive pairings of the US with a conditioned stimulus (CS, e.g., scent and/or colour) a response may occur as a proxy of learning if a stimulation with the CS elicits a PER in the absence of any US stimulation (Bitterman et al., 1983; Takeda, 1961).

We tested the effect of different types of bimodal stimulation (visual and olfactory) on bees' performance and latency time. We presented the stimulation as either synchronous or asynchronous at different intensities. We also tested the effect of the order of presentation in the asynchronous condition to see if it influenced the bees' response. Our goal was to determine if there is a temporal window of integration and if it interacts with stimulus intensity. If honey bees integrate bimodal stimulation based on predictions of the temporal window of integration (Colonius and Diederich, 2004; Meredith and Stein, 1983; Rowland and Stein, 2008; Stanford and Stein, 2007), we would expect to observe a higher PER performance for synchronous bimodal stimuli compared to asynchronous stimuli. Such predictions state

that the degree of multisensory integration might depend on the timing of unimodal stimuli, with simultaneous presentation having a higher chance of integration (Colonius and Diederich, 2004). We further aimed to investigate the potential interaction between intensity and a temporal integration window. Hence, we tested whether stimulus intensity modulates the effect of temporal synchrony on the probability of falling within a temporal window of integration. We based this hypothesis on the prediction that the processing speed and level of integration of the temporal window might be affected by unimodal features such as stimulus intensity (Balkenius and Balkenius, 2016d; Colonius and Diederich, 2004; Meredith and Stein, 1983; Stanford and Stein, 2007; Stein and Stanford, 2008). As both operate simultaneously during multimodal integration, the PoIE and the temporal window can be expected to interact. Moreover, we studied the impact of temporal order of unimodal stimuli during asynchronous stimulation. Analysing performance differences between two orders of stimulation (olfactory, then visual stimulation vs vice versa; i.e., OV vs VO) helped us determine whether bees can distinguish stimulus order and if temporal asymmetries or modality lags affect their ability to integrate stimuli in a temporal window (Keetels and Vroomen, 2012). Finally, we investigated the potential temporal and intensity effects during memory retention tests, aiming to determine whether the effects observed during the acquisition phase persisted 24 hours later. Specifically, we sought to identify whether there was differentiated recall between synchronicity and intensity levels during the memory test, indicating further crossmodal interactions caused by temporality and intensity.

Our study suggest the presence of a specific temporal period during learning and memory when honey bees integrate bimodal stimuli. This integration is influenced by stimulus intensity in a manner consistent with the principle of inverse effectiveness. Our results may indicate that lower intensity asynchronous stimuli were processed with greater temporal flexibility than their more intense counterparts. This heightened temporal sensitivity could potentially impact the temporal window of integration during foraging tasks in honey bees.

MATERIALS AND METHODS

Animal handling

We employed Africanized honey bees (*Apis mellifera*) managed at the Universidad Nacional de Colombia in Bogota (≈2600m elevation). We collected worker bees leaving the hive (13:00h-16:00),

utilising an acrylic pyramid (Matsumoto et al. 2012). Individual bees were then ice anaesthetized (Jernigan et al. 2014) and harnessed into custom-made 3D-printed plastic tubes (Gil-Guevara et al., 2022). A few minutes after recovery, all individuals were fed *ad libitum* with sucrose-water (1.5M). We kept harnessed bees at the laboratory overnight under controlled photoperiod and humidity (58%RH). The following morning before the onset of the experiments, we tested bees' motivation using the innate PER elicited by the antennal presentation of sucrose water. We solely included motivated individuals in the experiments. We released all surviving bees after finishing acquisition and memory retention tests. To ensure a single experimentation per individual bee within the hive, we tagged them with a small drop of enamel paint.

Automatized training device

We employed a rotary training device (Riveros and Gronenberg 2009; Jernigan et al. 2014; Riveros et al. 2020) modified to allow automatic and precise delivery of olfactory and visual stimuli (Gil-Guevara et al., 2022). The rotary setup (diam.: 0.52 m) contained 12 chambers, each one covered with aluminium foil tape on the inside to homogenize the light reflectance from a LED positioned below the chamber (**Fig 1a**). In addition, every chamber had two openings: at the front and the back, allowing the flow of pumped air through the enclosure and access for the experimenter to manually deliver the sucrose reward. The training device was connected to a computer-controlled system to deliver visual and olfactory stimuli at different intensities and temporal orders. The stimuli followed a pre-programmed sequence, (Gil-Guevara et al., 2022), controlled by a PC running Processing software (v. 3.5.3) (Reas and Fray 2015). The software read and executed the stimuli sequence after passing it to an Arduino Uno microcontroller (v. REV 3 SMD) using custom-made code implemented in Arduino (v. 1.8.7). The system consisted of a set of parallel electronic valves that allowed airflow from a pumping device into either one of two parallel glass tubes holding filter paper with a scent molecule at a particular concentration. The airflow reached the test chambers (flow rate: 1.08 L/min; Fluke VT Plus HF gas flow analyser) after mixing it with a parallel and constant flow of unscented air (flow rate: 0.33 L/min) aimed to minimize the possible effect of mechanical stimulation between the puffs of scent stimulation (**Fig 1a**). The system also controlled light intensity by automatically varying the electric current. In addition to executing the stimuli delivery, our software code also allows registering the time of behavioural events (latency of response, see below) by emulating a synchronized chronometer. The position of the harnessed bees inside the apparatus and the ubication of the LED light source (see **Fig 1a**) might have allowed a direct light illumination only at the lower portion of the bee's eyes. However, the differential distribution of LED light inside the chambers was not measured

(Gil-Guevara et al., 2022; Jernigan et al., 2014). We prepared olfactory stimulation by soaking a piece of filter paper (~10 x 4mm) with 10 μL with a molecule in solution at the corresponding concentration following the planned treatments within the corresponding glass container of the training device (see below and **Fig 1**). During each training session, we replaced the paper with the solution concentration after three consecutive rounds of airflows of the pumping device.

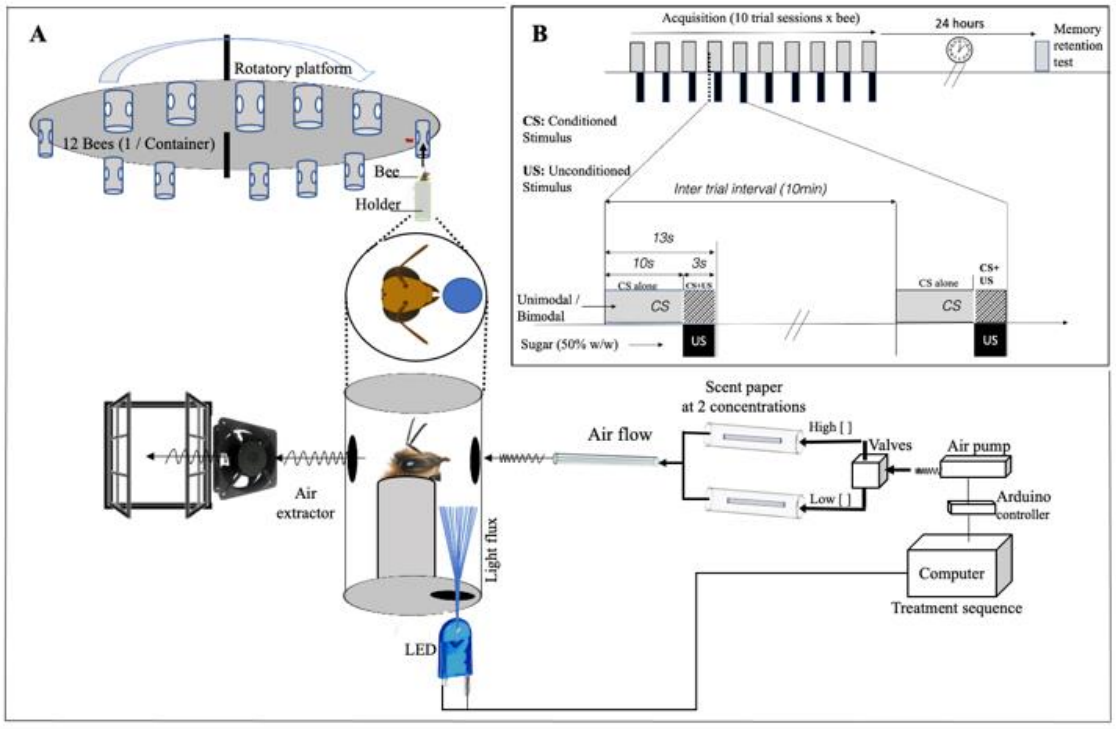

Fig. 1 A) Diagram of training apparatus: An electromechanical system accurately controlled olfactory and visual stimuli with varying concentrations/intensities. A PC connected to an Arduino Uno microcontroller delivered commands consisting of different combinations of scents and colour stimuli with specific timing onsets, volume fluxes, and LED intensity (see Fig 1C). The system was used with 12 bees per session. **B)** Graphic representation of the absolute conditioning procedure: bimodal stimuli (olfactory + visual) were presented in synch or asynchronous (OV/VO) at low and high intensities as the conditioned stimulus (CS). Each of the 12 bees received 10 consecutive trials, with 10s of CS stimulation followed by 3s of pairing with the unconditioned stimulus (US). The memory retention test was conducted 24h later. We registered PER as binary response and latency time (s) towards a sequence of unimodal stimuli (OV / VO) followed by a third stimuli that was always a bimodal in synch. During the memory retention test, all stimuli were presented without a reward (US).

Experimental stimuli: low and high intensity

To achieve low and high-intensity levels of visual and olfactory stimulation, we adjusted our experimental setup based on previous experiments where we trained bees using decreasing intensity levels aimed to reach near-threshold parameters (Gil-Guevara et al., 2022). In those experiments, the near low threshold level of visual and olfactory learning was determined as the minimum possible magnitude that allowed a level of conditioning that differed from a negative control and that simultaneously differed from the learning performance achieved with higher intensity magnitudes (Gil-Guevara et al., 2022). Therefore, following our previous studies on the effect of intensity levels on the conditioned response of Africanized honey bees, we determined the specific intensity levels used in the experiments described next.

Visual stimulation was supplied by a monochrome LED (blue; peak λ = 458 nm) that shone from below inside the chamber (**Fig 1a**) within a range of intensities from 0 to 45.7 µmol photons $m^{-2}s^{-1}$ (LI-COR portable spectroradiometer, model Li-1400, Lincoln, NE, USA). We determined the two levels for visual stimulation used in the present study as follows: *Low* intensity (4.6 µmol photons $m^{-2}s^{-1}$,10% of max intensity), and *High* intensity (45.7 µmol photons $m^{-2}s^{-1}$, 100% max intensity). To produce olfactory stimulation, we employed 1-Hexanol 98% (Sigma-Aldrich #H13303). To achieve the two levels of concentration, we either diluted the pure solution in mineral oil to achieve *low* concentration ($2x10^{-4}$ M; 0.025%) or we delivered the solution undiluted (7.8 M; 98%) for the *high* intensity olfactory stimuli. Lastly, to achieve bimodal stimulation, the electronic device was programmed to provide simultaneous visual and olfactory stimuli (as opposed to asynchronous, see below) at *Low* (odour 0.025% + blue light 10%), and *High* intensities (odour 98% + blue light 100%) see **Fig 1**.

Experimental design

We employed absolute conditioning, where a conditioned stimulus (CS) is paired with an unconditioned stimulus (US) or reward (see training procedure, below). We contrasted the effect of (i) stimulus intensity, (ii) synchrony level and (iii) delivery order of the elements of compound signals, on the learning performance of bees. Each bee received one of six different treatments (CS+): synchronous bimodal (visual + olfactory), or asynchronous at low and high intensities. When asynchronous, we balanced the presentation order of the compound elements (olfactory, then visual or visual, then olfactory, i.e., OV vs VO) (**Figs 1b, 2**). All combinations of treatments were delivered in a pseudorandom order within each experimental cycle. The bimodal synchronous stimuli (visual + olfactory) were delivered

during 13 s (see below). Bees trained with the asynchronous stimulation received the first element during the first 6.5 s. They immediately received the second component during the last 6.5 s, adding up a total of 13s of stimulation (**Figs 1b, 2**). For the memory retention test on each of the six acquisition treatments, we performed three successive tests in the surviving individuals 24h later (see below) providing unimodal (V, then O, and vice versa) and bimodal stimulation without a reward. For the first memory test, half of the individuals within each acquisition treatment received the visual stimulation first (unimodal), while the other half of experimental bees within that treatment, received the olfactory stimulus first. During the second memory retention test, we delivered unimodal stimuli in the complementary order (i.e., if the individual received olfactory stimuli first then, we delivered visual stimulation and vice versa). Finally, during the third memory retention test, we offered synchronous bimodal stimuli (**Fig 2**).

Training procedure

We employed classical conditioning of the PER, after modifying the original protocol (Takeda, 1961; Bitterman et al., 1983; Hori et al., 2006; Giurfa and Sandoz, 2012; Matsumoto et al., 2012), as described by Jernigan (2014) and Gil-Guevara et al (2022). First, before the onset of the learning procedure, where the presence of stimuli eventually serves to predict a reward, bees were acclimated for 15s to the training apparatus. Next, we trained bees to associate a visual/olfactory stimulus with a sugar-water reward. After the first 10s of conditioned stimuli presentation (CS+ = visual / olfactory in alternate order or in synch, see experimental design, above), we gently neared a micropipette holding a small drop of sugar-water (≈1µl; 1.5M; unconditioned stimulus, US) to the bee. The sugar-water was paired with the stimulus for the following 3 seconds, and the bee's PER response was recorded. Therefore, the training trial lasted for 15 seconds of acclimation, followed by 13 seconds of stimulus presentation, where the last 3 seconds were paired with the reward, and ended with a final lapse of 15 seconds before the next bee was positioned. Each subject was exposed to 10 trials at intervals of 10 min (see **Figs 1b, 2**). The memory retention test was conducted 24 hours later, exposing bees to a CS without providing any reward (see **Fig 2**). We tested bees for motivation and discharged from experiments if CS followed by US did not evoke a PER. Along trials for acquisition and memory retention, we registered the PER response and the latency time (s) to PER.

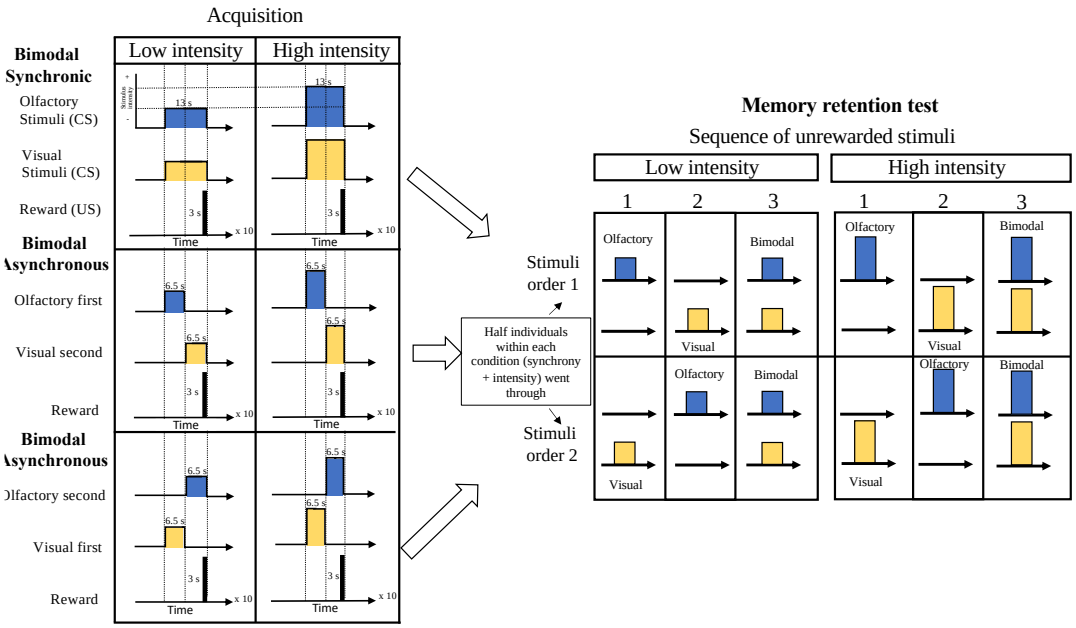

Fig. 2. Experimental design schematic: left panel: three types of stimuli were used during the acquisition phase, each followed by a reward. Top panel: synchronous bimodal stimuli presented at low and high intensities. Middle and bottom panels: asynchronous bimodal stimuli presented at low and high intensities with either the olfactory or visual stimulus presented during the first or last 6.5 seconds of the conditioned stimuli (CS). The reward was paired with the last 3 seconds of stimulation in all cases. The right panel shows the memory retention test, which involved a three-stage memory test to investigate the effects of synchrony level/order, intensity level during acquisition, and order of unimodal elements presented during the memory test on stimulus recall. Half of the individuals of each of the six treatments of the acquisition phase were tested with two alternate orders of unimodal stimuli (OV / VO). The third stimuli were always bimodal in synch. During the memory retention test, all stimuli were presented without a reward (US).

Statistical analyses

We studied the role of stimulus synchrony, modality order and intensity during the multimodal acquisition, by dividing the analysis into two phases: learning (acquisition) and memory retention. First, to understand the effect of the factorial combination of the three synchrony levels/modality orders at two intensity levels (**see Fig 2**) on the PER response during acquisition, we employed a Generalized Linear Mixed Model (GLMM). As independent variables in the model, we assigned synchrony/modality order (3 levels), intensity (2 levels), the interaction between them (Modality order X intensity), across the 10 training trials (the within-subject factors / repeated measures); the individual honey bees were included as the random factors. Similarly, to analyse how the latency time of bees was affected by these independent factors, we implemented a Linear mixed-effect model (LMM), an analysis that also allows to model random effects of subjects measured several times (repeated measures) but unlike GLMM does not assume that the distribution of response variable require a link function (e.g., binomial) (Bolker et al., 2009; Harrison et al., 2018; Pirk et al., 2013). Second, during the retention test, we asked whether the order of presentation of the unimodal elements affected the memory recall of the information acquired during learning. Therefore, we implemented the same statistical models (GLMM and LMM) for PER and latency time respectively, while adding the independent factor "modality order during memory" to the models. We executed the analyses in R v.4.0.3. (http://www.R-project.org/) employing the package "lme4" (Bates et al., 2015). This package allows carrying GLMMs with a binomial error distribution using the function glmer(), as well as implementing LMMs using the lmer() function. We revised adequate models using the Akaike Information Criterion (AIC). To test the effect of individual factors in the models we used $\chi 2$ analysis for both GLMMs and LMMs, using the function "Anova" of the car() package (Fox and Weisberg, 2019). Then, to determine where significant effects lay across the different levels of factors during the post hoc, we used the package emmeans() to obtain pairwise comparisons (Tukey HSD method with Bonferroni correction), estimated marginal means (EMMS), odds ratios, and predicted probabilities (https://github.com/rvlenth/emmeans).

