UNIVERSITE DE LIEGE
FACULTE DE MEDECINE VETERINAIRE
DEPARTEMENT DES MALADIES INFECTIEUSES ET PARASITAIRES
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Facteurs affectant la santé et la durabilité des colonies d'abeilles sans dard (Hymenoptera, Meliponini) en Équateur: interactions entre les pratiques de gestion, l'exposition aux pesticides, les ressources florales et les pathogènes

Factors affecting the health and sustainability of stingless bees (Hymenoptera, Meliponini) colonies in Ecuador: interactions between management practices, pesticide exposure, floral resources, and pathogens

Joseline Sofía OCAÑA CABRERA

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Abbreviations

AMPA	Aminomethyl-phosphonic acid
a.i.	Active ingredient
ABPV	Acute bee paralysis virus
AD	Anno Domini (after the birth of Christ)
AGROCALIDAD	Agency of the Regulation and Control of Plant and Animal Health (Ecuador)
AluI	Type I restriction endonuclease isolated from *Arthrobacter luteus*
ARES	Académie de recherche et d'enseignement supérieur
As	Arsenic
ASV	Amplicon Sequence Variant
BC	Before Christ
BQCV	Black queen cell virus
CBPV	Chronic bee paralysis virus
Cd	Cadmium
C I	Confidence Interval
CO_2	Carbon dioxide
COVID-19	Coronavirus disease of 2019
Cr	Chromium
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DWV	Deformed wing virus
DWV-A	Deformed wing virus genetic type A
EcoRI	Restriction enzyme from *Escherichia coli* strain R13
e.g.	*exampli gratia* (for example)
EPA	Environmental Protection Agency
ESPE	Escuela Superior Politécnica del Ejército (former name of the Universidad de las Fuerzas Armadas in Ecuador)
et.al	*et alia/aliae* (and others)
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FFF	Forest and Farm Facility
Firm-4	Phylum Firmicutes group 4
Firm-5	Phylum Firmicutes group 5
g	Grammes
GLY	Glyphosate
GMPs	Good Management Practices
h	Hours
Hg	Mercury
HpaII	*Haemophilus parainfluenzae* restriction enzyme
i.e.	*id est* (that is)
IAPV	Israeli acute paralysis virus
INEN	Servicio Ecuatoriano de Normalización
ITS2	Internal transcribed spacer 2
kg	Kilogrammes
km	Kilometre
LD_{50}	Median lethal dose
LC_{50}	Median lethal concentration
LOC	Level of concern
m a s l	Metres above sea level

MAATE	Ministry of Environment, Water, and Ecological Transition (Ecuador)
MAG	Ministry of Agriculture and Livestock (Ecuador)
mg	Milligrammes
mL	Millilitre
mm	Millimetre
MPs	Microplastics
N	North
N	Population size
n	Sample size
NDA	No data available
ng	Nanogrammes
NGO	Non-governmental Organization
Ni	Nickel
PAH	Polycyclic aromatic hydrocarbons
Pb	Lead
PCBs	Polychlorinated biphenyls
PET	Polyethylene terephthalate
pH	Potential of hydrogen
POPs	Persistent Organic Pollutants
PPP	Plant Protection Products
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PS	Polystyrene
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
RNA	Ribonucleic acid
RQ	Risk quotient
S	South
Sb	Tin
SB	Stingless bees
SEM	Scanning electron microscopy
Se	Selenium
Sn	Antimony
sp.	Species
US	United States
UTPL	Universidad Particular de Loja (Ecuadorian university)
Zn	Zinc
°C	Degrees Celsius
%	Percentage
$	Dollars
µL	Microlitre
µg	Microgrammes

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Abstract

Ecuador, located in the Neotropical region of South America, is a notable example of a megadiverse country, with approximately 200 species of stingless bees and more than 700 meliponicultors. The country is experiencing a significant growth in the practice of meliponiculture, concurrent with its status as a hotspot for native stingless bee diversity. As with other Hymenoptera, bees of the tribe Meliponini are experiencing a decline in population numbers, with this decline occurring due to a number of factors that either alone or in interaction reduce the number of their populations annually. The present study aims to provide empirical evidence on factors of anthropogenic origin, such as pesticides, human management, as well as those of natural origin, including pollen resources, pests, and pathogens, that influence the health status of stingless bees in Ecuador. In addition to the provision of guidance on actions to be taken, ensuring their positive impact on the conservation and reduction of stingless bee colony deaths.

By November 2024 (systematic review), a total of 15 pathogens and pests that affect the health of stingless bee colonies had been reported, as well as 26 contaminants that have lethal or sublethal effects on the lives of stingless bees (Study 1). The health of stingless bees in Ecuador is influenced by various factors, including the presence of pesticides and other contaminants in the environment, which can access the interior of the nest. Additionally, pests, some of which can become pathogenic, also pose a threat. In cerumen, a product of stingless bees, both for internal use in the nest and for application to humans, glyphosate and AMPA (a metabolite) were detected, along with heavy metals and metalloids (As, Cd, Cr, Ni, Pb) (Study 4). The estimated annual mortality rate of 15% for stingless bees farmed for honey production and sale (*Melipona*, *Tetragonisca*, *Scaptotrigona*, the latter genus depending on the country's climatic region) enabled the identification of areas for improvement through the application of good management practices (Study 5).

The type and diversity of pollen collected by bees affects their nutrition, which in turn impacts their immune system, their ability to resist pathogens, and their capacity to recover from stress caused by pesticides. Using two techniques — electron microscopy-morphometry (Study 2) and DNA barcoding (Study 3)— 27 plant families and 18 genera were identified, and up to 34 species, respectively. The first technique was based on pollen grain counts to determine the abundance of families and genera, with Melastomataceae (*Miconia*) and Asteraceae (*Bidens*) identified as the main sources of pollen. The most abundant species and genera present per sample for the second technique were *Prockia crucis*, *Coffea canephora*, *Miconia nervosa*, *Laurus nobilis*, *Theobroma* sp., *Miconia notabilis*, *Artocarpus* sp., *Croton* sp., *Euphorbia* sp., *Cecropia ficifolia*, *Mikania* sp., and *Ophryosporus* sp., in order of number of readings per ASV (Amplicon Sequence Variant).

The results of this integrated study provide a basis for the adoption of sustainable practices by meliponiculture farmers and policymakers, thereby supporting the survival of native stingless bees and preventing the overexploitation of these important pollinators in Ecuador's tropical regions.

Résumé

L'Équateur, un pays de l'Amérique latine, se distingue par une biodiversité remarquable, comme en témoigne la présence d'environ 200 espèces d'abeilles sans dard. En parallèle, le pays connaît une croissance significative de la pratique de la méliponiculture avec plus de 700 méliponiculteurs spécialisés dans l'élevage de ces abeilles. À l'instar d'autres hyménoptères, les abeilles de la tribu des Meliponini sont toutefois confrontées à un déclin de leur population. Ce déclin est attribuable à un ensemble de facteurs qui, isolément ou de manière conjointe, entraînent une diminution annuelle du nombre d'individus. L'objectif de cette étude est de fournir des preuves empiriques sur les facteurs anthropiques, tels que l'utilisation de pesticides et la gestion humaine, ainsi que sur les facteurs naturels, comme les ressources en pollen, les ravageurs et les agents pathogènes, qui influencent l'état de santé des abeilles sans dard en Équateur. Il s'agit également de fournir des conseils sur les mesures à prendre pour garantir leur impact positif sur la conservation et la réduction de la mortalité des colonies d'abeilles sans dard.

Jusqu'en novembre 2024, 15 agents pathogènes et ravageurs affectant la santé des colonies d'abeilles sans dard ont été signalés, ainsi que 26 contaminants chimiques ayant des effets létaux ou sublétaux sur ces abeilles (Étude 1). En Équateur, la santé des abeilles sans dard est influencée par divers facteurs, notamment la présence de pesticides et d'autres contaminants chimiques dans l'environnement. Ces substances chimiques peuvent pénétrer dans le nid des abeilles et affecter la santé de celles-ci ainsi que leur capacité à se reproduire. Par ailleurs, la présence de parasites, dont certains peuvent se transformer en agents pathogènes, représente une menace supplémentaire pour la santé animale. Des analyses ont révélé la présence de glyphosate et d'AMPA (un métabolite) dans le cérumen, un produit des abeilles sans dard destiné à un usage interne dans la ruche et à une application topique chez les humains. En outre, les métaux lourds et métalloïdes (As, Cd, Cr, Ni et Pb) ont été détectés (Étude 4).

Une analyse approfondie de la littérature scientifique a permis d'estimer le taux de mortalité annuel à 15 % chez les abeilles sans dard élevées pour la production et la vente de miel (*Melipona*, *Tetragonisca* et *Scaptotrigona*, ce dernier genre dépendant de la région climatique du pays). Cette étude a permis d'identifier les domaines à améliorer grâce à l'application de bonnes pratiques de gestion (Étude 5). Le type et la diversité du pollen récolté par les abeilles ont une incidence sur leur alimentation, qui influe à son tour sur leur système immunitaire, leur capacité à résister aux agents pathogènes et leur aptitude à se remettre du stress causé par les pesticides. Grâce à deux techniques

— la morphométrie par microscopie électronique (Étude 2) et le codage à barres de l'ADN (Étude 3) —, 27 familles de plantes et 18 genres ont pu être identifiés, ainsi que jusqu'à 34 espèces différentes.

La première technique consistait à compter les grains de pollen afin de déterminer l'abondance des familles et des genres. Les Melastomataceae (*Miconia*) et les Asteraceae (*Bidens*) ont été identifiées comme les principales sources de pollen. Les espèces et genres les plus abondants présents par échantillon pour la deuxième technique étaient *Prockia crucis*, *Coffea canephora*, *Miconia nervosa*, *Laurus nobilis*, *Theobroma* sp., *Miconia notabilis*, *Artocarpus* sp., *Croton* sp., *Euphorbia* sp., *Cecropia ficifolia*, *Mikania* sp., et *Ophryosporus* sp., dans cet ordre, selon le nombre de lectures de chaque variante de séquence d'amplicon (ASV). Les résultats de cette étude multidisciplinaire serviront de base à l'adoption de pratiques durables par les éleveurs d'abeilles mélipones et les décideurs politiques afin de maintenir la vie des abeilles indigènes sans dard et d'éviter la surexploitation de ces pollinisateurs importants dans les régions tropicales de l'Équateur.

Resumen

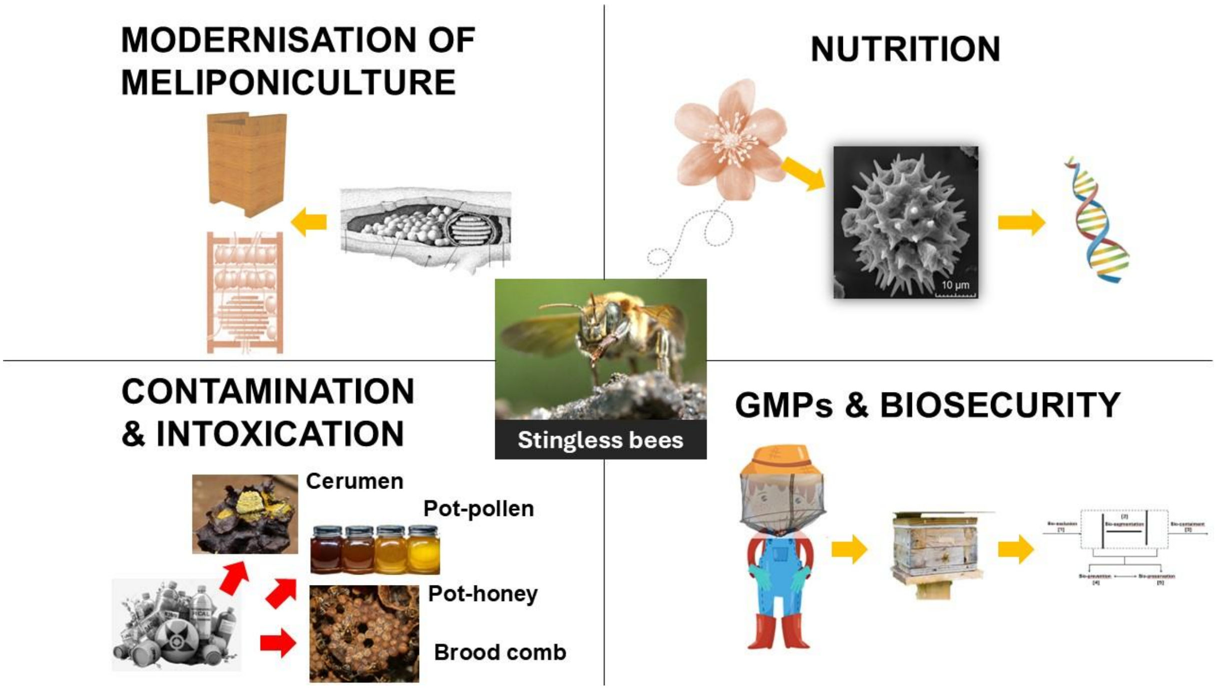
Con aproximadamente 200 especies de abejas sin aguijón y más de 700 meliponicultores, el Ecuador, en América del Sur (Neotrópico), se posiciona como un país megadiverso en cuanto a abejas nativas sin aguijón y, a su vez, atravesando un boom en la práctica de meliponicultura. Tal como otros himenópteros, las abejas de la tribu Meliponini se enfrentan a un declive y pérdida de colonias debido a diversos factores que, solos o en interacción, reducen el número de sus poblaciones anualmente. El presente estudio busca proporcionar información real sobre factores de origen antropogénico, por ejemplo, pesticidas, manejo, y aquellos de origen natural, por ejemplo, recursos polínicos, plagas y patógenos, que pueden influir en el estado de salud de las colonias de abejas sin aguijón de Ecuador. Así como proporcionar un direccionamiento sobre acciones a tomar que impacten positivamente en la conservación y reducción de muertes de colonias.

Hasta noviembre de 2024 (revisión sistemática) se reportó un total de 15 patógenos y plagas que afectan la salud de las colonias de abejas sin aguijón, así como otros 26 contaminantes con efectos letales y subletales para la vida de las abejas sin aguijón (Estudio 1). La salud de las abejas sin aguijón en Ecuador se ve influida por diversos factores, entre ellos la presencia de pesticidas y otros contaminantes en el medio ambiente, que pueden llegar al interior del nido. Además, las plagas, algunas de las cuales pueden convertirse en patógenos, también suponen una amenaza. En el cerumen, un producto de las abejas sin aguijón, tanto para uso interno en el nido como para aplicación en humanos, se detectaron glifosato y AMPA (un metabolito), junto con metales pesados y metaloides (As, Cd, Cr, Ni, Pb) (Estudio 4). La tasa de mortalidad anual estimada del 15% para las abejas sin aguijón que se crían para la producción y venta de miel (*Melipona*, *Tetragonisca*, *Scaptotrigona,* este último género en dependencia de la región climática del país) permitió identificar áreas de mejora mediante la aplicación de buenas prácticas de gestión en meliponicultura (Estudio 5).

El tipo y la diversidad del polen recolectado por las abejas afectan a su nutrición, lo que a su vez repercute en su sistema inmunitario, su capacidad para resistir a los patógenos y su capacidad para recuperarse del estrés causado por los pesticidas. Mediante dos técnicas —microscopía electrónica-morfometría (Estudio 2) y código de barras de ADN (Estudio 3)— se identificaron 27 familias de plantas y 18 géneros, y hasta 34 especies, como resultado respectivo de cada estudio. La primera técnica se basó en el recuento de granos de polen para determinar la abundancia de familias y géneros, identificándose Melastomataceae (*Miconia*) y Asteraceae (*Bidens*) como las principales fuentes de polen. Las especies y géneros de plantas más abundantes presentes en la muestra para la segunda técnica fueron *Prockia crucis*, *Coffea canephora*, *Miconia nervosa*, *Laurus nobilis*, *Theobroma* sp., *Miconia notabilis*, *Artocarpus* sp., *Croton* sp., *Euphorbia* sp., *Cecropia ficifolia*, *Mikania* sp. y *Ophryosporus* sp., en orden de número de lecturas por ASV (Amplicon Sequence Variant).

Los resultados de este estudio con enfoque integrado sirven de base para la adopción de prácticas sostenibles por parte de los meliponicultores y los reguladores políticos, apoyando la vida de las abejas nativas sin aguijón y evitando la sobreexplotación de estos importantes polinizadores en las regiones tropicales del Ecuador.

Graphical Abstract



General preamble

To date, a total of 605 species of stingless bees have been reported worldwide. The Neotropics rank first with over 400 species. Ecuador, located in South America, is considered a megadiverse country and contains three biodiversity hotspots: the Tropical Andes, the Choco rainforest, and the Yasuní National Park. This diversity includes approximately 200 species of stingless bees (Hymenoptera, Meliponini), the breeding and management of which dates back to indigenous cultures and is now a thriving agricultural practice for the country's 700-plus stingless beekeepers. In addition to the natural biotic factors that control the life of stingless bee species, abiotic factors, especially those resulting from their management, have begun to pose a serious threat to their health and conservation.

This study aims to improve our understanding of the various factors, both human-made and natural, that affect the health of Ecuador's stingless bees. **Study 1** compiled research on stingless bees published up to November 2024, which examined the presence of pests, pathogens, and chemicals, or other foreign substances, in stingless bee products and colonies. Four studies reported different bacteria as the cause of diseases that weakened stingless bee colonies to the point of death. Three studies reported viruses, while one study reported a microsporidium; the consequences were not fatal for the nests of stingless bees. Heavy metals and metalloids were also reported in stingless bee products, including honey, pollen, cerumen, geopropolis, and worker stingless bee bodies. Polycyclic aromatic hydrocarbons (PAHs) and microplastics were found in honey and the midguts of stingless bee larvae, respectively. Concentrations of organochlorine pesticide derivatives ranging from 3.8 to 645.08 µg/kg were present in pot-honey and pot-pollen.

Good management practices in meliponiculture should include the maximization of adequate nutrition for stingless bee colonies by ensuring access to quality floral resources. Studies 2 and 3 used two techniques to identify the plants that stingless bees use as pollen resources. **Study 2** used scanning electron microscopy and morphometry to identify 49 pollen types, classifying 27 families and 19 genera. **Study 3** employed DNA barcoding, using ITS2 and the rbcL gene as markers. This identified 34 species, demonstrating the effectiveness of this additional molecular technique. The results could be categorised by area and sampling time. Pollen species important to stingless bees and foraging patterns were identified in tropical rainforests and tropical dry forests, during rainy and non-rainy seasons in Ecuador.

Study 4 reported the detection of 0.02–0.2 mg of glyphosate and 0.028 mg of AMPA (a metabolite) per kilogram of cerumen from stingless bees. The study also reported concentrations of heavy metals that could pose a health risk to stingless bee workers and larvae, as well as to humans, especially in samples from the Highlands region. Adopting good practices when managing stingless bee colonies can help to reduce health risks. For example, strategic placement of meliponaries can decrease exposure to pesticides. Good practices can also reduce the incidence of diseases and parasites that are specific to stingless bees or those that are spread by contagion (usually from *Apis mellifera*).

A holistic approach will be adopted to address these issues and propose effective solutions that will improve the current situation of meliponiculture in Ecuador. **Study 5** revealed an overall compliance rate of 32.6% with good management practices in meliponiculture, attributable to the development of a barometer in conjunction with a network (spider web) that graphically illustrated the interaction between management domains and facilitated the identification of areas requiring enhancement. The areas of discussion included the environment and conservation, the use of personal protective equipment and biosecurity measures, and healthcare. The mortality rate was estimated at 15% per year for managed stingless bee colonies.

The results address concerns of global interest and impact regarding the decline of bees, bearing in mind that less well-known and less studied bee species may be even more vulnerable than other groups of bees. The decline of these pollinating insects in tropical regions would directly affect food security not only in areas where they are endemic, but also worldwide. These results can also help to guide both meliponiculture practitioners and decision-makers in the adoption of sustainable practices and policies to ensure the conservation of this bee group.

Introduction

1 Beyond the well-known honey bees

1.1 A brief history of Hymenoptera insects and their importance

Phylogenomic studies have determined that the diversification event of the order Hymenoptera occurred between two geological periods, the Carboniferous and the Triassic, approximately 329 - 239 million years ago (Peters et al. 2017). The former occurred in the Palaeozoic era, characterized by the formation of forests that gave rise to coal (Aretz et al., 2020), through the incomplete decomposition of swamp forest plant remains and the subsequent mixing of these organic remains with marine sediments. Coal was formed by the action of heat and pressure over time. The latter happened in the Mesozoic era, when the first dinosaurs appeared (Ogg et al. 2020).

Hymenoptera is one of the most diverse orders, comprising more than 153,000 insect species whose interactions include parasitic, predatory, and pollinating behaviours (Grimaldi and Engel 2005). Diversification processes have encompassed evolutionary transitions from solitary to eusocial life, accompanied by a strict division of labour and the establishment of castes (Quiñones and Pen 2017). The classification of the order Hymenoptera partly reflects these transitions, including the sawflies, suborder Symphyta, distinguished by the morphology of the ovipositor (Wutke et al. 2024), and the suborder Apocrita, mainly stingless parasitoids. The suborder Apocrita comprises the clade Aculeata, which includes stinging wasps, ants, and bees (Anthophila) (Brothers 2019).

Bees, together with sphecoid wasps (solitary and others with social remnants), constitute the superfamily Apoidea. Bees are dependent on pollen from flowering plants, mainly as a source of protein in their diet and for the development of the ovaries of oviparous females (Michener, 2007). Bees, with more than 20,000 described species in seven families, are the richest lineage of insects and a vital component of natural and agricultural ecosystems (Danforth et al., 2019; He et al., 2018). The monophyly of bees, families, and subfamilies is evidenced by the presence of a single common ancestor (Engel 2000). Utilising the General Heterogeneous Evolution on a Single Topology (GHOST) tree as a means to elucidate phylogenetic relationships, the families Andrenidae, Apidae, Colletidae, Halictidae, Megachilidae, Melittidae, and Stenotritidae are delineated (Almeida et al. 2012; Bossert et al. 2019; Michez, Patiny, and Danforth 2009).

The Apidae family comprises more than 5,700 species of bees, bumblebees, and carpenter bees (Melo and Gonçalves 2005). These insects are characterised by their social nature (Grüter, Balbuena, and Valadares 2023), robust physical appearance, and the presence of a thin tongue and elongated labial palps (proboscides) on their mouthparts (Engel 2005). These structures are specialised for the collection of nectar and pollen. The females of numerous species possess specialised structures on their hind legs (corbicula) that facilitate the transportation of pollen (Richardson et al. 2018). Some species are obligate kleptoparasites, a trait characterised by females not constructing their own nests (Straka and Bogusch 2007). The family Apidae comprises five subfamilies, of which the subfamily Apinae includes some of the best-known and most studied groups of bees: honey bees, bumble bees, stingless bees, and carpenter bees. The function for which bees are well-known is pollination, a process that benefits humans in various areas such as agriculture and ecosystem sustainability (Cortopassi-Laurino et al. 2006; Kwon 2008; Matsuzawa and Kohsaka 2021; Solís-Montero et al. 2023).

“No bees, no food” is an important phrase showing the human need for pollinator-dependent crops. Between 2001 and 2022, the total global cropland area exhibited growth of 80 million hectares, representing an estimated increase of 5%, thus reaching a total of 4,781 million hectares (Potapov et al. 2022). The geographical expansion of the area under cultivation has been most marked in parts of Africa, South America, and Southeast Asia. Agricultural practices that reduce crop diversification to monocultures threaten the lives of pollinating insects, reducing their natural habitat and exposing them to pesticides (Aizen et al. 2019).

Apis mellifera, commonly known as the honey bee or western honey bee, is a species that has been the focus of extensive research due to its role as a pollinator and its commercial value, the latter making it the most economically exploited species (Hristov, Neov, et al. 2020). Honey bees have been observed to establish colonies with an average population of 100,000 individuals (Danforth, 2007), and just eleven species of *Apis* have been described (Beekman and Oldroyd 2018; Crane 2009; Han, Wallberg, and Webster 2012). The distinguishing characteristics of *Apis mellifera* are as follows: queen bees can mate with multiple males, and they possess the capacity to fly to a new location, accompanied by a portion of the worker bees to establish a new colony (Abou-Shaara, Adgaba, and Al-Ghamdi 2021). The structure of the brood cells does not differ from food storage cells; however, a discernible distinction exists between cells of the male and worker bees (Zhang et al., 2024). Rearing queens requires a larger quantity of food of varying quality known as royal jelly (Lo, Beekman, and Oldroyd 2019). The primary defensive mechanism employed by honey bees involves the use of their stinger (Papa et al. 2022).

1.2 Stingless bees: a closer look

The number of species of stingless bees is estimated to be approximately 600, with population sizes ranging from 300 to 80,000 individuals (Engel et al. 2023). The vast majority of these bees inhabit durable, perennial nests, with some exceptions, such as colonies of Asian *Tetragonula laeviceps* and *Frieseomelitta silvestrii,* for which successful absconding has been observed after stressful factors (de Portugal Araujo 1963; Ribeiro and Bego 1994). Their body size ranges from 2 mm in stingless bees of the genus *Trigonisca* (Roubik, 2018) to 15 mm in species of the genus *Melipona* (Michener, 2007). Nesting sites include tree cavities, termite nests, wall cavities, or underground (Grüter, 2020b; Macedo et al., 2021; Roubik, 2006). Their defence mechanisms are varied, ranging from acid discharge, suicidal biting, joint attack, and the use of sticky resins (Grüter et al. 2016a; Shackleton et al. 2015). Their social habits are also varied in most species; colonies may have single queens (monogynous) and rarely multiple queens (facultatively polygynous, i.e., *Melipona bicolor*) (Bueno et al. 2023; Vollet-Neto et al. 2018). Workers may be completely sterile or lay eggs regularly (Garcia Bulle Bueno et al. 2020; Sommeijer, Chinh, and Meeuwsen 1999). Their age-related division into labourers (age polytheism) includes a soldier caste (Grüter et al. 2023).

The behaviour exhibited by queen bees of the stingless bee variety differs from that of honey bees in that mating is usually with a single male (Vollet-Neto et al. 2017). Concerning brood rearing, worker bees regurgitate larval food into an empty cell; this is called mass provisioning (Gilliam et al. 1985). Thereafter, the queen deposits an egg on the larval food, and the workers immediately seal the cell, maintaining this state until the stingless bee has completed its development and emerges (Grüter 2020b).

Two main reasons for queen development in stingless bees have been described: genetic factors and the protein content of the food larvae receive (Bueno et al. 2023). Except for species belonging to the genus *Melipona*, stingless bees distinctly differentiate queen cells within the brood chamber, while worker and male cells remain indistinguishable. In the absence of stingers, or in cases where stingers are atrophied and non-functional, stingless bees utilise their mandibles for defence, and resin to immobilise enemies (Grüter 2020a). Another characteristic of stingless bees is a reduced wing venation, which is probably the result of the small body size of the ancestors of these bees (Melo 2020). Further notable morphological characteristics include the penicillum, a robust, curved tibial tuft located at the base of the hind tibia, and the absence of an auricle (pollen press). Additionally, an expansion of the base of the hind basitarsus is evident (Melo 2020; Quezada-Euán et al. 2018) (Figure 1).

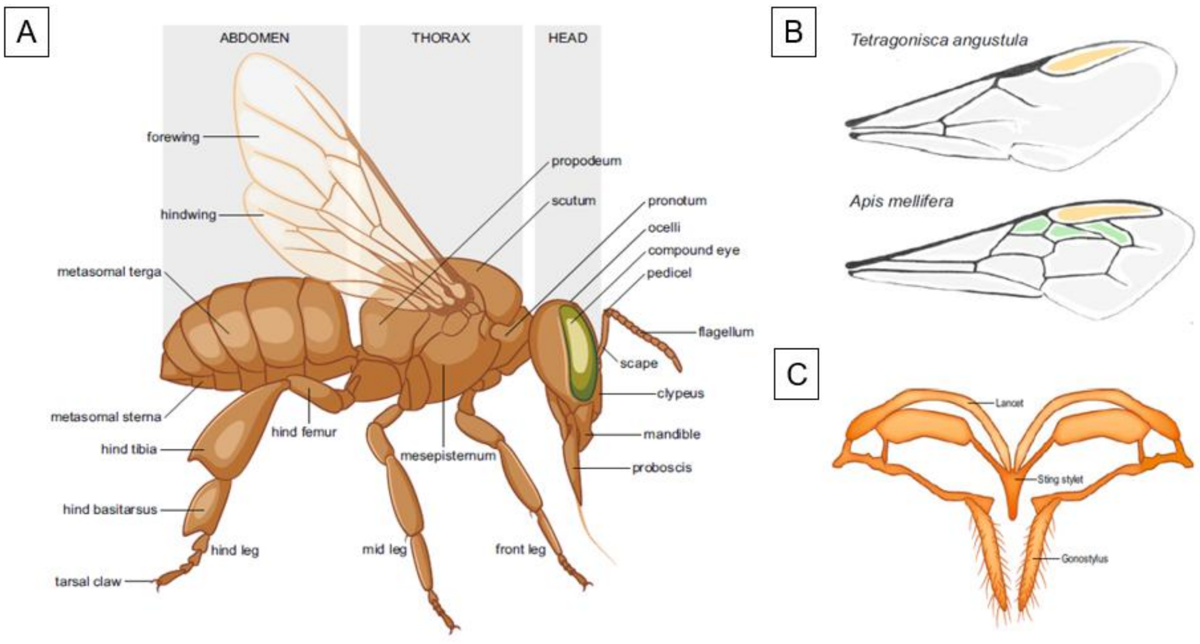


Figure 1. Main morphological features of stingless bees. A) Stingless bee worker morphology (illustration by Nadja Stadelmann). B) Reduced wing venation in the stingless bee (here *Tetragonisca angustula)* compared to the honey bees (here *Apis mellifera*). The marginal cell (orange) is often open, and the submarginal cells (green in *A. millifera*, sm1-sm3 from left to right) are weakly defined or not visible at all in stingless bees. C) Reduced sting apparatus in *Melipona*. The lancet (first valvula) and sting stylet (second valvula) are reduced to varying degrees in different stingless bee species. In *A. mellifera,* the lancet and sting stylet combine to form a functional sting (illustration by Nadja Stadelmann). Source (Grüter 2020c).