RESULTS

We captured and harnessed 486 worker bees and selected individuals that elicited a PER to a sucrose solution while excluding those that showed spontaneous responses to the conditioned stimuli before training. Our study included 300 bees across six treatments, which combined three levels of synchrony (asynchronous: ***a.*** olfactory, then visual stimulation (OV) or ***b.*** vice versa: visual, then olfactory (VO); ***c.***

synchronous: the bimodal elements were delivered simultaneously (O+V)) and two intensity levels each, low and high, as illustrated in **Fig 2**.

Effects of synchronicity, temporal order, and intensity level on PER response during acquisition

Bees learned the association across all treatments, shown by the increased PER probability through training trials (Binomial GLMM: trial effect: $\chi^2_{1,300} = 33.171$; $p < 0.0001$; **Fig 3a, b**; **Table S1a**). The probability of such conditioned response depended upon the synchronicity level (when asynchronous, on the modality temporal order: VO or OV) (GLMM: *synchronicity level* effect: $\chi^2_{2,300} = 38.945$; $p<0.0001$; **Table S1a**), and intensity level (GLMM: *intensity* effect: $\chi^2_{1,\ 300} = 7.726$; $p=0.0054$; **Table S1a**) of the stimuli. Importantly, we found that the associative strength of the stimuli depended on the interaction between synchronicity (and hence, on the temporal order of the asynchronous bimodal stimuli) and intensity level (GLMM: *synchronicity level* * *intensity*, interaction effect: $\chi^2_{2,\ 300} = 15.689$; $p<0.001$; **Table S1a**, **Fig 3a, b**).

Our exploration of this interaction revealed that the associative effectiveness of bimodal stimuli's synchronicity level depended on the intensity of their unimodal components. At low intensities, the PER performance induced by the synchronic bimodal stimulation was significantly higher than either asynchronous stimulation (*post hoc, at low intensities*: bimodal in-sync vs asynchronous olfactory, then visual (OV), Tukey: z ratio = 7.145; odds ratio = 8.2; estimate=-2.10; $p < 0.0001$; and bimodal in-sync vs asynchronous visual, then olfactory (VO), Tukey: z ratio = 3.720; odds ratio =2.93; estimate=-1.07; $p = 0.0006$; **Fig 3b**). On the contrary, at high intensities the difference in performance disappeared between synchronous and asynchronous stimulation (*post hoc, at high intensities:* bimodal in-sync vs asynchronous (OV), Tukey: z ratio = 1.708; odds ratio = 1.63; estimate=-0.49; $p= 0.2023$ (ns); bimodal in-sync vs asynchronous (VO), Tukey: z ratio = 1.570; odds ratio = 1.57; estimate = -0.45; $p=0.2586$ (ns); **Fig 3a**). Interestingly, only at low intensities, we found significant PER performance differences between the temporal orders of the asynchronous stimulation, with VO eliciting a higher response than OV (post hoc, at *low intensity*: OV *vs* VO, Tukey: z ratio = -3.559; odds ratio = 2.8; estimate = -1.03, $p=0.0011$; while at *high intensity*: OV *vs* VO, Tukey: z ratio = -0.145; odds ratio = 1.04; estimate = -0.04; $p=0.9885$(ns); **Fig 3a,b**).

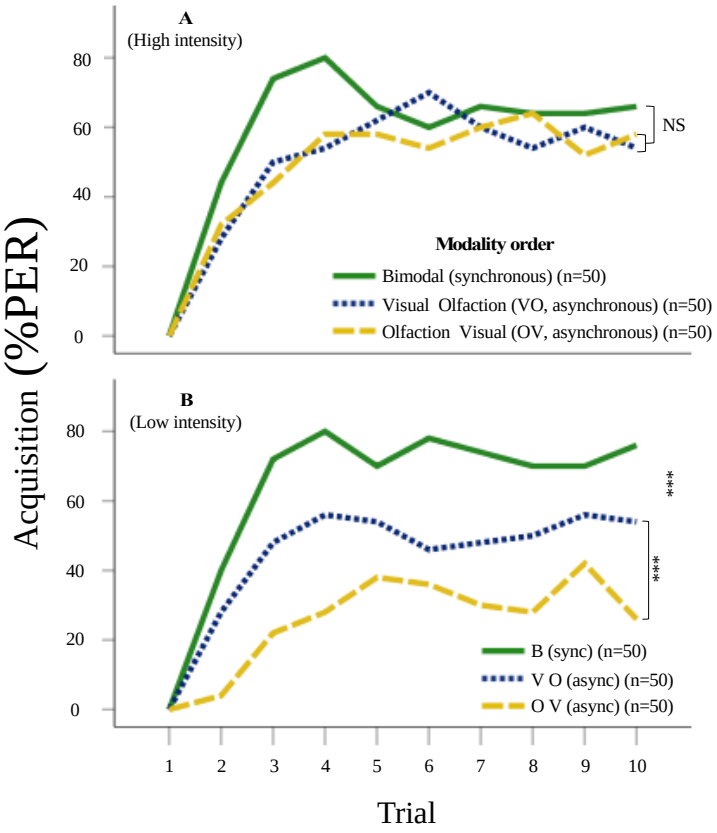

Fig. 3. Learning curves comparing the percentage of PER of honey bees for each modality order/synchrony level during acquisition, at high **(A)** or low **(B)** intensities. Only at low intensities, a significant difference was detected between synchronic and asynchronous stimulation, as well as between different orders of presentation of the asynchronous stimuli.

Effects of synchronicity level, temporal order, and intensity on latency time during acquisition learning

The speed of honey bee response during learning was influenced by both the synchronicity level and intensity of the conditioned stimuli (LMM, **Table S1b**). Although the latency time by bees mainly remained unchanged across the trials within treatments (LMM: Trial effect: $\chi^2_{1,300} = 0.018$; $p = 0.8927$; **Table S1b**), the latency time of the PER response was overall affected by the synchronicity level (LMM: synchronicity level effect on latency time: $\chi^2_{2,300} = 18.156$; $p < 0.0001$; **Table S1b**). Also, although the stimuli intensity by itself did not affect the latency time (LMM: Intensity effect: $\chi^2_{1,300} = 0.427$; $p = 0.5134$; **Table S1b**), it significantly interacted with the synchronicity level, influencing the latency time to exhibit a conditioned PER by bees (LMM: synchronicity level * Intensity effect: $\chi^2_{2,300} = 29.871$; $p = < 0.0001$; **Table S1b**).

To identify the contrasts of such interaction, we performed the corresponding post hoc tests. Responses to synchronous bimodal stimuli and asynchronous temporal order OV were observed at equivalent speeds across different intensity level (*post hoc, at low intensities*: olfactory, then visual (OV) *vs.* bimodal: Tukey: t ratio = -2.63; estimate = -0.76; SE=0.287; df =293; $p = 0.0243$ (ns); and at *high intensities:* Tukey: t ratio = -0.912; estimate = -0.23; SE=0.253; df = 239; $p = 0.6335$ (ns); **Fig 4**). However, at low intensities, the asynchronous presentation of visual followed by olfactory stimulus (VO) led to an average response time of 2 and 2.7 seconds slower than the response times to both the synchronous bimodal stimuli and the reverse asynchronous stimuli (OV), respectively (*post hoc, at low intensities*: (VO) *vs.* bimodal, Tukey: t ratio = 7.991, $p < 0.0001$; (VO) *vs.* (OV), Tukey: t ratio = -9.345, $p < 0.0001$; **Fig 4**). At high intensities, a similar trend was observed, with the synchronous bimodal stimuli and the asynchronous presentation of visual followed by olfactory stimulus (OV) inducing faster-conditioned responses than the reverse asynchronous stimuli (VO), with average response times that were 3.9 and 4.2 seconds slower, respectively (*post hoc at high intensities*: (OV) *vs.* (VO), Tukey t ratio = -16.328, p<0.0001; (VO) *vs.* bimodal, Tukey t ratio = 15.796, p<0.0001; **Fig 4**).

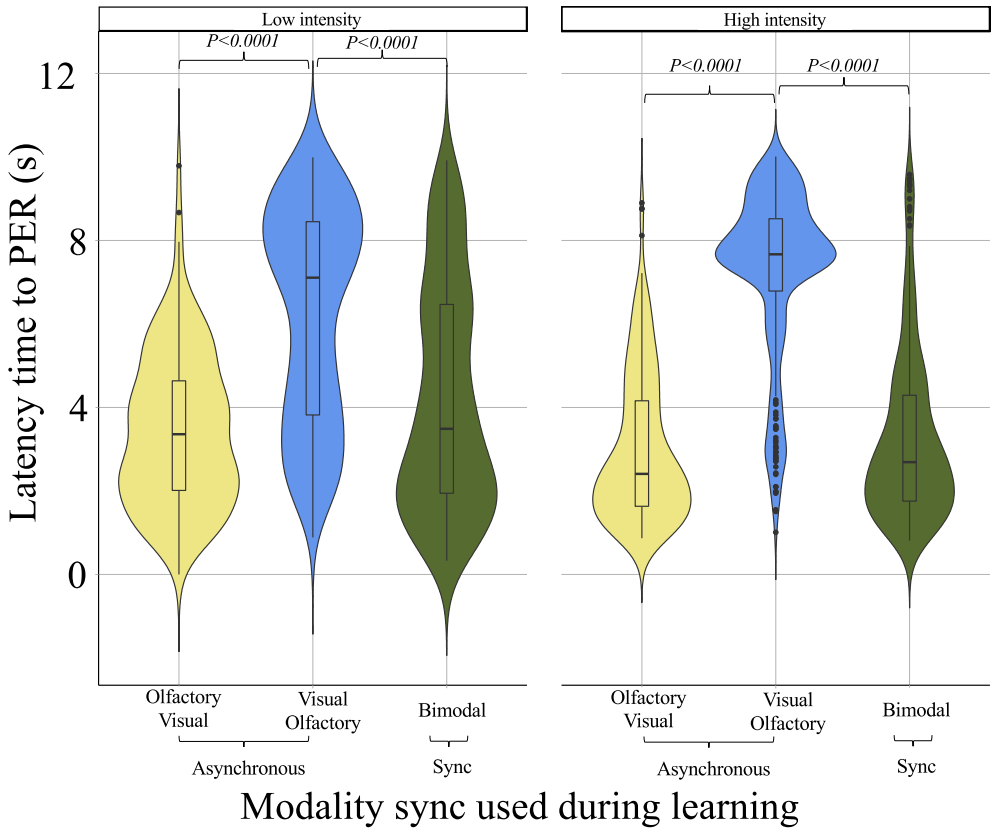

Fig. 4. Effect of the synchronicity level of bimodal stimuli and its intensity on the latency time (s) to elicit a conditioned PER in bees. Individuals responded faster to asynchronous bimodal stimuli led by the olfactory element and followed by the visual stimulus (OV), compared to the opposite temporal order (visual, then olfactory; VO) at both low and high intensities. Similarly, bimodal synchronous stimuli elicited faster responses than the asynchronous VO at either intensity. However, the bimodal stimulus in sync produced responses at equal speeds to those induced by the asynchronous stimulus, OV regardless of intensity level.

The effect of intensity levels within each type of synchronicity on the PER response during learning

We investigated the effects of intensity variation (low/high) within synchronous (O+V) and asynchronous (OV or VO) bimodal stimuli on PER and latency time performances (**Table S2**). The asynchronous temporal order of OV resulted in an increase in PER performance that was correlated with the intensity level (effect on PER response: GLMM, asynchronous OV, *low vs high* intensity effect: $\chi^2_{1,100} = 19.752$; $p<0.0001$; **Table S2a**; post hoc: Tukey z ratio = -4.444; estimate=-1.37; SE=0.308 $p<0.0001$). In contrast, varying intensity levels for the opposite asynchronous stimuli (VO) had no effect on bee PER performance (PER response: GLMM asynchronous VO, *low vs high* intensity effect: $\chi^2_{1,100} = 1.7427$; $p=0.186$; **Table S2b**). Similarly, the variation of the intensity levels for the synchronous bimodal stimuli did not produce a differential PER response in bees during learning (PER response: GLMM bimodal in-sync, *low vs high* intensity effect: $\chi^2_{1,100} = 0.7946$; $p=0.372$; **Table S2c**).

Surprisingly the latency time of bees for asynchronous temporal orders showed divergent trends with varying intensity levels. Intensity variations within the asynchronous temporal order OV did not affect the bees' latency time (Latency time: LMM (OV), *low vs high*-intensity effect: $\chi^2_{1,84} = 2.044$; $p=0.152$ **Table S2d**). On the contrary, for the opposite asynchronous temporal order (VO), the latency time of bees increased with the intensity level (Latency time: LMM (VO), *low vs high* intensity effect: $\chi^2_{1,95} = 17.094$; $p<0.00001$ **Table S2e**; post hoc: t ratio= -4.128; $p<0.0001$). Finally, increasing the intensity of the synchronous bimodal stimulation led to reduced latency times in bees, consistent with the observed pattern of improved PER performance (Latency time: LMM bimodal in-sync, low vs high intensity effect: $\chi^2_{1,96} = 10.563$; $p=0.0011$; **Table S2f**; post hoc: t ratio= 3.248; $p=0.0016$).

Effects of synchronicity, temporal order, and intensity on PER response during memory tests

After training bees with different bimodal synchronicity and intensity levels, we assessed memory retention after 24 hours, examining the impact of synchronicity and temporal order on retention and the relative influence of unimodal elements and presentation order. The likelihood of bees eliciting a conditioned PER response 24 hours after conditioning was affected by both the synchronicity level (and therefore, temporal order) experienced during the acquisition phase and the unimodal stimuli presented during the memory retention test (GLMM: *sync level during acquisition effect:* $\chi^2_{2,300} = 10.1941$; $p= 0.006$;

modality order during memory test effect: $\chi^2_{4,300} = 128.995$; $p < 0.0001$; **Table S3**). Moreover, the sync level employed during acquisition interacted with the order of the unimodal stimuli presented during the retention test, affecting memory performance (GLMM: *sync level during acquisition X modality order during memory test* effect: $\chi^2_{8,300} = 16.6176$; $p=0.034$). Although the model did not support the main effect of intensity (GLMM: *intensity effect*: $\chi^2_{1,300} = 1.4257$; $p = 0.232$; **Table S3**), the intensity level interacted significantly with the modality order during memory test *(*GLMM: *intensity* modality order during memory test* effect: $\chi^2_{4,300} = 21.7330$; $p < 0.0001$; **Table S3**). That is, the intensity level of stimuli during acquisition interacted with the temporal order of unrewarded stimulation during memory tests.

Remarkably, after conducting a posthoc test (**Table S4**), we found that bees trained with asynchronous temporal orders performed worse on memory tests at low intensities compared to those trained with synchronous bimodal stimuli, especially in response to unimodal olfactory stimulation (**Fig 5**; **Table S4**). Also, the asynchronous temporal orders elicited differential memory responses: bees trained with the asynchronous combination of visual-olfactory (VO) exhibited higher levels of conditioned PER than the opposite temporal order (i.e., VO>OV) (**Fig 5; Table S4**). Bees trained with high-intensity stimuli showed superior overall performance across all synchronicity treatments during memory tests in response to unimodal olfactory and bimodal stimulation; responses to visual stimulation at high intensities were reduced relative to low intensities (**Fig 5; Table S4**). Overall, memory tests showed that PER performance was mainly influenced by olfactory and synchronous bimodal stimulation, rather than visual stimuli regardless of training conditions (**Fig 5; Table S4**).

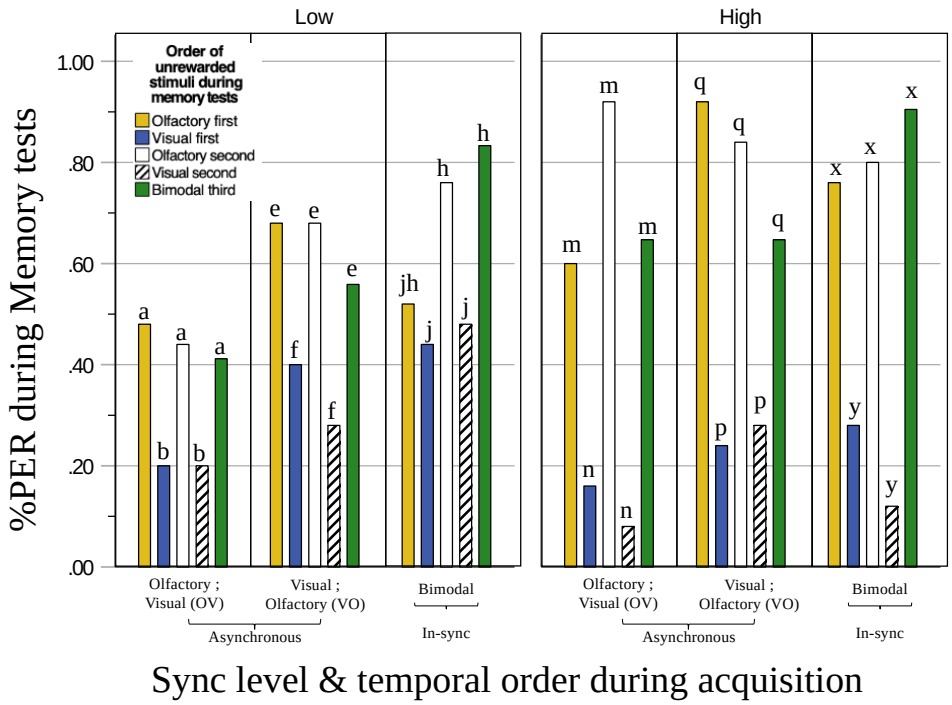

Fig. 5. The PER performance during memory retention in bees was influenced by the synchronicity level and intensity during training, as well as the modality used in retention tests. At low intensity, bees trained with asynchronous temporal orders exhibited lower PER performance than those trained with synchronous bimodal stimuli. Additionally, bees trained with the asynchronous combination of visual and olfactory stimuli performed better with the temporal order VO compared to OV. At high intensities, PER performance, was overall higher and was dominated by olfactory and bimodal stimulation regardless of the synchronicity level used during training.

Effects of synchronicity, temporal order, and intensity on latency time during the memory retention tests

The unrewarded modality used during memory tests affected bee latency time in retention (LMM: *Modality order during memory* effect: $\chi^2_{4, 238} = 95.638$; $p < 0.0001$; **Table S5A**). Also, the speed of the PER response was also affected by the order in which the visual stimuli were delivered during the memory test (**Table S5A**). The intensity level received during acquisition had an impact on the latency time observed during the memory test (LMM: *Intensity* effect: $\chi^2_{1, 238} = 4.308$; $p<0.05$). Despite this, the model did not find a significant relationship between the synchronicity level (synchronous or asynchronous, in either OV or VO order) employed during acquisition and the variation in latency time in the PER response elicited by bees during the memory test (LMM: Synchronicity level during acquisition effect: $\chi^2_{2, 238} = 2.435$; $p<0.296$; **Table S5A**).

We performed a posthoc test to contrast the effects of unimodal (olfactory, visual) and synchronous bimodal (O+V) stimuli and the order of presentation (first, second, or third) on the bee's reaction speeds of bees during three stages of the memory test (**Fig 6**). During memory test, bees showed a higher latency time to elicit a PER when presented with visual, compared to olfactory or bimodal stimulation, regardless of the order or intensity of the training stimuli (posthoc: Tukey method; **Fig 6**; **Table S5B**). Our study found that olfactory and bimodal stimulation elicited equally fast reactions at both low and high intensities. During the memory test, both olfactory and bimodal stimuli resulted in faster reactions compared to visual stimulation, regardless of intensity level (**Fig 6**; **Table S5B**). Our study found that higher-intensity stimulation, regardless of the modality during the retention test, or the synchronicity used during acquisition, tended to elicit faster PER reactions during memory tests (**Fig 6**; **Table S5B**).

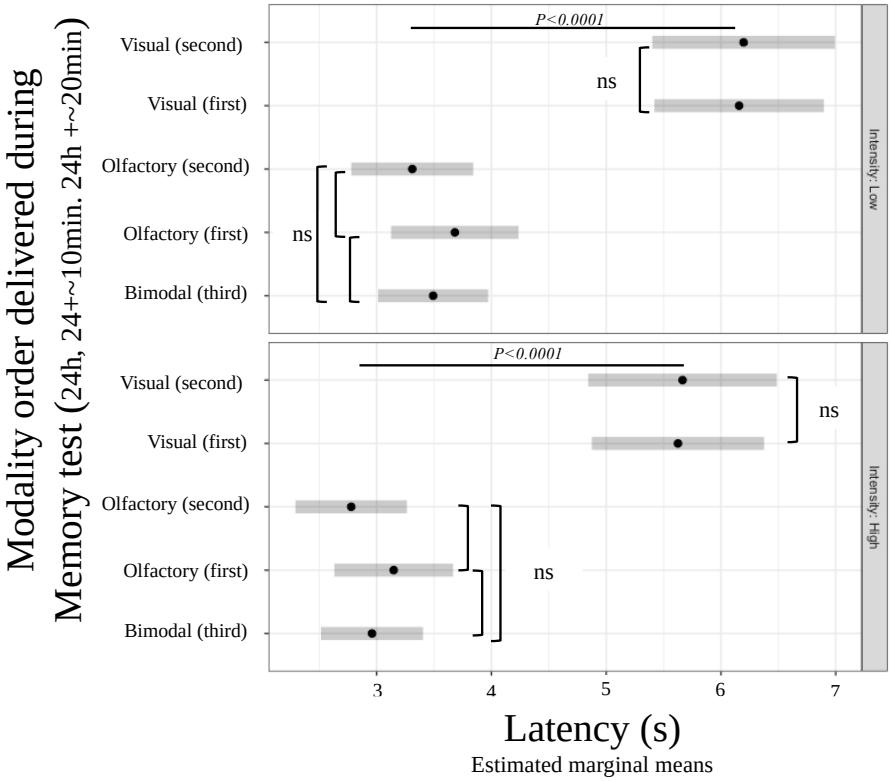

Fig. 6. The modality employed during the memory test had a significant overall effect on the latency time of bees. Visual stimulation consistently delayed the onset of the PER response compared to olfactory or bimodal stimulation. Modality had a stronger influence on reaction speeds during memory tests than the presentation order

DISCUSSION

Our objective was to determine whether the bees' ability to learn bimodal stimuli is consistent with a temporal window of integration and whether it interacts with stimulus intensity. We tested the possible interdependence between temporal synchrony and intensity levels during honey bees' bimodal learning and memory. We found that at low-intensity levels, bimodal synchronous stimuli produced higher PER performance than asynchronous stimuli, while there were no differences at high-intensity levels. Furthermore, only at low intensities, we observed differential PER performance among alternate temporal orders of asynchronous stimuli. These findings support the principle of inverse effectiveness and suggest an interaction with the temporal window of multisensory integration. We discuss the interaction among sensory intensity, synchronicity, and temporal order in multisensory integration and their impact on learning and memory.

Our study shows that the interplay of synchronicity level, temporal order, and stimulus intensity affects the conditioned PER. Consistent with previous research (Balkenius and Balkenius, 2016d; Gil-Guevara et al., 2022; Katzenberger et al., 2013; Meredith et al., 1987; Stein et al., 1988), synchronous bimodal stimulation leads to high PER performance. However, synchronous bimodal stimuli had a significantly greater effect than asynchronous stimuli, but only at low intensities (**Fig 3**), indicating that synchronous stimulation may selectively enhance perceptual processing at lower levels of sensory input. Moreover, only at low intensities, bees showed differential PER performance between the alternate temporal orders for asynchronous stimulation, likely due to the wider temporal window of integration. At high intensities, however, the temporal window might narrow, and bees may require a more precise temporal relationship between stimuli to integrate them effectively, resulting in similar PER performances regardless of synchronicity level or temporal order. Hence, our findings are consistent with the temporal window of integration hypothesis, which proposes a nervous system filter that determines if information from different sensory modalities is registered closely enough in time to trigger multisensory integration (Balkenius and Balkenius, 2016d; Colonius and Diederich, 2004; Meredith et al., 1987; Rowland and Stein, 2008). Our study builds upon previous findings (Keetels and Vroomen, 2012; Krueger Fister et al., 2016) and demonstrates a significant interaction between the principles of temporal order and inverse effectiveness. We discuss the implications for learning and memory tasks in bees.

At low intensities, bees showed lower learning performance in response to bimodal asynchronous stimuli compared to synchronous stimuli (**Fig 3**), possibly due to the low effectiveness of the two separate stimuli. The temporal window suggests optimal multimodal interaction occurring when two modalities are presented together, corresponding the obtained results with synchronous bimodal stimuli (**Fig 3**). Low-intensity asynchronous stimuli resulted in less effective learning in bees, but may provide greater temporal flexibility, resulting in differential PER performance between temporal orders. This suggests that bimodal signals can be learned over time, and not necessarily at the same time, albeit with a concomitant loss in associative strength. These findings are in accord with the idea that multisensory integration is dependent on the level of temporal asynchrony between stimuli and predicts that the probability of integration depends on unimodal features affecting the speed of processing, such as stimulus intensity (Balkenius and Balkenius, 2016d; Chandrasekaran, 2017; Colonius and Diederich, 2004; Ma and Pouget, 2008; Meredith et al., 1987). Low-intensity stimuli may facilitate a wider window of multisensory integration. This allows for higher temporal flexibility despite lower performance levels in response to stimulus asynchronies. This is expressed in the differential PER reactions observed. At these low intensities, maximal multisensory enhancement occurs when the bimodal inputs occur in synchrony (**Fig 3**). In contrast, higher intensities might lead to a narrower temporal window. This reduces temporal flexibility and prevents differential PER reactions towards different input orders (**Fig 3**). The strong learning performance induced by the olfactory component at these intensities may override that of the visual component, as suggested in previous work (Gil-Guevara et al., 2022; Riveros and Gronenberg, 2009b; Riveros and Gronenberg, 2009a; Riveros and Gronenberg, 2012). Also at high intensities, the relative advantage of bimodal synchronous over asynchronous stimulation is diminished (**Fig 3**), in a similar way in which the lack of advantage synchronous bimodal over unimodal olfactory stimulation (Gil-Guevara et al., 2022). High-intensity stimulation may evoke strong but redundant responses that reduce the relevance of integrating multiple sensory sources (Otto et al., 2013; Ross et al., 2007c; Stein and Stanford, 2008).

High-intensity multimodal stimuli may narrow the temporal window of integration, leading to a lower likelihood of integration (Colonius and Diederich, 2004; Krueger Fister et al., 2016; Meredith and Stein, 1983). This may be attributed to the increase in animal responses that typically accompanies stimulus intensity (Bhagavan and Smith, 1997; Gil-Guevara and Amézquita, 2020; Mackintosh, 1974; Wright et al., 2005). Therefore, at high intensities, the lack of PER enhancements for synchronous stimuli compared to asynchronous ones may reflect a conditioned response towards the more effective unimodal component rather than true bimodal integration (Gingras et al., 2009; Stanford et al., 2005; Stein and Stanford, 2008). It is possible that the latter applies to our results (**Fig 3**), implying the effect of an olfactory bias over visual

stimulation during associative learning in bees (Gerber and Smith, 1998; Nishino et al., 2018; Paoli and Galizia, 2021; Riveros and Gronenberg, 2009b; Riveros and Gronenberg, 2009a; Riveros and Gronenberg, 2012; Smith, 1991; Wright et al., 2009). This aligns with previous findings of reduced probability of multimodal integration during associative learning and memory tasks in bees at high intensities (Gil-Guevara et al., 2022).

Multimodal integration is expected to enhance reaction times and performance, particularly under optimal conditions such as synchronous stimuli at low intensities, as observed in our results (**Fig 4**) and previous studies (Gil-Guevara et al., 2022; Hebets and Papaj, 2005b; Leonard and Masek, 2014a; Riveros et al., 2020). However, our findings also revealed different latency patterns for asynchronous stimuli, with OV producing equivalent latencies to synchronous stimuli while VO temporal order resulted in slower latency times across intensities (**Figs 3 & 4**). These results suggest that latency time reactions did not always conform to predictions of the temporal window and inverse effectiveness across our experimental conditions. Similar inconsistencies in latency patterns in honey bees have also been reported previously in response to intensity (Gil-Guevara et al., 2022) and supports the idea that multimodal stimuli may elicit similar performance levels by controlling the salience of unimodal stimuli to ensure comparable effectiveness (Otto et al., 2013). Our present findings suggest that temporal asynchronies may also contribute to this variability in latency responses. Future research could investigate additional manipulations of intensity and temporal dimensions of bimodal stimuli to determine optimal conditions for balancing PER performance and latency, beyond the scope of our experiment. Our results demonstrate the interplay between synchrony and intensity level, revealing diverging trends in PER performance and reaction time (**Figs 3 & 4**). Synchronous bimodal stimuli led to high learning performance and faster reaction times at high-intensity levels, while the temporal order VO showed a higher PER performance but slower reaction times at low intensities. Conversely, the opposite order OV improved with intensity, revealing a differential effect of intensity on the two temporal orders. These contrasting trends may suggest different processing speeds depending on the temporal order.

The opposing trajectories of PER performance and reaction time in our experiment suggest a trade-off between speed and accuracy. Similar trade-offs have been observed in prior research on bees' foraging choices, where bees that choose rapidly sacrifice accuracy for speed, while those taking more time are more accurate (Chittka and Spaethe, 2007a; Chittka et al., 2003). The observed differences in PER performance can be explained by the relative proximity of each modality to the sucrose reward (**Fig 1**). The well-known differences in associative strengths between olfactory and visual modalities (Hori et al.,

2006; Jernigan et al., 2014; Menzel, 1981; Mota et al., 2011; Rose and Menzel, 1981; Szyszka et al., 2014; Warrant et al., 1996) would be sufficient to explain the observed differences in PER performance (**Fig 3**). However, this does not fully explain the obtained reaction time differences in which the temporal order VO was consistently slower than OV (**Fig 4**). Delays in processing time due to improvements in accuracy may explain the observed contradictory trajectories between PER performance and reaction time. While multimodal integration considers the speed-accuracy trade-off in temporal and intensity dimensions, pollinator foraging requires additional temporal considerations beyond this trade-off. The temporal configuration of bimodal signals is crucial for mimicking natural floral displays during free flight. A recent study on bumblebees found that an asynchronous visual component followed by an overlapping olfactory component was optimal for performance (Riveros, 2023). This bimodal structure enhances olfactory but not visual acquisition, and the synchrony effects depend on the signal's first component and structure, affecting response speed. While we do not analyse the effect of different levels of bimodal component overlap, this is a potential avenue for future research. Our current work adds to previous findings on the optimal structure of bimodal stimuli by highlighting the role of synchronicity and intensity in learning and memory. However, our findings suggest that synchronous bimodal stimulation: 1) is faster across intensity than asynchronous order, VO; 2) elicits response speeds equivalent to those of the opposite temporal order, OV; and 3) speeds up with intensity (**Fig 4**). These results support the idea of a real-time temporal window of integration, which has been reported in previous studies on cats and moths (Balkenius and Balkenius, 2016d; Meredith and Stein, 1986a; Stein and Stanford, 2008; Stein et al., 1988). Furthermore, our study found that these learning patterns were long-lasting, as evidenced by consistent PER performances and reaction times throughout the memory retention tests.

Effects on memory

During our memory retention test, bees reacted similarly to the acquisition stage. During the retention test, PER and latency performance was influenced by the interaction between conditions used during acquisition (synchrony and intensity levels), and by the specific modality orders used during the memory test (**Fig 5; Tables S3, S4 & S5**). This finding suggests that the timing of modality cues plays an important role in the bees' ability to form and retrieve memories. We found that bees that have experienced scents and colours synchronously, can posteriorly recall both modalities after presenting them in isolation, with varying associate strength. This latter was true even for asynchronic stimulation but with lower learning and recalling scores. We also found that intensity level influences the strength of such

recollections (**Fig 5; Table S6**). This might be compatible with research on insects suggesting that cross-modal associative recall is like that of humans, where smell or sound can trigger memories of crossmodal associated events (McLaren and Mackintosh, 2002; Srinivasan et al., 1998). We found that the scent was more strongly recalled than the colour in isolation, regardless of intensity and congruency during training or temporal order of stimulation during tests. Bimodal congruent training resulted in higher PER recollections after a bimodal stimulation during the memory test. Consequently, visual stimulation was recalled less, especially at high intensities, which aligns with previous studies on bee cognition (Gil-Guevara et al., 2022; Menzel, 1981; Rose and Menzel, 1981). We also found some lasting effects of the intensity level was evident during the memory test (**Fig 6; Tables S4 & S5B**). The higher stimulation salience in both conditioning and memory testing led to faster bee reactions, indicating that signal-to-noise problems during foraging (Chittka and Spaethe, 2007a; Chittka et al., 2003; Giurfa, 2022; Leonard and Masek, 2014a; Leonard et al., 2011b; Leonard et al., 2011a) can affect bimodal signals irrespective of congruency level or temporal order.

Taken together, our findings are consistent with neurophysiological studies on moths, which have shown that olfactory and visual stimuli interact within the mushroom body, following the temporal window of integration and inverse effectiveness rule (Balkenius and Balkenius, 2016). This also aligns with studies of the critical role of the honey bee mushroom body in integrating sensory information and forming memories (Avalos et al., 2021; Ehmer and Gronenberg, 2002; Erber, 1978; Gronenberg, 2001; Homberg and Erber, 1979; Hussaini and Menzel, 2013; Kirschner et al., 2006; Menzel, 2014; Menzel and Giurfa, 2001; Sommerlandt et al., 2016; Strube-Bloss and Rössler, 2018; Strube-Bloss et al., 2011, 2016). Specifically, the output neurons of the mushroom body exhibit cross-modal integration by showing increased neural activity in response to bimodal stimuli (Strube-Bloss and Rössler, 2018). Our results help connect neural mechanisms with adaptive behaviour during learning and memory tasks by showing that multimodal integration principles act jointly.

Foraging bees rely on multisensory integration to process information about the location and quality of flowers. Our results support the temporal rule and inverse effectiveness principles, which might allow for more accurate and faster decision-making (Chittka and Spaethe, 2007a; Chittka et al., 2003). Visual cues can aid and interact with olfactory signals in several ways, providing important information such as nectar guides and species identity (Dyer et al., 2011; Gerber and Smith, 1998; Gil-Guevara et al., 2022; Harrap et al., 2019; Hempel de Ibarra et al., 2014; Jernigan et al., 2014; Lawson et al., 2017a; Mansur et al., 2018; Mota et al., 2011; Riveros et al., 2020). However, signals from a distance are more likely to

be temporally dissociated and have a larger decay in intensity (Gil-Guevara and Amézquita, 2020; Katzenberger et al., 2013; Wiley, 2006). Therefore, bees may benefit more from associating congruent bimodal stimulation emitted from the same flower or patch when detecting them at a distance. Once closer to the flower reward, olfaction guides primary navigation while visual cues provide essential secondary information (Chittka and Spaethe, 2007c; Leonard and Masek, 2014a; Mota et al., 2011; Riveros, 2023; Solvi et al., 2020). Despite method limitations, PER conditioning may test the temporal window of integration in bees with greater precision through gap variation. Future research should address this question.