Extant research on eusocial bees revealed that, as of 2020, a mere 6% of published research focused on stingless bees. The gaps in our knowledge of their biology, health, and management need to be highlighted. The latter is known as Meliponiculture or stingless beekeeping, defined as the care and breeding of native stingless bees for mostly commercial purposes, which has spread rapidly in recent years in the tropical and subtropical areas of the planet where they live (Quezada-Euán et al. 2022).

1.2.1. Stingless bee taxonomical features and classification

In conjunction with bees of the tribe Apini, Euglossini, and Bombini, the stingless bees of the tribe Meliponini comprise the clade of corbiculate bees. The corbicula is a widened, depressed, smooth, and largely hairless structure located on the apical prolateral surface of the metatibia (Michener, 1999). Long setae are usually present along the border, forming a concave space that is used to transport resources (Engel et al. 2023). Some of the characteristic features of

Meliponini include simple pretarsal claws, general reduction of the sclerites associated with the sting complex as well as the external grooves of the mandibles, loss of the metatibial spurs, presence of arolia, absence of supra-alar carina, and the presence of a jugal lobe on the hind wing (Engel et al., 2020, 2021, 2023; Michener, 1990; Michener, 2007).

A classification of stingless bees, encompassing regionally differentiated genera and subgenera, was informed by the most recent expert review (Engel et al. 2023). The distribution of New World (American continent) stingless bees extends from 34.89°S in Uruguay and Argentina to 27.03°N in Mexico. They have been found at exceptional elevations up to 4000 metres above sea level (m.a.s.l) in the Peruvian and Bolivian Andes (Camargo and Pedro 2007; Roig-Alsina and Alvarez 2017). All Neotropical stingless bees are classified within the subtribe Meliponina, with 474 species and 26 extant genera (Hartfelder 2008) (Figure 2).

Old-world meliponines are classified within the subtribe Hypotrigonina. The Old-World stingless bees comprise species that are distributed across Africa, Madagascar, and Asia. The Afrotropical Meliponini are distributed from Senegal to Eritrea, along the southern Sahel, and as far south as KwaZulu-Natal in South Africa. This group is characterised by its low level of diversity, with 33 species and eight genera being recognised (Eardley and Urban 2010). The group under consideration also comprises 98 species and 11 genera distributed across South and Southeast Asia, the Indomalaya region, Papua New Guinea, and Australia (Rasmussen 2008) (Figure 3).

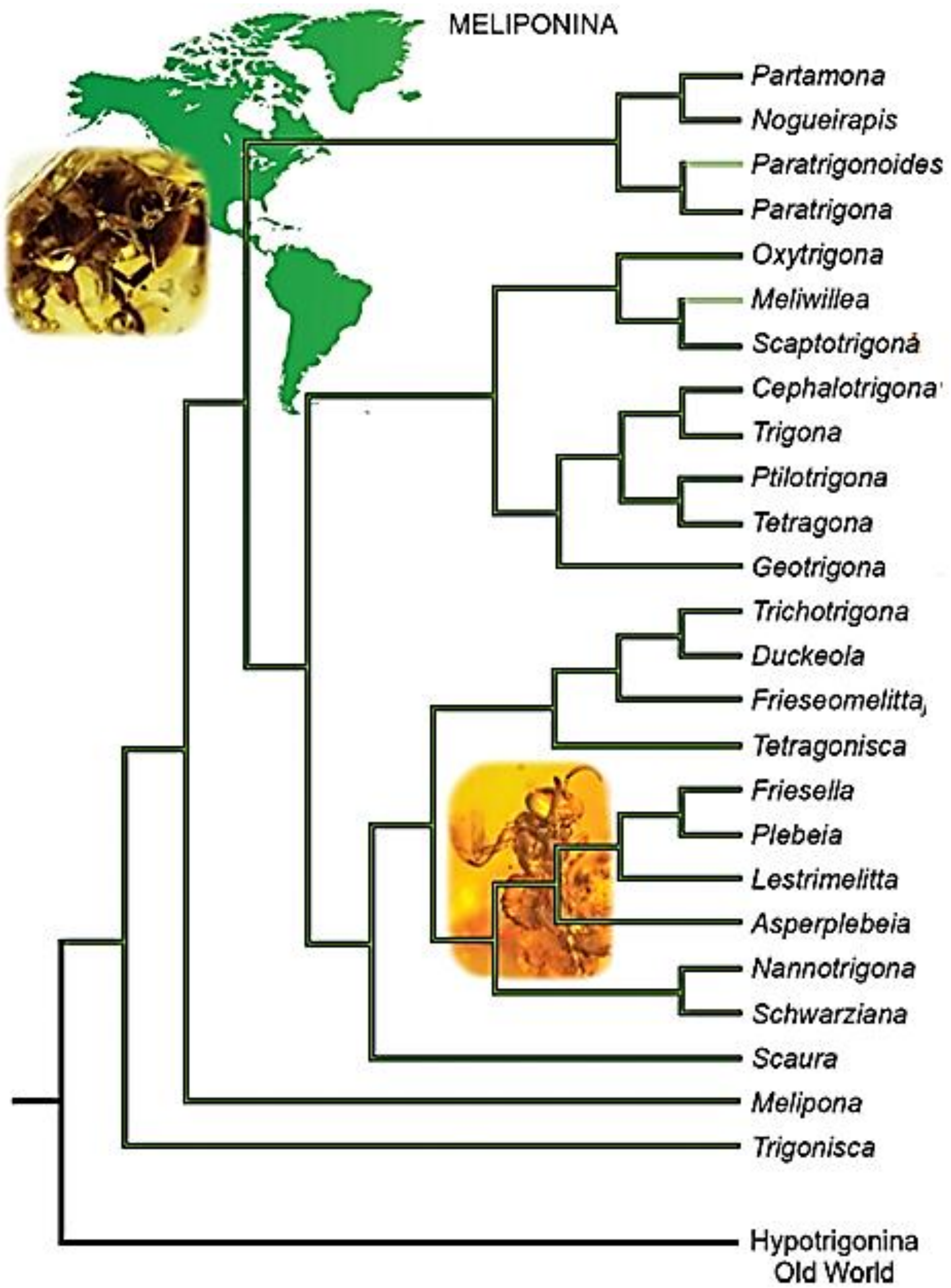


Figure 2. **Phylogeny of subtribe Meliponina (New World Meliponini)** modified from Engel et al. (2023), summarized from Rasmussen & Cameron (2010), with hypothesized placements of *Paratrigonoides* Camargo & Roubik and *Meliwillea* Roubik, Loco Segura, & Camargo. Representative fossil bees (from top to bottom, not to the same scale): *Cretotrigona prisca* (Michener & Grimaldi) in Maastrichtian New Jersey amber, *Proplebeia silacea* (Wille) in Miocene Chiapas amber. Images of fossil bees from Engel (2000); Engel et al. (2021).

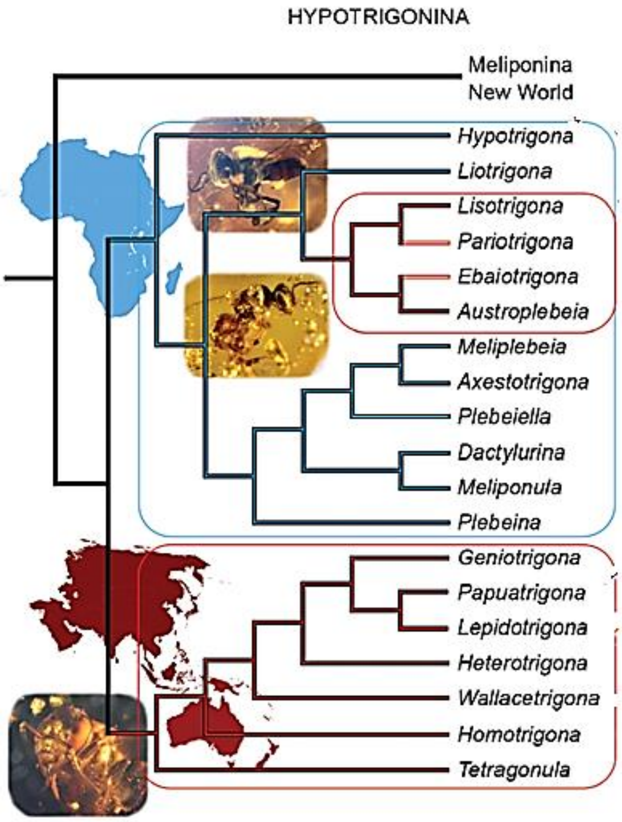


Figure 3. Phylogeny of subtribe Hypotrigonina (Old World Meliponini) modified from Engel et al. (2023), summarized from Rasmussen & Cameron (2010), with hypothesized placements of *Pariotrigona* Moure and *Ebaiotrigona* Engel & Nguyen. Blue branches indicate African lineages, while red branches indicate Southeast Asian-Malesian-Papuasian-Australian lineages. Representative fossil bees (from top to bottom, not to the same scale): *Liotrigona (Tapheiotrigona) aethiopica* Engel in Miocene Ethiopian amber, *Liotrigonopsis rozeni* Engel in Eocene Baltic amber, *Tetragonula (Tetragonula) florilega* Engel in Miocene Zhangpu amber. Images of bees from Engel (2001); Engel et al. (2021); Engel & Aber (2022); Rasmussen et al. (2017).

1.2.2 General internal and external composition of stingless bee nests

The inaccessible and resistant structures of stingless bee nests gained importance for the survival of these insects, as the functionality of the sting apparatus was lost. Internally, stingless bee nests are generally constituted of cerumen, which is a material made of a mixture of wax produced by the bees themselves from the dorsal tergal glands, along with saliva and resins, and other sticky plant substances (Massaro et al. 2011). The incorporation of resins provides strength to this material and protection to the individuals due to the antimicrobial and antifungal properties that plant resins can have (Shanahan and Spivak 2021).

Construction activities represent a specific role in the life cycle of stingless bees, as brood cells are never reused like in honey bees, but are built from scratch for each new egg (Layek et al. 2024). Furthermore, the protective cerumen and batumen sheets are continuously constructed and modified to ensure the colony's protection. Batumen is a robust material composed of a mixture of wax, resin, mud, plant material, and, in certain species, animal faeces (Shanahan and Spivak 2021). The function of batumen within the nest is twofold: to provide protection and to limit the nesting space (Roubik, 2006), which can be rearranged according to the nest's growth needs. In stingless bee species (i.e., *Geotrigona* and *Partamona*) that build their nests on the ground, batumen layers play a significant role in the process of waterproofing (Wille and Michener 1973). Batumen can be categorised according to composition form and use. Exposed batumen constitutes the hard outer layer that surrounds and protects exposed and semi-exposed nests. Batumen plates are defined as those that envelop and protect nests within a cavity. Lining batumen constitutes a thin, continuous, resinous-looking covering layer that is placed over the rough inner surfaces of the hollow cavities where the bee nests are located. Laminate batumen, characterised by its layered composition and the presence of channels, enables the bees to navigate and facilitates air circulation and water evaporation (Roubik, 2006; Shanahan & Spivak, 2021; Wille & Michener, 1973). Batumen is also referred to as geopropolis, and is characterised by the bees' mixture of mud and clay with propolis (Roubik, 2023). The entrances to the nests of stingless bees can vary from being small and inconspicuous, comparable in size to the head of a bee, to large, ornate tubes with a length of up to 1.5 m, as observed in colonies of *Lepidotrigona terminate* (Bänziger, Pumikong, and Srimuang 2011). The specific architecture of the entrance facilitated the swift and efficient location of the nest by foraging bees, who can enter at high velocity and subsequently move into the interior of the nest. Tube-shaped entrances can harden, soften, or flex over time. In the case of the *Nannotrigona* species, the area near the external opening is characterized by softness and flexibility, a property that facilitated the closure of the entrance during nocturnal hours. The presence of resins acts as a repellent to ants or spiders (Grüter 2020b; Tichit et al. 2020) (Figure 4).

The involucrum is a structure formed by several soft cerumen layers that separate and protect the brood chamber from the rest of the nest. The occurrence of this material is subject to intraspecific variations (i.e., *Austroplebeia australis* and *A cincta*, or in some American *Plebeia* species), the presence of constant and high temperatures, and the symbiosis of certain species with termites and ants (i.e., *Scaura latitarsis*, *Trigona cilipe*s) (Dollin, Dollin, and Sakagami 1997).

The involucrum is not a completely closed structure. The multiple layers of cerumen create passageways that allow the workers to enter and exit the brood chamber. These passageways also serve to protect the brood by creating a labyrinth against flies or parasitic bees (Oldroyd & Pratt, 2015; Roubik, 2006).

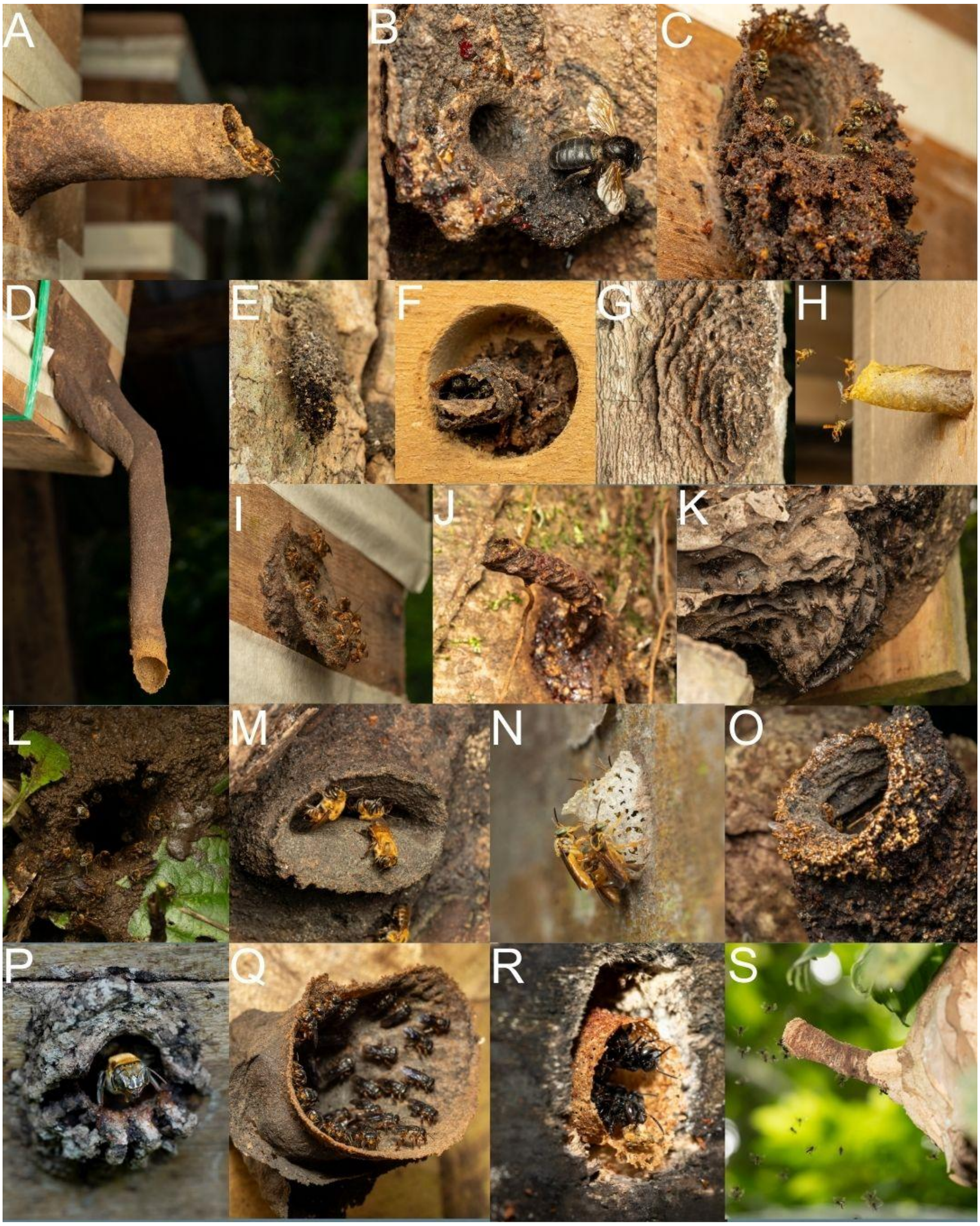


Figure 4. Types of entries of different stingless bee genera in Ecuador. Stingless bees' nests from the Ecuadorian Choco region. **A)** *Scaptotrigona* sp.1. **B)** *Melipona fallax*. **C)** *Nannotrigona* sp. **D)** *Scaptotrigona* sp.2. **E)** *Plebeia* sp. **F)** *Aparatrigona* sp. **G)** *Trigonisca* sp. **H)** *Tetragonisca* sp. **I)**

Scaptotrigona sp.3. **J)** *Tetragona* sp. **K)** *Oxitrigona chocoana*. Stingless bees' nests from the Ecuadorian Amazon: **L)** *Partamona* sp. **M)** *Melipona eburnea*. **N)** *Tetragonisca angustula*. **O)** *Trigona* sp. **P)** *Melipona grandis*. **Q)** *Scaptotrigona* sp.4. **R)** *Frieseomelitta nigra*. **S)** *Scaura* sp. Photographer Alfonso Jimenez ©.

Brood cells are individual and consist of soft cerumen (Menezes et al. 2015). There are three main types of cell arrangements: horizontal combs, vertical combs, and clustered cells. The arrangement is not species-specific because only minor obstruction or changes in construction behaviour are required to change the arrangement from discs to spirals (Cepeda 2006). Food storage pots are placed around the brood chamber, and far enough away from the brood chamber, when they are intended for colony reserves. The pots are made of soft cerumen and, depending on the season, there may be a few pots, sufficient for the survival of the nest, or hundreds of pots (Rasmussen et al. 2024) (Figure 5), usually used for commercialisation in meliponiculture.

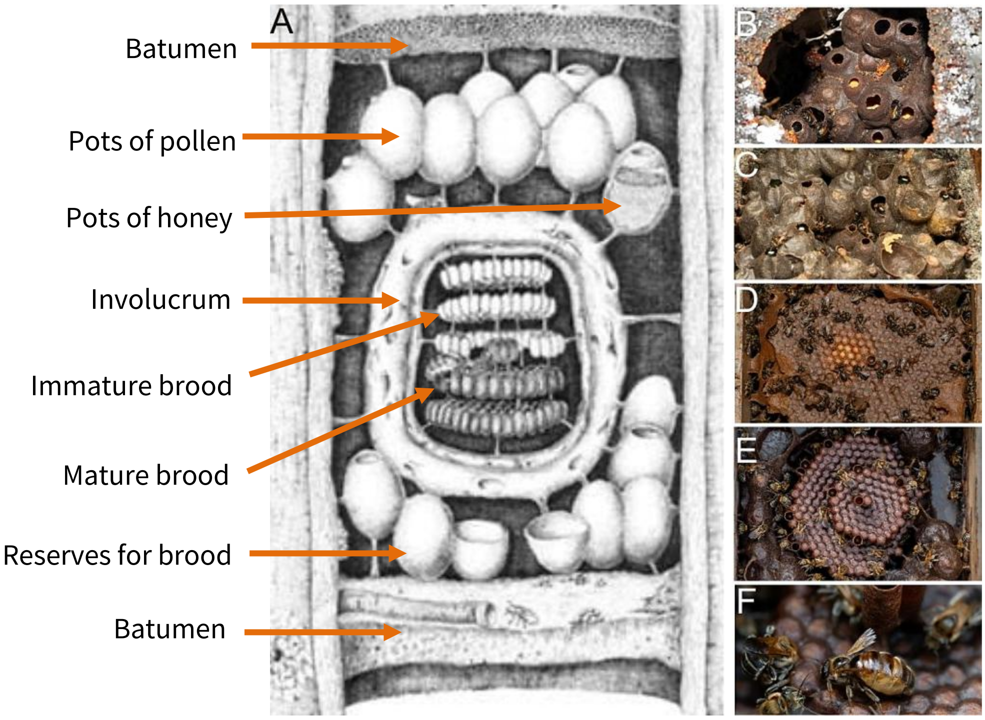


Figure 5. Amazonian stingless bees' nest structure. A) Schematic drawing of a natural nest of *Melipona* bees. Modified from Venturieri (2009). **B)** Batumen, honey pots, and pollen pots of *M. titania*. **C)** Pot-honey and pot-pollen of *M. eburnea*. **D)** Maturation of discs combs covered by the involucrum of *M. illota*. **E)** Comb structure of *M. grandis*- **F)** Queen of *M. grandis*. Photographer Alfonso Jimenez ©.

Some species of stingless bees do not store food reserves; rather, they are known to procure them by stealing food from other colonies or by invading nests with available resources (Roubik, 2006). The practice of storing honey in pots among tropical bees may have evolved as a strategy to reduce competition, to facilitate the accumulation of food reserves, and to gather materials useful for nest building, or for enduring periods of scarcity (Roubik, 2023). The quantity of honey varies according to the species and the time of year, ranging from a few grams to several kilograms (<5kg) per year (Duangphakdee et al. 2024).

The main components of stingless bee honey are: carbohydrates (±70%), sugars (fructose, and glucose (>60%) (Souza et al. 2006), trehalulose (13 - 44%) (Fletcher et al. 2020)), water (19.9 – 41.9%), proteins (0.07 – 1.19%), minerals (0.01 – 0.33%) (Deliza and Vit 2013), organic acids, phenolic compounds (phenolic acids and flavonoids), amino acids, enzymes, and vitamins (Biluca et al. 2016). Table 1 outlines the key differences between apiculture and meliponiculture.

Table 1: Comparative table of the main characteristics of beekeeping and meliponiculture

Unique features	Beekeeping	Meliponiculture
Bees and size	*Apis mellifera* (12 – 15 mm)	Meliponines (2 – 20 mm)
Managed species	1 species	± 50 sp (honey producers)
Method of defence	Injection of venom through the sting	Bites, acid injection, stacking, sticky resins
Derived products	Honey, pollen, bee bread, wax, propolis	Pot-honey, pot-pollen, cerumen, geopropolis/propolis
Honey production and price	25 kg/hive/year ±13 €/kg	500 mL – 2L/nest/year 51-171 €/L (60 – 200 USD/L) Depends on species/region
Storage and harvesting of honey	Honey combs Protection + smoke	Honey pots Syringe + sterilized materials
Main use of honey	Food	Nutraceutical, dermatology, and natural medicine
Objectives of the practice	Honey production	Honey production, conservation, entomotourism, and cultural heritage

1.2.3 Distribution and habitat of stingless bees

Stingless bees are a lineage currently restricted to the tropics of the planet, which expanded northwards during the Paleogene, probably in association with rising temperatures in the

Northern Hemisphere, as evidenced by the fossil *Cretotrigona prisca* (Almeida et al. 2023). The origin of the entire clade of corbiculate bees (i.e., bumblebees, orchid bees, honey bees, and stingless bees) was Neotropical (Martins et al., 2014). The Australian continent is home to approximately 11 species (Halcroft, Spooner-Hart, and Dollin 2013), while tropical Africa and Asia are home to around 131 species (Chakuya et al. 2022; Rattanawannee et al. 2019). The Neotropics, Central and South America, the Caribbean, and parts of North America are particularly rich in stingless bee species, with 474 species recorded in these regions (Engel et al. 2023).

The geographical distribution of stingless bee species encompasses a wide range of ecosystems, including tropical rainforests, cloud forests, and arid landscapes (Ayala, 1999; May-Itzá et al., 2022; Reyes-González et al., 2014). Their altitudinal range extends from sea level to elevations of up to 4,000 metres within the Andes Mountain range (Roig-Alsina and Alvarez 2017). The distribution of these organisms across the continent is of significance, as they play an indispensable ecological role in the pollination of both native wild plants and crops (Toledo-Hernández et al. 2022). Their nesting habits are highly variable, ranging from underground nests, tree cavities, and from the base of trees up to more than 30 metres high. These nests are exposed or semi-exposed to termite, ant, or wasp nests in which stingless bees coexist and develop beneficial mutualisms and symbioses (Beyene et al., 2024; Macedo et al., 2021; Reinhard Jesajas et al., 2023; Roubik, 2006; Siqueira et al., 2012).

Rainforests, including the Amazon and Cochó, offer sustenance to flower visitors throughout the year (Kajobe and Echazarreta 2005), with temperature fluctuations between 21 and 30 °C. Conversely, dry forests may experience prolonged periods of water deficit, characterised by elevated levels of solar radiation and average annual temperatures reaching up to 30 °C. These extreme conditions may restrict the availability of floral resources to short and unpredictable periods, presumably imposing a high selective pressure on bees (Ayala et al., 2013; Maia-Silva et al., 2018). *Melipona subnitida*, for instance, has been observed to survive periods of scarcity and low rainfall by modulating brood production, focusing on high-yield floral resources, and exhibiting enhanced thermotolerance in foragers (Hrncir et al. 2019).

The cloud forest is considered to be an uncommon forest type, representing a mere 1.2% of the forests in the Americas (Gould et al. 2024). This forest harbours elevated levels of plant and animal endemism and is a crucial source of water. The populations of plants and animals that are characteristic of this ecosystem may have limited dispersal, leading to differentiation. An exemplification of this phenomenon is observed in *Partamona bilineata*, a rarely managed stingless bee, which showed a pattern of differentiation associated with elevation changes. *Partamona bilineata*, a resilient stingless bee species in terms of pollination services, has a low effective population size, which represents a potential vulnerability to future environmental changes (Landaverde-González et al. 2017).

The range of internal nest temperatures for stingless bees varies between 25 and 40 °C, and this variation is attributable to external temperatures. Stingless bees employ a variety of behavioural strategies to modulate their nest temperature. These include wing fanning and the collection of water (Vollet-Neto, Menezes, and Imperatriz-Fonseca 2015). Fluctuations in the internal ambient temperature of the nest can have a deleterious effect on the production rates of brood cells, with the potential to reduce these rates by up to 50-60% in conditions of low temperature, while elevated temperatures compromise adult morphology, immunity, and survival (Quezada-Euán et al. 2024).

Ecuador is characterised by the presence of four distinct natural regions. The Costa or Litoral region is characterised by a tropical humid climate in the northern coastal provinces of Ecuador and a tropical dry climate in the southern coastal provinces. The region's mean annual temperature is between 23 °C and 26 °C. Concerning its topography, the region is predominantly characterised by plains and alluvial lowlands, in addition to low-lying mountain ranges. The elevation of the region varies from sea level to approximately 800 m.a.s.l (Chinacalle-Martínez et al. 2021; Epler and Olsen 1993). The Inter-Andean or high-mountain region is characterised by a temperate mountain climate, with variations in accordance with altitude – that is, microclimates. The mean temperature of the region is typically between 10 °C and 20 °C. The topography of the region is characterised by the presence of the Andes Mountain range, which comprises two branches, the Western and Eastern Andes, giving rise to fertile inter-Andean valleys, plateaus, and depressions. The range of altitudes is from 1,500 to 6,300 m.a.s.l (Coblentz and Keating 2008; Moreno et al. 2018).

The Amazon region is characterised by a tropical climate, with high temperatures and abundant rainfall throughout the year, typically ranging from 2,000 to 4,000 mm. The temperature of the region ranges from 23 °C to 27 °C. The region is distinguished by extensive plains that are covered by tropical rainforest. The topography is characterised by the presence of sedimentary and alluvial soils, which are intersected by fast-flowing rivers. The elevation of the region varies between 200 and 1,200 m.a.s.l (Espinoza Villar et al. 2009; Krahenbuhl 2010). The Galapagos Islands are characterised by a tropical oceanic climate, influenced by ocean currents. Their temperature range is from 22 °C to 30 °C. The islands are of volcanic origin and thus possess a mountainous terrain, featuring volcanic cones, plateaus, and coastal plains. The elevation of the region varies significantly, ranging from sea level to a maximum altitude of 1,707 m.a.s.l (Wolf Volcano on Isabela Island) (Chirico and Warner 2005; Roell, Phillips, and Parent 2021).