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Supplementary information for Chapter 2.
Stimulus intensity modulates the temporal window of integration during multimodal learning and memory in honey bees

Table S1. A) Generalized linear mixed model (GLMM) of the effect of Modality order (synchrony level), intensity and Trial on the probability of eliciting a PER response during the acquisition phase of associative conditioning experiments on bees. **B)** A linear mixed model (LMM) that explores the change in the latency time (s) as a function of the modality order (synchrony level), intensity level, and trials during acquisition. Significance levels are assessed after Bonferroni's correction

A) GLMM Acquisition Model for PER

PER response ~ Modality Order *X* Intensity + Trial + (1 | individual honeybee)

	Estimate	Std.error	z value	*P-value*
Intercept	-1.567	0.225	-6.962	**<0.0001**
(Modality order) **Visual, then Olfactory**	1.030	0.289	3.559	**<0.001**
(Modality order) **Bimodal**	2.105	0.295	7.145	**<0.0001**
(Intensity) **High**	1.330	0.290	4.584	**<0.0001**
Trial	0.084	0.015	5.759	**<0.0001**
(Modality order) **Visual, then Olfactory X** (Intensity) **High**	-0.989	0.405	-2.440	**<0.05**
(Modality order) **Bimodal X** (Intensity) **High**	-1.614	0.411	-3.925	**<0.0001**

B) LMM Acquisition Model for Reaction time (s)

Latency time ~ Modality Order *X* Intensity + Trial + (1 | individual honeybee)

	Estimate	Std.error	df	t value	*P-value*
Intercept	3.468	0.260	528.5	13.357	**<0.0001**
(Modality order) **Visual, then Olfactory**	2.794	0.299	322.9	9.349	**<0.0001**
(Modality order) **Bimodal**	0.756	0.287	295.4	2.631	**0.009**
(Intensity) **High**	-0.368	0.295	316.9	-1.250	0.212
Trial	0.003	0.019	1503.0	0.135	0.893
(Modality order) **Visual, then Olfactory X** (Intensity) **High**	1.389	0.394	294.8	3.529	**0.0005**
(Modality order) **Bimodal X** (Intensity) **High**	-0.526	0.382	269.8	-1.375	0.1702

Table S2. Summary of the GLMM and LMM models comparing the PER (tables a, b and, c) and latency time (d, e and, f) performances between low and high intensities for each synchronicity level (O; V, V; O and Bimodal in sync) during the learning phase of the experiments.

a) **GLMM model contrasting PER response induced by O; V at low and high intensities during acquisition**

=PER ~ Olfactory; Visual (low) + Olfactory; Visual (High) + Trial + (1 | Individual bee)

Synchronicity level (Coefficients)	Estimate	SE	z. val.	*p*
(Intercept) Olfactory; Visual (Low)	-1.815	0.269	-6.736	<0.0001
Olfactory; Visual (High)	1.369	0.308	4.444	<0.0001
Trial	0.124	0.026	4.724	<0.0001

b) **GLMM model contrasting PER response induced by V; O at low and high intensities during acquisition**

=PER ~ Visual; Olfactory (low) + Visual; Olfactory (High) + Trial + (1 | Individual bee)

(Intercept) Visual; Olfactory (Low)	-0.506	0.216	-2.344	0.0191
Visual; Olfactory (High)	0.320	0.243	1.320	0.1868
Trial	0.081	0.024	3.393	0.0007

c) **GLMM model contrasting PER response induced by Bimodal in sync at low and high intensities during acquisition**

=PER ~ Bimodal in sync (low) + Bimodal in sync (High) + Trial + (1 | Individual bee)

(Intercept) Bimodal in sync (Low)	0.751	0.270	2.786	0.0053
Bimodal in sync (High)	-0.286	0.321	-0.891	0.3727
Trail	0.048	0.027	1.790	0.0735

d) **LMM model contrasting Latency (s) induced by O; V at low and high intensities during acquisition**

=Latency (s) ~ Olfactory; Visual (low) + Olfactory; Visual (High) + Trial + (1 | Individual bee)

Synchronicity level (Coefficients)	Estimate	SE	t. val.	*p*
(Intercept) Olfactory; Visual (Low)	0.358	0.250	1.430	0.16
Olfactory; Visual (High)	3.188	0.242	13.163	<0.001
Trial	-0.009	0.031	-0.297	0.77

e) **LMM model contrasting Latency (s) induced by V; O at low and high intensities during acquisition**

=Latency (s) ~ Visual; Olfactory (low) + Visual; Olfactory (High) + Trial + (1 | Individual bee)

(Intercept) Visual; Olfactory (Low)	-1.014	0.268	-4.135	<0.001
Visual; Olfactory (High)	6.847	0.245	25.528	<0.001
Trial	0.075	0.036	2.094	0.04

f) **LMM model contrasting Latency (s) induced by Bimodal in sync at low and high intensities during acquisition**

=Latency (s) ~ Bimodal in sync (low) + Bimodal in sync (High) + Trial + (1 | Individual bee)

(Intercept) Bimodal in sync (Low)	0.901	0.277	3.250	<0.001
Bimodal in sync (High)	3.589	0.261	13.761	<0.001
Trail	-0.043	0.031	-1.389	0.17

Table S3. Summary of a binomial GLMM model the PER response of honey bees during the three phases of the memory retention test (see methods). The model test three coefficients: Synchronicity level received during acquisition (three levels), intensity level (two levels) and modality order received during the three-phase memory test (five levels). Model fit: link function (logit); marginal / conditional R2=0.27/0.67, AIC = 887.6, ICC = 0.55, individual honeybees denoted as random effects=300. Follow up chi-square test shown in text (see methods, results). Significance values following a Bonferroni's correction.

GLMM Memory Model for PER

PER response ~ Sync level during acquisition X Intensity X Stimuli order during memory test + (1 | individual honeybee)

	Estimate	**Std.error**	**z value**	***p***	
Intercept	-0.31805	0.53279	-0.597	0.5505	
(Sync level / acquisition) Visual; Olfactory	0.78672	0.74485	1.056	0.2909	
(Sync level / acquisition) Bimodal	2.55136	0.79338	3.216	**0.0013**	**
(Intensity) High	1.25342	0.74617	1.68	0.093	.
(Stimuli order during memory) Olfactory first	-0.01766	0.70605	-0.025	0.98	
(Stimuli order during memory) Olfactory second	0.29068	0.70521	0.412	0.6802	
(Stimuli order during memory) Visual first	-1.2775	0.79036	-1.616	0.106	
(Stimuli order during memory) Visual second	-1.85207	0.78221	-2.368	**0.0179**	*
(Sync level / acquisition) Visual; Olfactory X (Intensity) High	-0.7047	1.06413	-0.662	0.5078	
(Sync level /acquisition) Bimodal X (Intensity) High	-0.7617	1.15318	-0.661	0.5089	
(Sync level /acquisition) Visual; Olfactory X (Stimuli order/memory) Olfactory first	0.83453	1.00805	0.828	0.4077	
(Sync level /acquisition) Bimodal X (Stimuli order/ memory) Olfactory first	-1.97827	1.03368	-1.914	0.0556	.
(Sync level/ acquisition) Visual; Olfactory X (Stimuli order/ memory) Olfactory second	0.07647	0.99274	0.077	0.9386	
(Sync level / acquisition) Bimodal X (Stimuli order / memory) Olfactory second	-1.1277	1.06594	-1.058	0.2901	
(Sync level /acquisition) Visual; Olfactory X (Stimuli order/ memory) Visual first	0.04648	1.05423	0.044	0.9648	
(Sync level /acquisition) Bimodal X (Stimuli order/ memory) Visual first	-1.52605	1.10165	-1.385	0.166	
(Sync level / acquisition) Visual; Olfactory X (Stimuli order/ memory) Visual second	0.2057	1.07481	0.191	0.8482	
(Sync level / acquisition) Bimodal X (Memory order/ memory) Visual second	-0.39267	1.08997	-0.36	0.7187	
(Intensity) High X (Stimuli order/ memory) Olfactory first	-0.08701	0.98397	-0.088	0.9295	
(Intensity) High X (Stimuli order/ memory) Olfactory second	1.25342	1.15299	1.087	0.277	
(Intensity) High X (Stimuli order/ memory) Visual first	-1.80566	1.12002	-1.612	0.1069	
(Intensity) High X (Stimuli order/ memory) Visual second	-1.99599	1.27553	-1.565	0.1176	
(Sync level / acquisition) Visual; Olfactory X (Intensity) High X (Stimuli order/ memory) Olfactory first	1.30259	1.57583	0.827	0.4085	
(Sync level / acquisition) Bimodal X (Intensity) High X (Stimuli order/ memory) Olfactory first	1.12973	1.48293	0.762	0.4462	
(Sync level / acquisition) Visual; Olfactory X (Intensity) High X (Stimuli order/ memory) Olfactory second	-0.24704	1.58481	-0.156	0.8761	
(Sync level / acquisition) Bimodal X (Intensity) High X (Stimuli order/ memory) Olfactory second	-1.71249	1.63791	-1.046	0.2958	
(Sync level /acquisition) Visual; Olfactory X (Intensity) High X (Stimuli order/ memory) Visual first	0.52254	1.53858	0.34	0.7341	
(Sync level /acquisition) Bimodal X (Intensity) High X (Stimuli order/ memory) Visual first	0.49111	1.59437	0.308	0.7581	

(Sync level/ acquisition) Visual; Olfactory X (Intensity) High X (Stimuli order/ memory) Visual second	1.23114	1.65307	0.745	0.4564
(Sync level / acquisition) Bimodal X (Intensity) High X (Stimuli order/ memory) Visual second	-0.80646	1.78064	-0.453	0.6506

Table S4. Post hoc contrasts following a GLMM model contrasting the effect of the synchronicity levels employed during acquisition, and the order of presentation on the PER of honey bees during a three-phase memory test. Confidence level used: 0.95. Results given on the log odds ratio scale; p values obtained using the Tukey HSD method.

Contrasts between order of stimulation during memory tests	**Sync level during acquisition:**									
	Low intensity					**High intensity**				
	Olfactory; Visual (O; V)					**Olfactory; Visual (O; V)**				
	estimate	**SE**	**z. ratio**	***p***		**estimate**	**SE**	**z. ratio**	***p***	
Olfactory first - Visual first	1.334	0.486	2.746	0.048	*	3.305	0.523	6.317	0.000	****
Olfactory first - Olfactory second	-0.336	0.482	-0.698	0.957		-1.649	1.024	-1.610	0.491	
Olfactory first - Visual second	1.430	0.414	3.456	0.005	**	3.755	0.510	7.356	0.000	****
Olfactory second - Visual first	1.671	0.419	3.986	0.001	***	3.687	0.497	7.415	0.000	****
Olfactory second - Visual second	1.766	0.493	3.581	0.003	**	4.137	0.585	7.074	0.000	****
Visual first - Visual second	0.095	0.497	0.192	1.000		0.450	0.557	0.808	0.928	
Bimodal third - Olfactory first	0.359	0.396	0.907	0.894		-0.180	0.429	-0.420	0.994	
Bimodal third - Olfactory second	0.023	0.404	0.057	1.000		-1.544	0.912	-1.693	0.438	
Bimodal third - Visual first	1.694	0.413	4.100	0.000	***	3.125	0.441	7.086	0.000	****
Bimodal third - Visual second	1.789	0.413	4.330	0.000	****	3.575	0.484	7.386	0.000	****
	Visual; Olfactory (V; O)					**Visual; Olfactory (V; O)**				
Olfactory first - Visual first	1.334	0.486	2.746	0.048	*	3.305	0.523	6.317	0.000	****
Olfactory first - Olfactory second	-0.336	0.482	-0.698	0.957		-0.382	0.551	-0.694	0.958	
Olfactory first - Visual second	1.430	0.414	3.456	0.005	**	3.755	0.510	7.356	0.000	****
Olfactory second - Visual first	1.671	0.419	3.986	0.001	***	3.687	0.497	7.415	0.000	****
Olfactory second - Visual second	1.766	0.493	3.581	0.003	**	4.137	0.585	7.074	0.000	****
Visual first - Visual second	0.095	0.497	0.192	1.000		0.450	0.557	0.808	0.928	
Bimodal third - Olfactory first	0.359	0.396	0.907	0.894		-2.032	0.999	-2.035	0.249	
Bimodal third - Olfactory second	0.023	0.404	0.057	1.000		-1.374	0.833	-1.649	0.466	
Bimodal third - Visual first	1.694	0.413	4.100	0.000	***	3.125	0.441	7.086	0.000	****
Bimodal third - Visual second	1.789	0.413	4.330	0.000	****	3.575	0.484	7.386	0.000	****
	Bimodal in-sync					**Bimodal in-sync**				
Olfactory first - Visual first	0.808	0.860	0.940	0.881		3.305	0.523	6.317	0.000	****
Olfactory first - Olfactory second	-0.336	0.482	-0.698	0.957		-0.382	0.551	-0.694	0.958	
Olfactory first - Visual second	0.249	0.706	0.352	0.997		3.755	0.510	7.356	0.000	****
Olfactory second - Visual first	1.671	0.419	3.986	0.001	***	3.687	0.497	7.415	0.000	****

Olfactory second - Visual second	1.766	0.493	3.581	0.003	**	4.137	0.585	7.074	0.000	****
Visual first - Visual second	0.095	0.497	0.192	1.000		0.450	0.557	0.808	0.928	
Bimodal third - Olfactory first	1.996	0.755	2.644	0.063		-0.180	0.429	-0.420	0.994	
Bimodal third - Olfactory second	0.023	0.404	0.057	1.000		-0.562	0.464	-1.210	0.746	
Bimodal third - Visual first	1.694	0.413	4.100	0.000	***	3.125	0.441	7.086	0.000	****
Bimodal third - Visual second	1.789	0.413	4.330	0.000	****	3.575	0.484	7.386	0.000	****

Table S5. A. LMM model to test the effects of Synchronicity level during acquisition, Intensity and the modality order employed during memory tests on latency time. Significance values following a Bonferroni's correction. **B.** Posthoc contrasts after the LMM model contrasting the difference in the latency time between orders of presentation of the unrewarded stimuli during the memory retention test.

A. LMM Memory Model for Reaction time (s)

Latency time ~ *Synchronicity level during acquisition* X *Intensity* + *Modality order during Memory* + (1 | individual honeybee)

	Estimate	Std.error	t value	*p*
(Intercept) (Synchronicity level / acquisition) Olfactory; Visual	3.2484	0.4132	7.862	**<0.001**
(Synchronicity level / acquisition) Visual; Olfactory	0.7491	0.4966	1.508	0.13
(Synchronicity level / acquisition) Bimodal	-0.0167	0.4811	-0.035	0.97
(Intensity) High	-0.3852	0.4958	-0.777	0.44
(Modality order during memory) Olfactory first	0.1889	0.2908	0.65	0.52
(Modality order during memory) Olfactory second	-0.1816	0.2763	-0.657	0.51
(Modality order during memory) Visual first	2.6654	0.3886	6.859	**<0.001**
(Modality order during memory) Visual second	2.7052	0.4226	6.401	**<0.001**
(Synchronicity level / acquisition) Visual; Olfactory X (Intensity) High	-0.5682	0.6586	-0.863	0.39
(Synchronicity level / acquisition) Bimodal X (Intensity) High	0.1263	0.6399	0.197	0.84

B. Posthoc contrasts

Low intensity

Contrasts between order of stimulation

Modality	**Order of presentation**		**Modality**	**Order of presentation**	**estimate**	**SE**	**df**	**t. ratio**	***p***
Bimodal	Third	-	Olfactory	First	-0.238	0.450	311	-0.529	0.984
Bimodal	Third	-	Olfactory	Second	0.396	0.441	342	0.9	0.897
Bimodal	Third	-	Visual	First	-2.216	0.546	330	-4.057	**0.001**
Bimodal	Third	-	Visual	Second	-1.568	0.569	340	-2.757	**0.048**
Olfactory	First	-	Olfactory	Second	0.634	0.508	408	1.249	0.723
Olfactory	First	-	Visual	First	-1.978	0.605	396	-3.267	**0.010**
Olfactory	First	-	Visual	Second	-1.330	0.581	268	-2.287	0.152
Olfactory	Second	-	Visual	First	-2.612	0.555	274	-4.711	**<.0001**
Olfactory	Second	-	Visual	Second	-1.964	0.613	401	-3.204	**0.013**
Visual	First	-	Visual	Second	0.648	0.696	391	0.932	0.885

High intensity

Bimodal	Third	-	Olfactory	First	-0.318	0.390	337	-0.815	0.926

Bimodal	Third	-	Olfactory	Second	-0.114	0.367	300	-0.309	0.998
Bimodal	Third	-	Visual	First	-3.203	0.614	329	-5.214	**<.0001**
Bimodal	Third	-	Visual	Second	-4.019	0.797	371	-5.043	**<.0001**
Olfactory	First	-	Olfactory	Second	0.205	0.426	409	0.48	0.989
Olfactory	First	-	Visual	First	-2.885	0.653	384	-4.421	**0.000**
Olfactory	First	-	Visual	Second	-3.701	0.793	324	-4.666	**<.0001**
Olfactory	Second	-	Visual	First	-3.089	0.613	297	-5.039	**<.0001**
Olfactory	Second	-	Visual	Second	-3.905	0.812	388	-4.81	**<.0001**
Visual	First	-	Visual	Second	-0.816	0.950	379	-0.859	0.912

CHAPTER 3

COMPLEMENTARY ROLES OF OCTOPAMINE AND DOPAMINE DURING OLFACTORY, VISUAL, AND BIMODAL LEARNING AND MEMORY IN BUMBLE BEES (*BOMBUS IMPATIENS*)

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ABSTRACT

Sensory processing and multimodal integration are crucial in shaping behaviour, especially in tasks involving learning and memory, such as foraging and pollination. Moreover, the strength of the associative value of those sensory inputs depends on a neuronal substrate modulated by biogenic amines. These influence synaptic interactions in a concentration- and location-dependent manner, influencing sensory sensitivity and reward systems. In bees and other insects, octopamine (OA) enhances olfactory and visual appetitive learning, while dopamine's (DA) role has traditionally been linked to aversive learning. Recent evidence has sparked controversy regarding its potential role in appetitive conditioning. While research on biogenic amines in appetitive learning has primarily centred around olfactory and, to some extent, visual learning, the role of biogenic amines in multimodal learning remains unknown. In this study, we examined the learning and memory performance of bumble bees fed with several doses of OA and the DA agonist 6,7-ADTN. The bees were subjected to olfactory (O), visual (V), and bimodal (O+V) conditioning using the proboscis extension response (PER) protocol. We found concentration-dependent and opposing effects of OA and DA on PER conditioning. Higher doses of OA improved performance across all modalities, while DA had inhibitory effects depending on the stimulus modality and dose. The convergent pathways for multiple sensory modalities, along the required conditions for processing, integration, and dose-dependent effects of amines, presents opportunities for precise modulation in the context of prediction error during both appetitive and aversive learning.

Keywords: PER conditioning, biogenic amines, unimodal, multimodal, neuromodulation

INTRODUCTION

Behavioural flexibility relies on cognitive processes shaped by past and present contexts. During learning and memory-dependent behavioural tasks, external signal properties such as intensity and spatiotemporal relationships critically influence both unimodal and multimodal integration (Chandrasekaran, 2017; Gil-Guevara and Amézquita, 2020; Gil-Guevara et al., 2022; Gingras et al., 2009; Otto et al., 2013; Stein and Stanford, 2008). Nevertheless, the inner neuromodulatory environment may play an equally critical role (Giurfa, 2006). Animal nervous systems employ evolutionary conserved biogenic amines, neuropeptides, and neurotransmitters to achieve modulation of behaviour (Marder, 2012). In insects, biogenic amines influence task specialization, motivation states and sensory sensitivity (Barron et al., 2002; Cabirol et al., 2018; Smith et al., 2013; Søvik et al., 2015). Learning and memory are particularly influenced by biogenic amines (Osborne, 1996; Scheiner et al., 2006; Søvik et al., 2015). Octopamine (OA) and dopamine (DA), found in relatively high amounts in the nervous system of adult honey bees, modulate the sensitivity of sensory receptors and interneurons (Scheiner et al., 2002). Compelling evidence suggests that biogenic amines modulate reinforcement properties during appetitive and aversive conditioning, thereby contributing to learning and memory formation (Giurfa, 2006; Hammer, 1993; Hammer and Menzel, 1998; Perry and Barron, 2013; Scheiner et al., 2006).

In the honey bee brain, unimodal and multimodal projections converge with reward pathways mediated by biogenic amines (Hammer, 1993; Hammer and Menzel, 1995; Hammer and Menzel, 1998; Kropf and Rössler, 2018; Menzel, 2001; Mobbs, 1982; Strube-Bloss and Rössler, 2018). Research on reward learning in honey bees addresses the convergence of pathways for conditioned stimulus (CS) and unconditioned stimulus (US). CS processing begins with chemoreceptors projecting to the antennal lobe (AL), followed by local interneurons and projection neurons that synapse with intrinsic neurons in the mushroom bodies (MBs) and the lateral protocerebral lobe (PL) (Fahrbach, 2006; Hammer and Menzel, 1995; Hammer and Menzel, 1998; Menzel and Bitterman, 1983; Mobbs, 1982; Perry and Barron, 2013). The MBs' Kenyon cells (KC) receive olfactory input and output via long clustered axons (Gronenberg, 2001; Mobbs, 1982). In the US pathway, sucrose-sensitive taste receptors and olfactory sensory afferents converge in the subesophageal ganglion (SOG) through the release of octopamine into the AL glomeruli, which responds to odour and associative changes during conditioning (Hammer and Menzel, 1995; Hammer and Menzel, 1998; Mobbs, 1982). On the other hand, the MBs receive multisensory projections (olfactory, visual, mechanosensory) organized in the main input structures of the MBs, further anatomically subdivided in anatomic subdivisions, the lip, collar, and basal ring, receiving olfactory, visual and both modalities

respectively (Galizia and Rössler, 2010; Gronenberg, 2001; Hussaini and Menzel, 2013; Mobbs, 1982; Sandoz, 2011; Strausfeld, 2002). The output neurons of these structures enable higher-order multimodal computations (Ehmer and Gronenberg, 2002; Rybak and Menzel, 1993). Recent research has identified varying proportions of output MB neurons responding to unisensory versus multisensory stimuli (Strube-Bloss and Rössler, 2018).

The convergence of sensory processing and reward systems in the honey bee MBs aligns with the reward prediction error signal (Rescorla and Wagner, 1972), a primary mechanism for classical conditioning. This signal encodes neural activity changes and enables the assessment of the disparity between predicted and obtained rewards across trials under specific conditions (Barron et al., 2010; Hammer, 1997; Hammer and Menzel, 1998; Menzel, 2001; Perry and Barron, 2013; Waddell, 2013). While in vertebrates and some invertebrates DA signalling is the key modulator of behavioural responses to rewards (Hazy et al., 2010; Schultz, 1998), in insects OA is thought of as a primary modulator of behaviour and physiology, exerting a greater influence on reward learning and reward responses than DA (Barron et al., 2010; Hammer and Menzel, 1998; Scheiner et al., 2002).

In insect models, OA is a strong modulator of appetitive olfactory associations (Barron et al., 2010; Scheiner et al., 2002; Vergoz et al., 2007) and serves as the neurotransmitter for reinforcement signalling in the brain (Farooqui et al., 2003; Hammer, 1993; Hammer and Menzel, 1998). It may replace sucrose rewards in olfactory conditioning and restores learning motivation in satiated bees (Hammer and Menzel, 1998; Scheiner et al., 2006). OA mediates consolidation and recall of olfactory memory, becoming an essential component of neural representations (Farooqui et al., 2003). It enhances olfactory appetitive learning performance by increasing responsiveness to the reward (Schilcher et al., 2021). OA has also shown to influence the visual responses by enhancing direction-specific antennal responses to moving stripe patterns and modifying motion-sensitive neurons in the visual system (Kloppenburg and Erber, 1995; Scheiner et al., 2006; Schilcher et al., 2021). It also enhances visual appetitive learning and increases the maximum receptor response in honeybees, leading to increased walking speed towards different light sources (Mancini et al., 2018; Schilcher et al., 2021). In bumble bees, higher doses of OA enhance visual associative learning compared to honey bees (Muth et al., 2023).

In contrast, DA brain dopamine microinjections impaired proboscis extension in appetitive conditioning (Mercer and Menzel, 1982). Importantly, DA is thought to primarily facilitate reinforcement signalling in aversive learning in bees, predominantly influencing punishment pathways rather than appetitive olfactory

learning (Vergoz et al., 2007). Pharmacological studies using DA antagonists inhibits aversive but not appetitive olfactory associations (Tedjakumala et al., 2014; Wright et al., 2010). Nevertheless, its specific role in appetitive learning remains controversial. For example, it was also found that DA impairs the formation of long-term appetitive olfactory memory in bees, with no observed impact during acquisition or on short-term memory (Klappenbach et al., 2013).

Dopamine (DA) receptors are typically categorized into two groups based on their impact on cAMP signalling: D1-like receptors boost cAMP levels upon activation, while D2-like receptors typically either have no impact on this signalling molecule or reduce cAMP production (Perry and Barron, 2013). Honey bee dopamine (DA) receptors can be classified into three distinct classes based on their structure and function. AmDOP1, a member of the invertebrate DA receptor class (DOP1), closely resembles vertebrate D1-like receptors and is constitutively active, elevating cAMP levels even without an agonist. AmDOP2 also activates cAMP signalling and additionally couples to calcium. Interestingly, AmDOP2 shares a closer evolutionary relationship with the honey bee octopamine receptor (AmOA1) than with AmDOP1 or AmDOP3, forming a unique group of invertebrate DA receptors (INDRs). On the other hand, AmDOP3 is a D2-like DA receptor, typically reducing cAMP levels upon DA activation, but it exhibits constitutive activity, which increases basal cAMP levels (Barron et al., 2010; Perry and Barron, 2013; Søvik et al., 2015).

In parallel, research in Drosophila melanogaster suggests that the interpretation of DA as exclusively acting in aversive learning might be too simplistic (Barron et al., 2010; Perry and Barron, 2013; Søvik et al., 2015; Waddell, 2013). A substantial body of research supports the idea that DA mediate reinforcement signalling for both appetitive and aversive learning in olfactory and visual domains (Burke et al., 2012; Kim et al., 2007; Liu et al., 2012; Selcho et al., 2009). Advanced genetic tools available in Drosophila offer precise insights, supporting the notion that while OA is essential for reward learning in insects, DA signals also impact reward responses (Barron et al., 2010; Perry and Barron, 2013; Søvik et al., 2015; Waddell, 2013). In support of such trend, recent work on honey bees found that both OA and DA antagonists impair visual discrimination performance, indicating their role in modulating appetitive visual learning in honeybees (Vieira et al., 2018). Similarly, during visual discrimination tasks at particularly high DA doses both OA and DA signalling play a role in mediating either appetitive sucrose signalling or the association between colour and sucrose reward during visual discrimination tasks (Mancini et al., 2018). Finally, also in honey bees, a motivation system (wanting) is activated by high DA agonistic

concentrations in the bee brain via an increased appetite during foraging behaviour and had enhanced appetite olfactory learning (Huang et al., 2022).

While extensive research has been conducted on the role of biogenic amines in appetitive learning, most studies have focused on olfactory learning, with limited attention given to visual appetitive learning (Giurfa, 2003b; Giurfa, 2006; Giurfa and Sandoz, 2012; Mancini et al., 2018; Muth et al., 2023; Vieira et al., 2018). However, the involvement of biogenic amines in multimodal learning remains largely unexplored. Therefore, our objective was to assess and compare the modulatory effects of the octopaminergic and dopaminergic systems on appetitive olfactory, visual, and bimodal learning and memory. Here, we tested whether OA enhances appetitive learning and memory retention across olfactory, visual, and bimodal stimulation. Based on the well-established role of OA in mediating sucrose reinforcement in the reward system (Hammer and Menzel, 1995; Hammer and Menzel, 1998; Mobbs, 1982), we predicted an associated enhancement in the overall associative strength of olfactory and visual learning with increasing OA dosage. Similar trends during bimodal learning may suggest a shared OA pathway among unimodal and multimodal neurons, as well as its involvement in the reward system (Hammer and Menzel, 1995; Hammer and Menzel, 1998; Mobbs, 1982). While the role of dopamine in appetitive signalling may be less clear (Barron et al., 2010; Hammer and Menzel, 1995; Hammer and Menzel, 1998; Perry and Barron, 2013; Scheiner et al., 2002; Søvik et al., 2015), the absence of an effect on the associative strength of olfactory, visual, or bimodal appetitive learning and memory suggests that our manipulation of dopamine may not impact learning and memory formation. Conversely, considering previous evidence suggesting that dopamine reduces sucrose responsiveness (Scheiner et al., 2002) if dopamine does play a role during appetitive learning, it is plausible to predict that dopamine's involvement in appetitive learning may result in a decreased associative strength observed during unimodal (olfactory or visual) as well as bimodal learning and memory tasks. For both systems (OA and DA) concentration variations linked to specific modalities may offer insights into modality-specific associative strength requirements.

To address these questions, we conducted our study using foragers of the bumble bee *Bombus impatiens*, known for their ability to learn associations between stimuli of different modalities (unimodal or bimodal) and a reward (Riveros, 2023; Riveros and Gronenberg, 2009b; Riveros and Gronenberg, 2012; Riveros et al., 2020). We employed the proboscis extension response (PER) protocol (Giurfa and Sandoz, 2012; Matsumoto et al., 2012) which involves training restrained bees while allowing a precise stimulus delivery (Leonard and Masek, 2014a), to investigate learning and memory. The PER protocol recapitulates a natural

situation where bees extend their proboscis in response to sensory stimulation (antennae, tarsi) with floral nectar (Menzel and Bitterman, 1983; Takeda, 1961). During training, the PER is conditioned by pairing a sweet sucrose solution (US) with an odour/colour (CS). Repeated pairings lead to the CS eliciting a PER in the absence of the US, a proxy for learning. This widely used protocol has been employed to study learning and memory capabilities (Giurfa and Sandoz, 2012). We administered various concentrations of octopamine (OA) and a dopamine (DA) agonist to bumble bee foragers and evaluated their learning performance and response latency across 24 treatments. These treatments included two biogenic amines, each at four concentrations, and three modalities (olfactory, visual, and bimodal). Additionally, we evaluated their memory performance in a test conducted 24 hours after conditioning.

We found concentration-dependent and opposing effects of the two biogenic amines on PER conditioning. High doses of OA improved PER performance across all modalities, while DA showed inhibitory effects at different doses depending on the stimulus modality. Low and moderate doses of DA inhibited olfactory stimulation, while visual stimulation showed inhibition only at moderate doses, and bimodal learning appeared more affected by higher doses of DA. Thus, these amines have contrasting effects on appetitive learning and memory: OA enhances performance while DA inhibits it in a dose-dependent manner, emphasizing their crucial roles in modulating appetitive learning across sensory modalities.

MATERIALS AND METHODS

Bumble bees' maintenance and collection

We housed four colonies of the North American bumble bee *Bombus impatiens* Cresson 1963 (Koppert Biological Systems in Howell, MI, USA), under indoor conditions (21.14°C, SD = 1.05; 12h:12h photoperiod). To maintain consistent pollen and sugar suitability for optimal learning performance of bumble bees during their growth cycle, we used two pairs of colonies sequentially, starting with the first pair and switching to the second pair after the 8th week of colony establishment. Pollen was supplied *ad libitum* in the nest boxes, while each colony was connected to a set of 20-cm plastic tubes leading to two separate foraging cages each, where the bees gathered the 0.5 M sucrose solution (Riveros, 2023). We collected foraging bumble bees from feeder cages, carefully ice-anesthetized them, and harnessed them using custom plastic tubes (Gil-Guevara et al., 2022; Jernigan et al., 2014; Riveros, 2023; Riveros et al., 2020). After recovery, harnessed bees were feed ad libitum with a micropipette using sucrose-water (1.0 M) and left undisturbed overnight. The next morning, 12 hours after the last feed, we tested bumble bees for motivation using the PER elicited by antennal stimulation with sucrose solution (1.0 M) allowing us to select individual bees to be used in the following absolute conditioning experiments.

Training apparatus

We employed a training apparatus designed to individually manipulate 12 harnessed bees within chambers attached to an acrylic rotary platform with a diameter of 0.52m (Gil-Guevara et al., 2022) modified from (Jernigan et al., 2014; Riveros et al., 2020). Each rotating chamber was illuminated from below by blue LEDs (see "training stimuli", below) and had aluminium foil-coated walls to homogenize and diffuse the light, reducing potential differences in light across different regions of the bees' eyes (Gil-Guevara et al., 2022; Jernigan et al., 2014; Riveros, 2023; Riveros et al., 2020). Two openings in each chamber facilitated olfactory stimulation flow (refer to "training stimuli" below) and sucrose reward delivery. Our training device integrates an electromechanical system that enables precise and automated delivery of olfactory and visual stimuli, as well as the ability to quantify the timing of behavioural events (Gil-Guevara et al., 2022; Gil-Guevara & Riveros 2023, unpublished). The training device was computer-controlled and delivered pre-programmed visual and olfactory stimuli (Gil-Guevara et al., 2022). Processing software (v. 3.5.3) (Reas and Fry, 2014) on a PC controlled the sequence, which was executed by custom code on an

Arduino Uno microcontroller (v. REV 3 SMD) using Arduino (v. 1.8.7). The system allows full control of the light onset and intensity by automatically varying the electric current. Moreover, the device featured parallel electronic valves that directed airflow from a pumping device into two glass tubes containing scents at specified concentrations. This airflow mixed with a constant unscented airflow (flow rate: 0.33 L/min), resulting in a total flow rate of 1.08 L/min (Fluke VT Plus HF gas flow analyser) into the test chambers and minimizing any mechanical stimulation between scent puffs (Gil-Guevara et al., 2022). Olfactory stimulation was achieved by saturating a filter paper (~10 x 4mm) with 10 μL of the molecule solution at the designed concentration (see below) and device glass container (see Fig M1). Between each training session, we replaced the filter paper with corresponding solution concentration after three consecutive rounds of airflows from the pumping device.

Training stimuli

A monochrome LED emitting blue light at a peak wavelength of 458 nm was positioned inside the chamber (see above) to provide unimodal visual stimulation. The intensity of the light was measured at 45.7 μmol photons $m^{-2}s^{-1}$ using a LI-COR portable spectroradiometer (model Li-1400, Lincoln, NE, USA). For unimodal olfactory stimulation, we employed 1-Nonanol (Sigma-Aldrich # 131210) and delivered and undiluted solution (9.8 mol l^{-1}; 98%). Finally, to accomplish bimodal stimulation, the electronic apparatus was precisely configured to deliver concurrent visual and olfactory stimuli (denoted as O+V).

Delivery and dosage of biogenic amines

Before the onset of the conditioning experiments, and immediately after the motivation test, bees were assigned to receive either Octopamine or a Dopamine agonist at four concentrations (a sucrose control, low, mid, and high) in a balanced random manner. We opted for oral administration of biogenic amines to bumble bees given the simplicity and effectiveness of non-invasive methods (Barron et al., 2007; Scheiner et al., 2002). We utilized Octopamine hydrochloride (OA; Sigma-Aldrich, O0250) and created four concentrations by dissolving it in a 1.5M sucrose solution. The control consisted of a sucrose-water solution, the lowest dose OA treatment received by bees consisted of 0.2μg (10^{-4} M), the mid dose was 2.0 μg (10^{-3} M) and the highest dose was 20 μg (10^{-2} M) (Scheiner et al., 2002). We also employed the dopamine receptor agonist 6,7-ADTN hydrobromide (Cayman Chemical, 23866) (hereafter, 6,7-ADTN) and created four additional concentrations by dissolving the solution in water and then adding it to a 1.5M sucrose solution to obtain experimental concentrations. Again, the control group received sucrose-water

solution (0.0μg), while the low concentration group received 0.2 μg (10^{-4} M), the mid concentration group received 2.0 μg (10^{-3}M) and the high concentration group received 20μg (10^{-2} M) of DA 6,7-ADTN (Huang et al., 2022; Scheiner et al., 2002; Schilcher et al., 2021). We fed the harnessed bees 10μl of the respective solution 10 minutes before the onset of the absolute conditioning experiments.

Experimental design

Our objective was to compare how the learning and memory performance of bees was affected by (i) modality, (ii) biogenic amine, and (iii) concentration. We were especially interested in revealing potential interactions among these factors. We utilized absolute conditioning, which involves the pairing of a conditioned stimulus (CS) with an unconditioned stimulus (US) or reward. Each bumble bee was assigned to one of the 24 unique treatments (CS+), which varied in terms of modality (olfactory, visual, or bimodal), biogenic amine (either octopamine or dopamine receptor agonist 6,7-ADTN) and concentration level (negative control, low, mid, or high concentration).

Training procedure

We used classical conditioning of the proboscis extension reflex (PER), with modifications to the original protocol (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Hori et al., 2006; Matsumoto et al., 2012; Takeda, 1961) as described elsewhere (Gil-Guevara et al., 2022; Jernigan et al., 2014; Riveros, 2023). Before beginning the process of absolute conditioning, which involves using stimuli to anticipate a reward, the bees were acclimated to the training apparatus for 15 seconds. Next, during the first 10s we presented bees with the conditioned stimuli (CS = visual, olfactory, or bimodal) and then, during the following 3s we carefully approached a micropipette with a small drop of sugar-water (≈1μl; 1.5M; unconditioned stimulus, US), pairing the stimuli with the reward, while recording the PER response. In this way, we trained the bees to associate the CS (unimodal or bimodal) with the sugar reward (US). The training concluded with a 15-second break before positioning the next bee. Bees were tested for memory retention 24 hours later by presenting them to a CS without a reward. We tested their motivation and excluded those that did not display a PER response to the CS followed by the US. The PER response and latency time were registered during acquisition and memory retention trials.

Statistical analyses

We investigated the role of modality, biogenic amine type, and concentration on unimodal and bimodal learning and memory. We used a Generalized Linear Mixed Model (GLMM) to examine the factorial impact of three modalities presented to bumble bees with two different biogenic amines at four concentrations on the PER response during acquisition (see Fig M2). The GLMM used PER as binary response variable (link function), and included modality (3 levels), biogenic amine (2 levels), and concentration (4 levels) were included as independent variables, along with the interaction terms (modality x amine x concentration) and 10 training trials (within-subject factors) and individual bumble bees as random factors. We used a Linear Mixed Model (LMM) to examine the independent and combined effects of the modality, biogenic amine, and concentration factors on the latency time during acquisition. This model can incorporate the repeated measures of subjects as random effects, but unlike GLMM do not require a link function for the response variable (Bolker et al., 2009; Harrison et al., 2018; Pirk et al., 2013). To analyse the effect of these factors on the PER response during the memory retention test, we implemented a similar GLMM model (excluding trials variation), while to examine the latency time during memory test, we employed a Linear mixed effects model (LME) that allows linear random effect (Bolker et al., 2009; Harrison et al., 2018). We run the analyses in R v.4.0.3. (http://www.R-project.org/) using the package "lme4" (Bates et al., 2015). We executed GLMMs, LMMs and LMEs using the functions glmer(), lmer() and lme(), respectively. We selected models based on Akaike Information Criterion (AIC) and used $\chi 2$ analysis to test individual factors' effects, with the "Anova" function from the car() package for both GLMMs and LMMs (Fox and Weisberg, 2019). To identify significant effects among factor levels in the post hoc analysis, we utilized the emmeans() package. This allowed us to obtain pairwise comparisons, estimated marginal means, odds ratios, and predicted probabilities using the Tukey HSD method with Bonferroni correction (https://github.com/rvlenth/emmeans).

RESULTS

Out of the 520 individual bees subjected to training, 16 were excluded due to a lack of PER before the experiments. Therefore, we used a total of 504 bees, which belonged to one of three modalities (olfactory, visual, or bimodal) and two biogenic amines (octopamine or dopamine agonist), each with four concentrations (control, low, mid, and high). Treatments were evenly distributed (n=21 each), resulting in a balanced design with 24 groups. The mean head width, which served as a proxy for body size, was not

significantly different across treatments (ANOVA: $F_{23, 504} = 0.275$, p=0.98). Therefore, body size can be ruled out as a possible explanation for any observed differences across treatments.