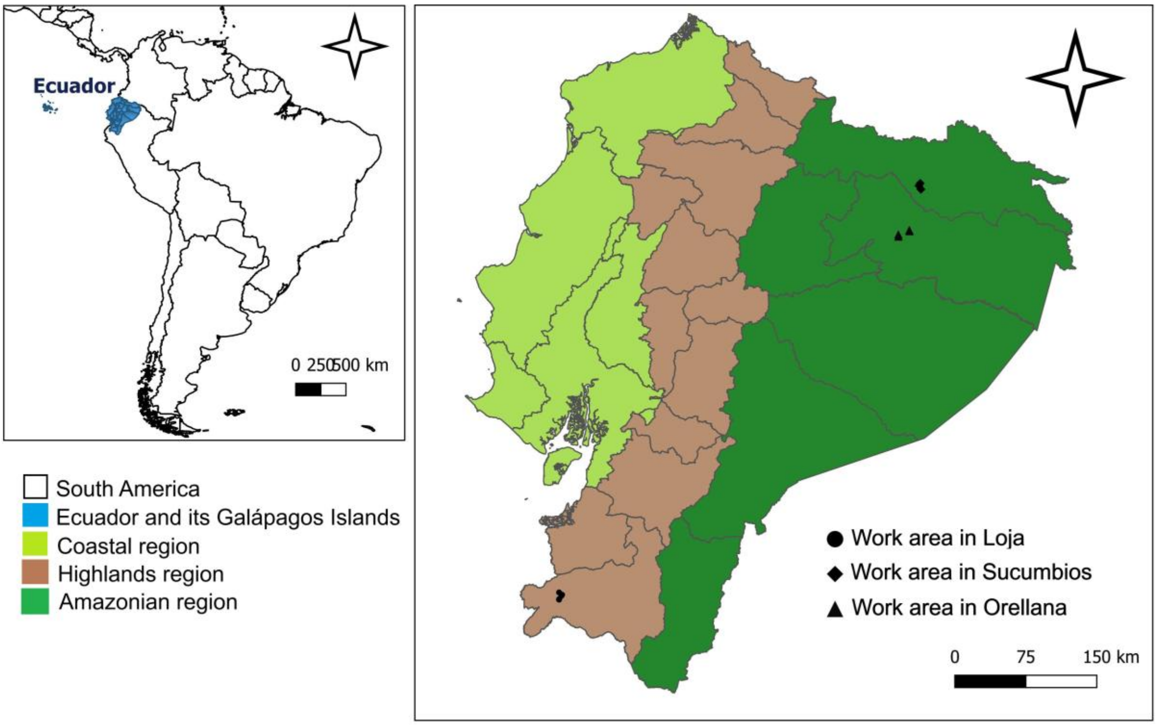


Figure 6. Geographical location of Ecuador and natural regions. Ecuador is divided into four natural regions. The coastal region is mostly warm and borders the Pacific Ocean. The Inter-Andean region is dominated by the Andes Mountain range and has a cold climate. The eastern region is mostly covered by tropical rainforests (part of the biggest Amazon). The insular region comprises the Galápagos Islands, home to species found nowhere else in the world. In addition, the three main areas where this thesis was developed are shown (Loja, Sucumbios, and Orellana provinces). The author's own work.

2 Ecological role of stingless bees

2.1 Pollen, a key resource for stingless bees

Pollen is the main source of protein for bees. The nutritional composition of pollen varies greatly among plant species and can provide carbohydrates, proteins, lipids, fibre, ash, sugars, amino acids, vitamins, terpenes, steroids, or carotenoids (Belina-Aldemita et al., 2019; El Ghouizi et al., 2023; Frias et al., 2016; da Silva et al., 2014). The availability of pollen resources is directly influenced by the seasonality of the region, leading to changes in the dynamics of colonies. During periods of pollen scarcity, colonies undergo a phenomenon of brood cessation, and worker lifespan is doubled (Hrncir, Jarau, and Barth 2016; Vaidya et al. 2023). The quality of pollen exerts a significant influence on the survival rate and pre-pupal weight of bees. Furthermore, its digestibility has been shown to impact larval growth and the developmental stage (Nicolson and Human 2013; Pang et al. 2022; Steijven, Steffan-

Dewenter, and Härtel 2016). The pollen is then referred to as "bee bread" when it is combined with the salivary enzymes of worker bees, regurgitated honey, and subjected to fermentation by local microbes. At this stage of the process, and during the storage of the bee bread in pots, the presence of beneficial microbes has been observed. These microbes are directly related to the microbiota present in the gut of stingless bees, which has implications for the health of the individuals in the colony and the properties of the honey (Mohammad, Mahmud-Ab-Rashid, and Zawawi 2021; Nicolson and Human 2013; Vásquez et al. 2012).

Bees can use the smell of pollen to locate flowers of the same species. The extraction of pollen from flowers by bees is predominantly facilitated by the basitarsus and the proboscis. Large-bodied stingless bees, i.e., *Melipona*, can extract pollen by buzzing or buzz pollination, a movement achieved by contracting their wing muscles at high speed on the flower to release pollen from anthers (Cooley and Vallejo-Marín 2021). In addition, certain species of stingless bees, such as *Trigona*, have been observed to damage anthers or other floral tissues to reach pollen or nectar. In such instances, it appears that the plant does not have the benefit of pollination (Rego et al. 2018). The bees pack the pollen into their corbicula with the assistance of their front and middle legs, while simultaneously mixing the pollen grains with regurgitated liquids. Within the nest, the pollen packages are deposited in pots, which can be placed separately or mixed with the pots of honey (Mohammad et al. 2021; da Silva et al. 2024). Pollen is an essential component of the nest diet, providing sustenance for the brood and young adults over a period of several months (McFrederick et al. 2012).

The analysis of the specialisation of bees for polliniferous plants is a valuable tool for the expansion of knowledge in the following areas: the natural history of bees, their evolution, studies on bee diet, and their use as pollinators of certain crops. The tribe Meliponini constitutes a eusocial group of bees for which a polylectic lifestyle is generally accepted; however, certain species appear to be more specialized than others, according to palynological data (Vossler 2018). Stingless bee genus such as *Melipona* show clear preferences for certain flowers, regardless of their availability (Antonini, Costa, and Martins 2006; Ocaña-Cabrera et al. 2022). These preferences are attributable to the benefits that stingless bees obtain by reducing interspecific competition, because they focus on resources unattended by *Apis* sp., or simply because they can select flowers that produce nectar and pollen in greater abundance (Balamurali et al., 2018; Martins et al., 2023).

Across the three geographic regions of the Neotropics, the Afrotropics, and the Indo-Malayan-Australasian tropics, the families Fabaceae, Asteraceae, Rubiaceae, Malvaceae, Euphorbiaceae, Arecaceae, Lamiaceae, and Myrtaceae consistently appear as the main plants foraged by stingless bees (Aleixo et al., 2013; Antonini et al., 2006; Basari et al., 2021;

Bobadoye et al., 2016; Faria et al., 2012; Forster et al., 2023; Miranda et al., 2015; Ocaña-Cabrera et al., 2025). However, some divergence among stingless bees in the Indo-Malayan-Australasian and Neotropical regions is attributed, in part, to the distinct co-evolutionary histories between the bees and the respective regional floras (Bulle Bueno et al. 2023).

These bees exhibit both the capacity to pollinate native flora and the ability to pollinate invasive plants. Furthermore, their resilience renders them effective pollinators of certain crops with massive flowering phenology, such as coffee (*Coffea*), mango (*Mangifera*), passion fruit (*Passiflora*), and guava (*Psidium*) (Cruz et al. 2005; Giannini et al. 2015; Suhri et al. 2022, 2022). As an example, the densification of stingless bees, including *Nannotrigona testaceicornis* and *Tetragonisca angustula*, for pollination of strawberry plantations during the summer months in the northwest of Brazil, enhances fruit quality and commercial value, due to a 15% increase in fruit weight and a reduction in deformity (Miranda et al., 2024).

Beyond their role as pollinators of crops of economic interest, stingless bees also pollinate plants endemic to the tropics and subtropics, thereby closely assisting in the conservation of native forests. In economic terms, the value of maintaining a forest near a farm in Costa Rica was estimated at US$60,000 per year, which is comparable to the value of other land uses (Ricketts et al. 2004).

2.2 Foraging behaviours in stingless bees

In the context of foraging, visual cues play a crucial role in a forager's ability to identify flowers at a distance. In such environments, the visual characteristics of the flowers, as well as the stingless bees’ physiological responses to them, are key factors in the foraging process. These include colour preferences that are both innate and modified by previous experiences (Romero-González, Solvi, and Chittka 2020), as well as visual acuity (MaBouDi et al. 2025), spatial resolution (Raine and Chittka 2007), and light sensitivity of the bees' eyes (Giurfa et al. 1995). All of these characteristics are particularly relevant in complex visual habitats, such as tropical rainforests, where they enable the stingless bees to identify flower patches in such a biodiverse environment (Hrncir et al. 2016).

Once a food source has been detected, foraging stingless bees must recognise flowers on the same plant that still offer resources. This is achieved through the cues (chemical signals) left by previous foragers (Espadas-Pinacho et al. 2023). How bees react to scent marks (i.e., attraction or avoidance) constitutes a process that foraging bees quickly learn through the course of each new foraging flight (Roselino, Rodrigues, and Hrncir 2016) (Figure 7).

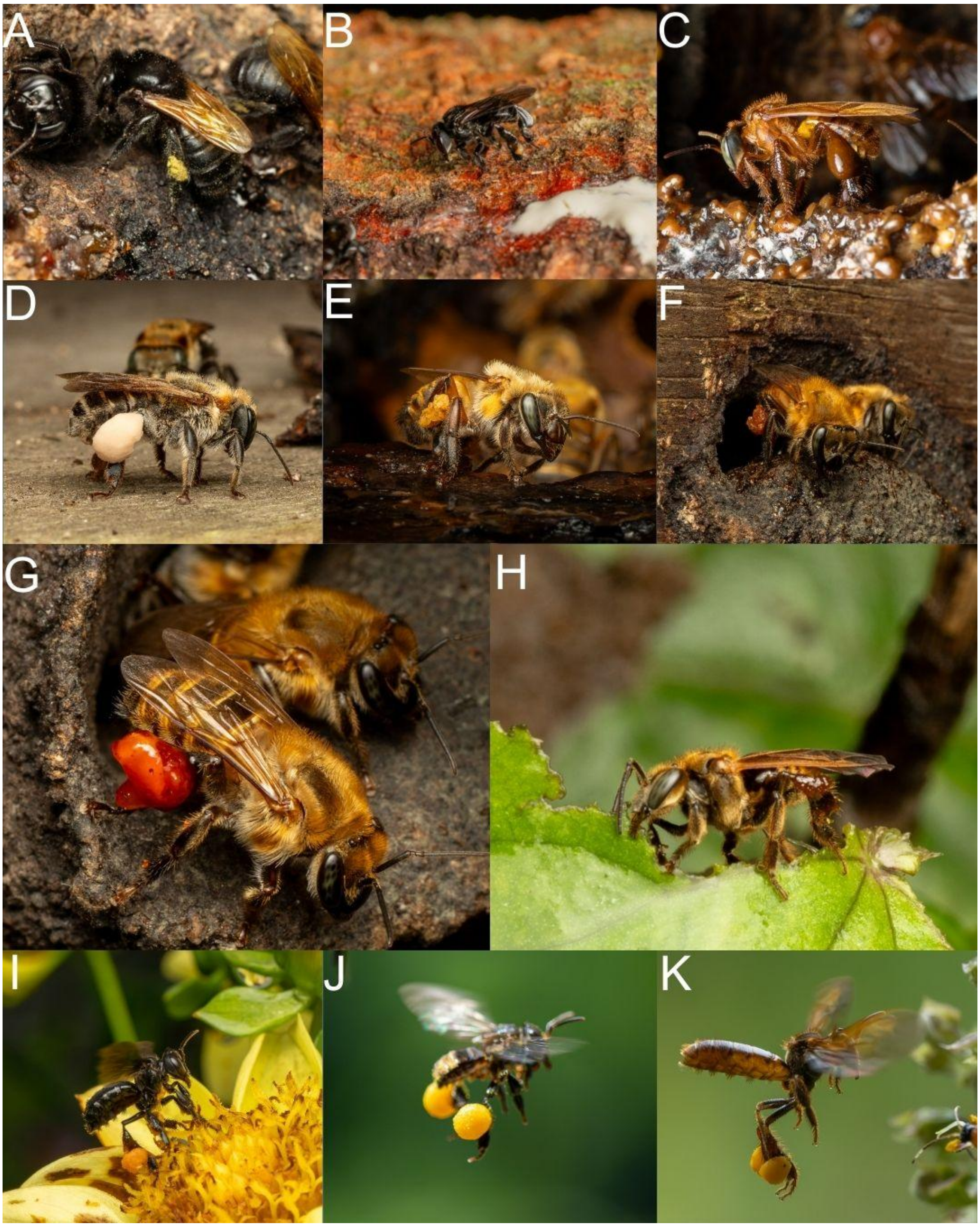


Figure 7. **Ecuadorian stingless bees' foragers**. **A)** *Melipona fallax* with resins in the corbicula. **B)** *Trigona* sp.1., collecting white resins. **C)** *Trigona* sp.2., with mud in the corbicula. **D)** *M. grandis* with white pollen in the corbicula. **E, F,** and **G)** *M. eburnea* with resins in their corbiculae. **H)** *Trigona* sp.3, cutting a leaf. **I)** *Trigona spinipes* with sunflower pollen. **J)** *Scaptotrigona* sp., and **K)** *Tetragona* sp., with pollen in their corbiculae. Photographer Alfonso Jimenez ©.

Chemical communication is based on pheromone markings in and near the resource-providing food flower (Bortolotti and Costa 2014). In the case of certain *Melipona* species, the development of recruitment strategies has enabled these species to avoid the attraction of other insects to these pheromones. Large numbers of nestmates are guided to food plots without the use of an odour trail (Flaig et al. 2016), thus ensuring food for their nests. In the context of maximising food gathering for the nest, other strategies employed by stingless bees include the consideration of climatic conditions (Maia-Silva et al., 2015; da Silva et al., 2019), the presence or absence of competitors (dos Santos et al., 2021), and the abundance and quality of resources (Basari, Ramli, and Mohd Khairi 2018; Villagómez, Keller, et al. 2024). Climatic conditions, including air temperature and precipitation, may constrain the foraging activity of other species (Keppner and Jarau 2016).

However, in the case of stingless bees, certain species have been observed to gather resources under such conditions, thereby avoiding competition with other insects for food sources (Leonhardt et al. 2016). In environments characterised by a high abundance of food sources, certain stingless bees can avoid competition for a resource and move to alternative flower patches, foraging efficiently and ensuring their return to the nest (Schorkopf 2016).

The capacity of stingless bees to adjust their foraging behaviour, at least in part, in response to the quantity of food stored in the nest, has been documented (Maia-Silva et al. 2015; Schorkopf 2016). In contrast to *A. mellifera*, an environmental generalist, members of the Meliponini tribe are predominantly located within relatively restricted geographical areas (Lee, Duwal, and Lee 2016). This characteristic renders them resilient insects with regard to food selection and foraging strategies.

2.3 Importance of stingless bees in the maintenance of tropical biodiversity

Stingless bees are of significant importance to the health of natural terrestrial ecosystems due to their role as pollinators of plants native to the tropics. In recent years, these bees have been highlighted as commercial pollinators of some crops (Atmowidi et al. 2022; Del Sarto, Peruquetti, and Campos 2005; Slaa et al. 2006; Wahizatul Afzan Azmi et al. 2017), and in their management and breeding, known as meliponiculture or stingless beekeeping.

In recent studies, researchers have documented that as of 2023, stingless bees worldwide have been recorded to visit flowers belonging to at least 221 different plant families and 1,476 genera (Bulle Bueno et al. 2023; Campbell et al. 2019; Giannini et al. 2015). Notably, several of these families represent the most diverse plant groups within tropical regions, including, but not limited to, Fabaceae, Asteraceae, Rubiaceae, Malvaceae, Lamiaceae, Euphorbiaceae, Arecaceae, Poaceae, Apocynaceae, and Melastomataceae (Bulle et al. 2021). Stingless bees from all geographical regions visit a range of non-native species from their preferred plant families (crops, timber, fibres, medicinal and ornamental) in addition to native plants (herbs, trees, shrubs, vines, lianas, epiphytes) (Meléndez Ramírez, Ayala, and Delfín González 2018).

In the context of foraging for sustenance, stingless bees establish intricate networks of interactions with floral species (Figure 8A). These interactions can encompass a spectrum of mutualisms (Gruchowski-Woitowicz, da Silva, and Ramalho 2024), where pollination occurs and the maintenance of diversity happens, to antagonisms (Rego et al. 2018; Reyes-González and Zamudio 2020), where resources are amassed without conferring any benefit to the plant, including fruiting (Absy, Rech, and Ferreira 2018). To fully understand pollination as an example of mutualism, studies of pollen niche breadth are used, among others, which vary in floristic composition according to geographic region (Figure 8B-8C). A 37-year study in the Brazilian Amazon revealed that niche breadth has decreased, possibly due to a mismatch in the timing of the life cycles of bees and their floral resources, and changes in the species composition of these resources (Pimenta et al. 2025). In addition to showing that larger bees collect a smaller variety of pollen (Costa et al., 2008), the study demonstrated that urban spaces cause stingless bees to expand their niche. This suggests that long-term changes to the pollen niche of these important pollinators could have serious consequences for plant-pollinator networks in the tropics.

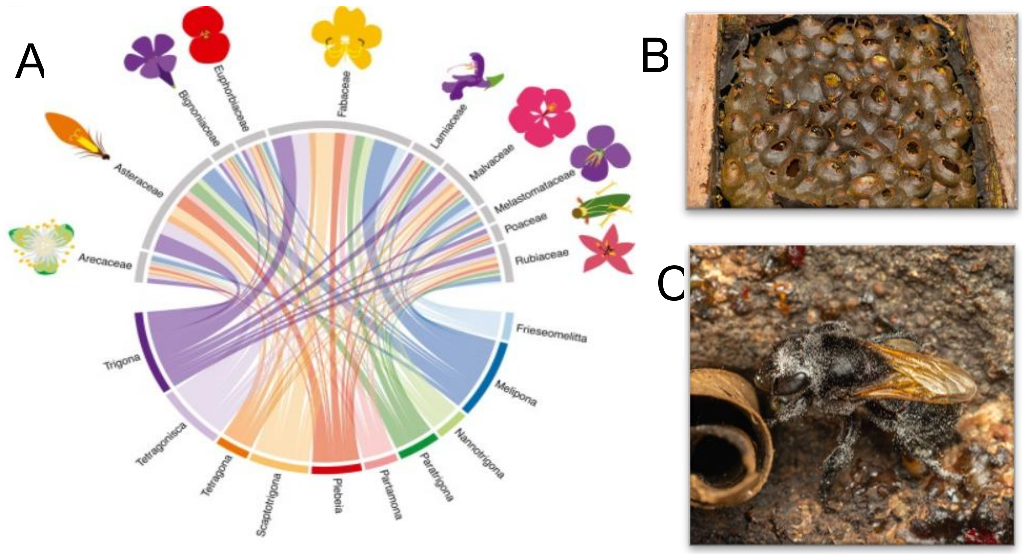


Figure 8. **Pollination services of stingless bees. A)** Diagram of visitation networks of stingless bees to flowering plants in the Neotropical region. Source (Bulle Bueno et al. 2023). **B)** Pollen and honey storage of *Tetragona* sp. in a wooden box. **C)** *Melipona fallax* covered by white pollen. Photographer Alfonso Jimenez ©.

The foraging activities of stingless bees influence not only plant reproduction and floral biology through pollination but also the dispersal of microbes (Roubik, 2023). When collecting and packaging resources such as pollen or resins, stingless bees' foragers come into contact with microbes that are specific to the resources they visit (de Paula et al. 2021a). If these interactions are advantageous, they may be conserved over time and found in the gut microbiota of stingless bee species (Salomón et al. 2024). For plants, microbial dispersal can be advantageous, as many bacteria and fungi have been found to improve resistance to diseases and pests, mitigate abiotic stress, and improve photosynthetic capacity and nutritional status by enhancing nutrient acquisition from the soil and increasing nutrient supply (i.e., through nitrogen fixation) (Harman et al. 2021).

The complexity of interactions that certain stingless bee species have established includes mutualism within a parasitism within a mutualism, as described by David Roubik in a province of the Ecuadorian Amazon (Roubik, 2021). Stingless bees of the genus *Nanoplebeia*, bees of the genus *Cecropia*, opportunistically create a pollination syndrome called melittophilia, co-opting ant plants and co-evolving with *Cryptostigma* (Coccidae) as obligate nest inhabitants from which they obtain wax and honeydew (Kondo and Roubik 2022).

3 Meliponiculture is rooted in inherited practices

Meliponiculture is the practice of managing stingless bees for commercial purposes, primarily for the sale of pot-honey. It is the second most prevalent bee management practice after apiculture and is becoming increasingly popular for agricultural purposes (Armas-Quiñonez et al. 2020; Jha and Dick 2010). Stingless bees are a well-documented example of social bees and are generally considered true generalists, foraging on up to 100 plant species (Wayo et al. 2020). However, individuals have been shown to specialise in a single floral species over time, a behavioural trait commonly referred to as 'floral constancy'. This behaviour is thought to lead to assortative mating of visited plants and thus more efficient pollination (Pangestika, Atmowidi, and Kahono 2017).

Meliponiculture, like apiculture and bombiculture (started in Belgium in 1989, rearing bumblebees in artificial nests for commercial pollination (Kwon 2008)), is a rapidly expanding practice in tropical countries. In addition to pollination services, meliponiculture provides products such as honey, propolis, resins, cerumen, and pollen, and is becoming a means of supporting and improving human nutrition and health (Barbiéri and Francoy 2020). The use and propagation of stingless bees is also indicative of cultural values. The practice of meliponiculture is the result of extensive interaction between humans and these social insects. Stingless bees hold cultural significance in various indigenous tropical communities. This ranges from the utilitarian to the mythological and encompasses a wide array of disciplines, including food, crafts, religion, and medicine. However, these cultural values are under threat, primarily due to external factors such as the loss of the stingless bees' natural habitat and the loss of ancestral knowledge that has not yet been documented by the scientific community (Quezada-Euán et al. 2018) (Figure 9).

In early pre-Columbian societies, cerumen was utilised in the fabrication of exquisite jewellery, predominantly composed of pure gold. Cerumen was an essential material for craftspeople, necessitating a constant and reliable supply. This placed significant demands on the individuals tasked with the procurement of the cerumen. This necessity may have promoted the adoption of more organised meliponiculture, which is a straightforward illustration of the economic principle of supply and demand (Jones, 2013). Cerumen and propolis were utilised as a binding agent in the manufacture of arrows, toys, and ceremonial objects in several tribes in Mesoamerica (Stearman et al. 2008a). In Australia, cerumen was also used to paint human and animal figures on rock walls. Some of these images have been dated to 2000 before Christ (BC) (Jones 2013).

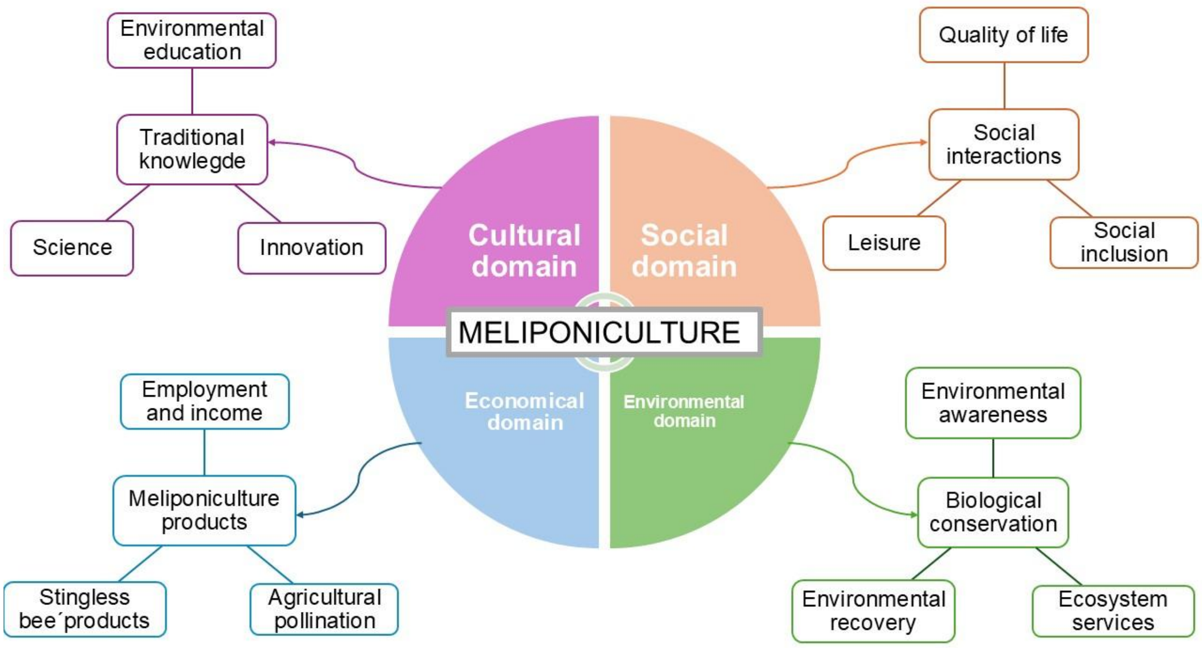


Figure 9. **Domains of Meliponiculture.** Modified from Barbiéri & Francoy (2020).

In some Aboriginal communities in Australia, insects such as stingless native bees played a central role in the development of their traditional culture. An example of this is the rich vocabulary in the Kune and Rembarrnga languages to differentiate between species of the genera *Tetragonula* and *Austroplebia* (Si and Turpin 2015). Young hunters and honey gatherers undertook the task of locating stingless bee nests to feed on the combs and extract honey. In addition, a cloth soaked in honey was transported back to the community to produce sugar water, which was then utilised in culinary practices (Perichon and Schouten 2021). In northwestern Australia, certain communities have a tradition of offering honey as a symbol of strengthening family and community relationships. Nowadays, pot-honey continues to be utilised as a conventional therapeutic agent, whilst the therapeutic benefits of stingless bee larvae have been demonstrated in the treatment of flu symptoms, skin irritations, and wound healing (Fijn 2014).

In certain zones of the Indo-Malayan region, such as Thailand, meliponiculture is considered an industry and includes over 700 practitioners, some of whom have over 15 years' experience. In the provinces of Chanthaburi and Trat alone, over 5,000 colonies are managed, with an estimated honey value of US$37–47 per kilogramme — ten times the value of *Apis* honey — and an annual production of 500 grams per nest. Cerumen has a higher market demand and value than honey in this area, with an estimated value of US$47–62 per kilogramme and an estimated production of 200 grams per nest per year (Rattanawannee et al. 2019). This industry is developing domestic and international markets in countries such as Taiwan and Japan (Chuttong, Chanbang, and Burgett 2014).

In the Afrotropics, hunters and meliponicultors in Kakamega, a town in Kenya, have worked together for a long time. In the past, the hunters would allow the colonies of stingless bees to die out once they had collected the honey. Nowadays, however, the hunters help the beekeepers to obtain colonies and prevent their collapse. Honey has a long history of use in ceremonies and cultural traditions. Non-food uses include using honey from *Meliponula lendliana* in circumcision ceremonies for medicinal purposes. Honey derived from *M. ferruginea* has been known for its aphrodisiac effects, while honey produced from *M. togoensis* is widely used to relieve dysentery and treat stomach ailments, as well as being used as a deworming agent (Héger et al. 2023).

3.1 Ancestral practices of stingless beekeeping in Latin America (Neotropics)

Meliponine husbandry has existed in Mesoamerica for more than 2,000 years. The earliest evidence of meliponiculture is found in ceramic effigy hobones (hollow logs arranged horizontally), and massive limestone discs from the Late Formative period (300 BC - 100 Anno Domini (AD)). In addition, detailed descriptions of the anatomy of the *Melipona beecheii* bee were found in the Maya culture, as well as knowledge related to stingless beekeeping (Paris et al. 2020; Źrałka et al. 2018). Approximately 20 indigenous groups, predominantly reported in Mexico, have a close relationship with stingless bees. Among these groups, there are variations in the manner in which they interact with bees, and a diversity of worldviews in which this interaction is immersed (Aldasoro-Maya et al., 2021; Ayala et al., 2013; Quezada-Euán et al., 2018).