Modality, Biogenic Amines, and Concentration interact to shape bees PER conditioning acquisition

Bees learned the association, as indicated by an increased PER probability across training trials, regardless of the specific treatment combination (Binomial GLMM: *Trial effect*: $\chi^2_{9,504} = 574.373$; $p<0.001$; see model on **Table S1A**). Our results not only demonstrate learning across trials, but also highlight the significant impact of stimulus modality (GLMM: *Modality effect*: $\chi^2_{2,504} = 7.177$; p=0.028), the biogenic amines (GLMM: *Amine effect*: $\chi^2_{1,504} = 5.649$; p=0.017) and concentrations employed (GLMM: *Concentration effect*: $\chi^2_{3,504} = 19.100$; $p<0.001$) on the probability of the PER conditioning response. Importantly, the effect of the biogenic amine on PER performance depended on concentration (GLMM: *Amine x Concentration effect:* $\chi^2_{3,504} = 19.597$; $p<0.001$). We also observed concentration-dependent interactions between changes in PER across trials, modality, and amines (GLMM: *Trial x Concentration effect*: $\chi^2_{3,504} = 9.752$; $p<0.021$; *Modality x Amine x Concentration effect*: $\chi^2_{6,504} = 11.237$; $p<0.081$; *Modality x Concentration x Trial effect*: $\chi^2_{6,504} = 13.453$; $p<0.036$). The impact of the two biogenic amines on PER conditioning was modulated by concentration, with overall opposite effects observed between OA and DA administration (post-hoc test results in **Table S2; Fig 1**). High doses of OA improved PER performance across all modalities, whereas low or moderate doses did not have a significant effect (**Fig R1**). Conversely, the effects of DA delivery on learning were more complex, varying depending on the stimulus modality employed. Bees trained with olfactory stimulation showed significant inhibition of PER performance with low and moderate doses of DA, but not with high doses. The PER inhibition for bees trained with visual stimuli was only significant for mid dosages. Finally, DA inhibition of bimodal learning was more apparent at higher dosages, however, such inhibition was not significantly different from the performance obtained with lower DA dosing (**Fig 1**).

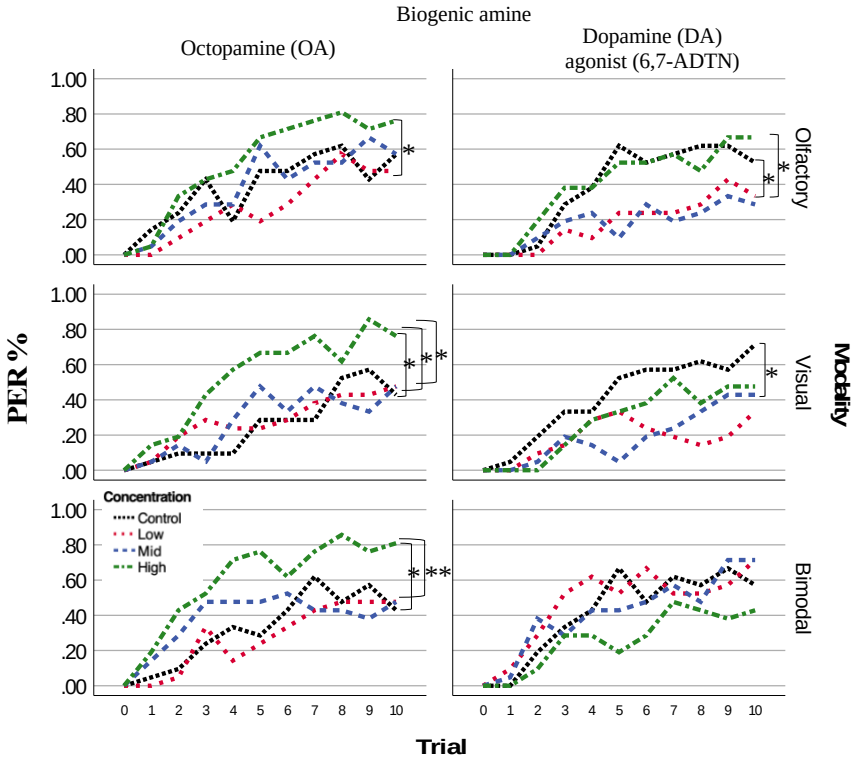

Fig. 1. Performance curves showing the percentage of conditioned proboscis extension reflex (PER) exhibited by bumble bees during 10 trials of absolute conditioning. The left panels of the figure display the performance of bees treated with octopamine (OA), while the right panels show the performance of bees treated with the dopamine agonists (DA, 6,7-ADTN). The top, middle, and bottom panels display the learning performance of bees exposed to olfactory, visual, and bimodal cues, respectively. The colour of the lines in each panel indicates the biogenic amine concentration: black represents the control concentration, while red, blue, and high correspond to the low, mid, and highest dosages, respectively. Significant differences are indicated.

Modality, amine, and concentration effects on PER latency time during acquisition

Stimulus modality and amine concentration influenced the speed of bumble bees' response during the acquisition (LMM: *Modality effect:* $\chi^2_{2,504}$ = 9.665; p=0.008; *Concentration effect:* $\chi^2_{3,504}$ = 14.425; p=0.002; see model **Table S1b**), while latency also changed across trials (LMM: *Trial effect*: $\chi^2_{9,504}$ = 4.167; p=0.041). The amine factor alone did not significantly affect the bees' latency time (LMM: *Amine effect*: $\chi^2_{1,504}$ = 1.517; p=0.218), nor interacted with concentration to affect the bees' response speed (LMM: *Amine x Concentration*: $\chi^2_{3,504}$ = 6.780; p<0.079). Similarly, Modality did not influence bees' latency across trials (LMM: *Modality x Trial*: $\chi^2_{2,504}$ = 4.948; p<0.084). Moreover, we did not find an interaction effect between modality and individual amine (LMM: *Modality x Amine effect*: $\chi^2_{2,504}$ = 0.1792; p=0.914). Our analysis revealed that DA agonist treatment significantly affected olfactory cue learning in bees, with response speed increasing as DA concentration increased. No significant effects were detected for other treatments (see **Table S3** and **Fig 2** for post hoc results). However, the effect of amine and concentration interaction on latency time varied depending on the modality (**Fig 2**). Bees under visual stimulation tended to exhibit faster reaction times with increasing DA concentration (**Fig 2; Table S3**), whereas no clear trend was observed in bees trained with bimodal cues (**Fig 2; Table S3**). Bees trained with olfactory cues showed progressively delayed responses as OA concentration increased, while those trained with bimodal cues exhibited faster reactions at higher OA concentrations (**Fig 2; Table S3**). Meanwhile, bees trained with visual cues displayed slower reaction times at mid OA concentrations (**Fig 2**).

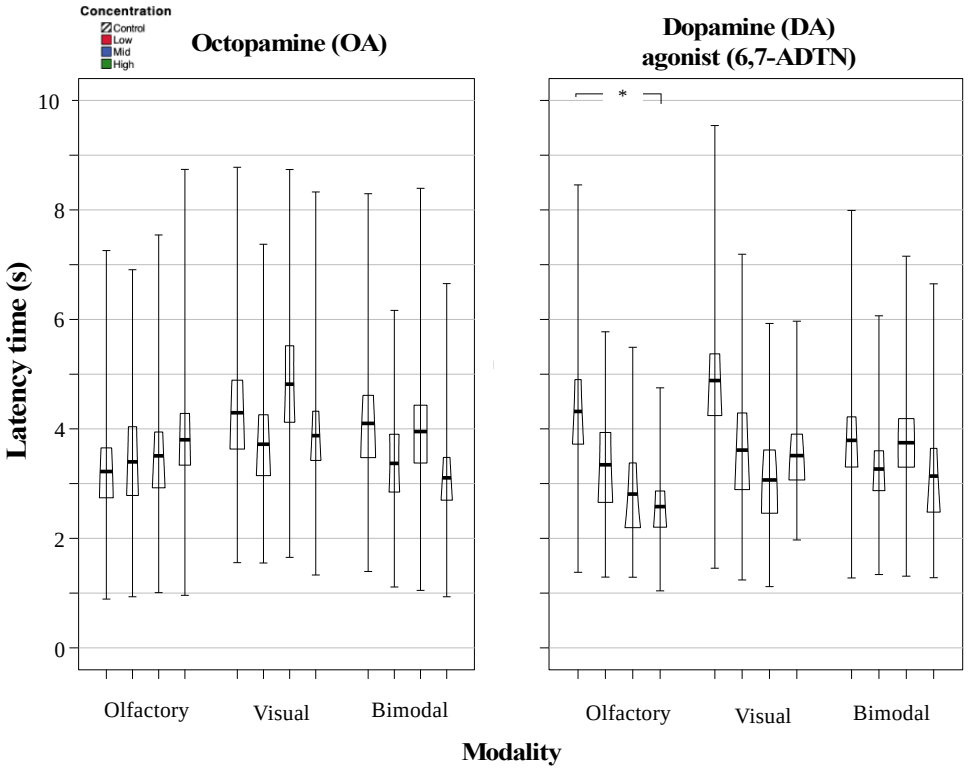

Fig. 2. Violin plots show the distribution of latency time (s) during acquisition phase for eliciting conditioned PER response in bumble bees. The left panel displays latency time of bees treated with octopamine (OA), while the right panel shows the performance of bees treated with dopamine agonist (DA -6,7ADTN). The colour of the lines in each panel corresponds to the biogenic amine concentration, with black indicating the control concentration and red, blue, and high representing low, mid, and highest dosages, respectively. Bees were conditioned to respond to olfactory, visual, and bimodal cues after receiving the respective amine dosages. Statistical differences are indicated (see Table S1 and results).

Effects on the PER response and latency time during memory retention test

During the memory retention test, the bumble bees' PER performance was influenced by the sensory modality used during training (GLMM, *modality effect:* $\chi^2_{2,504} = 16.810$; $p<0.0001$; **Table S4a**), as well as by the interaction between the amine identity and its concentration (GLMM, *Amine x Concentration effect:* $\chi^2_{3,504} = 14.625$; $p=0.002$). Interestingly, concentration alone was also found to be a significant factor, while amine alone did not render a significant effect (GLMM, *concentration effect:* $\chi^2_{2=3,504} = 15.278$; $p=0.002$; *amine effect:* $\chi^2_{1,504} = 0.0041$; $p=0.949$). We found that bumble bees treated with OA and trained with either unimodal or bimodal cues exhibited a concentration-dependent increase in PER recall, with a significant improvement in PER performance at high, relative to low OA concentrations. Notably, the most significant improvement in PER performance induced by higher OA dosage was observed in bees trained with bimodal stimulation (post hoc test **Table S5**; **Fig 3**). In contrast, while bees treated with OA showed a concentration-dependent improvement in PER recall, bees treated with DA exhibited a sharp decline in memory performance at low and mid dosages for unimodal tasks. However, in contrast to unimodal tasks, the inhibition of memory induced by DA did not affect the recall of bimodal tasks in bumble bees (post hoc test **Table S5**; **Fig 3**).

Finally, in contrast to the acquisition phase, the memory retention test showed no significant effects of the experimental factors on the latency time of bumble bees (see LMM model in Table S4b). Neither modality, amine, nor concentration had a significant impact on the latency time during the retention test (ANOVA: $F_{2,161} = 1.88$, $p = 0.15$; $F_{1,161} = 0.02$, $p = 0.87$; $F_{3,161} = 2.30$, $p = 0.07$; respectively; see post hoc test, Table S6).

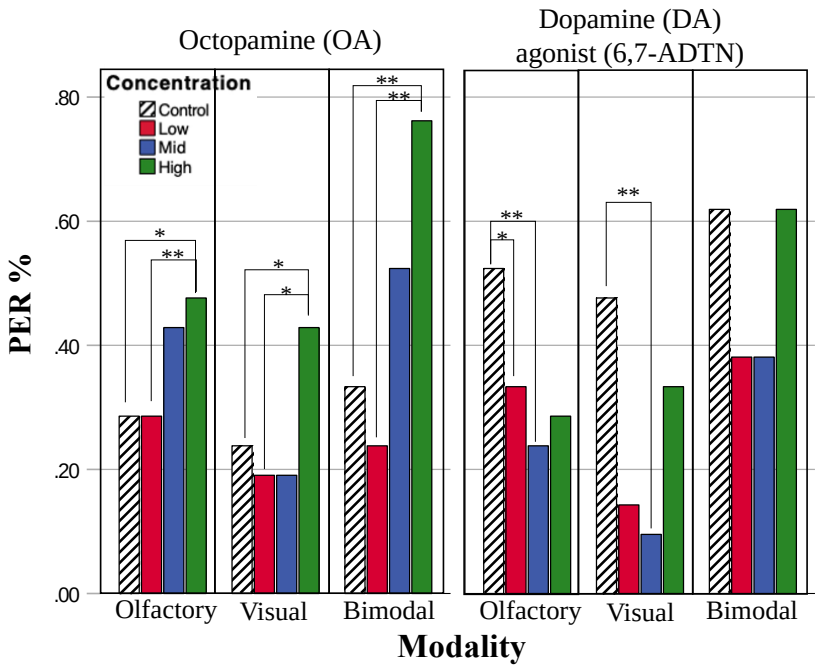

Fig. 3. Performance of bumble bees during the memory retention test represented as the percentage of conditioned PERs for each combination of biogenic amines and dosage concentrations after stimulation with the same unrewarded cue used during acquisition trials, 24 hours earlier. Statistical differences are indicated as *p<0.05; **p<0.01.

DISCUSSION

Our objective was to determine the roles of octopamine (OA) and the dopamine agonist 6,7-ADTN during unimodal (olfaction and vision) and multimodal (bimodal) appetitive learning and memory in bumble bees, while exploring possible interaction effects between amine dosage and the sensory modalities used. Our findings suggest that both OA and DA agonist 6,7-ADTN might have complementary roles on PER conditioning, irrespective of the sensory modality employed during appetitive associative learning. Overall, our results are consistent with the well-established role of octopamine in appetitive learning for invertebrates, especially in the context of olfactory conditioning. Moreover, our results extend this role to include enhancement of visual and bimodal learning in bumble bees. However, contrary to the traditional view of the dopaminergic system having an exclusive role in aversive learning in insects, we present complementary evidence to recent research implying a dopaminergic role during appetitive olfactory, visual, and probably bimodal learning. Notably, our results indicate a modulatory role of both biogenic amines during appetitive learning across modalities, with concentration-dependent and modality-specific effect sizes during PER conditioning.

OA at high concentrations generally enhanced unimodal and bimodal learning and memory, whereas low doses of 6,7-ADTN inhibited olfactory learning and significantly reduced its memory recall at low and mid concentrations. Mid-dosages had the most significant impact on inhibiting visual learning and memory. While the highest dosage of 6,7-ADTN showed the strongest inhibitory effect during bimodal learning, memory recall was mostly inhibited at the low and mid dosages; however, despite the observed trends, the effects during bimodal stimulation were not significant. Our study underscores the significance of context-specific effects of biogenic amines in exploring the neural mechanisms underlying associative learning and memory. Our findings reveal that the octopaminergic and dopaminergic systems play complementary roles in modulating appetitive learning, not limited to unimodal olfactory and visual contexts but also expanding to multimodal learning situations.

Octopaminergic and dopaminergic roles during appetitive olfactory learning and memory

Our results revealed both concentration-dependent and opposing effects on olfactory learning following the administration of OA and 6,7-ADTN. First, we found a significant enhancement in PER performance during olfactory learning and memory following the administration of OA, which is consistent with

previous research. Notably, this effect was only observed at the highest concentration tested (Figs 1, 3). OA has been shown to enhance olfactory learning in bees (Balfanz et al., 2014; Farooqui et al., 2003; Hammer and Menzel, 1998; Scheiner et al., 2002; Scheiner et al., 2006) and bumble bees (Muth et al., 2023) and equally relevant, evidence suggested that OA affects reward learning among other physiological roles (Hammer, 1993; Hammer, 1997; Perry and Barron, 2013; Roeder et al., 2003). Our results (Figs 1 and 3) are consistent with previous studies showing that OA enhances appetitive olfactory learning, memory formation, and retrieval (Scheiner et al., 2002; Scheiner et al., 2006). However, unlike the U-shaped dose-response curve reported for biogenic amines, we found a significant increase in PER performance only at the highest OA concentration (fig 1). The difference in the dose-response pattern could be due to variations in the method of drug administration, or even differences in body size relative to honey bees, as discussed below.

In contrast, our data also indicate that 6,7-ADTN impaired olfactory learning and memory in bumble bees at low and mid dosages with no significant changes observed at the highest dose (Fig 1 and 3, tables S2, S5). These results in bumble bees align with previous research on honey bees, where both DA and 6,7-ADNT impaired olfactory memory consolidation while blocking DA receptors enhances it (Klappenbach et al., 2013). These authors injected honey bees into the thorax utilizing the same dosage used here. Interestingly, they found that 6,7-ADTN impaired memory only during retention, not during acquisition. They reported that at 24 hours, significant memory impairment was observed only at the highest dose (10^{-2} M), while lower doses (10^{-3} M and 10^{-4} M) showed no significant effect. Mid-concentrations only had an effect after 72 hours. Conversely, our results on bumble bees showed memory impairment at low and mid dosages at 24 hours, but not at the highest dose (memory was not tested beyond 24 hours) (Fig 3). A possible explanation for the observed acquisition and memory differences is the drug administration method. Oral feeding and injection may have differentially affected the rate of access to the bee brain, impacting the time range of action of biogenic amines (Barron et al., 2007; Scheiner et al., 2002). Aside from differences in experimental methods and design, species-specific factors such as body size, variations in DA receptor subtypes, and neural circuitry may also contribute to the observed dose-dependent disparities (Sadd et al., 2015; Watanabe and Sasaki, 2022). Future research on bumble bees' neurophysiological mechanisms may aid in solving such disparities. However, the overall pattern is consistent: DA or its receptor agonist 6,7 ADTN applied before training has a detrimental effect on olfactory appetitive long-term memory and here, we also report its detrimental effect during acquisition. Indeed, recent research supports a role for dopamine in the appetitive learning of honey bees, extending beyond its typical aversive role. Higher levels of dopamine (beyond the doses tested here of 6,7-ADTN

and DA at 1.54 $x10^{-2}$ M and 2.612 x 10^{-2} M, respectively) improved sucrose solution's incentive value, individual appetitive responsiveness, and discriminant olfactory learning and memory retrieval, indicating dopamine's crucial role in reward motivation and acquisition (Huang et al., 2022). Our results on the impairment of 6,7-ADTN during olfactory learning and memory in bumble bees are consistent with this trend.