In recent years, there has been a notable increase in the practice of meliponiculture. In response to this growth, proponents of ancestral knowledge or "saberes ancestrales" have identified two trends within this practice. On one hand, there is the group of breeders focused solely on the commercialisation of stingless bee products and the modernisation of hives and harvesting practices. On the other hand, some meliponicultors prioritise ensuring access to products for medicinal purposes, both for personal use and for the benefit of the community. These practitioners recognise the significance of honey production for the well-being of the bees and the importance of the symbiotic relationship between stingless bees and the environment (Aldasoro Maya et al. 2023).

In 2007, a proposal was made that sought to address the practice of meliponiculture following agroecological principles, thereby encompassing the economic, social, ecological, political, cultural, and ethical dimensions of sustainability. The proposal emphasises the maintenance of stingless beekeeping in preserved areas, agroforestry systems, and locations free from agrochemicals. The sustainable meliponiculture approach is predicated on the adaptation to the biodiversity of existing species and agroecosystems, thus avoiding the introduction of exotic species and the extraction of tree colonies that are key to the survival of the colony (Teixeira, 2007).

In Bolivia, the Yuqui people, an indigenous community inhabiting the Chapare region, utilise a mixture of cerumen and vegetable resin in the fabrication of hunting arrows (Stearman et al. 2008b). The Siriono people, an indigenous group inhabiting the Amazonian region, engage in a unique dietary practice. They consume stingless bee larvae, along with the larval food and cerumen, which is the substance from which the cell is formed. Their diet is further complemented by pot-pollen and pot-honey, which are incorporated into their fermentation process to produce traditional beverages (Lehm et al. 2004). The utilisation of cerumen for the production of textiles has been observed in Ayoreo communities located in the southern Bolivian region (Adler et al. 2023).

In Ecuador, a community of approximately 160 Afro-Ecuadorian families and families from the Awá and Eperaara indigenous nationalities in the coastal area of the country is known to protect the native forest where stingless bees live and sell *Melipona* sp., pot-honey only when they need money for health or education, respecting its biological, cultural, economic, and medicinal value (MDG Fund 2013). In the ethno-ecological community of Pablo López de

Oglán Alto, Arajuno canton, Pastaza province in the Ecuadorian Amazon, a total of 15 names of native stingless bees were documented, of which 13 were in Kichwa, one in Spanish, and one of mixed Kichwa-Spanish origin, such as “Putan mishki asesina”, demonstrating a past and persisting connection between the Ecuadorian Quichua community and stingless bees (Paredes-Bracho 2022). It was generally reported that stingless beekeeping in Ecuador has developed traditionally, particularly in seasonally dry areas such as Manabí, Guayas, Loja, and El Oro (Espinosa 2018). In the country, there is a paucity of information regarding studies of ancestral practices of meliponiculture, which should be a priority for future research in the anthropological area.

3.2 The advancement of stingless beekeeping in Ecuador

Meliponiculture, the practice of raising stingless bees in captivity, mainly honey-producing species (Venturieri et al., 2007), has recently experienced a surge in popularity. This development can be attributed to the emergence of several initiatives that utilise this practice as a means of production, an added value in agro-ecological systems, and in Ecuador, largely due to the COVID-19 epidemic that affected the economy of families, especially in rural areas. Meliponiculture, understood as an economic activity, requires the establishment of regulatory frameworks and public policy approaches. Such strategies ought to accord primary importance to the conservation of stingless bee species, and as far as possible, to the social and cultural values attached to this practice (Barbiéri and Francoy 2020).

The high demand for stingless bee products and high price have led to the perception of meliponiculture as a lucrative venture. However, this has led to an increase in the collection of nests from their natural distribution areas and the unnecessary extraction of nests from the forest (Quezada-Euán and Alves 2020). The relocation of stingless bee colonies to urban areas could constitute an additional risk to colony survival (dos Santos et al., 2022) and merits further study. In addition, social networks that promote commercialisation create the impression that keeping bees as resources or pets and promoting honey and other commercial products at high prices are inherently desirable (Carvalho 2022). The ecological network of bee populations and their associated organisms and ecological processes is rarely considered in the context of nature (Quezada-Euán et al. 2022).

A major factor contributing to the distortion of management methods in meliponiculture is the inexperience and lack of qualifications of the trainers. The combined effects of inexperienced training, deteriorating management, and the lack of continuous monitoring of implemented projects or initiatives by non-governmental organizations (NGOs) or government agencies are destructive for meliponiculture (Jaffé et al. 2015a). Management is a factor that can affect the life of stingless bees if good practices are not applied. In order to ensure that this practice does not lead to over-exploitation and the extinction of stingless bees, there is a necessity for the formulation of enhanced management proposals.

In Ecuador, a study has been proposed that involves the use of a wooden box to enhance the management of *Melipona* (Michmelia) *rufiventris* Lepeletier, 1836, within the coastal zone. The standardised box, crafted from *Cordia alliodora* (laurel), has been demonstrated to enhance honey and pollen production by 185% and 200%, respectively (Richard et al. 2019). The study also revealed that the initiative facilitated the management, division, multiplication, and harvesting practices of the families in the designated study area.

In addition to the development of species-specific hive designs for stingless bees and the implementation of good management techniques for efficient production and sustainable colony health (Ocaña-Cabrera et al., 2025a), further research is required to ensure the long-term survival of these important pollinators. Proposals have been formulated with integrative approaches, which aim to rescue traditional knowledge and merge it with modern scientific methods for efficient and sustainable management, including the conservation of stingless bee populations (Aldasoro Maya et al. 2023). In terms of disease and parasite control, prevention and control strategies have been proposed (Real-Luna et al. 2023). Educational initiatives and outreach efforts aim to promote sustainable meliponiculture (Imbernon et al. 2022).

In Ecuador, one of the main reasons for practising meliponiculture is to sell honey. To this end, it was necessary to establish requirements that guarantee the quality of the product intended for human consumption. In this regard, the Ecuadorian Standardisation Institute (INEN) made an attempt in 2014 to include requirements for pot-honey, which is produced by stingless bees, in the existing norm (INEN 1988). However, the revision process took a year, and the parameters could not be standardised or added to the norm due to inconsistencies in the laboratory analysis. Ecuador currently has no regulations for stingless bee honey. Meliponiculture is not recognised as an agricultural practice by the relevant bodies of the country: the Ministry of Agriculture and Livestock (MAG), the Ministry of the Environment, Water and Ecological Transition (MAATE), and the Agency for the Regulation and Control of Plant and Animal Health (AGROCALIDAD).

Thanks to the work of civil society collectives, projects developed by universities and NGOs have made some progress, although it is not legally recognised. This includes two booklets on the rights of nature that mention stingless bees (Colectivo en Defensa de los Polinizadores 2025; Recalde-Vela et al. 2023). There have also been advances in the generation of scientific information on the characterisation of honey and pollen, the description of stingless bee species and their practices, among others (Cabezas-Mera et al., 2024; Cabezas-Mera et al., 2023; Guerrini et al., 2009; Ocaña-Cabrera et al., 2022, 2024; Ocaña-Cabrera et al., 2025; Ocaña-Cabrera et al., 2025b; Paredes-Bracho, 2022; Richard et al., 2019; Roubik, 2018; Vit et al., 2018).

3.2.1 Honey-producing stingless bees

The following stingless bee genera are known to produce honey and are used in commercial meliponiculture in Ecuador: *Melipona*, *Scaptotrigona*, *Geotrigona*, and *T. angustula*. which, despite producing less honey than *A. mellifera*, their honey is highly valued for its flavour and medicinal properties (Faleiros-Quevedo and Francoy 2022; Gadge et al. 2024). The following genera can be added to the list of honey-producing species: in the Amazonian zone, *Tetragona* and *Ptilotrigona*, whilst in the high-mountain zone, typified by dry forest and cloud forest, *Cephalotrigona* and *Partamona* (Izabely Nunes Moreira et al. 2023).

Honey is the main commercial product of stingless bees, which produce honey for extra storage. The subject has inspired innovative applications in a range of sectors, including high-end catering (haute cuisine), in both raw and prepared forms. In this context, the stingless bee´s honey is likened to wine due to its unique sensory characteristics (Zawawi et al. 2022). As with honey, pollen-producing species have long been used as a condiment in culinary contexts (Maicelo-Quintana et al. 2024). Cerumen has been utilized as a constituent in the manufacture of scented candles and as a substitute for kerosene (Rasmussen et al. 2024). Finally, production of propolis garnered attention in the pharmacological sector due to its antibiotic effects (Araújo et al. 2010) and cytotoxic activity against four human cancer cell lines (KB, HepG2, Caco-2, and SK-MEL-28) (Vongsak, Chonanant, and Machana 2017).

In several Latin American countries (i.e., Mexico, Colombia, Brazil, Peru, Ecuador), the market price of meliponine honey ranges from $40 to $200 or more per litre (Quezada-Euán et al. 2018). In contrast, *A. mellifera* honey is generally priced between $5 and $15 per litre. The value of stingless honey is determined by various factors, including the species of origin, the method of production, regional availability, demand, and certifications such as medicinal and organic certifications, among others.

The elevated price of stingless bees´ honey is primarily attributed to its limited annual yield, which typically ranges from 200 millilitres to 2 litres of honey per colony per year. Additionally, the cultural and ecological value of stingless bees´ honey enhances overall value, as the breeding and management of these species promote the conservation of native plant species and the maintenance of biodiversity through pollination. Despite the existence of reports regarding the production and marketing of this honey, there are no global regulations such as the Food Codex or the Food and Drug Administration (FDA) for this product (Ávila, Beux, Ribani, et al. 2018).

3.2.2 Non-honey-producing stingless bees

Studies have documented that some stingless bee genera (i.e., *Plebeia*, *Trigonisca, Trigona*, *Paratrigona*, *Lestrimelitta*) produce only negligible quantities of honey. Observed, further indicate that certain species of stingless bees store nectar for immediate consumption, rather than engaging in a large-scale honey production (Villagómez et al., 2024). In certain instances, the very small size of stingless bee colonies and their pot-honey, coupled with their distinctive and highly aggressive behaviour, makes honey extraction from them unfeasible.

The evolution of these bees has been characterised by a shift in priorities, with a focus on ecological efficiency superseding the production of honey. The value of these non-honey-producing stingless bees lies in their role in pollination, a process vital to the functioning of ecosystems. The specialisation of certain species in pollinating specific floral varieties is such that even *A. mellifera* is unable to effectively pollinate, thus contributing to the maintenance of ecosystem balance and the genetic diversity of plants (Chuttong et al. 2022; Kakutani et al. 1993). This process facilitates the crossbreeding of different varieties, a key aspect in the resilience of forests, jungles, and agroecosystems against pests, diseases, and climate change.

Stingless bees that are not notable for their production and commercial meliponiculture play a role in the sustenance of other species by their participation in trophic chains. The limited products they store serve as a source of nourishment for a diverse array of animal species, including birds, reptiles (Roubik, 2023), and other insects (Boff & Somavilla, 2024; Chui et al., 2023; Roubik, 1989). Their activity also helps to maintain plant populations that provide shelter and food for other living organisms (Fernando 2012).

Stingless bee species, which are not usually associated with honey commercialisation, have been valued and conserved for other purposes, such as meli-tourism, a practice of entomotourism. Meli-tourism generally involves honey-producing species for the harvest of products to sell, while non-honey-producing species are used for educational purposes, biodiversity conservation (Lemelin 2020), and cultural practices preservation (Nicolas et al. 2022). This activity helps to diversify income for families and communities, as it fosters community-based tourism (Echenique-Diaz and Mizota 2019).

3.3 Stingless bee by-products as bioindicators of environmental health

A growing body of research has focused on the potential of bioindicators derived from stingless bees´ products, such as honey, propolis, cerumen, and pollen, to provide insights into the ecological health of the environment. These substances are of particular interest due to the distinctive ecological functions and the sensitivity of stingless bees to environmental changes (Real-Luna et al. 2023).

The utilisation of stingless bee products as bio-indicators is advantageous for several reasons. Firstly, these bees and their products are distributed extensively throughout tropical and subtropical ecosystems (Ramalho 2004). Secondly, stingless bees exhibit high nest fidelity, which ensures that their collection range remains largely constant. Finally, they are highly sensitive to pollutants.

3.3.1 Propolis

Bees in the tribe Meliponini collect sticky, resinous materials that are secreted by plants (Figure 10). They use these materials (propolis) in their nests for construction and support (Shanahan and Spivak 2021), to fortify entrances and repel invaders (Roubik, 2006), and to regulate and control the microbial communities inside the nest (Roubik, 1989). The chemistry of stingless bee propolis must be identified, not only because it is increasingly being used as a natural remedy, food additive (Rocha et al. 2023), or antioxidant (Pazin et al. 2017), but also because it is closely related to the viability of stingless bee nests (Shanahan and Spivak 2021). The chemical diversity of propolis is closely related to plant biodiversity, opening a channel to use this material as a bio-indicator of the environment.

Propolis is frequently analysed for the presence of heavy metals, polycyclic aromatic hydrocarbons (PAH), and other pollutants (Dobrinas, Birghila, and Coatu 2008; Moret, Purcaro, and Conte 2010; Rozman et al. 2022; Simsek et al. 2021). Urban environments and areas surrounded by industrial activity can be sources of unnatural substances, such as asphalt, tar, paints, and mineral oils, which bees collect when local vegetation cannot meet the needs of the nest (Sarapa et al. 2025). These abiotic stresses can alter the composition of the propolis, introducing pollutants into the nest and affecting the health of the colony's inhabitants, as well as posing a risk to human health.

Figure 10. **Pot storage of plant resin** in a nest of *Ptilotrigona lurida*, Iranduba Municipality, Amazonas Province, Brazil. (Photographer: ©R.M.O. Alves) from (Vit, Wang, et al. 2024).

3.3.2 Cerumen

Stingless bees' wax, one of the components of cerumen, has a lower melting point compared to honey bees' wax (Koedam et al. 2002). Cerumen contains a much higher percentage of hydrocarbons: ~60 to 90% versus ~14% in *A. mellifera*, and less esters: ~6% to 25% versus ~35% in *A. mellifera* (Gupta et al. 2014; Rocha et al. 2023). The amount of resin, the other major component of cerumen, varies according to the species and building material inside the nest, but can be higher than 40% (Roubik, 2006). Cerumen is organized in deposits and later retrieved for various construction activities inside the nest. Alternatively, the substance under consideration is obtained directly from a worker secreting wax and subsequently combined with resin that is also stored inside the nest in various piles, which are usually located near the entrance (Shanahan and Spivak 2021).

The cerumen found within the nests is reused by the workers. For instance, some of the cerumen extracted from cells that are no longer occupied is removed and reused elsewhere (Rasmussen et al. 2024). Due to factors including its capacity for re-use and its composition, the substance is considered a valuable long-term reservoir of lipophilic (fat-loving) contaminants such as persistent organic pollutants (POPs). Cerumen stays in the hive for extended periods, which allows lipophilic pollutants sufficient time to aggregate and become concentrated, thereby providing a historical record of exposure (Chuttong et al. 2023; Layek et al. 2024).

Lipophilic contaminants are defined as pesticides (e.g., DDT, chlorpyrifos), herbicides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and heavy metals. These contaminants can bind to organic molecules, which by their chemical nature tend to partition into waxy, oily substances such as cerumen.

3.3.3 Honey

Stingless bees´ honey contains a greater proportion of water than *A. mellifera* honey (Esa et al. 2022a). This higher water content facilitates the proliferation of hydrophilic symbiotic microorganisms (i.e., osmophilic yeasts) involved in sugar fermentation. The fermentation process is key in the preservation of honey, imparting the product with less sweet and more acidic flavours (Ngalimat et al. 2019). The microbiota and secondary metabolites present in honey have been shown to possess medicinal properties, as they are enriched with compounds (i.e., antimicrobial compounds) derived from vegetable resin, a constituent of the cerumen from which pots are made (Nordin et al. 2018).

Stingless bees are capable of foraging over a wide geographical area and collecting nectar, pollen, and resins from a wide variety of plants. Consequently, the honey produced by these bees is capable of reflecting the area's botanical diversity, as well as the presence of pollutants, pesticides, or heavy metals in the local flora (Sarapa et al. 2025). The presence of residues of pesticides (Pinheiro et al. 2020), heavy metals (Okeola, Oluade, and Liad 2020), and microplastics (Ibrahim et al. 2025) in stingless bee honey has been demonstrated, thus rendering it a bioindicator. The incidence of antibiotic residues in honey from stingless bees is low because antibiotics are not commonly used in Meliponiculture. However, environmental or cross-contamination (i.e., via water or floral sources) cannot be ruled out, which may lead to trace levels of these compounds being detected (Souza et al. 2006). Meliponine species exhibit heightened sensitivity to environmental alterations, including pollution and degradation of their habitat (for example, deforestation, urbanization, climate change, and the availability of floral resources) (Ferreira et al., 2024; Miotelo et al., 2021; Quezada-Euán et al., 2024). The honey produced by these stingless bees may contain, as *A. mellifera* traces of pollutants, thus serving as an organic 'record' of the quality of the environment (Souza et al. 2006).

The chemical composition of a stingless bee´s honey depends on the quality of the plants and soil from which the substance originates. Consequently, the regular analysis of its composition can provide indications regarding the presence of contaminants or imbalances within the ecosystem. Their honey reflects very localised environmental conditions, making it very accurate for monitoring specific ecosystems (Biluca et al. 2016).

The decline in honey production by stingless bee colonies, or the presence of chemical indicators of stress or contamination in their honey, is often indicative of broader environmental issues. These include the deterioration of biodiversity (Brühl and Zaller 2019), the excessive utilisation of pesticides (Ghimire and Woodward 2013), and/or the contamination of water and soil resources (Zhou, Li, and Achal 2025).

3.3.4 Pollen

Pollen has been demonstrated to be a reliable indicator of the presence of agrochemicals (Del Hierro et al. 2016). *A. mellifera* covers stored pollen with a layer of propolis, possibly as a cleaning strategy and with implications for the reduction of larval mortality, when the pollen has been contaminated with certain pesticides, namely the fungicide chlorothalonil (vanEngelsdorp et al. 2009).

Metals and metalloids (As, Cd, and Pb) were detected in the pollen of *T. angustula* in Brazil (de Oliveira et al., 2017). As well as at least one organochlorine pesticide in eight (22.22%) pollen samples of *S. mexicana* (Ruiz-Toledo et al. 2018). Pollen analysis results show it as a useful bio-indicator of environmental contamination by pesticides, given the long distances that bees travel (Oliveira et al., 2016). A study in Italy estimated that the majority of pesticide-contaminated pollen for *A. mellifera* came from areas outside the intensive cultivation sites where the bees were placed, as well as from urban sites and gardens, where the unprofessional use of pesticides also posed a risk to bees. Other sources of contaminated pollen were woody and weed plants located near the main cultivation area. Initially, it can be concluded that the contamination of pollen collected by the bees is due to the drift, resuspension, and wind transport of contaminated pollen grains or the direct application of pesticides to weeds (Mair and Wolf 2023).

4 Potential risks to stingless bees´ health and survival

4.1 Plant sources of intoxication

As with other pollinators (Barmaz et al. 2012; Nicholson and Egan 2020; Stanley and Preetha 2016), Meliponini bees are exposed to a number of risks with regard to their plant sources. While plants constitute their primary food source (in the form of pollen and nectar), there is a possibility that some of them may also pose risks, whether directly or indirectly. Certain species of plants are capable of producing nectar or pollen that contains natural toxins, which have the potential to be detrimental to bees. The presence of *Rhododendron* sp. and *Tilia* sp. has been demonstrated to result in symptoms including disorientation, reduced life expectancy, and, in extreme cases, death (Mitrović et al. 2023; Zoltani 2012). Certain species of plants are capable of producing secondary metabolites, which are then ingested by bees via nectar. Unfortunately, chronic exposure to elevated concentrations of compounds, such as alkaloids, has the potential to exert a deleterious effect on the health of stingless bees (Yan et al. 2022).

Spathodea campanulata Beauv. (Bignoniaceae), has been widely introduced to non-native areas, from Equatorial and Western Africa (Sutton, Paterson, and Paynter 2017), for its extensive use in ornamental and landscaping purposes. It has spread from Australia and Brazil to Puerto Rico and several Pacific islands (Francis and Lowe 2000). The Invasive Species Specialist Group has listed it as one of the 100 worst invasive alien species in the world (Lowe et al. 2000) because it endangers local plant biodiversity by inhibiting its development. The flowers of this plant produce potentially toxic nectar with insecticidal properties, which can cause high mortality rates among several species of hymenopterans (Queiroz, Contrera, and Venturieri 2014a). A study in Brazil revealed that 98.1% of insects found dead in 86 *S. campanulata* flowers were bees (Apidae and Meliponini tribes), primarily *Trigona spinipes* (50%) and *Partamona helleri* (24%) (Castagnino et al. 2024). While in Argentina, the species of stingless bee with the highest mortality rate in flowers of the same plant was *Scaptotrigona jujuyensis* (30%) (Ayala et al., 2024).

The life-threatening mucilage of the aforementioned bee species is not attractive to the stingless bee *Melipona scutellaris*. In a Brazilian study, 30 *M. scutellaris* colonies were part of the study site, yet no dead specimens were found on *S. campanulata* flowers (Castagnino et al. 2024). This suggests that these stingless bees tend to favour alternative food sources, thus avoiding the African tulip death trap.

4.2 Unethical stingless bee nest handling

Several deleterious effects have been identified in the practice of stingless beekeeping. Firstly, there is the somewhat hazardous improvisation of standardised types of wooden nest boxes for technical nest management (Jailani et al. 2019). This entails a trial-and-error stage that must be adapted to the specific area and species being managed. Secondly, the ambition to obtain honey and establish a stingless bee nest can lead to the adoption of management methods that often overlook biological considerations. This is further compounded by the inexperience of the beekeepers (Quezada-Euán et al. 2022). This results in the swift dissolution of recently established colonies.

Recently, there has been an increasing number of observations of *Cephalotrigona* species being used in Mexico (Quezada-Euán and and González-Acereto 2001) and of *Geotrigona* species, also known as ground bees, in Ecuador. These two species and those of the genus

Partamona cannot adapt to wooden hives due to their subterranean nesting habits, which result in poor thermoregulatory capacity. Experiments involving the transfer of *Cephalotrigona* colonies to wooden hives have demonstrated that colonies frequently diminish in size and perish within several months (Quezada-Euán et al. 2022). The colonies, i.e., *Geotrigona,* are known to produce substantial and palatable honey, a characteristic that renders them appealing to meliponicultors. However, the majority of colonies perish when transferred to conventional hives (Oliveira et al. 2013). Or they perish when their honey is extracted directly from the nest on the ground, without any care by honey harvesters or honey hunters.

The development of efficient and rapid methods of colony propagation is in response to the growing demand for stingless bee products, which are increasingly reliant on wild stingless bee populations and extractive practices (Eleutério, Rocha, and Freitas 2022; Menezes, Vollet-Neto, and Fonseca 2013). Nevertheless, the efficacy of these nest-splitting methodologies remains contentious, and their implementation would substantially mitigate the escalating risk of depletion of wild colonies.

The criteria employed for the maintenance of honey bees are frequently applied to stingless bees, without consideration of their proven biology. Rational methods of stingless bee beekeeping are not being applied correctly (Quezada-Euán and Alves 2020). These methods originate from the experimentation of other meliponicultors and are shared on social media. Such methods are generally subject to trial-and-error and have often not been tested using any scientific method, resulting in the loss of stingless bee colonies. One such example is the control of phorid flies, the most common and serious stingless bee pest. The utilization of vinegar traps for the management of flies (typically implemented following the transfer or division of a colony) is a recommended practice, albeit exclusively in instances of moderate to substantial infestation (Silva Correia et al. 2024). New meliponicultors are now using vinegar traps permanently, both inside and outside the hives. The persistent sour odour is likely to attract flies, which is the opposite of the desired effect.

Brazil has a law against the illegal trade of wildlife. However, 33 species of stingless bees are traded online without complying with legal requirements, with anonymity being a key factor in the success of this trade (Carvalho 2022). The risks associated with moving colonies include the potential spread of diseases. Moving colonies of stingless bee species out of their natural habitat can lead to changes in symbionts and in the colony’s internal temperature, affecting species that cannot tolerate these new conditions and causing their loss. Even if they survive, there is a risk that they will become invasive species in their new environment (dos Santos et al., 2022). One consequence of colony movement and survival is a reduction in genetic diversity, which can lead to inbreeding or a genetic bottleneck involving the production of diploid males (Cook & Crozier, 1995; May-Itzá et al., 2021; Zayed, 2009). These effects could have long-term consequences for the survival of the species, although highly inbred populations of stingless bees in Brazil are known to have remained viable for many years (Alves et al. 2011).

The effects of stingless bee colony management that does not consider the bees' biology or sustainable practices, and that is not regulated, are still being revealed. However, the impact of poor management practices on the death and loss of stingless bee colonies is evident (Figure 11).

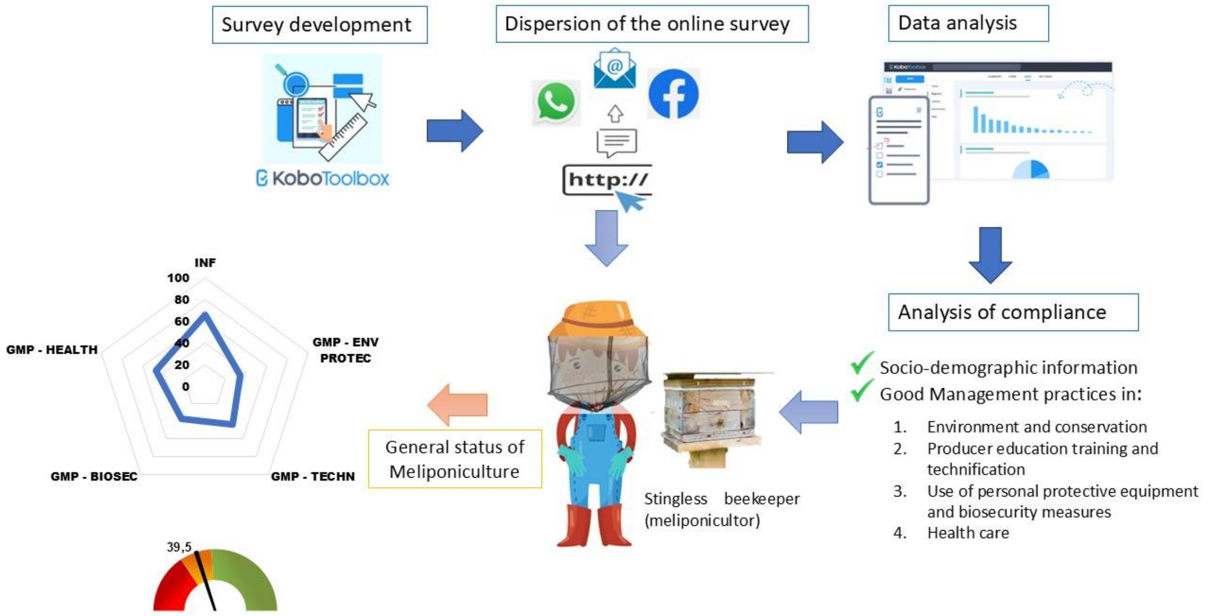


Figure 11. Information gathering flow for the evaluation of the application of good management practices in meliponiculture

4.3 Pest and pathogen threats

To date, research on the pathogens of stingless bees is limited but can be summarized in: viruses, bacteria, fungi, and parasites (i.e., arthropods). The potential for undiscovered infectious agents to be present in various species or populations remains to be elucidated.

Abiotic factors, such as the anthropogenic movement of stingless bee colonies to different geographical regions, carry the risk of spreading diseases and/or pathogens (Goulson and Hughes 2015). This should be considered a significant criterion during the implementation and evaluation of meliponiculture. The practice of feeding stingless bees with honey and pollen from honey bees is becoming increasingly prevalent. The discovery of the etiological agent of the European foulbrood, *Melissococcus plutonius*, in feeding stingless bee colonies with *A.*

mellifera honey and pollen (Teixeira et al., 2020) is a clear example of how pathogens spread from one species to another. In Mexico, deformed wing and black cell viruses have been identified in *S. mexicana* and *M. beecheii* in the Yucatan Peninsula (Fleites-Ayil et al. 2021; Guzman-Novoa, Hamiduzzaman, Anguiano-Baez, Correa-Benítez, Castañeda-Cervantes, and N. I. Arnold 2015). Biotic factors, such as floral resources, serve as pathways for disease transmission (Iwasaki & Hogendoorn, 2022) (Figure 12), which are generally beyond the capacity of beekeepers to monitor or manage effectively.

Figure 12. *Apis mellifera and Frieseomelitta nigra* collecting pollen from a palm of the genus *Pseudophoenix.* Photographer Jorge Ramírez-Pech.

4.3.1 Viruses

Viral diseases have been reported in species of stingless bees and are now considered a significant health concern. The first documented case of virus infection was the detection of acute bee paralysis virus (ABPV), which originally affects honey bees and has been found in 10 colonies of *Melipona scutellaris* in Brazil (Ueira-Vieira et al. 2015). However, the study did not confirm that this virus was pathogenic to this species of stingless bee, nor that this infection caused its death. Other factors, such as changes in environmental conditions, must be considered. The researchers also raised the possibility that phorid flies could act as viral vectors for stingless bees, in the same way that *V. destructor* acts for honey bees.

The honey bee viruses deformed wing virus (DWV) and black queen cell virus (BQCV) have also been reported in *Scaptotrigona mexicana* (Guzman-Novoa, Hamiduzzaman, Anguiano-Baez, Correa-Benítez, Castañeda-Cervantes, and N. Arnold 2015). The researchers identified the introduced honey bee, *A. mellifera*, as a potential source of this infection, given that it shares the same ecosystem as the stingless bee in which both viruses were detected. They also identified pollen foraged by both bee species as a potential source of viral loads in colonies. In Argentina, two previously mentioned viruses (ABPV and DWV type A) and a new one, Israeli acute paralysis virus (IAPV), were detected in four other stingless bee species: *Tetragonisca fiebrigi*, *Plebeia droryana*, *Plebeia emerinoides*, and *Trigona spinipes* (Alvarez et al. 2018). However, it should be noted that the presence of viral RNA in an individual does not necessarily mean an active infection (Singh et al., 2010).

An association was found between the level of chronic bee paralysis virus (CBPV) infection and climatic variables such as temperature and relative humidity in *Tetragona elongata* over a period of four months (two spring and two summer months) in Brazil (Guimarães-Cestaro et al. 2020). While in Mexico, it was documented that DWV-A and BQCV replicate in the stingless bee *Melipona colimana*, demonstrating that this species is a host rather than a casual vector (Morfin et al. 2021a). The varying viral loads of DWV and BQCV in *M. colimana* and *A. mellifera*, in the same study, suggest that the pathogenicity of these viruses may depend on the defence mechanisms of the respective bee species.

4.3.2 Bacteria

The first report of brood disease in stingless bees involved isolating the bacterium *Lysinibacillus sphaericus* (Firmicutes, Bacillaceae) from worker and queen larvae, brood cell provisions, and pot-honey of *Tetragonula carbonaria,* in Australia. The bacterium's pathogenicity was confirmed, with the first symptoms of brood disease appearing 22 days after infection (Shanks et al. 2017a). The symptoms associated with this disease included reduced foraging activity on an ideal summer day, slow or absent worker mobility, a small and non-uniform brood structure with few brood cells, and rough, dark-coloured inner materials.

In Brazil, honey and pollen from the *A. mellifera* bee are commonly used by meliponicultors to supplement the diet of stingless bee colonies. A study detected the bacterium *Melissococcus plutonius*, which causes European foulbrood, in stingless bee colonies of the genus *Melipona*, fed with honey bee products, and documented the symptoms associated with brood death and colony loss. This suggests that European foulbrood is more virulent in stingless bees (Teixeira et al., 2020).

Studies of the microbiota of stingless bees have revealed the presence of coliforms, including *Acinetobacter baumanii*, *Escherichia coli*, *Alcaligenes faecalis,* and *Enterobacter cloacae*, in samples taken from the inner surface of the nest of four species of Brazilian stingless bees

(*Frieseomelitta varia*, *T. angustula*, *Melipona quadrifasciata*, *Trigona spinipes*) (Sousa 2021a). The study results shed light on the types of materials that the stingless bees collect and bring into the nest, as well as highlighting the risk of contamination of honey and other products intended for human consumption. Another study identified Group U and Z Firmicutes, as well as Acetobacteraceae, in healthy and unhealthy adult individuals of *Melipona quadrifasciata* in Brazil. This was done as a means of elucidating an unknown annual colony collapse disorder (Díaz et al. 2017).

4.3.3 Fungi

The detection of *Nosema ceranae*, a microsporidian parasite that has only recently been identified as a honey bee pathogen, has been documented in stingless bee samples of *Tetragonisca fiebrigi*, *Scaptotrigona jujuyensis*, *T. angustula*, *Melipona fasciculata*, *Melipona quadrifasciata anthidioides*, *Melipona marginata*, *Melipona rufiventris*, and *Melipona mandacaia* from Argentina and Brazil (Porrini et al. 2017). Another study of stingless bees (*N. testaceicornis*, *T. angustula,* and *Tetragona elongata*) sharing a foraging area with *A. mellifera* in Brazil detected *N. ceranae* spores. However, these spores were not found in the bees' midguts, suggesting that they were unaffected but could act as vectors for the microsporidian. The researchers potentially linked the increased presence of spores in autumn and winter to a higher defecation frequency of the stingless bees and the scarce food resources available in the field, which increases the sharing of plant species between stingless bees and honey bees (Guimarães-Cestaro et al. 2020).

Nosema ceranae is the most prevalent disease among adult *A. mellifera* bees (Goblirsch 2018). In Australia, a study found a prevalence of 20% in five *T. hockingsi* nests over five months. It also found that this microsporidium was transmitted effectively (67%) through flowers visited by infected honey bees and healthy stingless bees. The study also reported a 2.96-fold increase in the mortality rate of *T. hockingsi* when fed sucrose and *N. ceranae* spores (Purkiss and Lach 2019).

The use of entomopathogenic fungi as bioinsecticides for pest control poses a new threat to the life and health of stingless bees. An isolate of *Beauveria bassiana* (conidia/mL) applied to the dorsal surface and in contact with newly emerged *Melipona scutellaris* bees was found to be virulent, causing mortality at low doses (Conceição et al. 2014). The effects of direct contact of three bioinsecticides formulated from *Beauveria bassiana*, *Metarhizium anisopliae,* and *Cordyceps fumosorosea* on three Brazilian stingless bees, *Melipona quadrifasciata*, *Plebeia droryana,* and *Scaptotrigona bipunctata* indicated differential tolerance and significant differences in mortality, according to the species (Faita, Pereira, and Poltronieri 2024).

4.3.4 Parasites: Arthropods

A significant challenge in meliponiculture is the infestation by flies of the species *Pseudohypocera kerteszi* (Diptera: Phoridae) (Figure 13A-13B). These flies are attracted primarily to weak stingless bees' colonies and can develop rapidly within the host colonies, consuming the stored pollen, where larvae and pupae also develop due to the marked acidity acquired by acetic fermentation by bacteria of the genus *Acetobacter* (Song et al. 2022). In a matter of days, the colony undergoes a process of dissolution. The fly *Apocephalus apivorus* was found in males of the stingless bee species *Trigona dorsalis* and *Cephalotrigona capitata* during their mating gatherings (Brown 1997).

Lestrimeliita stingless bee genera employ a deception mechanism involving the chemical similarity of their cuticular profiles to those of their selected host, enabling them to "plunder" the nest sources due to their natural condition as kleptobiotic stingless bees (Vázquez et al. 2022) (Figure 13C). The true problem of *Lestrimelitta* attacks materialises after plundering, as the scent of the open fermented pollen pots attracts the Phoridae fly, a pest that has previously been explained.

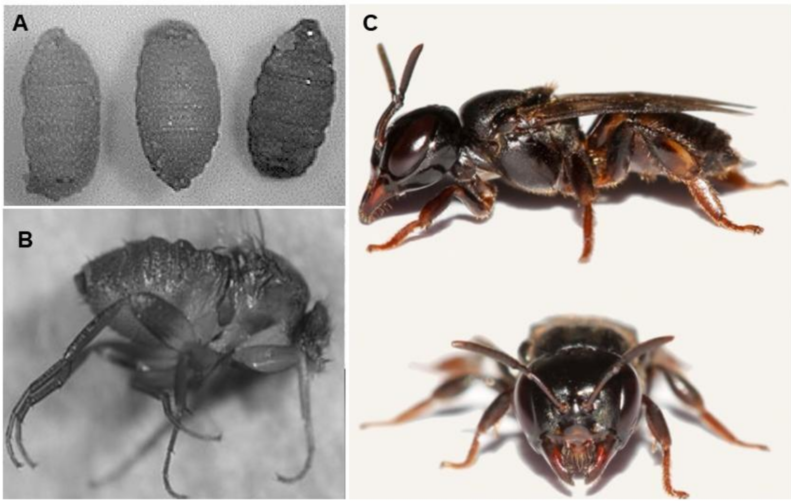


Figure 13. Main plagues for stingless bees. *Pseudohypocera kerteszi* **A)** Three pupae of different age; left: young pupa, middle: middle-aged pupa, right: mature pupa**.** Source (Robroek et al. 2003)**. B)** Female of *P. kerteszi.* **C)** *Lestrimelitta limao* (Smith). Photographer Cristiano Menezes ©.

Given the significant challenges posed by *Varroa* to beekeeping (Traynor et al. 2020), mite infestation is a key concern in Meliponiculture. However, no cases of *Varroa* mite infestation have been documented in stingless bees, although infestation by other mites has been reported. In 2009, an infestation of *T. angustula* and *Frieseomelitta varia* colonies by the mite

Pyemotes tritici was reported in Brazil, resulting in the death of four colonies and one colony, respectively. This infestation was caused by the movement of colonies, the transfer of infected combs, and subsequent poor management practices (Menezes et al. 2009).

Two reports of mite infestation in stingless bees from India occurred in 2013. Initially infesting pot-pollen and then spreading to brood cells, *Carpoglyphus lactis* (L.) caused the collapse of *Trigona iridipennis* colonies in one month (Karuppasamy, Muthuraman, and Jayaraj 2013). In addition, an infestation of *Pyemotes* sp. was reported in *Tetragonula iridipennis* colonies under experimental conditions. These parasitic mites were found lodged in the intersegments of the queen bee, as well as in the brood cells of the pupae. The infested colony died within one month, attracting predators to stored food and abandoned building materials and causing *Pyemotes* sp. to spread to other nearby colonies (Vijayakumar and Jayaraj 2013).

Leptus mites have also been reported parasitising stingless bee species (Schwarziana quadripunctata, *Trigona spinipes*, *Melipona torrida*, *Scaptotrigona depilis*), with prevalence ranging from 6.25% for *T. spinipes* to 14% for *S. depilis* (Martínez et al. 2024). The study suggests that the infestation originated in honey bee populations that shared the same floral resources in the area. Furthermore, the researchers propose that the intensive technification of stingless bee colonies may enhance the spread of pathogens, parasites, and diseases, as was the case with honey bees (Fontana et al. 2018).

4.4 Agrochemicals exposure

The indiscriminate use of agrochemicals represents a growing threat to the sensory and cognitive abilities of foraging bees. Exposure to sub-lethal doses of pesticides has been demonstrated to compromise the neuronal plasticity of stingless bees during ontogenesis (Farder-Gomes et al. 2024). This has been shown to result in a reduction in the brain volume of worker stingless bees, particularly in the mushroom bodies and optic lobes, which has a detrimental effect on their foraging performance (Hrncir et al. 2016). The effects of exposure to lethal doses may include a reduced feeding rate, reduced locomotion, changes in foraging behaviour, and memory loss. For example, the deadly dose of thiamethoxam for *Melipona interrupta* Latreille, 1811, is three times lower than for *Apis mellifera* Linnaeus, 1758 (Campos et al., 2024), clarifying the importance of species-specific studies on stingless bee species and more comprehensive toxicological experiments.

Beyond the effects of classified agrochemicals on the target organism, this section aims to highlight the impacts of pesticide cocktails, which provide a more realistic approximation of what occurs in the fields where stingless bees reside. A study conducted in Brazil aimed to evaluate the impacts and sublethal effects of combinations of the pesticides imidacloprid (IMD), pyraclostrobin (PYR), and glyphosate (GLY) on the behaviour, morphology, and physiology of fat body cells of *Melipona scutellaris*. The observed effects included a reduction in motility, as well as morphological changes in the fat body, such as vacuolisation. In addition, alterations were observed in cell shape and nuclear morphology, as well as an increase in the production of altered oenocytes and trophocytes (Farder-Gomes et al. 2024). Another study of *M. capixaba* investigated the lethal and sub-lethal toxicity of multiple agrochemicals, such as glyphosate and thiamethoxam, using contact and ingestion exposure bioassays, as well as feeding repellency bioassays. The study revealed that the effects varied depending on the agrochemical. Thiamethoxam caused high mortality irrespective of the exposure route or dose, as well as impaired flight ability at the lowest contact doses. Glyphosate caused high mortality following oral exposure and impaired flight ability following contact exposure (Gomes et al. 2023). Table 2 summarises the LD50 and LC50 estimates for various pesticides in different species of stingless bees.

Table 2: Median lethal dose (LD_{50}) and lethal concentration (LC_{50}) of various Plant Protection Products (PPP) on different species of stingless bees.

Stingless bee species	LD_{50} topical route	LC_{50} oral exposure	PPP	Reference
Frieseomelitta varia	NDA	0.00068 µg a.i./µL (648h)	Thiamethoxam	(de Souza et al. 2024)
Melipona quadrifasciata (larvae)	NDA	Lethal to all	Glyphosate	(Seide et al. 2018)
		Sublethal: altered development	Cry1F, Cry2Aa toxins	
Melipona scutellaris	0.025 µg a.i./stingless bee (6h)	0.0039 µg a.i./µL (6h)	Imidacloprid	(Costa et al., 2024)
	0.0006 µg a.i./stingless bee (48h)	0.011 µg a.i./µL (48h)	Fipronil	(Lourenço et al. 2012)
	NDA	5.43×10^{-7} µg a.i./µL (264h)	Thiamethoxam	(Miotelo et al. 2022)
Plebeia catamarcensis	NDA	0.000408 µg a.i./µL (24h)	Thiamethoxam	(Paula et al. 2023)
Scaptotrigona bipunctata	9.37×10^{-5} µg/stingless bee	3.1×10^{-6} µg a.i./µL	Fipronil	(de Carvalho et al. 2024)

Scaptotrigona postica	0.00054 µg a.i./stingless bee	0.00024 µg a.i./µL	Fipronil	(Jacob et al. 2013)
	NDA	0.00011 µg a.i./µL (24h)	Thiamethoxam	(Maloni et al. 2025)
Scaptotrigona xanthotricha	NDA	0.0001848 µg a.i./µL	Thiamethoxam	(Quiroga et al. 2017)
	NDA	0.0008162 µg a.i./µL	Fipronil	
Tetragonisca angustula	2×10^{-5} µg a.i./stingless bee (48h)	NDA	Malathion	(Mena et al. 2023)
	0.0004 µg a.i./stingless bee (48h)	NDA	Fipronil	
	9.73×10^{-4} µg a.i./µL	1.27×10^{-3} µg a.i./µL	Thiamethoxam	(Stuchi et al. 2023)
	6.67×10^{-6} µg a.i./µL	1.66×10^{-2} µg a.i./µL	Malathion	
	NDA	0.006664 µg a.i./µL	Thiamethoxam	(Quiroga et al. 2017)
		0.0001864 µg a.i./µL	Fipronil	
Tetragonisca fiebrigi	0.002 µg a.i./µL	0.107 µg a.i./µL	Fipronil	(Stuchi et al. 2022)

NDA: no data available. h: hours. µg: micrograms. a.i. active ingredient. µL: microlitres

Plants that have been treated with systemic insecticides (i.e., neonicotinoids) may contain residues of these chemicals in their nectar and pollen (Gierer et al. 2024). The exposure of stingless bees to these chemicals results in several adverse outcomes, including impaired foraging and learning behaviour (Aguiar et al. 2023) and an increased susceptibility to disease (Farder-Gomes et al. 2024), with a subsequent death of workers following the colony collapse (Rosa et al. 2016). Experimental evidence of pesticide toxicity suggests that ingestion of pollen and nectar contaminated with neonicotinoids and organophosphates can result in damage to larval health. The effects of exposure of the larvae of the stingless bee *Scaptotrigona bipunctata* to different doses of chlorpyrifos (an organophosphate compound) demonstrated that exposure resulted in the production of lighter, smaller, and deformed adult workers (Dorneles et al. 2021). The exposure to neonicotinoid products during the larval stage also results in alterations to the brain (Miotelo et al. 2021). The ingestion of contaminated pollen or nectar, in conjunction with the presence of invasive plant species, engenders nutritional stress, compromised immune systems, and diminished brood production in stingless bees. This phenomenon is attributed to the deterioration in the nutritional quality of sustenance sources (Trinkl et al. 2020) (Figure 14).

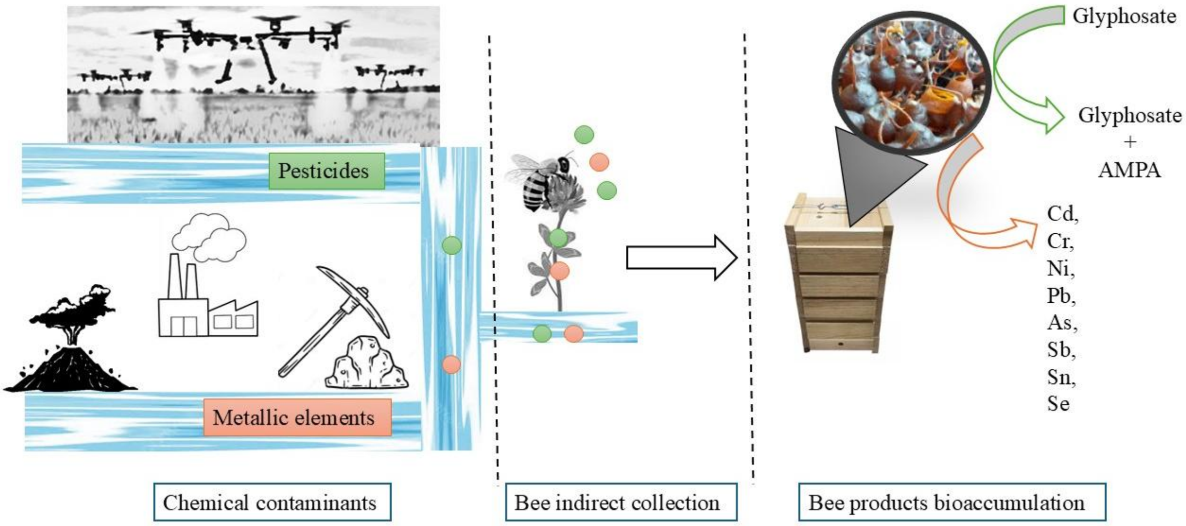


Figure 14. Scheme of the main chemical contamination sources of stingless bee by-products.

4.5 Climate change

Stingless bees exhibit a high degree of sensitivity to temperature fluctuations (Vollet-Neto et al. 2015). In conditions of elevated or reduced temperature, the capacity of these organisms to forage, reproduce, or even survive may be diminished (Quezada-Euán et al. 2024). The modelling of possible scenarios based on an increase in greenhouse gases predicts that stingless bees (*Frieseomelitta paupera*, *Melipona eburnea*, *M. favosa*, *Nannotrigona gaboi, N. melanocera*, *N. schultzei*, *Paratrigona eutaeniata, P. opaca*, and *Scaura longula*) will lose between 60% and more than 80% of their habitable area. This will force those that adapt to move to higher elevations or cause the extinction of those that do not, as well as the loss of plants that depend on pollination by specific insects (Gonzalez et al. 2022). For those who survive, the consequences would be a collective inability to obtain resources, resulting from the diversion of energy from workers to other essential needs. This may result in reduced nest resilience. In addition, warmer temperatures have been demonstrated to contribute to the accelerated dissemination of disease and parasites, rendering stingless bees particularly vulnerable (Ostwald, da Silva, and Seltmann 2024).

Alterations to flowering cycles may be precipitated by climate change (Dorji et al. 2020). In the event of flowers flourishing at an abnormally early or late point in the annual cycle, stingless bees may encounter a paucity of sustenance. This phenomenon is referred to as a “phenological mismatch” (Gérard et al. 2020; Kehrberger and Holzschuh 2019). Furthermore, the alteration of cycles of drought and rainfall has the potential to deplete resources such as water, nectar, and resins that are essential for the survival of stingless bees (Frigero et al. 2025).

A correlation has been identified between reduced precipitation and decreased forest moisture levels, which has resulted in an escalating risk of forest fires (Ma et al. 2024; Szpakowski et al. 2021; Xu, Huang, and He 2022). This situation has been further aggravated by deforestation (Leite-Filho et al. 2021). This, in turn, has the potential to result in the destruction of tropical forests and the habitat of numerous stingless bee species. A reduction in the area of forest cover has the consequence of a diminution in the number of nesting sites and a reduction in the availability of sustenance (Gonzalez et al. 2021). In addition to the loss of nesting sites and floral resources as a result of a chain of effects due to climate change, there is also an increased level of competition for resources (Angelella, McCullough, and O'Rourke 2021), as well as the spread of invasive species into stingless bee ecosystems (Goulson 2003).

In the context of climate change, efforts to mitigate its impacts can, on occasion, result in maladaptation (Schipper 2020). For instance, large-scale tree planting (Rana and Varshney 2023) and indoor farming, if not meticulously planned, have the potential to be counterproductive. The deployment of inappropriate plant species in unsuitable locations has the potential to adversely impact pollinators by displacing local flora. Greenhouse crops frequently have to remove existing habitat and depend on introduced bees that compete with wild bees (Osterman et al. 2021).

5 Epidemiology applied to stingless bees' knowledge

Epidemiology is defined as the study of disease in a population (Ahlbom 2020). In this context, the term 'disease' refers to any deviation from perfect health. When it comes to bee health, it is necessary to use a combination of direct measurements (observation and verification of the health status of bees or colony through biological or clinical evidence) and indirect measurements (assessments and pattern search in environmental factors, management factors, and production or behavioural indicators) to provide a comprehensive evaluation of a colony's health status (Steinhauer and vanEngelsdorp 2017). The primary goal of epidemiology is to prevent disease, which involves evaluating prevention strategies and ensuring that proven best management practices are adopted.

The application of epidemiological principles to the study of stingless bees (meliponines) involves the utilisation of scientific methodologies to comprehend the geographical distribution and factors that influence the prevalence of diseases within these populations. This approach would involve analysing the epidemiology of diseases, focusing on identifying the demographic characteristics ('who'), the geographical distribution ('where'), temporal patterns ('when'), and causal factors ('why') associated with these diseases (Ruegg 2006).

The identification of risk factors involves research on factors that increase the likelihood of stingless bees becoming unwell (Van Engelsdorp et al. 2013). Such factors may include studies on management practices, the type of flowers visited by the stingless bees (nutrition), climate change, competition with other bees, natural pests, pathogens, and their ecological dynamics.

To ascertain the probability of disease transmission or a disturbance of perfect health in stingless bee colonies, further research is required to elucidate the role of specific management practices (Ocaña-Cabrera 2025a), including deliberate feeding, the movement of colonies beyond their natural range (Quezada-Euán et al. 2022), their utilisation and exploitation as pollinators in monocultures, and the associated risks (i.e., pesticides) (Bogo et al. 2025).

The development and design of strategies that contribute to control and prevention programmes based on epidemiological data is imperative, as well as the development of ongoing health surveillance programmes for disease outbreaks or pest attacks (Ocaña-Cabrera 2025b), effective treatments, and, above all, improvements in environmental protection to conserve them. Epidemiology provides scientific knowledge on policy issues through analytical and macro-epidemiological approaches, taking into account the economic, legal, cultural, and biological aspects of the population under study (Hueston 2003).

6 Principal methods used in the thesis

6.1 Scanning electron microscopy

Scanning electron microscopy (SEM) is a sophisticated technique that involves scanning the sample surface with an electron beam and collecting the emitted signals to form high-resolution images (Kröner and Hirsch 2019). SEM is a foundational instrument within a wide range of scientific and technological research domains, owing to its capacity to procure intricate morphological and compositional data (Sacco et al. 2025). The SEM is initiated by the emission of electrons from a source (tungsten filament or a cold field-emission gun), which are then accelerated and focused into a fine beam by electromagnetic lenses. The electron beam traverses the sample in a zigzag pattern, thus ensuring comprehensive coverage and enabling the acquisition of high-resolution images. Upon impact of the beam with the sample, a series of interactions ensues, encompassing the emission of secondary electrons (SE), which yield detailed images of surface topography and exhibit heightened sensitivity to morphological variations. This non-invasive imaging technique enables magnification ranging from 20x to approximately 30,000x, with a spatial resolution of 50–100 nm (Troisi et al. 2024).

6.2 DNA Barcoding

The process of DNA barcoding involves the utilisation of a standardised DNA region for the identification of taxa (Antil et al. 2023). This technique is employed by ecologists to assist in the identification of species composition within environmental samples (Valentini, Pompanon, and Taberlet 2009). Short DNA fragments have been shown to persist in the environment (Arsenault et al. 2025), thus enabling the assessment of local biodiversity from soil (Orgiazzi et al. 2015), water (Królikowska et al. 2024), or faecal samples (Malik et al. 2024). Accuracy, speed, and reliability have been added to DNA barcoding thanks to Next-generation Sequencing Technology (Purty and Chatterjee 2016). This method improves taxonomic accuracy by allowing several DNA barcode regions (nuclear and mitochondrial markers) to be sequenced simultaneously from a single sample (Sonet et al. 2018).

6.3 Biodiversity index

Alpha diversity is usually used to estimate the variety and number of species in a specific environment at the local scale (Willis 2019). The richness (number of taxonomic groupings), uniformity (distribution of group abundances), or both, of an ecological community can be summarised using alpha diversity (Kitikidou et al. 2024). Alpha diversity analysis of amplicon sequencing data is the standard initial step in microbial ecology for evaluating environmental differences (Finn 2024).

Faith's phylogenetic diversity, which can be considered a phylogenetic generalisation of species richness, is calculated by adding together the branch lengths of a phylogenetic tree connecting all the species in a study set. Like species richness, Faith's phylogenetic diversity ignores species abundance (Chao, Chiu, and Jost 2016). For conservation applications, the existence or lack of a species is all that matters, or all that can be inferred from the available information. For conservation purposes, a set of phylogenetically diverse species is considered more biologically diverse than a set of closely related species. These phylogenetic differences can be based directly on evolutionary history, in the form of taxonomic classification or well-supported phylogenetic trees (Pavoine, Baguette, and Bonsall 2010). Phylogenetic diversity measures quantify the amount of evolutionary history preserved by a given set of species (Cavender-Bares, Ackerly, and Kozak 2012).

There is controversy surrounding the correct application of biodiversity indices, with some suggesting that they should be used according to the situation. The Shannon diversity index is more sensitive to rare vegetation cover types (Cassol, Ibañez, and Bustamante 2025). However, in landscapes where a single dominant type of terrestrial vegetation is of interest, the Simpson diversity index may be preferable (Nagendra 2002).

An indicator of how similar two collections of elements are to one another is the Jaccard similarity index. It is applied to qualitative data (e.g., species lists) and compares two places according to the presence or absence of species. It is predicated on the notion that two places are more alike if they share more species (Lakićević and Srđević 2018).

6.4 Chemical risk assessment

The risk quotient (RQ) is the ratio of a point estimate of exposure to a point estimate of consequences. The United States Environmental Protection Agency (EPA) uses it primarily to evaluate the ecological risk posed by pesticides. The estimated environmental concentration is referred to as exposure. An LC50, or other effect level or endpoint derived from ecotoxicity experiments, is referred to as toxicity (Karki et al. 2024). After the estimation, it is contrasted with the EPA's Level of Concern (LOC). Generally, a risk is considered acceptable if its RQ is lower than the LOC.

A human health risk assessment is the process of estimating the nature and likelihood of adverse health effects in humans who may be exposed to chemical substances, environmental contaminants, or other stressors in contaminated environmental media, now or in the future. It involves determining the toxicity of the hazard, identifying how and where people are exposed, and estimating the nature and likelihood of adverse health effects. The objective is to inform decisions on risk management, the development of cleanup strategies, and the protection of the health of specific populations (Zhang et al. 2023).

Objectives

General objective

The general objective of this study is to provide an enhanced comprehension of the multifaceted and imbricated factors, both anthropogenic and natural in origin, that influence the health status of Ecuador's stingless bees. A holistic approach will be adopted to address these issues and propose effective solutions that will lead to an improvement in the current conditions.

Specific objectives

The study will firstly conduct a comprehensive review of studies on stingless bees in the Tropics to ascertain widely reported diseases, agrochemicals, and other abiotic particles in the predominantly affected stingless bee species (**study 1**).

As part of these study areas, on the nutritional side, the pollen sources of the main stingless bee genera used as honey producers were identified (**studies 2 and 3**). In the domain of agrochemicals, traces of glyphosate, its metabolite aminomethylphosphonic acid (AMPA), metals, and metalloids were detected in cerumen, a by-product of stingless bees that serves as a bio-indicator of environmental health (**studies 4 and 1**).

The last study aims to provide a comprehensive account of the status of the application of good management practices in Meliponiculture in Ecuador, to describe the status quo, and then characterise it in focus areas. The study will also develop tools to facilitate a comprehensive understanding of all areas and to identify possible areas for improvement. Another objective of this study was to estimate the mortality rate of managed stingless bee nests and to relate the practices associated with this loss (**study 5**).