OA and 6,7-ADTN effects during visual learning and memory

We also compared the effects of OA and DA agonist 6,7-ADTN on appetitive visual conditioning and once again, we observed opposite effects between these two neuromodulator systems. Only bumble bees that received the highest dose of OA showed significant improvement in visual learning, and this effect was sustained through the memory retention test conducted 24 hours later (see Fig 3). This finding is in line with prior research demonstrating the arousing role of OA in insects general (Perry and Barron, 2013; Roeder et al., 2003), including honey bees (Mancini et al., 2018; Scheiner et al., 2006; Schilcher et al., 2021) and bumble bees in particular (Muth et al., 2023). Studies on honey bees have shown that a low dose ($4x10^{-7}$M) of the OA antagonist epinastine enhances visual discrimination tasks, while higher doses ($4x10^{-5}$ or $4x10^{-3}$M) hinder them (Vieira et al., 2018). In addition, Schilcher et al. (2021) found that in honey bees, a dose of 10^{-3} M OA, enhances the electroretinography receptor response, while a higher dose of 10^{-2} M is required for an increased walking speed towards light sources. Our study in bumble bees is consistent with these findings, as we did not observe an enhancing effect at OA concentrations of 10^{-4} or 10^{-3}M, but instead found that a higher 10^{-2}M dose was necessary to elicit a PER response during acquisition and memory (Fig 1). Consistently, recent studies on the effect of OA during visual learning in bumble bees, also have reported that higher doses than those observed in honey bees were required to enhance gustatory responsiveness and visual associative learning (Muth et al., 2023).

Following the opposite neuromodulator trend between OA and DA agonist 6,7-ADTN, we found that the latter impaired visual appetitive learning. Although all doses elicited reduced PER responses during visual learning and memory, only bumble bees treated with the mid-dose showed significant impairments in PER performance relative to control groups (Fig 1 and 3). Previous studies on the neuromodulation of visual appetitive learning in honey bees have shown a strong connection between the visual and dopaminergic systems, suggesting their involvement in the pathways for visual stimulus-reward association (Mancini et al., 2018; Vieira et al., 2018). Although the blockage of DA receptors employing brain injections of very

small amounts of the DA antagonist Flupentixol ($2x10^{-7}$ M) allowed visual discrimination learning, higher doses ($2x10^{-5}$ and $2x10^{-3}$ M) impaired it (Vieira et al., 2018). Interestingly, topical application of Flupentixol at $2x10^{-2}$ M concentration on the bee thorax was insufficient to block DA receptors and hinder visual discrimination; instead, to impair it, a higher dosage of $2x10^{-1}$ M was required (Mancini et al., 2018). Additionally, honey bees topically treated with two high concentrations of DA (0.105 and 1.05 M) were able to learn the discrimination between rewarded and non-rewarded stimuli, but their performance did not differ significantly from the control group (Mancini et al., 2018). The overall message of these studies is that enhancing DA signalling improved visual retention in the highest DA dose while blocking it with the highest dose of Flupentixol impaired both visual learning and retention. Since we observed impairment of the DA agonist 6,7-ADTN in visual learning (Fig 1) and memory retention (Fig 3), one possible interpretation of our results is that they contradict the aforementioned pattern (Mancini et al., 2018; Vieira et al., 2018). Alternatively, our results do not oppose such a trend, but they rather complement it. We believe this latter to be more likely: across studies, a general trend emerges despite variations in drug administration, molecule type, behavioural task, and species-specific differences: small doses of DA antagonists impair visual learning, slight increases in DA agonists still impede visual retention and memory (this study), while larger DA increases have a significant enhancing effect. Our results suggest that optimal dosage for learning may vary between different types of learning, and the effects of different dosages of DA agonist and antagonist are not linear, as evidenced by the varied effects of mid dosages across experiments. Thus, our results support the idea that DA signalling modulates visual appetitive learning.

OA and 6,7-ADTN effects during bimodal learning and memory

Pollinators' sensory reality is multimodal, therefore integrating multiple sources of information is essential for learning and memory tasks during foraging (Kulahci et al., 2008; Leonard and Masek, 2014a; Riveros et al., 2020). Our recent evaluation of the multimodal integration principles in honey bees and bumble bees revealed that effective integration depends on specific intensity (Gil-Guevara et al., 2022) and timing conditions (Gil-Guevara and Riveros, 2023 under review; Riveros, 2023), ultimately shaped by external factors of the signal. Here, we aimed to assess the role of biogenic amines as part of the physiological inner state of bees during multimodal learning and memory tasks. The PER performance of bumble bees during bimodal appetitive learning and memory indicates that both octopaminergic and dopaminergic systems have a similar impact as during unimodal olfactory and visual learning. Our study revealed a significant enhancement in the bimodal PER performance of bees treated with the highest OA dose compared to the

control group during both acquisition and retention (Fig 1 and 3). Multimodal enhancement is regarded both as an essential characteristic of multimodal communication and dependent on the spatiotemporal features and salience of unimodal components (Gil-Guevara and Riveros, 2023 under review; Gil-Guevara et al., 2022; Riveros, 2023; Stein and Stanford, 2008). Therefore, the substantial increase in PER performance of bees treated with the highest OA dose beyond the bimodal control suggests a boosting effect of the OA system during bimodal learning and memory. On the same token, the DA agonist 6,7-ADTN caused a decrease in the PER performance, particularly at higher doses, which though non-significant, indicates a reversal of the enhancing effect of multimodal signals during acquisition, while during memory low and mid dosages persist to impair recall (Fig 1 and 3). We interpret our results as suggesting a concentration-dependent balance between the octopaminergic and dopaminergic systems, which may fine-tune the associative value of both unimodal and bimodal sensory stimulation. The notion might be consistent with the reward prediction error signal, as suggested by Rescorla and Wagner (1972), widely assumed as a mechanism within Pavlovian conditioning (Barron et al., 2010; Burke et al., 2012; Hazy et al., 2010; Perry and Barron, 2013; Waddell, 2013). Therefore, the modulatory interplay of biogenic amines during unimodal and bimodal learning may be as fundamental as the multimodal integration abilities allowing an adaptive sensory plasticity.

Cross-modal interactions typically result in multimodal enhancement if specific spatiotemporal and intensity parameters are met. The extent of the integration is indicated by the underlying neural computations, ranging from large enhancements due to additive and superadditive effects to subadditive or even multimodal depression (Chandrasekaran, 2017; Ma and Pouget, 2008; Meredith and Stein, 1983; Stanford and Stein, 2007; Stein and Stanford, 2008; Stein et al., 1988). It might be plausible to predict an interaction between external traits of cross-modal signals and those of the inner neuromodulator environment, which could impact multimodal computations (e.g., additive, superadditive, subadditive). It may be worth further investigating the extent to which the octopaminergic and dopaminergic systems influence these computations during learning tasks in bees, given the existing support from anatomical, neurophysiological, and neuropharmacological convergence. Mushroom body (MB) output neurons (ON) in bee brains integrate across visual, olfactory, and bimodal modalities (Strube-Bloss and Rössler, 2018; Strube-Bloss et al., 2016) within specific regions of the MB (Ehmer and Gronenberg, 2002; Gronenberg, 2001; Mobbs, 1982; Strausfeld, 2002). In turn, significant convergence of octopaminergic and dopaminergic circuits occurs in relevant brain regions, such as the MB, driving both aversive and appetitive learning (Perry and Barron, 2013; Sabandal et al., 2020; Scheiner et al., 2006; Schilcher et al., 2021).

Effects on latency time during acquisition and memory

During acquisition, we found independent effects of modality and amine concentration on bumblebee latency time during PER conditioning. However, we did not observe any significant interactions between amine identity and concentration, or between modality and concentration, in influencing latency time. Consequently, we found no clear concentration effect on a modality basis during the acquisition task, except for olfactory learning where the DA agonist 6,7-ADTN increased PER response speed with increasing concentration (Fig 2). Our study revealed a non-significant trend towards a speed-up effect by DA during visual learning. Additionally, only olfactory learning exhibited a slight increasing trend under the influence of OA, although this trend was not statistically significant. Higher doses of biogenic amines may be required to affect the reaction time of bees during visual and olfactory learning. This may also hold true for detecting lasting effects during memory retention tests using oral feeding for amine delivery.

Furthermore, we did not find any trends in latency for our bimodal learning task, despite the suggested changes in reaction time as one of the potential enhancements resulting from bimodal signalling and integration (Hebets and Papaj, 2005b; Leonard and Masek, 2014a). However, it has also been suggested that this multimodal effect is expected when the individual components of a multimodal stimulus have comparable levels of performance. This implies that an acceleration in latency time response may result after adjusting for the salience of the unimodal elements of the composed stimuli to ensure a similar performance among them (Otto et al., 2013). To investigate the effects of biogenic amines on reaction time during multimodal learning tasks, future research should aim to control the saliency of unimodal stimuli and ensure equivalent performance levels between visual and olfactory modalities

Our results support the idea that feeding biogenic amines can be an effective alternative to injection or topical application (Barron et al., 2007; Scheiner et al., 2002). Feeding is a non-invasive drug delivery method that produced clear results without harming bees, who may not exhibit strong visual learning under stressful conditions (Mancini et al., 2018). However, while simple non-invasive treatment methods like oral and topical administration effectively deliver biogenic amines to honey and bumble bees (Muth 2023), feeding may lead to uneven distribution of biogenic amines, unlike tissue-directed injections (Barron et al., 2007; Scheiner et al., 2002). Although it can be challenging to directly compare results from studies using different administration methods, they can still provide valuable insights into the effects of different treatments on different insect taxa. Performing comparative delivery methods in bumble bees can enhance our understanding of how their responses may differ from other species.

Conclusions

The effects of the tested biogenic amine systems on unimodal and bimodal PER conditioning were dependent on the concentration levels. Overall, opposite effects were observed between OA and DA administration. High doses of OA improved PER performance across all modalities, whereas the effects of DA delivery on learning were more complex and varied depending on the stimulus modality employed. These observations suggest that different biogenic amines may have complementary roles in learning and memory processes. Bimodal learning and memory may be influenced by the same forces acting during unisensory learning, crossmodally unifying the amine roles during learning tasks. Our present study on bumble bees extends the understanding of how octopamine and dopamine complementarily modulate appetitive learning and memory during unimodal and bimodal tasks. Given the convergence of multiple sensory modalities, the necessary conditions for processing, integration, as well as the dose-dependent amine action, there may be opportunities for precise modulation within the context of prediction error in both appetitive and aversive learning.

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Supplementary information for Chapter 3.
Complementary roles of octopamine and dopamine during olfactory, visual, and bimodal learning and memory in bumble bees (*Bombus impatiens*)

Table S1. A) Summary of the binomial Generalized linear mixed model (GLMM) of the effect of Modality (olfactory, visual, or bimodal) and biogenic amine (octopamine -OA or dopamine agonist -DA) at four concentration levels (control, low, mid, and high) on the probability of eliciting a PER response during the acquisition phase (across 10 trials; repeated measures) of the associative conditioning experiments on bumble bees (Model fit: link function (logit), conditional R^2=0.67; AIC = 4734.52; BIC=5054.26; ICC= 0.57; individual bees denoted as random effects, n= 504). **B)** Linear mixed model (LMM) exploring the change in latency time (s) as a function of modality, biogenic amine and concentration, across the training trials during acquisition (Model fit using the REML method; t-test adjusted using Satterthwaite's method; AIC= 8488.02; individual bees as random effects n=387).

A) GLMM Acquisition Model

Formula:

PER (Response) ~ Modality (3) X Amine(2) X Concentration(4) X Trial + (1| individual bee) (random effect)

Independent (Fixed effects)	**Estimate**	**SE**	**Z val.**	***p***	
Intercept (Modality: Olfaction; Amine: OA; Concentration: Control)	-2.196	0.639	-3.437	**0.001**	****
Visual	-1.754	0.977	-1.794	**0.073**	*
Bimodal	-0.547	0.907	-0.603	0.547	
DA antagonist (6,7, ADTN)	-0.344	0.892	-0.386	0.700	
Low [ ]	-1.986	0.999	-1.989	**0.047**	**
Mid [ ]	-0.596	0.921	-0.647	0.518	
High [ ]	-0.096	0.902	-0.107	0.915	
Trial	0.298	0.069	4.349	**0.000**	****
Visual * DA antagonist (6,7, ADTN)	1.517	1.329	1.142	0.254	
Bimodal * DA antagonist (6,7, ADTN)	0.236	1.288	0.183	0.855	

B) LMM Acquisition Model

Formula:

Latency time (Response) ~ Modality (3) X Amine (2) X Concentration (4) X Trial + (1| individual bee) (random effect)

Estimate	**SE**	**DF**	**t value**	***p***	
3.236	0.718	1375	4.506	**0.000**	***
2.324	1.260	1618	1.844	**0.065**	.
1.205	1.108	1580	1.087	0.277	
1.586	1.095	1553	1.447	0.148	
0.731	1.203	1593	0.608	0.544	
0.339	1.053	1456	0.322	0.747	
0.434	0.967	1370	0.449	0.654	
0.025	0.089	1670	0.283	0.777	
-2.340	1.684	1608	-1.390	0.165	
-0.881	1.567	1595	-0.562	0.574	

Visual * Low [ ]	2.684	1.428	1.880	**0.060**	*	-3.412	1.809	1604	-1.886	**0.059**	.
Bimodal * Low [ ]	0.829	1.396	0.594	0.553		-0.319	1.763	1627	-0.181	0.857	
Visual * Mid [ ]	1.276	1.372	0.930	0.352		-1.257	1.740	1605	-0.722	0.470	
Bimodal * Mid [ ]	1.888	1.290	1.464	0.143		-0.131	1.524	1458	-0.086	0.931	
Visual * High [ ]	2.103	1.322	1.591	0.112		-0.965	1.554	1544	-0.621	0.535	
Bimodal * High [ ]	1.112	1.287	0.864	0.388		-1.318	1.407	1457	-0.937	0.349	
DA antagonist (6,7, ADTN) * Low [ ]	-0.960	1.510	-0.636	0.525		-1.177	1.982	1637	-0.594	0.553	
DA antagonist (6,7, ADTN) * Mid [ ]	-0.450	1.340	-0.336	0.737		-3.725	1.727	1518	-2.157	**0.031**	*
DA antagonist (6,7, ADTN) * High [ ]	-0.459	1.287	-0.356	0.721		-2.761	1.468	1492	-1.880	**0.060**	.
Visual * Trial	0.135	0.105	1.281	0.200		-0.131	0.155	1781	-0.844	0.399	
Bimodal * Trial	0.032	0.096	0.335	0.737		-0.020	0.139	1654	-0.144	0.885	
DA antagonist (6,7, ADTN) * Trial	0.066	0.095	0.702	0.483		-0.074	0.138	1694	-0.538	0.590	
Low [ ] * Trial	0.184	0.111	1.666	**0.096**	*	-0.072	0.146	1653	-0.494	0.621	
Mid [ ] * Trial	0.107	0.101	1.059	0.290		0.005	0.130	1676	0.036	0.972	
High [ ] * Trial	0.199	0.105	1.904	**0.057**	*	0.032	0.118	1635	0.268	0.789	
Visual * DA antagonist (6,7, ADTN) * Low [ ]	0.161	2.055	0.078	0.937		3.672	2.724	1613	1.348	0.178	
Bimodal * DA antagonist (6,7, ADTN) * Low [ ]	3.272	2.017	1.622	0.105		-0.870	2.557	1613	-0.340	0.734	
Visual * DA antagonist (6,7, ADTN) * Mid [ ]	-2.748	2.022	-1.359	0.174		5.306	2.628	1593	2.019	**0.044**	*
Bimodal * DA antagonist (6,7, ADTN) * Mid [ ]	-0.358	1.859	-0.193	0.847		3.456	2.279	1495	1.516	0.130	
Visual * DA antagonist (6,7, ADTN) High [ ]	-2.840	1.890	-1.502	0.133		3.459	2.297	1597	1.506	0.132	
Bimodal * DA antagonist (6,7, ADTN) * High [ ]	-1.417	1.870	-0.757	0.449		2.686	2.149	1524	1.250	0.212	
Visual * DA antagonist (6,7, ADTN) * Trial	-0.070	0.144	-0.489	0.625		0.225	0.209	1745	1.077	0.282	

Bimodal * DA antagonist (6,7, ADTN) * Trial	0.049	0.140	0.351	0.726		-0.098	0.197	1654	-0.499	0.618	
Visual * Low [ ] * Trial	-0.290	0.156	-1.854	**0.064**	*	0.290	0.220	1734	1.317	0.188	
Bimodal * Low [ ] * Trial	-0.087	0.152	-0.574	0.566		-0.124	0.217	1690	-0.572	0.568	
Visual * Mid [ ] *Trial	-0.200	0.149	-1.346	0.178		0.176	0.216	1748	0.817	0.414	
Bimodal * Mid [ ] * Trial	-0.288	0.138	-2.088	**0.037**	**	-0.095	0.190	1675	-0.501	0.616	
Visual High [ ] * Trial	-0.189	0.150	-1.255	0.210		-0.057	0.191	1732	-0.297	0.766	
Bimodal * High [ ] * Trial	-0.019	0.153	-0.127	0.899		-0.065	0.173	1626	-0.378	0.706	
DA antagonist (6,7, ADTN) * Low [ ] * Trial	-0.017	0.167	-0.104	0.917		0.060	0.241	1727	0.248	0.804	
DA antagonist (6,7, ADTN) * Mid [ ] * Trial	-0.212	0.144	-1.471	0.141		0.257	0.220	1787	1.164	0.245	
DA antagonist (6,7, ADTN)* High [ ] * Trial	-0.107	0.146	-0.733	0.464		0.050	0.182	1641	0.274	0.784	
Visual * DA antagonist (6,7, ADTN) * Low [ ] * Trial	-0.136	0.226	-0.603	0.546		-0.426	0.337	1755	-1.264	0.206	
Bimodal * DA antagonist (6,7, ADTN) * Low[ ] * Trial	-0.222	0.223	-0.995	0.320		0.291	0.314	1708	0.927	0.354	
Visual * DA antagonist (6,7, ADTN) * Mid [ ] * Trial	0.399	0.221	1.807	**0.071**	*	-0.738	0.326	1778	-2.263	**0.024**	*
Bimodal * DA antagonist (6,7, ADTN) * Mid [ ] * Trial	0.314	0.201	1.559	0.119		-0.167	0.288	1735	-0.582	0.561	
Visual * DA antagonist (6,7, ADTN) * High [ ] * Trial	0.130	0.213	0.611	0.541		-0.266	0.283	1692	-0.938	0.348	
Bimodal * DA antagonist (6,7, ADTN) * High [ ] * Trial	-0.134	0.216	-0.619	0.536		0.063	0.265	1625	0.237	0.812	

Table S2. Post-hoc comparison of PER response in bees across amine concentration levels for each stimulation modality, based on the GLMM PER acquisition model (see Table S1A; Fig R1).