Experimental section

Experimental section

Study 1:

Environmental Sources of Possible Associated Pathogens and Contaminants of Stingless Bees in the Neotropics

Insects 2025, 16, 350

Joseline Sofía Ocaña-Cabrera, Sarah Martin-Solano, Claude Saegerman

Preamble

The existing research based on the pathogens associated with diseases affecting stingless bees is limited. The reported effects of this phenomenon include brood loss and annual death, mostly in a specific *Melipona* species, especially in areas of Brazil. Contaminants of anthropogenic origin have been found to accumulate in stingless bees and their products, mainly pot-honey.

The causal microorganisms of different diseases reported on stingless bees were bacteria, viruses, and a combination of fungi and bacteria. Heavy and rare metals, polyethylene (PET), and polystyrene (PS) microplastics, polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides were also quantified in various matrices derived from stingless bees, including bee bodies and the midguts of larvae. These reports were obtained following a systematic review of 30 articles published by November 2024.

In some cases of pathogen contamination, the source was honey bee-derived food provided to stingless bee nests. Of all the reports, only three did not refer to managed nests, i.e., stingless bees that were being managed in modern wooden hives. Two of these did not specify whether the nests were wild or in wooden boxes, and the last one was a laboratory trial. Regarding synthetic or chemical pollutants, the sources are varied; therefore, research is needed into the impact of contaminants and pathogens on the physiology and health of stingless bees or in their colonies. Honey bee references should not be used in the context of lethal or sublethal doses of chemical contaminants in other bee species.

insects

MDPI

Review

Environmental Sources of Possible Associated Pathogens and Contaminants of Stingless Bees in the Neotropics

Joseline Sofía Ocaña-Cabrera [1], Sarah Martin-Solano [2,†] and Claude Saegerman [1,*,†]

[1] Research Unit of Epidemiology and Risk Analysis Applied to Veterinary Sciences (UREAR-ULiège), Fundamental and Applied Research for Animal and Health (FARAH) Center, Faculty of Veterinary Medicine, University of Liège, Quartier Vallée 2, Avenue de Cureghem 6, B43a, Sart-Tilman, 4000 Liege, Belgium; jocana@doct.uliege.be

[2] Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Av. Gral. Rumiñahui S/N, Sangolquí 171103, Ecuador; ssmartin@espe.edu.ec

* Correspondence: claude.saegerman@uliege.be; Tel.: +32-4-366-45-79

† These authors contributed equally to this work.

Simple Summary: The Meliponini tribe of bees, which are distributed in tropical and subtropical climates around the world, play an important role in pollination. It is imperative to ascertain the microorganisms and contaminants that impact them, which may also be of human origin, to implement preventive measures for their conservation. A comprehensive investigation was conducted into agents associated with stingless-bee diseases and contaminants, as well as their origin and spread. The presence of bacteria and viruses associated with a particular syndrome that results in the death of colonies of the *Melipona* species has been identified. Contaminants found in materials inside the nest, as well as in the products derived from stingless bees and destined for human consumption, were indicative of the quality and health of the environment surrounding the nests, increasing the vulnerability of the bees. It is imperative to expand research efforts to explore the health of bees in greater depth from a One Health perspective and to elucidate how biotic and abiotic factors pose threats to the lives of stingless bees, both individually and in combination with other factors.

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Abstract: Stingless bees are crucial for pollination and support diverse ecological relationships, offering economic benefits and contributing to enhanced crop yields. Their tropical pollinator status makes them highly sensitive to environmental changes and disruptions, which could affect their survival, as well as to pathogens that threaten their health. The lack of comprehensive research and the scattering of reports make it difficult to identify pathogens and contaminants. This review aims to provide an overview of diseases in stingless bees, examine chemical contaminants in their products, and explore threatened sources. Using the PRISMA flowchart, a total of 30 articles from 2009 to 2024 concerning pathogens and contaminants in stingless bees were retrieved. A total of 15 pathogens and 26 pollutants affect life expectancy and survival rate of stingless bees (mainly the genera *Melipona* and *Tetragonisca*) were identified in five major areas of the Neotropics, including Brazil, Mexico, Costa Rica, Australia, and Asia. Studies indicated that the bacterial genera *Pseudomonas*, *Melissococcus*, and *Lysinibacillus* are affecting the survival of stingless bees, particularly their brood, and contributing to annual colony deaths. Heavy metals, polycyclic aromatic hydrocarbons (PAHs), and microplastics have been detected in by-products of stingless bees, especially honey. Epidemiological research is crucial, including studies on pathogens associated with diseases, the effects of contaminants on bees, and the development of quality guidelines for stingless-bee products.

Keywords: Meliponini; threat; agrochemicals; disease agents; preventive measures; One Health

1. Introduction

Stingless bees (Hymenoptera, Apidae, Meliponini) are a large and ecologically vital group of eusocial bees, with over 600 species, predominantly found in tropical regions [1]. They play an important role in the environment by affecting how plants reproduce and supporting different relationships between plants and insects [2,3]. Stingless bees also help with pollination, which can increase crop yields by almost 40% [4]. This makes them important for the economy. Stingless bees produce honey that has special medicinal properties [5], such as antimicrobial and antioxidant benefits [6]. This honey fights off germs and protects cells; it is valuable in the food, pharmaceutical, and cosmetic industries [7,8].

Stingless bees exhibit significant ecological and behavioral diversity [9], with different species showing various foraging strategies [10], colony sizes, and nesting behaviors [11]. The production and management of stingless-bee products, including honey and cerumen, have traditionally been part of local economies, particularly in tropical regions like Latin America and Asia [12–15] where specific species are cultivated for honey production. As interest in stingless-bee cultivation (meliponiculture) grows, the industry supports biodiversity conservation and offers a sustainable source of income for stingless-bee keepers, empowering rural communities. The economic and environmental importance of stingless bees underscores their potential as a cornerstone for both ecological preservation [16,17] and socio-economic development in tropical countries.

Stingless bees play a crucial role in pollination and in maintaining biodiversity in tropical and subtropical ecosystems [10]. Their foraging behavior and diet breadths are closely tied to forest cover [18], with species richness being higher near forest edges [16]. However, deforestation and habitat fragmentation threaten their persistence, as smaller species are particularly vulnerable [18]. Climate change poses additional risks [19,20], disrupting their developmental cycles, their social behaviors [21], and overall survival [22–24], which can further jeopardize ecosystems. As tropical pollinators, stingless bees are highly sensitive to environmental changes, making their conservation vital for maintaining biodiversity and ecosystem services in a warming world.

Factors like scent influence stingless-bee foraging behavior [25], along with the color [26], location, and temperature of flowers [27]. While they prefer feeders closer to the nest based on scent, their color preferences appear random [28]. Foraging decisions are also socially driven; returning bees share olfactory and gustatory information with nestmates [29], influencing future food choices. Additionally, many stingless-bee species rely on plant resins for nest building and defense. Species with a higher resin intake tend to be more active [30], highlighting the critical role of resins in their behavior and nest maintenance [31].

A major threat to the well-being of stingless-bee colonies is the transmission of parasites from other insects that interact with them while collecting food and materials from the same plants in the field [32]. Deformed wing virus (DWV) is currently among the most widespread insect pathogens on the planet, and its propagation has been linked to infestations of *Varroa* mites in honey bees [33]. The virus was classified into three distinct genotypes: DWV-A, DWV-B, and DWV-C. The latter has been identified as the most prevalent in the stingless bee *M. subnitida* in Brazil [34], and co-infections involving multiple genotypes, and the black queen cell virus has also been documented [35,36].

Bacterial strains of *Lysinibacillus sphaericus* [37], as well as fungal pathogens such as *Melissococcus plutonius* [38] and *Nosema ceranae* [39], have also been reported in stingless bees. The health implications of nests infected with these pathogens encompass various adverse outcomes, including brood mortality, diminished worker population [40], and the repercussions of Colony Collapse Disorder (CCD) [41], which can be exacerbated by pesticide exposure.

The increasing demand for land for monoculture crops, combined with unsustainable agricultural practices, has led to the degradation of natural habitats. This phenomenon is primarily attributable to the elevated demand for pesticides, which exert a direct impact on bees and other pollinators [42,43]. The direct effects of pesticides on stingless-bee species are size- and species-dependent, owing to the presence of specific detoxification mechanisms [44]. For instance, in *Melipona scutellaris*, alterations in the foraging-bee ascent rate and changes in heterochromatin were observed after topical exposure to fipronil [45].

Exposure to agrochemicals not only affects individual bees but also extends to products derived from the nest. In a region of Italy, an analysis of honey bees' bee bread revealed the presence of 63 different pesticide residues, some of which were detected at levels that exceeded the risk threshold established for bees [46]. However, expanding pesticide risk assessment studies to non-*Apis* bee species remains a contentious issue among researchers [44,47,48], primarily due to differences in species biology. A significant gap in the existing body of toxicological research on stingless bees is highlighted by the limited number of studies conducted in Latin America. Notably, more than 80% of the published research focuses on Brazilian species, underscoring the paucity of studies on other species and the limited evaluation of crop pest products [49].

The characterization of contaminants and pathogens impacting stingless bees constitutes the initial step in determining the direction of priority research and identifying knowledge gaps. The study of pesticide contamination of stingless-bee products is particularly important to conservation efforts and sustainable agricultural practices. A comprehensive understanding of these impacts is essential for fostering improved land-management practices and more-sustainable agricultural techniques, which in turn can positively impact local economies as well as the domains of agriculture and stingless-bee keeping or meliponiculture. This systematic review has two main objectives: (1) to analyze diseases associated with pathogens in stingless bees and (2) to examine chemical contaminants present in their products. Additionally, recommendations based on the One Health approach will be proposed to mitigate these impacts.

2. Materials and Methods

The literature review on environmental contaminants affecting stingless bees in the Neotropics was conducted from 1 to 28 November 2024 under the Preferred Reporting Items for Systematic Reviews and Meta-Analysis for Scoping Reviews (PRISMA-ScR) 2018 checklist and the PRISMA 2020 flowchart.

A search was conducted in the Google Scholar and PubMed databases, using the following keywords and Boolean operators to find indexed articles: stingless bees AND Brazil OR Mexico OR Costa Rica OR Australia OR Asia, AND honey OR cerumen OR resins OR propolis, AND contaminant OR heavy metals OR neonicotinoids OR pesticides OR pathogens OR disease. These terms were used to retrieve all relevant publications, regardless of their year of publication. The selection of countries was based on their significant role in meliponiculture research and development within the Neotropics.

Exclusion criteria included the following: (i) language other than English, Portuguese, or Spanish, (ii) a focus on beekeepers, honey bees, wasps, and other Hymenoptera, (iii) duplicates between the two databases, (iv) information that is exclusively concerned with the methodology or the development of new methodologies, (v) toxicity studies, or (vi) literature of a comprehensive and overarching nature.

The data from the selected articles were compiled into a database to assess sampling efforts, measured by the number of publications. The database included records of contaminants in stingless-bee products from Neotropical countries (sample numbers and prevalence), the most frequently monitored native bee species, and habitat types (primary forest, secondary forest, disturbed areas, unspecified locations, urban areas, and agricultural zones).

3. Results

This systematic review was conducted following the PRISMA guidelines (see Figure 1), allowing for the identification of pathogens (n = 15) and contaminants (n = 26) affecting stingless-bee survival in five selected primary areas representative of the Neotropics. The sampling effort (n = 30) covered key regions, including Brazil (n = 21), Mexico (n = 2), Costa Rica (n = 1), Australia (n = 4), and tropical and subtropical Asia (n = 2).

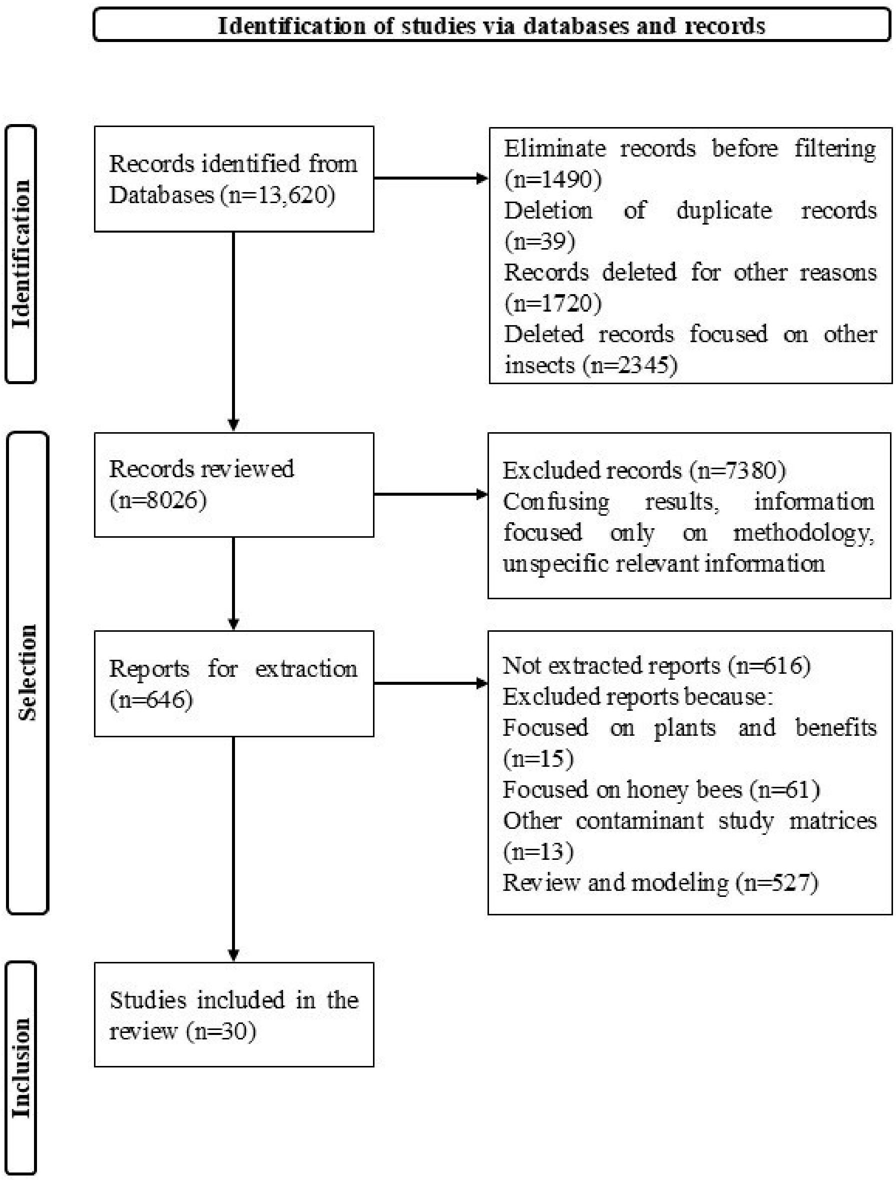

Figure 1. PRISMA 2020 flowchart.

Studies conducted in geographically distinct regions—Australia, Brazil, and Mexico—reported the presence of pathogens and contaminants in various genera and species of stingless bees, as well as in diverse nest by-products such as honey, pollen, geopropolis, wax, and brood. In the tropical zone of the western hemisphere, Brazil had the highest number of studies reporting pathogens and pollutants. In contrast, Australia had the highest number of studies in the eastern hemisphere.

Diseases in stingless-bee brood of bacterial origin have been reported from distant areas such as Brazil and Australia, although different bacterial species have been reported in each case. While diseases associated with viruses have been reported exclusively in Brazil, this may be attributable to the level of research conducted in that country, without excluding the possibility of detection in other areas of Latin America where stingless bees are distributed. Concerning contaminants, Mexican reports have indicated the presence of trace amounts of pesticides, while Brazilian reports primarily concern trace metals in stingless-bee products, as do Australian reports.

3.1. Study Matrices for Pathogens and Pollutants in Stingless Bees

Adult foraging bees constituted the main sample (59%, 7/13) in detecting seven of the eight pathogen-associated diseases listed in Table 1. In certain studies, these individual bees were used together with honey (15%, 2/13), brood (7%, 1/13), worker and queen larvae (7%, 1/13), brood cell provisions (7%, 1/13), and pollen (7%, 1/13) from several stingless-bee species.

Table 1. Summary of pathogens (bacteria, fungi, and viruses) that are affecting stingless bees.

Stingless-Bee Species	Study Matrix	Disease/Pathogen	Study Prevalence	Detection Method	Habitat/Season	Country	Publication
Melipona quadrifasciata	Unhealthy and healthy adult individuals	Unknown annual colony collapse syndrome Disorder Firmicutes Group U (23%), Firmicutes Group Z (23%), and Acetobacteraceae (16%)	52 positives/76 samples = 0.68	PCR and Illumina MiSeq sequencing to analyze the variable region V1-V3 of the 16S rDNA gene	Managed nest Summer	Brazil	[50]
Melipona marginata *Melipona quadrifasciata* *Melipona mandacaia* *Melipona compressipes* *Melipona rufiventris* *Melipona mondury*	Brood, pollen, and honey	European Foulbrood *Melissococcus plutonius* Brood (66%), pollen (6%), honey (33%)	18 positives/30 mixed samples = 0.6	PCR and Sanger sequencing and fragment analysis applications, to analyze 16S rDNA gene	Managed nest distributed in an open and roofed area, in an orchard Spring	Brazil	[38]
Tetragonula carbonaria *Austroplebeia australis*	Workers and queen larvae, brood cell provisions, and honey pots	Bacterial brood disease *Lysinibacillus sphaericus* (Firmicutes, Bacillaceae) strains	Not specified	Characterization and pathogenicity by microbiology. PCR of the 16s rDNA gene, and cloning. Multilocus sequence typing (MLST) analysis	Managed colonies Summer	Australia	[37]
Melipona subnitida	Workers	Deformed wing virus variants DWV-A and DWV-C The average total viral loads per bee was 8.8×10^7	21 stingless-bee positive/100 (10 pools of 10) = 0.21	RT-PCR of total RNA	Managed colonies Spring	Brazil	[34]
Tetragonisca fiebrigi *Scaptotrigona jujuyensis* *Tetragonisca angustula* *Melipona fasciculata* *Melipona quadrifasciata anthidioides* *Melipona marginata* *Melipona rufiventris* *Melipona mandacaia*	Adult individuals	Nosemosis *Nosema ceranae*	7 positives/8 species = 0.87	Duplex PCR of the 16S rRNA locus	Managed and wild colonies. Sampling over 5 years in Argentina, and one year in Brazil	Argentina and Brazil	[39]

Table 1. *Cont.*

Stingless-Bee Species	Study Matrix	Disease/Pathogen	Study Prevalence	Detection Method	Habitat/Season	Country	Publication
Melipona quadrifasciata	Healthy and diseased forager bees	Tailed viruses (Caudoviricetes)	Not specified	DNA and RNA metagenomic	Not specified	Brazil	[51]
Frieseomelitta varia *Tetragonisca angustula* *Trigona spinipes* *Melipona quadrifasciata*	Adult individuals	Unknown annual syndrome *Pseudomonas* sp. *Sphingomonas* sp. *Escherichia coli* *Alcaligenes faecalis*	Not specified	PCR of the 16S rRNA gene (V3/V4 regions) and the MiSeq sequencing system	Managed colonies Spring–Summer	Brazil	[52]
Nannotrigona testaceicornis *Tetragonisca angustula* *Tetragona elongata*	Adult individuals	*Nosema ceranae* Acute bee paralysis virus (APBV) (10.8%) Deformed wing virus (DWV) (5.1%) Black queen cell virus (BQCV) (5.1%)	Histology detected spores in 100% stingless-bee bodies. Not detected in the midgut by PCR Viruses were found in 23.4% of stingless-bee samples.	Duplex PCR of 16S ribosomal gene RT-qPCR of mRNA from stingless bees	Managed nests Autumn–winter	Brazil	[53]

The species belonging to the genera *Melipona* (58%, 15/26) and *Tetragonisca* (15%, 4/26) have attracted the most interest. The stingless-bee species included in the studies are listed in order of increasing to decreasing presence: *Melipona quadrifasciata*, *Melipona marginata*, *Melipona rufiventris*, *Melipona mandacaia*, *Tetragonisca angustula*, *Tetragonisca fiebrigi*, *Scaptotrigona jujuyensis*, *Frieseomelitta varia*, *Trigona spinipes*, *Nannotrigona testaceicornis*, *Tetragona elongata*, and finally, two Australian stingless-bee species: *Tetragonula carbonaria* and *Austroplebeia australis*.

Most samples were obtained from domesticated or managed nests (88%, 7/8), while only one study reported sampling from both managed and wild nests. Furthermore, the predominant sampling season in pathogen-associated-disease studies was summer–spring, with only one study sampling during autumn–winter.

The matrices used for detecting contaminants (Table 2) in stingless-bee nests included honey (46%, 6/13), geopropolis (15%, 2/13), pollen (15%, 2/13), wax (7%, 1/13), individual bees (7%, 1/13), and larvae midguts (7%, 1/13). Most samples were obtained from domesticated nests (77%, 7/9), while one study was developed under laboratory conditions (bioassay). The predominant season for sampling was summer, and the focus was on long-term sampling, ranging from one to four years in duration.

Table 2. Summary of contaminants found in stingless-bee by-products.

Stingless-Bee Species	Study Matrix	Contaminant [Min–Max]	Habitat/Season	Country	Publication
Tetragonisca angustula	Honey and pollen	As [1.70 ± 0.01–361.30 ± 18.88] $\mu g\ kg^{-1}$ Cd [0.11 ± 0.01–1.64 ± 0.01] $\mu g\ kg^{-1}$ In [0.08 ± 0.01–0.53 ± 0.29] $\mu g\ kg^{-1}$ Pb [1.20 ± 0.01–463.31 ± 35.16] $\mu g\ kg^{-1}$	Not specified	Brazil	[54]
Melipona scutellaris	Geopropolis	Cr [6.5–39.0] $mg\ kg^{-1}$ Cu [1.9–8.4] $mg\ kg^{-1}$ Mo [0.6–2.5] $mg\ kg^{-1}$ Ni [0.8–6.8] $mg\ kg^{-1}$ Pb [1.6–8.9] $mg\ kg^{-1}$ Zn [1.2–21] $mg\ kg^{-1}$ Cd [0.2–1.2] $mg\ kg^{-1}$	Managed nests Urban environment Sampling over one year	Brazil	[55]
Partamona helleri	Larvae midguts	500 ng/bee of plastic microparticles of polystyrene (PS), and polyethylene terephthalate (PET) 10 µg/bee of nanoparticles of a metal oxide (titanium dioxide—TiO_2)	Bioassay (laboratory conditions)*	Brazil	[56]
Melipona quadrifasciata	Honey	0.1 to 2.6 particles per honey mL of microplastics (primarily composed of polypropylene)	Managed nests Built-up and vegetated areas	Brazil	[57]
Tetragonula carbonaria	Bees, honey, and wax	As [12–140] $\mu g\ kg^{-1}$ Pb [11–2050] $\mu g\ kg^{-1}$ Mn [410–46,400] $\mu g\ kg^{-1}$ Zn [490–73,000] $\mu g\ kg^{-1}$	Managed nests Summer	Australia	[58]

Table 2. *Cont.*

Stingless-Bee Species	Study Matrix	Contaminant [Min–Max]	Habitat/Season	Country	Publication
Scaptotrigona bipunctata *Tetragonisca angustula* *Melipona quadrifasciata* *Tetragonisca weyrauchi* *Tetragona clavipes* *Scaptotrigona postica* *Melipona marginata*	Honey	Ca [0.70 ± 0.06–123.92 ± 1.49] μg g^{-1} Mn [0.66 ± 0.06–41.92 ± 4.67] μg g^{-1} Mg [1.60 ± 0.25–351.48 ± 9.58] μg g^{-1} Fe [13.04 ± 0.39–363.77 ± 6.41] μg g^{-1}	Managed nests Atlantic Forest, and Amazon River Sampling over 4 years	Brazil	[59]
Tetragonisca angustula *Scaptotrigona depilis* *Scaptotrigona postica* *Melipona quadrifasciata* *Scaptotrigona bipunctata* *Melipona marginata* *Melipona bicolor*	Honey	1.4 to 23.3 μg kg^{-1} of polycyclic Aromatic Hydrocarbons (PAHs)	Managed nests Native forests and industrial areas Summer	Brazil	[60]
Melipona quadrifasciata anthidioides	Geopropolis	Al [20,414.40–36,911.1] mg kg^{-1} As [4.37] mg kg^{-1} Cr [17.41–38.07] mg kg^{-1} Ni [2.28–21.74] mg kg^{-1} Pb [3.45–8.55] mg kg^{-1} Sb [1.34–1.64] mg kg^{-1} Sn [4.92–16.14] mg kg^{-1}	Managed nests Summer	Brazil	[61]
Scaptotrigona mexicana	Honey and pollen	Organochlorine compounds: Heptaclor [96.4–645.08] μg kg^{-1} γ-HCH [8.8–207.15] μg kg^{-1} α-HCH [3.8–4.79] μg kg^{-1} β-HCH [26.1–68.41] μg kg^{-1} p,p′-DDE [25.1–34.1] μg kg^{-1} Heptachlor epoxide [18.1–21.68] μg kg^{-1} α-Endosulfan [51–59.12] μg kg^{-1} p,p′-DDT [99–440.78] μg kg^{-1}	Managed nests Sampling over one year	Mexico	[62]

Legend: Bioassay (laboratory conditions)*; Al = aluminum, As = arsenic, Ca = calcium, Cd = cadmium, Cr = chrome, Cu = copper, Fe = iron, In = indium, Mg = magnesium, Mn = manganese, Mo = molybdenum, Ni = nickel, Pb = lead, Sb = antimony, Sn = tin, Zn = zinc. HCH = hexachlorocyclohexane, DDT = dichlorodiphenyltrichloroethane, DDE = dichlorodiphenyldichloroethylene.

The *Melipona* (38%, 8/21), *Scaptotrigona* (28%, 6/21), and *Tetragonisca* (19%, 4/21) species were the most prevalent genera in contaminant studies. The following species were involved in contaminant studies: *M. scutellaris*, *M. quadrifasciata*, *M. marginata*, *M. bicolor*, *S. bipunctata*, *S. postica*, *S. mexicana*, *Tetragonisca weyrauchi*, *T. angustula*, *Partamona helleri*, and one Australian representative species: *T. carbonaria*.

3.2. Bacterial, Fungi, and Viral Pathogens of Stingless Bees

Four diseases associated with bacteria were reported: Unknown annual colony collapse syndrome Disorder, European foulbrood, unknown annual syndrome (Brazil), and bacterial brood disease (Australia). The following groups of bacteria were attributed to stingless-bee or nest damage: Firmicutes Group U and Group Z, and Acetobacteraceae. The bacterial genera mentioned were *Pseudomonas* sp. and *Sphingomonas* sp., while the bacterial species identified were *M. plutonius*, *L. sphaericus*, *Escherichia coli*, and *Alcaligenes faecalis*. The stingless-bee species in which the most prevalent reports of diseases related to bacteria were documented were *Melipona* in regions of Brazil and *Tetragonula* in Australia.

The only microorganism in the fungal kingdom was *N. ceranae*, which causes nosemosis. The viruses reported were deformed wing virus (DWV) variants A and C, tailed viruses (Caudoviricetes), acute bee paralysis virus (APBV), and black queen cell virus (BQCV). The only two pathogens reported in two studies were *N. ceranae* and deformed wing virus. The genus *Melipona* had the highest number of reports of fungi- and virus-associated diseases in Brazil, followed by the genera *Tetragonisca*, *Scaptotrigona*, *Nanotrigona*, and *Tetragona*.

The prevalences among samples positive for pathogens were estimated to range from 21 to 87% of the total number of samples examined in each study (see Table 1). The pathogen with the highest percentage prevalence was *N. ceranae*, a microsporidium that causes a disease known as nosemosis. A case of co-infection was reported in a brooding sample of *M. marginata*, in Brazil, where the microsporidium *N. ceranae* and the bacterium *M. plutonius* together caused European foulbrood.

3.3. Anthropogenic Contaminants in Stingless-Bee By-Products

The following metals have been identified in various stingless-bee nest matrices from specific regions of Brazil and Australia. Notably, in all studies, these metals exceeded the detection and quantification limits of each laboratory: aluminum (Al), arsenic (As), calcium (Ca), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), indium (In), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), antimony (Sb), tin (Sn), and zinc (Zn), as well as nanoparticles of titanium dioxide (TiO_2). Honey, geopropolis, wax, and bees were presented as the main matrices for detecting trace amounts of these metals. The metals that were found to be of most concern were arsenic and lead in the honey samples, and aluminum and chromium in the geopropolis samples.

Compounds belonging to the organochlorine pesticide group—including heptachlor, endrin, gamma-hexachlorocyclohexane (γ-HCH), dichlorodiphenyltrichloroethane (DDT), and dichlorodiphenyldichloroethylene (DDE)—were also reported to be the most concerning contaminants, in honey and pollen from *Scaptotrigona mexicana* in Mexico.

Compounds derived from the oil industry or the burning of organic matter the polycyclic aromatic hydrocarbons, were reported in honey from the *Melipona*, *Scaptotrigona*, and *Tetragonisca* genera of stingless bees in Brazil. The compounds identified included light polycyclic aromatic hydrocarbons (PAHs), such as fluorene, phenanthrene, anthracene, and heavy PAHs, including benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene, and dibenz[a,h]anthracene.

The presence of plastic microparticles of polystyrene (PS), polyethylene terephthalate (PET), and polypropylene was identified in honey from *M. quadrifasciata* and in the midguts of *P. helleri* larvae—both of which are Brazilian stingless-bee species.

4. Discussion

4.1. Occurrence and Reporting of Pathogens in Stingless Bees

Nosema ceranae, an epidemiologically significant parasite of honey bees [63], possesses spores that are highly resistant and spread via the oral–fecal cycle [64]. Cross-contamination in nature is likely due to the overlap in spatial distribution, range, and feeding resources among insects [65]. Specifically, within the order Hymenoptera, the transmission of this pathogen can occur via flowers through shared used by pollinators [66], but pathogens can also be transmitted through behaviors such as the theft of honey and pollen [67], the usurpation of nest sites, and the dispersal of spores by certain insectivorous birds [68]. These events facilitate the host-hopping of the pathogen and the subsequent territorial spread of the disease.

In the Argentine province of Misiones, colonies of *T. fiebrigi* and *S. jujuyensis*, which were positive for *N. ceranae*, were located near honey-bee hives, suggesting inter-species contact due to robbing behavior. In contrast, *N.-ceranae*-positive colonies in Argentina's Chaco province were farther from managed honey-bee hives, indicating another form of contact with the spores [39].

In southeastern Brazil, the bacterium *M. plutonius* and symptoms of European foulbrood (EFB) were reported for the first time in *Melipona* species [38]. In areas where beekeeping and meliponiculture coexist, managed honey bees (*Apis mellifera*) and stingless bees (Meliponini) likely share plant resources, increasing the risk of pathogen and parasite transmission [69]. Furthermore, the notion that certain beekeeping practices can be extrapolated to meliponiculture has emerged as a potentially hazardous approach, such as the utilization of *A. mellifera* supplements for the feeding of stingless-bee colonies [70].

Melissococcus plutonius can modify its physiological state to ensure its survival [71]. This ability may represent a significant adaptive trait, enabling it to survive on honey-bee products and infect stingless-bee broods. It is important to note that the increased prevalence of EFB symptoms in Brazilian stingless bees can be attributed to elevated environmental stress factors, including a reduction in natural foraging habitat [24], and increased exposure to chemicals [47]. The effects of the EFB on unsealed stingless-bee larvae manifest in symptoms of illness, and a subsequent elimination due to hygienic behavior and worker inspection, indicative of social immunity [40]. In the case of *M. scutellaris*, colonies experienced a rapid collapse, followed by a phorid attack.

Even though not all stingless-bee pathogens are transmitted from honey bees, the bacterium *Lysinibacillus sphaericus* has been reported in two endemic stingless-bee species in Australia. This has resulted in a reduction in colony populations and a failure of the workers to maintain hive structures, which has had a direct effect on brood rearing [37].

Virus families such as Dicistroviridae, Parvoviridae, and Circoviridae have been reported in diseased stingless bees [72]. Nevertheless, there is still a considerable gap in investigating viruses associated with these eusocial bees. Metagenomic studies of honey bees have identified viruses originally described in plants, a subgroup of Aphid Lethal Paralysis Virus (ALPV), Israel Acute Paralysis Virus (IAPV), and Lake Sinai Virus (LSV) [73]. Initially, researchers hypothesized that these viruses were only environmental contaminants introduced into the nest through pollen and nectar collected by bees. However, subsequent research has revealed that these viruses may be utilizing the bees as hosts, as evidenced by the example of tobacco ringspot virus (TRSV), which, in addition to infecting *Apis mellifera*, has also been found to replicate in this bee species [74].

A holistic approach is recommended for studying viral communities in managed and wild bee species, including their food plants. Additionally, considering geographical context can provide deeper insights into bee–virus–plant interactions [75].

Viruses were present in the following products as well: BQCV in the bee pollen of *A. mellifera* and a protein-based commercial ration (prepared with the same *Apis mellifera* bee-pollen from the South of Brazil), and ABPV in the powder of *A. mellifera* bee-pollen (purchased from the Northeast of the Country) [76].

DWV-A and BQCV have been detected in two stingless-bee species in Mexico, with prevalences of 1% and 15%, respectively. In *M. beecheii*, experimental inoculation of pupae and adults with RNA viruses showed negative effects on colonies [69]. In the case of *M. colimana*, both viruses were found naturally in adult bees and replication of these viruses was demonstrated [36], making this species a possible host and vector for both viruses.

4.2. Bees and Nest By-Products as Bioindicators of Environmental Health

The use of honey-bee hives to study environmental pollutants has revealed that the bees themselves provide a more accurate reflection of environmental health than hive products [77]. In the case of stingless bees, the Australian species *T. carbonaria*, with a foraging range of 0.3–0.7 km [78], has been studied as a small-scale bio-indicator of trace elements in different landscapes [58]. The influence of *M. quadrifasciata*, *M. scutellaris*, and *T. bhingami* on metal accumulation in their geopropolis, particularly lead, has been documented [77].

Several studies have used bee products to detect and quantify contaminants. Propolis, pollen, and wax are more suitable for studying toxic metals than honey. As is illustrated in the present systematic review, more studies on metal detection utilized stingless-bee honey compared to other products. In instances where the objective was to evaluate the quality of the environment and nest health, bees emerged as the optimal study matrix. Conversely, if the concern pertains to food safety, the utilization of bee nest products was advocated, given their designated purpose for human consumption.

The presence of heavy metals, including lead (Pb) and cadmium (Cd), has been documented in trace amounts in bee specimens as well as in other matrices such as propolis, pollen, and honey [79–81]. These metals in the nest result from various factors, including the environmental quality of worker-bee foraging areas. In meliponiculture, nest-product harvesting avoids using steel utensils that could release traces of Al, Zn, or Fe. The honey or pollen pots are crafted from cerumen, a malleable (resins plus wax) material that opens without force to facilitate the collection of honey or pollen. In contrast, tools such as crowbars or spatulas are employed to access the nest, given that batumen, a comparatively rigid material, or propolis, in certain stingless-bee species, occasionally necessitates the application of force or support to displace the floors of the technical nest.

If the species of pollen and nectar plants are exposed to chemical contaminants, the final composition of the honey and bee pollen produced in those locations may contain contaminating elements [80]. Other anthropogenic sources of environmental metals include vehicle emissions [82,83], mining- and industrial-waste smelting [84,85], and residual leaded petrol, which persists in the environment [86,87].

Iron (Fe) and aluminum (Al) are among the most prevalent elements in the Earth's crust [88]; however, concerns arise when these elements are found in concentrations exceeding 20 and 2 mg/kg, respectively [89,90]. These concentrations represent the permissible limits for these elements in food. While there is no global regulation for products derived from stingless bees intended for human use or consumption, the presence of concentrations above the reference concentrations in honey or geopropolis suggests a potential food safety concern.

In the context of the bumblebee area, the bees were exposed to arsenic oxide, cadmium chloride, or chromium oxide in a sucrose solution. The results indicated that significant amounts of cadmium ($CdCl_2$ 10.3 mg/L) were accumulated in the bodies of the exposed bees. However, no accumulation of chromium or arsenic was observed [91]. While it is improbable that foraging bumblebees or other bees will encounter lethal concentrations of these metals in the field, it is imperative to comprehend how sublethal concentrations influence colony functionality, given the observed variation in bee responses to different metal species. As an example, laboratory experiments with worker bees (*Apis cerana cerana*) demonstrated that chronic exposure to low-level concentrations of Cd resulted in a significant reduction in the number of antioxidant gene transcripts. Additionally, Cd inhibited the transcription of immune-related genes and altered the structural characteristics of bacterial and fungal communities within the bee gut [92].

Pesticides and heavy metals have been shown to induce changes in the composition of the microbiome, cellular damage in the midgut tissue, and a disruption of the peritrophic membrane in honey bees [93]. The latter physiological effects may increase the susceptibility of social insects to intestinal or bacterial pathogens. Conversely, the impact of plastic microparticles has been associated with a decline in intestinal microbiota, modifications in the expression of genes associated with oxidative damage and detoxification, and alterations in the cognition and nervous system of honey bees [94].

In the case of stingless bees and other contaminants, analytical investigations were conducted in Brazil on *M. subnitida* honey samples from urban and rural areas. The analysis yielded positive results for the presence of organophosphorus compounds. Subsequent comparative analyses of pesticide frequencies revealed no significant disparities between the urban and rural zones [95]. Indeed, a preceding study in a nearby region detected residual levels of chlorpyrifos and monocrotophos in the water [96], with water and soil being other sources of contaminants for stingless-bee nests. Furthermore, it is imperative to implement comprehensive pesticide control strategies, encompassing both field and bee health measures. Additionally, research is necessary to substantiate the potential lethal threat to bees posed by exposure to banned pesticides.

The presence of polycyclic aromatic hydrocarbons (PAHs) in the environment is attributable to a variety of sources, including pyrogenic products resulting from the incomplete combustion of organic materials [97]. Petrogenic sources include petroleum by-products and coal distillation [93]. Biogenic sources are synthesized by biological entities during the slow biological conversion of organic materials [97]. For the honey of *M. marginata*, the results showed contaminants from pyrogenic sources. For the honey of species such as *M. bicolor*, *T. angustula*, and *S. postica*, the PAH contaminants were related to petrogenic sources. This study ranked PAH contamination using the ratio ΣCOMB/Σ16 PAHs. ΣCOMB is defined as the sum of Fluoranthene, Pyrene, Benz[*a*]anthracene, Chrysene, Benzo[*b*]fluoranthene, Benzo[*k*]fluoranthene, Benzo[*a*]pyrene, Indeno[1,2,3-*cd*]pyrene, and Benzo[*ghi*]perylene, while Σ16 PAHs represents the total of all 16 analyzed PAHs.

In this particular case, the location of the colonies in two different Brazilian locations was not shown to directly influence the results [60], and although the results showed two types of PAH sources, both come from anthropogenic activities.

4.3. Good Management Practices (GMPs) in Meliponiculture

It is recommended that good management practices in meliponiculture be adopted and applied to mitigate the risks of contamination, pollution, and pathogens. Certain stingless-bee species and the social wasp *Polybia scutellaris* have been observed robbing nests that still contain honey in cells or pots after the meliponiculture harvest season [39]. Therefore, it is recommended to conduct a thorough honey harvest and clean the nest, including its internal structures.

Feeding stingless bees with *A. mellifera* pollen and honey poses a health risk, as these products can carry diseases. Understanding the susceptibility of different bee species is crucial for assessing the impact of pathogens on their survival. Stingless-bee microbiomes may offer resistance to pathogens and diseases. Further research is recommended.

Another aspect of GMP in meliponiculture is bio-compartmentalization, a biosecurity procedure used to limit the spread of diseases among bees [98]. In practice, colonies should be well-spaced in open areas, maintaining at least 2 m between nests to prevent diseased bees from entering healthy colonies [99].

To combat/fight against diseases in stingless bees, queen replacement is practiced producing pathogen-resistant brood, along with selective breeding for hygienic behavior [100]. This selection must be carried out with the utmost diligence and in strict accordance with the guidelines established by the respective national health authorities.

Due to the lack of global regulations for stingless-bee honey as a food supplement [101], some studies, such as the one identifying PAHs in honey [60], have classified it as a special medical-purpose food. This classification allows for the comparison of contaminant concentrations and highlights potential human health risks associated with consuming PAH-contaminated honey.

However, at the regional level, there have been proposals for the establishment of standards with a view to their application in the regulation of stingless-bee honey. Such proposals have been made in the following countries: Bahia in Brazil (2014), Malaysia (2017), Tanzania (2017), Indonesia (2018), Argentina (2019), Australia (2024), and Thailand (2024). According to Vit et al. [102], now is the ideal time for stingless-bee honey regulations to be elevated to an international level, such as Codex Alimentarius. The first step must be the adoption of good management and sustainable practices in meliponiculture. Initiatives in this respect have been taken in Latin American countries, like Bolivia, Brazil, and Colombia.

These countries have incorporated legal measures into their national laws intending to reduce risks to domesticated stingless bees [103].

4.4. One Health Approach

A "One Health" approach, which integrates the fields of environmental health, animal health, and human well-being, should be a critical component of stingless-bee management. This approach ensures sustainable meliponiculture practices by recognizing the interconnected nature of these fields. Stingless bees play a crucial role in maintaining biodiversity in tropical zones, enhancing crop yields, and producing honey with different applications in medicines, cosmetics, and foodstuffs. Therefore, their conservation is essential for ecosystem resilience and food security. Sustainable management strategies for these bees involve protecting natural habitats, minimizing pesticide exposure, and promoting diverse and native floral resources to support colony health.

The availability of plant species for stingless bees depends on land management. This management falls under political rulers' jurisdiction. Environmental education programs, as well as reforestation, propagation, and seed rescue, are ways of working with communities directly [104]. Engaging local communities in educational and conservation efforts fosters resilience against climate change and habitat loss, ensuring the long-term viability of stingless-bee keeping.

Adopting biosecurity measures, along with responsible harvesting techniques and hygienic nest management, has been linked to a lower risk of pathogens spreading, benefiting stingless bees and their nest-by-product consumers. The implementation of additional preventive measures, such as the tracking and monitoring of the anthropogenic or environmental impacts on stingless bees, has the potential to be advantageous. This is because both the bees and the stingless-bee keeper can serve as an early warning system for environmental degradation and/or the presence of human health risks [105].

The care and management of stingless bees, as well as beekeeping, contributes to sustainability and promotes community living while stimulating local food production and a better understanding of ecosystems [106]. Moreover, this One Health cycle is completed with the human consumption of honey or pollen, or even the use of propolis and its derivatives in local medicine. Indeed, to ensure the quality of these products, the care of plants useful to bees should be the starting point.

5. Conclusions

A paucity of research exists on the pathogens associated with diseases affecting stingless bees. The reported effects include brood loss and annual death in a specific *Melipona* species.

Contaminants of anthropogenic origin have been found to accumulate in stingless-bee products at levels higher than those permitted in other matrices with which stingless-bee products can be compared.

The establishment of optimal practices and biosecurity measures in meliponiculture is imperative as an economic activity to support communities in tropical regions. This is crucial to mitigate risks to the survival and well-being of these species, which are confronted with natural enemies that are still being described.

The establishment of regional and global quality guidelines for stingless-bee by-products is imperative to ensure food security and product quality for both human consumption and other uses, such as nutraceuticals.

Further research is necessary to determine the impact of contaminants and pathogens on the physiology of stingless bees. It is imperative to avoid using honeybee references for lethal or sublethal doses of chemical contaminants in other bee species

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Experimental section

Study 2:

Pollen preferences of stingless bees in the Amazon region and southern highlands of Ecuador by scanning electron microscopy and morphometry

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Joseline Sofía Ocaña-Cabrera, Jonathan Liria, Karla Vizuete, Cristina Cholota-Iza, Fernando Espinoza-Zurita, Claude Saegerman, Sarah Martin-Solano, Alexis Debut, Jorge Ron-Román

Preamble

The results of the 2018 survey demonstrated that stingless bees do not collect pollen from a single plant species, although there is evidence of a predilection for certain plant families. A total of 46 pollen types were identified through scanning electron microphotographs, exhibiting a prevalence greater than 10% in the three designated study provinces in Ecuador. These pollen types were categorised into six families and seven genera within the province of Sucumbios during April. There were 19 families (14 genera) in Orellana from August to December, and 10 families (8 genera) in Loja in September. These provinces were selected to encompass two of the three provinces with the highest number of registered meliponaries (Loja and Orellana), and due to the prior contact established by the project technician with these communities.

Miconia (Melastomataceae) was identified as the primary pollen source, as it was present in all three study areas. The Melastomataceae family was the most abundant in the Amazonian tropical rainforest provinces, while the Asteraceae family was the most abundant in the tropical dry forest Highlands province. These were followed by the families Alismataceae, Piperaceae, Fabaceae, Burseraceae, and Molluginaceae.

This survey constitutes a demonstration of the significance of morphometric analysis in supporting the differentiation of taxa between provinces and meliponaries for pollen grains of the Melastomataceae and Asteraceae plant families. These results suggested the existence of more than one species belonging to the same genus in both *Miconia* and *Bidens*.

The diversity indices indicated high richness but low uniformity in the abundance of each family identified. The calculations were conducted based on each pot serving as the unit of measurement. This finding indicated that, within each pot, stingless bees store a single type of pollen, the classification of which is determined by its floral origin and, probably, its nutritional quality.

The utilisation of stingless bee pot-pollen as a study unit for floral diversity research in specific regions serves as an effective complement to ecological studies. In addition, the utilisation of stingless bee nests as sentinels can serve to indicate the health of the environment, mainly in tropical regions. In Ecuador, this study constituted a preliminary contribution to the establishment of a pollen database for tropical dry forest and Amazonian regions. The utilisation of pot-pollen and other stingless bee products is proposed for the identification of contaminants, given the location of the meliponaries close to oil exploitation, livestock farms, and crop fields.

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RESEARCH ARTICLE

Pollen preferences of stingless bees in the Amazon region and southern highlands of Ecuador by scanning electron microscopy and morphometry

Joseline Sofía Ocaña-Cabrera[1,2,3], Jonathan Liria[4], Karla Vizuete[5], Cristina Cholota-Iza[1,2], Fernando Espinoza-Zurita[2], Claude Saegerman[3☯]*, Sarah Martin-Solano[1,2☯], Alexis Debut[5☯], Jorge Ron-Román[1,6☯]

1 Laboratorio de Biotecnología Animal, Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Pichincha, Ecuador, **2** Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Pichincha, Ecuador, **3** Research Unit of Epidemiology and Risk analysis applied to Veterinary Sciences (UREAR- ULg), Fundamental and Applied Research for Animal and Health (FARAH) Center, Department of Infections and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liege, Liege, Province of Liège, Belgium, **4** Grupo de Investigación en Población y Ambiente, Universidad Regional Amazónica IKIAM, Tena, Napo, Ecuador, **5** Laboratorio de Caracterización de Nanomateriales, Centro de Nanociencia y Nanotecnología, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Pichincha, Ecuador, **6** Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería Agropecuaria, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Pichincha, Ecuador

☯ These authors contributed equally to this work.
* claude.saegerman@uliege.be

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Abstract

Stingless bees are effective pollinators of native tropical flora. Their environmental service maintains flow of pollen through pollination, increase reproductive success and influence genetic structure in plants. The management of stingless bees "meliponiculture", is an activity limited to the countryside in Ecuador. The lack of knowledge of their managers about pollen resources can affect the correct maintenance/production of nests. The objective is to identify botanical families and genera of pollen grains collected by stingless bees by morphological features and differentiate potential species using geometric morphometry. Thirty-six pot pollen samples were collected from three Ecuadorian provinces located in two climatically different zones. Pollen type identification was based on the Number, Position, Character system. Using morphological features, the families and genera were established. Morphometry landmarks were used to show variation for species differentiation. Abundance, diversity, similarity and dominance indices were established by counting pollen grains, as well as spatial distribution relationships by means of Poisson regression. Forty-six pollen types were determined in two study areas, classified into 27 families and 18 genera. In addition, it was possible to identify more than one species, classified within the same family and genus, thanks to morphometric analysis. 1148 ± 799 (max 4211; min 29) pollen grains were counting in average. The diversity showed a high richness, low dominance and similarity between pollen resources. Families Melastomataceae and Asteraceae, genera

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Miconia and *Bidens*, were found as the main pollen resources. The stingless bee of this study are mostly generalist as shown the interaction network. The results of the present survey showed that stingless bees do not collect pollen from a single species, although there is evidence of a predilection for certain plant families. The diversity indexes showed high richness but low uniformity in the abundance of each family identified. The results of the study are also meaningful to the meliponiculture sector as there is a need to improve management practices to preserve the biodiversity and the environment.

Introduction

Stingless bees of the tribe Meliponini belong to the group of corbiculate bees as they possess an anatomical structure on the hind legs known as the pollen baskets [1] or "corbicula" [2] specialized in the storage and transport of pollen and resins [3] from plant to the nest. The main morphological characteristics are: reduction of the sting, little venation on forewings and reduction of the penicillum [2]. Based on the degree of speciation of these insects, South America is presumed to be the territory of origin and the starting point for their spread in Asia and Africa [4].

In Ecuador, the most recent study on native bees reported 132 species, classified in 23 genera, distributed in the 24 provinces of the country [5]. Being a megadiverse country, it is important to study meliponids to understand their ecological interactions and their importance in Ecuadorian forests.

The combination between the intertropical location and the altitudinal gradient caused by the Andes mountain range creates a diversity of habitats that give rise to distinct floristic elements [6]. The number of vascular plant species in Ecuador exceeds 17,000 [7]. Over the last few years, harmful environmental effects have been observed in wild bees, leading to a decrease in the plant-pollinator ratio and consequently, with a decrease in plant diversity [8]. Agriculture, mechanical and chemical treatments are examples of activities that has affected the natural habitat of wild bees [9]. In 2014, FAO proposed topics on which efforts should be focused due to the lack of information on pollination as an environmental service, especially in Latin America and the Caribbean [10].

Stingless bees build their nests in various cavities (abandoned bird nests, cement blocks or under the ground) [11], with different nesting features such as the shape of the nest entrances or protection of the brood discs (exposed or protected by a layer of wax or involucre) [12]. The nest is protected and divided by batumen, a composition of mud, faeces, resins, or plant material such as seeds, mixed or not with wax [13]. Internally, nests are made of cerumen a construction material composed of a mixture of wax, resins and other sticky substances of plant origin [14, 15]. It also serves as an antimicrobial [16] and antifungal [17] protection. Several layers of cerumen form the involucre, a structure that surrounds, protects and maintains temperature [13, 18, 19], in the brood chamber of the nest. Nest food storage is in pots made of soft cerumen that are located outside the involucre, round and small for honey pots, large and elongated for pollen pots [20].

In the tropical zone of America more than 500 species [1] of stingless bees plays an important role as pollinators in maintaining the forest diversity [21] ensuring its reproduction thanks to the dispersion of pollen [22]. Moreover, stingless bees also maintain the stability of economically important crops in agroforestry projects [23, 24] and help to reestablish the ecological balance in invaded endemic areas [25, 26].

Stingless bees meet the need for pollination of endemic plants, thanks to their species diversity and adaptive capacity [27]. For instance, the genera *Bombus*, *Centris*, *Eulaema*, *Melipona* and *Cylocopa* use a specific mechanism for pollen collection, their thoracic muscles vibrate inside the flower releasing pollen that sticks to the body [28]. Native bees visit flowers that *Apis mellifera* cannot because of their larger size compared to *Melipona* species [29]. Stingless bees visit between 15–20% of angiosperm plants [30] their pollination services cover between 30,000–50,000 species [31]. In developing countries, meliponiculture, manage and care of stingless bees, continues to be an informal economic activity, or even considered a hobby because there is not much scientific knowledge or standardized processes about it [32].

The health status of the stingless bee colonies is dependent on the great diversity of plants from which they obtain resources [33]. Their foraging activity depends on the floral availability of the environment in which they live, which is specialized temporarily when a certain plant species offers attractive resources [34]. Pollination service provided by bees increases reproductive success in plants, influences the genetic structure of populations and plays a major role at the evolutionary level [9, 35, 36]. In the specific case of *Apis mellifera*, its floral constancy promotes geitonogamy and reduction of genetic exchange [37]. It is important to know if this behavior also occurs in stingless bees or if their role as pollinators maintains biodiversity.

Pollen, as the object of study, is the male gamete of phanerogamous plants, formed by a vegetative cell that has sperm cells enclosed in a cytoplasm surrounded by an external wall or exine [38]. Pollen is the main source of protein and carbohydrates for stingless bee brood, adult bees and queens [33]. It is used inside the nest for the production of honey and bee bread [39]. At palynological level, the national databases lack sufficient information but it exists a database in constantly update that includes some Ecuadorian plant information [40]. As a guide in this survey a compilation of information about botanical families used by stingless bees was developed (**S1 Table**) [41–91].

Pollen origin analysis are generally conducted by light microscopy techniques [92]. Distinguishing pollen characteristics requires exceptional instruments, for instance, a Scanning Electron Microscope (SEM), that allows through microphotography to describe shape and morphological size characteristics of pollen to classify it into a family, a genus or even a species level [93, 94]. The lack of information on pollen description and identification is related to the lack of information on bee-plant interactions and food source plants in Ecuador. It makes it necessary to generate scientific data that will benefit meliponicultors (managers of stingless bees).

The objectives of the survey were: i) to identify the families and genera of plants used as food sources for stingless bees in three provinces of Ecuador through morphological analysis of pollen grains (pg) collected from meliponid nest pots. This allowed us to ii) determine the preference of native bees for certain Ecuadorian plant families in two climatologically different areas, as a tool for species conservation and to contribute to the knowledge of meliponicultors.

Materials and methods

Study design and sampling

In this cross-sectional survey, two provinces of the Amazon region (Orellana and Sucumbios) and one province of the southern highlands region (Loja) of Ecuador were defined as sampling regions (Fig 1) (Table 1). During field work, small scale meliponicultors were contacted in the three areas. In absence of a sampling frame, the field visits were guided by a local field technician with expertise in Meliponiculture. Meliponicultors were given an informative survey and signed a consent for the collection of pollen samples from their nests in April, August-September and December 2018.

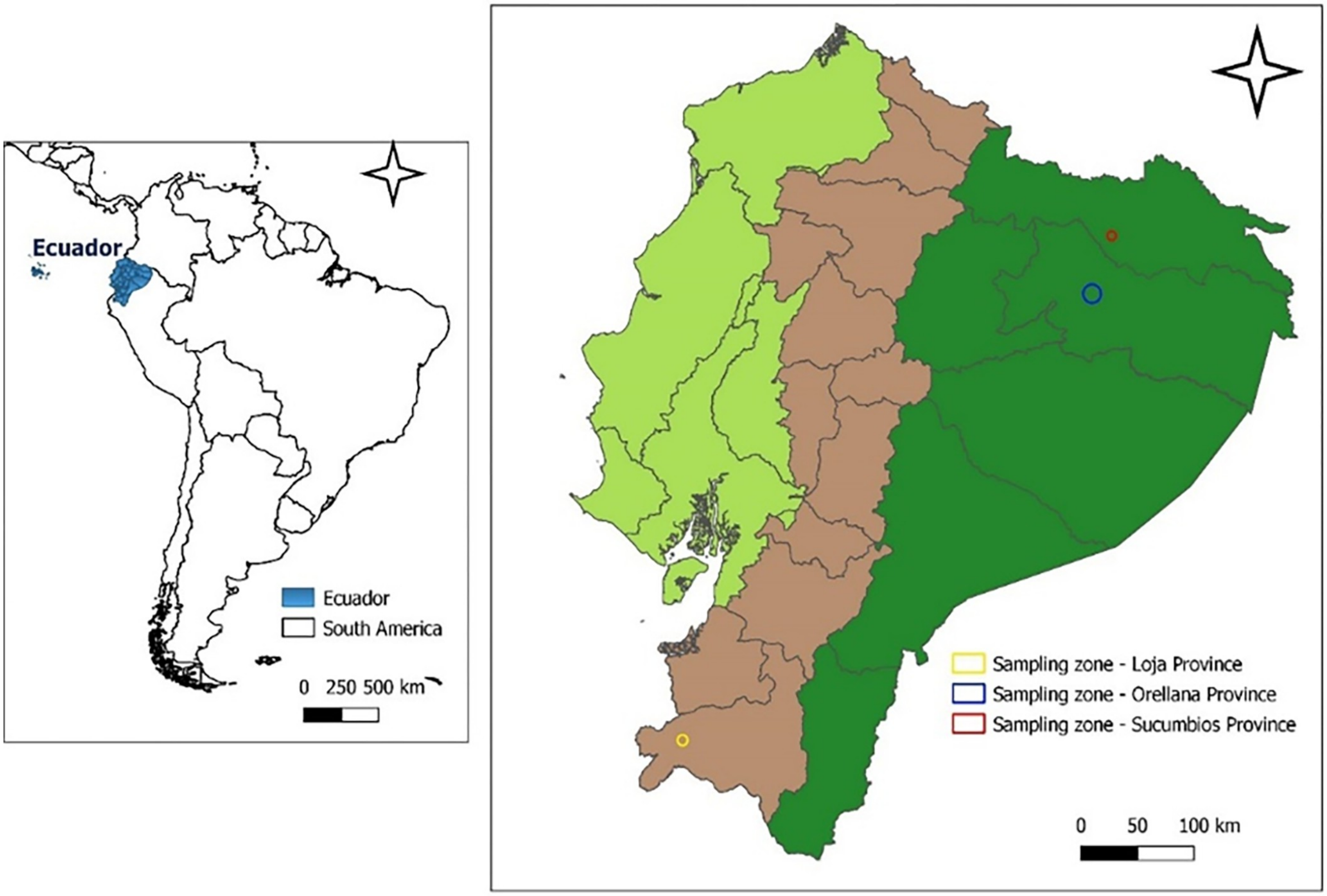

Fig 1. Geographical location of the study areas, in three provinces of Ecuador. Layers were obtained from: http://www.efrainmaps.es. Carlos Efraín Porto Tapiquén. Geography, GIS and Digital Cartography. Valencia, Spain, 2020 for America layer, Instituto Geográfico Militar, 2008, Base nacional escala 1:1'00.000 for Ecuador layer.