Octopamine (OA)

Concentration contrast			Olfactory					Visual					Bimodal				
			Estimate	SE	z. ratio	*p*		Estimate	SE	z. ratio	*p*		Estimate	SE	z. ratio	*p*	
Control	–	Low	0.974	0.720	1.351	0.530		-0.118	0.730	-0.162	0.999		0.625	0.719	0.869	0.821	
Control	–	Mid	0.009	0.705	0.013	1.000		-0.164	0.727	-0.226	0.996		-0.293	0.710	-0.412	0.976	
Control	–	High	-1.001	0.705	-1.419	0.487		-2.065	0.715	-2.887	**0.020**	*	-2.005	0.715	-2.807	**0.026**	*
Low	–	Mid	-0.964	0.721	-1.338	0.539		-0.046	0.729	-0.063	1.000		-0.917	0.726	-1.264	0.586	
Low	–	High	-1.974	0.721	-2.739	**0.031**	*	-1.947	0.717	-2.717	**0.033**	*	-2.630	0.730	-3.604	**0.002**	**
Mid	–	High	-1.010	0.706	-1.432	0.480		-1.901	0.714	-2.662	**0.039**	*	-1.713	0.721	-2.374	**0.082**	·

Dopamine agonist (DA)

[ ] Contrast			Olfactory					Visual					Bimodal				
Control	–	Low	2.029	0.753	2.695	**0.035**	*	1.524	0.723	2.107	0.151		-0.371	0.706	-0.526	0.953	
Control	–	Mid	1.625	0.731	2.224	0.117		2.004	0.748	2.679	**0.037**	*	-0.044	0.704	-0.063	1.000	
Control	–	High	0.047	0.704	0.067	1.000		1.107	0.718	1.541	0.413		1.194	0.724	1.649	0.351	
Low	–	Mid	-0.404	0.785	-0.515	0.956		0.480	0.768	0.625	0.924		0.327	0.702	0.466	0.966	
Low	–	High	-1.982	0.760	-2.606	**0.045**	*	-0.417	0.739	-0.564	0.943		1.565	0.722	2.168	0.132	
Mid	–	High	-1.578	0.739	-2.136	0.142		-0.897	0.763	-1.175	0.643		1.238	0.720	1.720	0.313	

Table S3. Post-hoc after LMM model for latency time (s) during acquisition (see Table S1B; Figure R2).

Octopamine (OA)

Concentration contrast			Olfactory						Visual						Bimodal					
			Estimate	SE	DF	z. ratio	*p*		Estimate	SE	DF	z. ratio	*p*		Estimate	SE	DF	z. ratio	*p*	
Control	–	Low	-0.253	0.624	365	-0.405	0.978		1.235	0.655	406	1.886	0.236		0.8896	0.63	364	1.413	0.492	
Control	–	Mid	-0.370	0.580	348	-0.638	0.920		-0.284	0.651	409	-0.435	0.972		0.3941	0.61	341	0.65	0.916	
Control	–	High	-0.644	0.558	316	-1.155	0.656		0.697	0.585	387	1.191	0.633		1.1083	0.56	329	1.99	0.194	
Low	–	Mid	-0.117	0.621	369	-0.189	0.998		-1.518	0.659	368	-2.302	**0.099**	.	-0.4956	0.65	338	-0.768	0.869	
Low	–	High	-0.392	0.600	340	-0.653	0.914		-0.538	0.594	341	-0.905	0.802		0.2186	0.6	327	0.365	0.983	
Mid	–	High	-0.274	0.554	319	-0.495	0.960		0.980	0.591	344	1.660	0.347		0.7142	0.58	302	1.243	0.600	

Dopamine agonist (DA)

[ ] Contrast			Olfactory						Visual						Bimodal					
Control	–	Low	0.528	0.702	393	0.752	0.876		1.168	0.656	396	1.781	0.284		0.609	0.570	307	1.068	0.710	
Control	–	Mid	1.652	0.673	376	2.456	**0.069**	.	1.332	0.680	406	1.958	0.206		0.072	0.570	314	0.125	0.999	
Control	–	High	1.785	0.578	310	3.090	**0.012**	*	1.431	0.617	345	2.321	**0.095**	.	0.433	0.635	342	0.682	0.904	
Low	–	Mid	1.124	0.796	412	1.413	0.492		0.164	0.755	459	0.217	0.996		-0.537	0.562	311	-0.956	0.774	
Low	–	High	1.257	0.717	368	1.753	0.298		0.263	0.699	410	0.377	0.982		-0.175	0.628	339	-0.279	0.992	
Mid	–	High	0.133	0.689	352	0.193	0.998		0.099	0.722	418	0.137	0.999		0.362	0.627	346	0.576	0.939	

Table S4. A) Summary of a binomial General linear mixed model (GLMM) for the PER response of bumble bees during the memory retention test 24 hours after training, testing the effect of amine dosage on the recall probability of unimodal and bimodal stimuli. Model fit link function (logit), AIC= 643; BIC=723; individual bumble bees denoted as random effects=504. **B)** Linear mixed effects model (Lme) to test the effect on the latency time of bumble bees during the memory retention test (model fit: AIC=881, BIC= 661; bumble bees entered as random effects = 185). Significance values following a Bonferroni's correction.

A) GLMM for PER during memory test

Formula:

PER (Response) ~ (Modality3 + Amine2 + Concentration4) ^2 + (1|Individual bee) (random effect)

Independent (Fixed effects)	Estimate	SE	z value	*p*	
Intercept (Modality: Olfaction; Amine: OA; Concentration: Control)	-0.90	0.41	-2.18	**0.029**	*
Visual	-0.24	0.54	-0.44	0.658	
Bimodal	0.17	0.52	0.33	0.741	
DA antagonist (6,7, ADTN)	0.98	0.47	2.10	**0.035**	*
Low [ ]	0.03	0.55	0.05	0.963	
Mid [ ]	0.60	0.54	1.12	0.264	
High [ ]	0.76	0.53	1.44	0.151	
Visual * DA antagonist (6,7, ADTN)	0.05	0.50	0.09	0.927	
Bimodal * DA antagonist (6,7, ADTN)	0.26	0.47	0.57	0.569	
Visual * Low [ ]	-0.59	0.71	-0.84	0.402	
Bimodal * Low [ ]	-0.31	0.66	-0.47	0.637	
Visual * Mid [ ]	-0.91	0.73	-1.24	0.213	
Bimodal * Mid [ ]	0.22	0.65	0.35	0.729	
Visual * High [ ]	0.22	0.65	0.34	0.737	
Bimodal * High [ ]	1.01	0.65	1.54	0.123	
DA antagonist (6,7, ADTN) * Low [ ]	-0.84	0.56	-1.50	0.134	

B) Linear mixed-effects model (lme) for latency (s)

Formula:

Latency time (response) ~ Modality (3) X Amine (2) X Concentration (4) + (1|individual honeybee) (random effect)

	Value	SE	df	T value	*p*	
	4.36	1.13	161.00	3.87	**0.000**	***
Visual	2.71	1.67	161.00	1.62	0.106	
Bimodal	0.19	1.53	161.00	0.12	0.904	
DA antagonist (6,7, ADTN)	2.54	1.40	161.00	1.81	**0.072**	.
Low [ ]	-1.43	1.59	161.00	-0.90	0.370	
Mid [ ]	1.57	1.45	161.00	1.08	0.283	
High [ ]	1.29	1.42	161.00	0.90	0.368	
Visual * DA antagonist (6,7, ADTN)	-3.34	2.06	161.00	-1.62	0.106	
Bimodal * DA antagonist (6,7, ADTN)	-3.10	1.90	161.00	-1.63	0.106	
Visual * Low [ ]	-0.68	2.44	161.00	-0.28	0.779	
Bimodal * Low [ ]	1.50	2.27	161.00	0.66	0.510	
Visual * Mid [ ]	-5.62	2.35	161.00	-2.39	**0.018**	*
Bimodal * Mid [ ]	-2.54	1.97	161.00	-1.29	0.200	
Visual * High [ ]	-1.99	2.09	161.00	-0.95	0.344	
Bimodal * High [ ]	-0.91	1.89	161.00	-0.48	0.631	
DA antagonist (6,7, ADTN) * Low [ ]	-1.37	2.08	161.00	-0.66	0.509	

DA antagonist (6,7, ADTN) * Mid [ ]	-1.84	0.56	-3.27	**0.001**	**	-4.24	2.08	161.00	-2.04	**0.043**	*
DA antagonist (6,7, ADTN) * High [ ]	-1.71	0.54	-3.20	**0.001**	**	-3.05	2.00	161.00	-1.53	0.128	
Visual * DA antagonist (6,7, ADTN) * Low [ ]	-	-	-	-		1.28	3.32	161.00	0.39	0.700	
Bimodal * DA antagonist (6,7, ADTN) * Low [ ]	-	-	-	-		2.17	2.91	161.00	0.75	0.457	
Visual * DA antagonist (6,7, ADTN) * Mid [ ]	-	-	-	-		5.12	3.51	161.00	1.46	0.146	
Bimodal * DA antagonist (6,7, ADTN) * Mid [ ]	-	-	-	-		5.87	2.76	161.00	2.12	**0.035**	*
Visual * DA antagonist (6,7, ADTN) High [ ]	-	-	-	-		2.88	2.86	161.00	1.01	0.316	
Bimodal * DA antagonist (6,7, ADTN) * High [ ]	-	-	-	-		3.84	2.59	161.00	1.48	0.140	

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Table S5. Post-hoc comparisons of the PER response in bumblebees across concentration levels of each biogenic amine for every stimulation modality, using the GLMM PER memory test (see Table S4A; Fig R3).

Octopamine (OA)

Concentration contrast			Olfactory					Visual					Bimodal				
			Estimate	SE	z. ratio	*p*		Estimate	SE	z. ratio	*p*		Estimate	SE	z. ratio	*p*	
Control	–	Low	-0.03	0.55	-0.05	0.991		0.57	0.61	0.92	0.793		0.29	0.55	0.52	0.955	
Control	–	Mid	-0.60	0.54	-1.12	0.679		0.30	0.62	0.49	0.960		-0.83	0.53	-1.56	0.402	
Control	–	High	-1.19	0.39	-3.09	**0.011**	*	-1.39	0.37	-3.09	**0.010**	*****	-1.77	0.55	-3.25	**0.006**	******
Low	–	Mid	-0.58	0.55	-1.06	0.715		-0.26	0.67	-0.39	0.979		-1.11	0.55	-2.03	0.176	
Low	–	High	-1.44	0.40	-3.63	**0.002**	**	-1.55	0.60	-2.60	**0.046**	*	-2.06	0.56	-3.66	**0.002**	******
Mid	–	High	-0.16	0.53	-0.30	0.990		-1.29	0.60	-2.16	0.133		-0.94	0.54	-1.74	0.304	

Dopamine agonist (DA)

[ ] Contrast			Olfactory					Visual					Bimodal				
Control	–	Low	1.12	0.38	2.92	**0.019**	*	1.41	0.59	2.39	**0.079**	·	1.13	0.54	2.10	0.154	
Control	–	Mid	1.38	0.40	3.47	**0.003**	******	2.14	0.64	3.33	**0.005**	**	1.01	0.53	1.90	0.228	
Control	–	High	0.95	0.53	1.79	0.279		0.73	0.53	1.37	0.517		-0.06	0.53	-0.12	0.999	
Low	–	Mid	0.42	0.57	0.74	0.879		0.74	0.69	1.06	0.713		-0.12	0.54	-0.21	0.997	
Low	–	High	0.13	0.55	0.25	0.995		-0.68	0.60	-1.14	0.667		-1.19	0.54	-2.20	0.123	
Mid	–	High	-0.29	0.56	-0.51	0.957		-1.41	0.65	-2.18	0.128		-1.07	0.54	-2.00	0.187	

Table S6. Post-hoc tests for contrasts in latency time to PER among concentration levels, biogenic amines, and modalities during the memory test.

Octopamine (OA)

Concentration contrast			Olfactory					Visual					Bimodal				
			Estimate	SE		z. ratio	*p*	Estimate	SE		z. ratio	*p*	Estimate	SE		z. ratio	*p*
Control	–	Low	1.432	1.59	161	0.9	0.805	2.116	1.85	161	1.145	0.663	-0.066	1.61	161	-0.041	1.000
Control	–	Mid	-1.566	1.45	161	-1.078	0.703	4.053	1.85	161	2.193	0.130	0.971	1.33	161	0.729	0.886
Control	–	High	-1.286	1.42	161	-0.904	0.803	0.702	1.54	161	0.456	0.968	-0.376	1.25	161	-0.301	0.991
Low	–	Mid	-2.998	1.45	161	-2.064	0.170	1.938	1.95	161	0.994	0.753	1.037	1.49	161	0.698	0.898
Low	–	High	-2.718	1.42	161	-1.91	0.228	-1.414	1.66	161	-0.854	0.828	-0.31	1.41	161	-0.219	0.996
Mid	–	High	0.28	1.27	161	0.221	0.996	-3.352	1.66	161	-2.024	0.184	-1.347	1.08	161	-1.247	0.598

Dopamine agonist (DA)

[ ] Contrast			Olfactory					Visual					Bimodal				
Control	–	Low	2.806	1.33	161	2.106	0.156	2.21	1.81	161	1.218	0.616	-0.86	1.24	161	-0.694	0.899
Control	–	Mid	2.674	1.49	161	1.799	0.278	3.177	2.13	161	1.488	0.447	-0.655	1.24	161	-0.529	0.952
Control	–	High	1.765	1.4	161	1.262	0.589	0.873	1.36	161	0.643	0.918	-1.164	1.08	161	-1.077	0.704
Low	–	Mid	-0.132	1.61	161	-0.082	1.000	0.967	2.52	161	0.384	0.981	0.205	1.38	161	0.149	0.999
Low	–	High	-1.041	1.53	161	-0.679	0.905	-1.338	1.9	161	-0.703	0.896	-0.304	1.24	161	-0.246	0.995
Mid	–	High	-0.909	1.67	161	-0.545	0.948	-2.304	2.21	161	-1.043	0.725	-0.509	1.24	161	-0.411	0.977

GENERAL CONCLUSIONS

This PhD thesis investigates the interplay between bimodal signal integration, multisensory processing, and the role of biogenic amines in learning and memory tasks of honey bees. The three chapters provide valuable insights into these interconnected concepts and on the mechanisms underlying bee's behaviour.

The first chapter focuses on the dependency of intensity, for the integration of bimodal signals during learning and memory tasks. Our findings demonstrate that successful integration of multimodal signals in honey bees during learning and memory tasks is influenced by the intensity levels of their individual components. This integration follows the principle of inverse effectiveness, akin to observations in vertebrates, suggesting comparable underlying neuronal computations. Notably, our study expands on previous knowledge by revealing not only the integration of vision and olfaction, but also an enhanced association between bimodal signals and a sucrose reward. This enhanced association is found to be dependent on the intensity levels of the individual components. Our findings align with phenomena observed in both unisensory and multimodal contexts across vertebrates. While the "cocktail party problem" describes how visual input aids in understanding speech in a noisy context, our findings support a "flower party effect" where the integration of visual and olfactory information enhances learning and memory at near-threshold intensities. These findings elucidate the intricate relationship between sensory modalities, signal integration, and reward processing in honey bees, providing valuable insights into honey bee remarkable learning and memory capabilities.

Building upon these findings on the role of intensity during bimodal integration, Chapter 2 explored the interaction between synchronicity, temporal order and intensity during learning and memory in honey bees. Our findings revealed interesting patterns in the bees' perceptual and behavioural responses. At low-intensity levels, relative to asynchronous stimulation, bimodal synchronous stimuli resulted in higher performance. However, at high-intensity levels, no significant differences were observed between asynchronous and synchronous stimulation. Additionally, we found that alternate temporal orders of asynchronous stimuli affected performance only at low intensities. That is, bees were performed differentially depending on the asynchronous order of presentation only at low intensities. Taken together these results support the temporal rule and inverse effectiveness principles, suggesting that bees exhibit enhanced perceptual processing when exposed to synchronous bimodal stimuli, particularly at lower levels

of sensory input. This indicates that the integration of multisensory information depends on the temporal synchrony between stimuli and the impact of unimodal features on processing speed, such as stimulus intensity. In addition, these effects were also detectable during memory retention indicating that honey bees learned differentially bimodal signals depending on the asynchronous temporal order relative to the reward. After recognizing the obvious differences between our experimental setup, in which bees were restrained, our findings suggest that bees may benefit more from associating congruent bimodal stimulation emitted from the same flower or patch when detecting them at a distance. As they approach the flower reward, olfaction becomes the primary guiding sense while visual cues provide secondary information for navigation. Our study contributes to our understanding of the interplay between synchronicity, temporal order, intensity, and bimodal integration in honey bees. These findings improve our understanding of the perceptual mechanisms and decision-making processes in insects, highlighting the adaptive strategies employed by bees in their foraging behaviour. Remarkably the fact that bimodal synchronicity is dependent on intensity level confirms the universality of the principles of multimodal integration across vertebrates and invertebrates.

My experiments employing bimodal stimulation in sync (Chapters 1, 2 and 3) and out of sync (Chapter 2) provide valuable insights into the complex nature of honey bee learning and memory, particularly when exploring the possibility of the existence of either blocking or overshadowing phenomena during multimodal integration. In chapter 2, the observed associative strength of olfactory stimuli over visual stimuli, regardless of temporal order, could be interpreted as a form of overshadowing, where the more salient olfactory stimulus 'overshadows' the less salient visual stimulus. However, the lack of selective retention based on temporal proximity to the reward in asynchronous configurations suggests that blocking is not a dominant factor in this context. Instead, an overshadowing effect could exist regarding the identity of the stimuli: that is, olfaction is the dominant sensory modality available to searching bees. Here, the overshadowing effect might occur as previously reported previously (Mota et al 2011; Riveros 2023): the visual stimuli may enlist attentional resources that enhance the olfactory learning, but this do not occur in the opposite order. That is, the sensory bias of honey bees towards olfaction, coupled with the presence of visual information in the VO order, could lead to overshadowing. Olfactory cues may dominate the association, overshadowing the visual cues, which might explain the observed differences in memory retention. On the other hand, the enhanced performance with synchronous bimodal stimuli indicates that the simultaneous presentation of stimuli may circumvent potential overshadowing effects. Nevertheless, the complex interplay between stimulus intensity, modality, and temporal configuration observed in my

study underscores the multifaceted nature of learning processes and highlights the need for further research to fully elucidate these phenomena.

Finally, chapter 3 investigates the effects of biogenic amines on unimodal and bimodal PER conditioning in bumble bees expanding our understanding of how octopamine (OA) and dopamine (DA) work in tandem to modulate appetitive learning and memory. The results suggest that the influence of octopamine (OA) and dopamine (DA) administration on learning and memory processes is concentration-dependent and exhibits distinct patterns. Higher doses of OA enhance PER performance across all modalities, including bimodal learning and memory, while the effects of DA delivery are more complex and depend on the specific stimulus modality. These findings suggest that OA and DA have complementary roles in learning and memory, possibly aiding in challenging the traditional view of them exclusively modulating different motivational systems in insects: appetitive and aversive. My work on the neuromodulation in bumble bees expands our knowledge of how octopamine and dopamine work together to modulate appetitive learning and memory in both unimodal and bimodal tasks. With the convergence of various sensory modalities and the specific conditions required for processing and integration, as well as the dose-dependent effects of these amines, there are potential opportunities for precise modulation in relation to prediction error during both appetitive and aversive learning scenarios.

Overall, the striking similarities observed between vertebrates and insects regarding multimodal integration principles and the involvement of biogenic amines underscore the universality of these mechanisms across different taxa. These findings contribute to our broader understanding of the neural mechanisms underlying learning and memory processes, emphasizing the adaptive strategies employed by animals in their foraging behaviour involving multiple modalities.