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Sample collection

Pollen grains stored in pots out of stingless bee nests were collected during the transfer from natural nests to a technical nest (Fig 2) by taking the pollen directly from the top floor (storage place of stingless bee pollen pots) of the box or technical nest. The selection of one pollen pot by nest was random. Only sealed pots were sampled. Three to six grams of pollen were

Table 1. Geographical location and number of the sampling zones.

Ecuadorian Region	Province	Location	Coordinates (UTM)[a]	Altitude (m.a.s.l.)[b]	Number of nests	Number of samples
Amazon	Sucumbios	Shushufindi	0°11'14"S[c] 76°38'42"W[d]	>262	4	6
Amazon	Orellana	Taracoa	0°26'08"S[c] 76°46'20"W[d]	>269	10	10
		Dayuma	0°40'14"S[c] 76°52'54"W[d]	>275	7	10
Southern highlands	Loja	Pindal	4°06'51"S[c] 80°06'28"W[d]	>801	10	10

[a] Universal Transversal Mercator coordinates system

[b] Meters above sea level

[c] South

[d] West.

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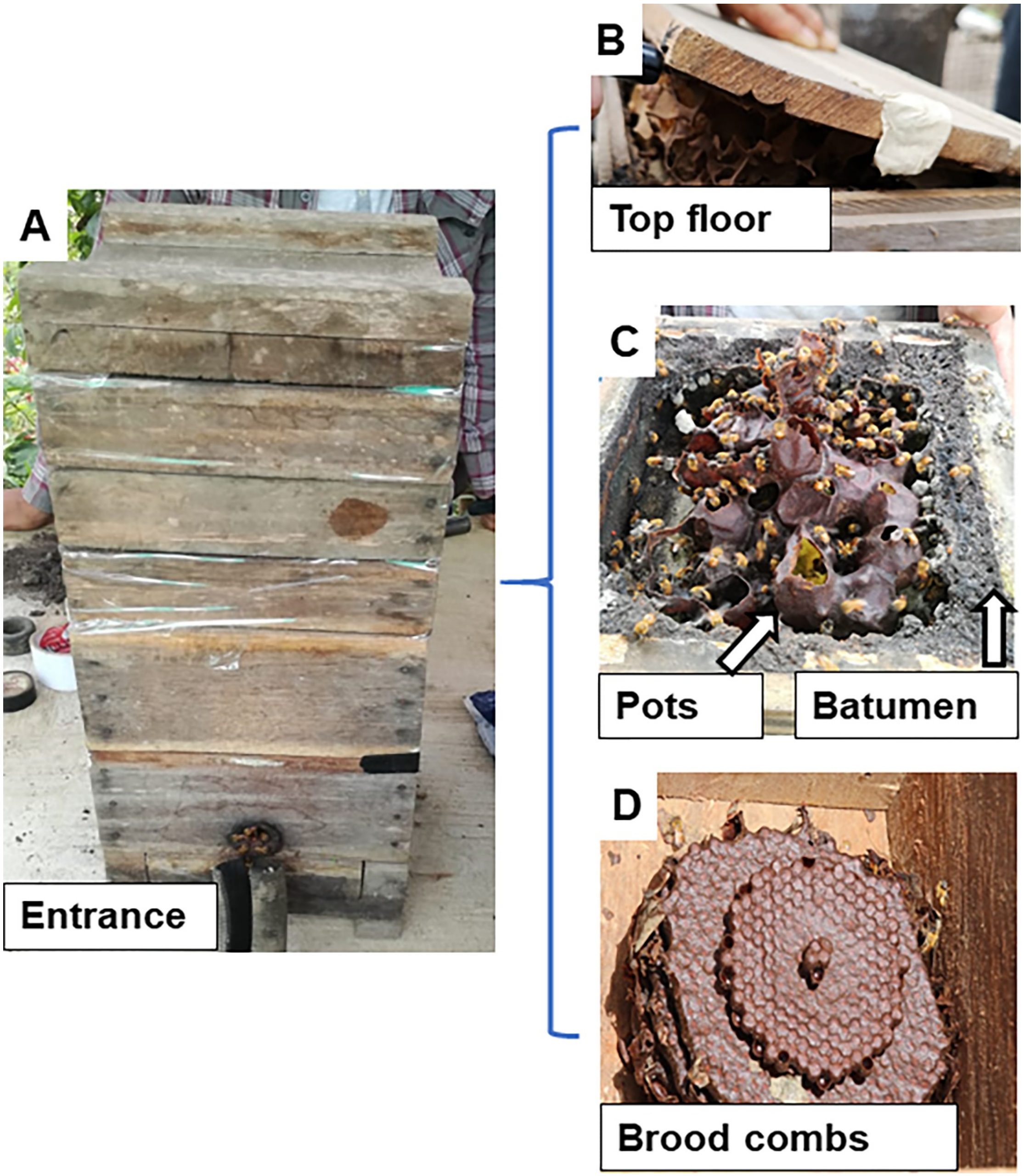

Fig 2. Technical nest of stingless bees in Ecuador. A) The nest consists in three main zones. B) top floor is the way by meliponicultor can open the nest and managed the bees. C) The storage zone cover by batumen (material made from resins and mug) contains honey and pollen pots. D) The brood chamber where different "plates or discs" are stacked in a column. *Note*: a natural nest has the same structure but inside a tree log.

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extracted from the pot with the help of a sterilized paddle; one paddle was used for each pot. The samples were stored at 4˚C in sterilized tubes, labelled with the following codes: H as the number of the meliponary, N as the number of the nest and then sealed until their transfer to the Animal Biotechnology laboratory at the Forces Armed University ESPE, Sangolquí, Ecuador.

Pollen as a product of a stingless bee nest is classified as a food supplement and/or medicinal product. The objective of collection was socialized with each owner, them approval was obtained by reading and signing an informed consent form.

Acetolysis and cleaning of pollen grains

Protocol of acetolysis [95] was used with modifications. To 0.1 g of pollen, 600 μL of washing solution was added composed of glycerol and hot distilled water (1:5). Solutions was then centrifuged at 2500 rpm for 10 min. The supernatant was discarded, and another wash was performed with 600 μL of hot distilled water; it was then centrifuged (250 rpm, 5 min) and the supernatant was discarded. To separate the sediment, 200 μL of warm distilled water was added, centrifuged (2500 rpm, 5 min) and the supernatant was removed and dried at room temperature (i.e., 20˚C) for 10 min in a laminar flow cabinet. 1 mL of acetolysis solution (sulfuric acid: glacial acetic acid, 1:9) was added and placed in a water bath at 70˚C for 20 min; centrifuged (2500 rpm, 10 min) and the supernatant was discarded.

Three drops of tween 20 plus 200 μL of warm distilled water were added, strongly vortexed and centrifuged (2500 rpm, 15 min). Subsequently, bacteria were eliminated using 12.5 μL of the Streptomycin antibiotic (20 μg/μL) and distilled water was added to complete 250 μL to wash the pollen grains. It was left in contact for 24 h, centrifuged (3500 rpm, 15 min) and the supernatant was removed. Samples were stored refrigerated until lyophilization.

Preparation and observation in scanning electron microscopy

The drying process by lyophilization was carried out in the equipment (ILSHIN, model FD 5508) for 24 h, at -62˚C and 1.2 Pa; samples that were placed in the equipment were previously frozen liquid nitrogen for 3 min. Once dry, the sample was dispersed on a copper foil glued to the aluminum sample holder. Sample was covered with a 20 nm layer of gold before being analyzed by SEM (TESCAN, model MIRA 3, field emission gun).

To count and identify pollen, microphotographies were taken, identifying an area of approximatively of 1 mm2 with uniform dispersion of pollen grains (at 200 x magnification). Each area was then divided into 16 quadrants (at 990 x magnification) for the respective count. Then, each pollen grain was photographed individually, with a magnification between 1600 and 15000 x, a parameter that was adjusted according to the size of each one (Fig 3).

Counting and morphological description

In each quadrat, pollen types were counted and differentiated manually, identifying each one with colors. To establish the abundance percentages [96] was considered. Pollen grains with abundance $\geq$ 10% were considered "really important" food sources. Pollen grains with abundance $\geq$ 90% are considered pollen sources of "temporal specialization" [97]. The following measurements were obtained from the individual photographs: polar and equatorial

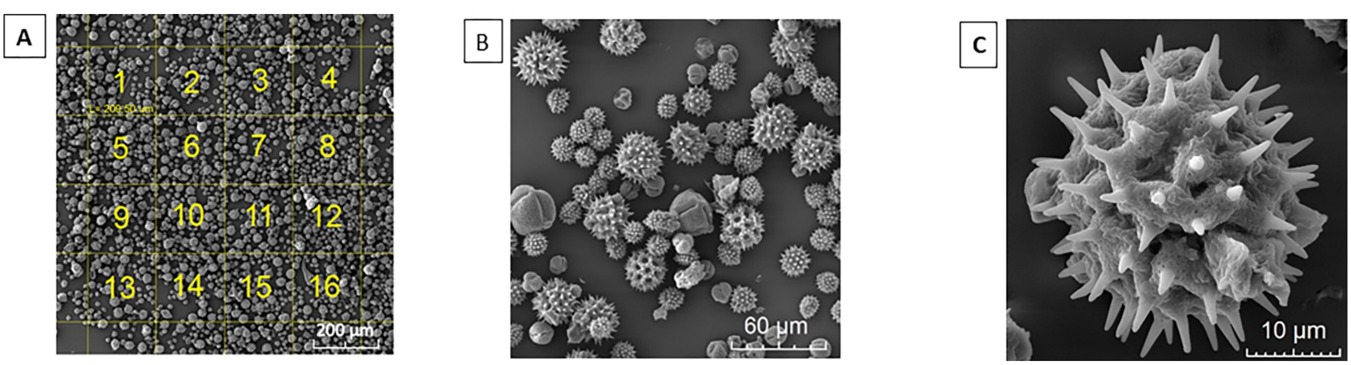

Fig 3. Scanning electron microscopy methodology. A) Area with homogeneous dispersion, 1 x 1mm. 200X; B) Counting quadrant 990X; C) Single photograph, magnification depending on pollen grain size (1.6–15 kx).

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diameter (μm), P/E (polar diameter/equatorial diameter ratio), area (μm^2) and perimeter (μm), by means of which size and shape could be defined [98].

Thanks to the magnification achieved with SEM, the characteristics of the exine, openings and ornamental elements of the pollen wall were identified, and using the NPC system (number, position of openings, and characteristic of the exine) [99], pollen grains were classified into pollen types.

Classification into family and genus

To classify pollen grains into family and/or genus, morphological descriptors and pollen type were used. For this purpose, scientific publications [100–103], books [12, 104, 105] and pollen databases were consulted: National Centre for Environmental Information [106], Apibotanica [107], the Global Pollen Project [108], Oreme [109], and mainly from Latin America [110]; to perform a comparison of measurements and shapes.

Morphometric analysis

Sections of the pollen grains that showed the greatest morphological variation were selected and these areas were marked with anatomic points or landmarks. Three photographs were used for each pollen grain, from which x, y coordinates were digitized using the TPSDIG program [111]. Conformational differences were analyzed in MorphoJ software, stated by [112] using Principal Component Analysis (PCA) as an exploratory technique to visualize the axes of greatest variation, and then Canonical Variables Analysis (CVA) to evaluate significant differences between the different groups (taxonomic categories) [113]. The pollen grains that were processed belong to the following families: Melastomataceae and Asteraceae. For the first family, six landmarks were selected (Fig 4A) in the equatorial zone that provided differential data based on the presence or absence of a pore and the width of the aperture. For the second family, eight landmarks (Fig 4B) were selected that differentiated the length of diameters and structures known as spicules.

An example of pollen grains that did not enter this analysis are the families Molluginacea and Piperacea, since the pollen grains are apolar and inapertured, being absent characteristics that allow their differentiation by means of landmarks.

Fig 4. Morphometric geometry methodology. A) Selected anatomical points for pollen from family Melastomataceae, genus *Miconia*; B) Selected anatomical points for pollen from family Asteraceae, genus *Bidens*. In the figures above each red point represent the specific position (coordinates) of one landmark or anatomical point, that surrounding the area which were used as potential indicator of species differentiation for pollen grains that belongs to the same family and genus.

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Distribution of pollen grains

In this study the Poisson distribution was used, which describes the probability with which an event can occur during a certain interval, whether of time, distance, area or volume [114]. The random variable of the distribution was the number of times that a certain pollen type appeared in the area (1 mm^2) of the SEM stub. R Commander software was used to verify that pollen grains counting follows Poisson distribution. In addition, Poisson Regression was also used to determine the spatial distribution of the families' plants found. The dependent variable was the number of pollen grains per pollen type (independent variable).

Calculation of biodiversity indexes

Based on pollen grains counting and the identification of families according to morphology, the Shannon index was calculated. It quantifies the specific biodiversity, that is, the non-uniformity of an area, taking into consideration the total number of families identified and the abundance of each one [115]. Simpson's index was also calculated, which represents the probability of randomly taking two individuals from a population and having them belong to the same species; it is an estimator of the dominance of certain plant species in a given area [116].

Finally, the Jaccard index was used to measure the degree of similarity in terms of families used as pollen resources inside the pot by nest, this index is a type of inverse measure to the diversity estimated by Shannon [117].

Network interactions

It was possible to establish preference relationships based on pollen grain counting between stingless bees and plant families. Stingless bee genus and species were identified in two parallel studies from this, using entomology [118] and molecular biology techniques for the identification of native bees by [119]. R Studio software was used to obtain the networks and the program developed by Dr. Dáttilo Wesley, PhD, of the Instituto de Ecología, A.C., Mexico [120].

Results

As explained in the methodology, the pollen grains (pg) that were completely analyzed were those that, during the initial count, exceeded 10% abundance in the selected stub area (i.e., important food sources). Thereafter, new percentages were established to provide an order, according to the study area.

The collection of 36 pollen samples from the three provinces located in the Amazon region and Southern highlands of Ecuador resulted in 2433 micro photographs, from which 54 pollen types (6533 pg) were identified in Sucumbíos, 84 pollen types (23579 pg) in Orellana and 49 pollen types (11232 pg) in Loja. This count made it possible to establish abundance percentages (Table 2) and to relate similar pollen types between provinces, identifying a total of 46 pollen types and 28 botanical families. Only 14 of the 28 families found could be classified "genus" at generic level, identifying 18 (Table 2), thanks to the morphological description of the different pollen types and the ornamentation of the exine (S2 Table).

Regarding morphometric analysis to identify species, significant differences were determined between pollen grains belonging to *Miconia* (Melastomataceae) at province level, between Loja—Sucumbios (discriminant function between Mahalanobis distance, p-value < 0.0001) and between Orellana—Sucumbios (p-value < 0.001); which means that there is more than one species of plants of the genus *Miconia* used as a pollen resource by stingless bees of these provinces. However, between the provinces of Orellana and Loja, there is no difference between the pollen grains (p-value < 0.08), which suggests that possibly, pollen grains

Table 2. Families, genera and their abundances of pollen grains identified by province.

		Abundances by province		
Family	**Genus**	**Sucumbíos**	**Orellana**	**Loja**
Alismataceae			+	+
Aizocaceae				+
Anacardaceae			+	
Arecaceae				+
Asteraceae	*Ageratum*			+
Asteraceae	*Bidens*		+	++
Asteraceae	*Iva*			+
Berberidaceae			+	
Bursecaceae	*Bursera*	+		
Bursecaceae	*Quercus*		+	
Cyperaceae	*Cyperus*		+	
Cytinaceae			+	
Euphorbiaceae			+	
Fabaceae	*Chamaecrista*		+	+
Fabaceae	*Inga*		+	
Lardizabalaceae			+	
Lythraceae	*Hemia*		+	
Loranthaceae		+		
Melastomataceae	*Miconia*	++++	++	+
Molluginaceae	*Mollugo*	+		
Molluginaceae			+	
Oleaceae	*Fraxinus*		+	
Papaveraceae			+	
Piperaceae			+	+
Plantaginaceae				+
Plumbaginaceae	*Plumbago*	+		
Poaceae			+	
Polygalaceae	*Polygala*			+
Rosaceae	*Prunus*	+		
Salisaceae	*Salix*	+		
Sapindaceae	*Paullinia*		+	
Vitaceae	*Cissus*	+		

Crosses represent 0–25% (+), 26–50% (++), 51–75% (+++), 76–100% (++++).

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belonging to the same species of *Miconia* were selected for analysis or that the area of the pollen grain does not act a discriminating parameter between plant species of these two provinces.

Pollen grains of *Bidens* (Asteraceae) were identified as resources only in the provinces of Orellana and Loja. There was a significant difference (discriminant function between Mahalanobis distance, p-value < 0.008) between those belonging to each province. It means that there is at least one species of *Bidens* collected by native bees. A significant difference was observed even among meliponaries of each province ($0.005 <$ p-value < 0.03), i.e., there are plants of several species of *Bidens*.

As shown in Table 2, the only botanical family that was present in the three study areas was Melastomatacea, which is why it was decided to choose this family as an independent variable

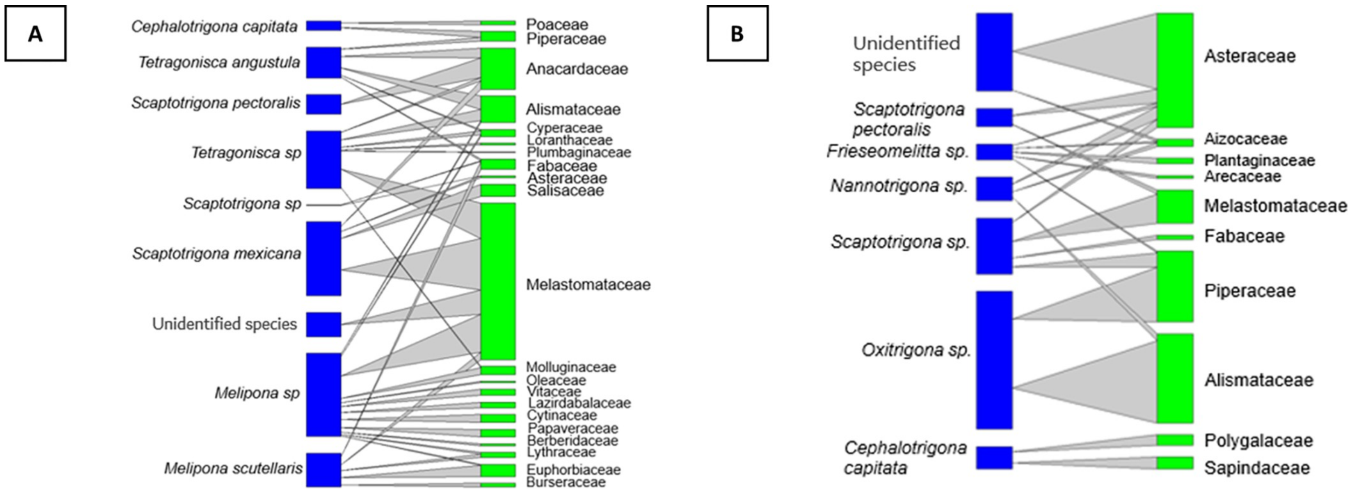

Fig 5. Bee-plant interaction network. A) Amazon region. **B)** Sierra South region.

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in the spatial distribution analysis using Poisson regression. It was verified that the pollen of this family is mostly used (< 70% of Sucumbios nests and < 30% of Dayuma nests) as a food resource (p-value < 2e-16) by stingless bees in the Amazon region. It was also verified that the collection of pollen grains of the Melastomataceae family is significantly related to the collection or storage of pollen of Burseraceae, Euphorbiaceae, Fabaceae and Molluginaceae (2e-16 < p-value < 0.001). In addition, interaction networks between the identified bee species and the families used as food resources were carried out, determining that *Melipona*, *Tetragonisca* and *Scaptotrigona* are the most generalist species of the Amazon Region (p-value < 0.001), as shown in Fig 5A. It is important to say that pollen grains of the Molluginaceae play an important role as a food resource (p-value < 0.001).

Asteraceae was the next family to have significance after the statistical analysis with Poisson regression, identifying that Asteraceae is the most important food resource for stingless bee nests in Pindal, Loja (p-value < 0.001). Moreover, if we compare with the rest of the families, if stingless bees collect pollen from Asteracea family plant is highly probably that also recollect pollen from Melastomataceae and Aizocaceae (p-value < 0.001) due to the floral spatial distribution within each meliponary. Regarding the interaction with bee species, it was obtained that *Nannotrigona* and *Frisiomelita* are closely related to the collection of Asteraceae pollen grains (both are small species) (p-value < 0.001), and *Frisiomelita* is the most generalist in Pindal—Sierra (p-value < 0.001) (Fig 5B).

The calculation of the diversity indexes (Shannon's index, H') of families identified per sample, resulted in low values, for Sucumbíos: 0.97 > H' > 1.72, for Orellana 1.86 > H' > 2.17, for Loja 2.22 > H' > 2.34. The data of the diversity indexes at sample level follow a normal distribution (Shapiro-Wilk, p-value = 0.12). There are no significant differences between the diversity indexes of the three zones (Kruskal-Wallis, p-value = 0.29).

There was no dominance (Simpson's index, λ) of a single family in any study area, with values of 0.23 > λ > 0.39 for Sucumbíos, 0.18 > λ > 0.22 for Orellana, λ = 0.17 for Loja. The similarity (Jaccard index, JI) between plant families found in the three study areas was low, with values between 0 > JI > 0.25. However, when comparing families found in provinces of the Amazon region, Sucumbíos and Loja, these presented a higher similarity index: 0 > JI > 0.43. However, this similarity was strongly higher between Orellana and Loja: 0 > JI > 0.66, provinces of different regions and climatic zones of the country (see S3 Table).

Discussion

The objective of the study was to identify the main families and genera of plants used as food sources by the stingless bees in two climatologically different regions of Ecuador. Twenty-eight families were found and identified into 19 genera. The existence of more than one species of the same genus was discriminated using geometric morphometry. Preference relationships were established between botanical family and stingless bee species. Values of diversity, dominance and similarity indexes were defined using pollen grain counts. Taxonomic classification was performed based on morphology and measurement of morphological parameters using microphotographs of pollen. In this sense, it is well known that exine shape and the generation of patterns on it, which are characterized by their high taxonomic specificity, are due to several protein subunits that are synthesized in the paternal myocyte and cellular interactions [130]. The shape of pollen grains and size, are used to reach at precise taxonomic identification, since generally, those pollen grains belonging to the same species have this constant characteristic, which is related to the chromosomal number of each plant [131]. Pollen can present a continuous wall or not, depending on the treatment given prior to microscopic observation. It can also expands when hydrated and the proximal poles (equatorial) fold if subjected to drying treatments such as acetolysis or natural fossilization processes [132]. On the other hand, the divergence in the wall and the variation in the measurements of the morphological parameters, possibly indicate a hybridization in the origin of the plant or the influence of the habitat in which it has developed [133].

Melastomataceae was presented as the only family in common for the three provinces of study, its pollen generally has apertures that classify it as psyllulate or rugulate and a heterocolpate or stephanocolpate conformation, characteristics that facilitate its existence in diverse climatological habitats [99]. In Brazilian forests, this family is typical as a source of pollen for stingless bees, since coevolution with bees of the genus *Melipona* has been verified [134]. Asteraceae occurred in two provinces, one in each region of the country. This family has the second-largest representation in Ecuador and its pollen is characterised by having spines or hooks on the exine [135], which facilitate its adhesion to the body of pollinators, thus ensuring its dispersal to other plants [131]. Molluginaceae previously reported in the Galapagos region [136] has pantocolpate pollen grains and spinulose exine [73] is considered one of the three families with the greatest preference for female bees in southern Africa [137].

Morphometric results of Melastomataceae, show the possible existence of more than two species belonging to the genus *Miconia*. However, it was not possible to identify the species since it is necessary a phenological study that relates the pollen of the nests with the pollen taken directly from plants. In terms of evolution, *Miconia* is known to be monophyletic in origin, and of the total number of species within this family, 25% have this origin [138]. *Miconia* is distributed throughout Latin America and have points of diversity in the Andes and in the humid forests of Brazil, which is why it is considered a genus of basic plants in all phytogeographic formations [139]. In Ecuador, these plants are used for medicinal, ornamental, food and construction purposes [140], but there is little information on their role as a pollen or nectary resource for stingless bees. The ecological importance lies in their high repopulation capacity in ecosystems altered by human intervention [141]. A degraded terrestrial ecosystem is recognized when there has been partial or total depletion of plants and soil nutrients [142]. Different studies on ecological restoration in southern provinces of the country caused by intensive cattle grazing, demonstrated the important role of *Miconia* plants in soil restoration, because it was observed growing after 2 years of total disappearance of plants and a great abundance after 10 years. This is mainly due because *Miconia* plants are good water catchers, maintaining soil moisture and fertility [143]. A common pattern of the sampled areas is the number of areas intervened by human, and in this study, it is evident how native bees have taken advantage of this natural ecological restoration, to obtain food resources and carry out, indirectly, the pollination that is supposed to have contributed to the restoration of these areas. Additionally, bees of the genus *Euglossa* temporarily specialize in collecting pollen from *Miconia chamissois* as a food source for their offspring [144].

The morphometric results for Asteraceae indicate significant differences between the pollen grains of *Bidens*, suggesting that there are also more than two species [145]. Plants of *Bidens* are used as forage for domestic animals, since their growth occurs in areas with abundant organic matter, near rivers or stream channels [146]. They are found in disturbed soils and are characterized by abundant nectar production during the summer season making them a very good source for bees [147].

Molluginacea and Piperacea pollen grains are unapertured and apolar [148]. According to the data and observations obtained, it is possible to raise hypotheses. The first one is that stingless bees classify pollen grains according to the species of origin and the second one, the pollen grains found in each pot may well be indicators of the spatial distribution pattern of plant species surrounding the nest. If a continuous habitable space is defined, it is possible to affirm that a population follows random, uniform or aggregate distribution patterns [149]. Investigations of this spatial dispersion allow the identification of inter-and intraspecific coexistence relationships, in addition to knowing the diversity of the ecosystem under study [150].

In this sense, there are families that presented significant degrees of relationship, Melastomataceae ~ Burseraceae, Euphorbiaceae, Fabaceae, Molluginaceae and Asteraceae ~ Melastomataceae, Aizocaceae, are possibly randomly distributed in the habitat outside the nest. The random distribution explains that each point in the environment is occupied by an individual and its presence does not affect the presence of another [151]. Pattern distribution of the rest of the families identified and that did not present a significant relationship, probably comply with a uniform distribution pattern, which is characterized by presenting a negative interaction expressed as competition for resources [152]. Melastomataceae and Asteraceae presenting an important significance in this study, probably follow an aggregate distribution in the space occupied by the bee nests, i.e., there is probably a positive attraction between individuals, at species level, since their distribution occurs by the formation of dense groups [151].

The existence of stingless bees and meliponiculture as such influence the maintenance of biodiversity thanks to the division and conservation of colonies or nests of these insects [152]. Stingless bees are the main pollinators and floral visitors of tropical native plants [47]. A complete specialization of bees towards plants is unlikely and is demonstrated in this study; stingless bees do not have a specialization towards certain plant [153–155], in addition the food source plants do not bloom throughout the year and therefore the same food source cannot supply the needs of the nest or colonies that have a perennial character [131, 156].

Abundance percentages reflect an order of preference [157–159]. Different authors have suggested the preference of stingless bees for specific plants according to their inflorescences. Such constancy is associated with the effectiveness of these insects as pollinators, since it facilitates both the harvest and the deposit of pollen grains, and ensures the reduction of contamination of stigmas with other pollen, optimizing the travels of worker bees to obtain nectar, pollen and resins [160]. Biologically, their polyilecty and great adaptability allow them to pollinate both endemic and introduced plants [47].

In Ecuador there are a lack of databases that can be used as starting point or loading information to contribute the information around the country. The potential of bees as bioindicators of the health environment is well known within biomonitoring, due to their foraging job [161]. Bees can storage in their nest large quantities of soil, vegetation, air and water residuals [162]. These characteristics turn bees in effective monitoring agents of zones affected by human practices.

Conclusions

The results of the present survey showed that stingless bees do not collect pollen from a single plant species, although there is evidence of a predilection for certain plant families. Forty-six pollen types were reported that complied with the characteristic of having abundance higher than 10%, i.e., a real food source, in the three study zones, which were classified into six families and seven genera for the province of Sucumbios during April, 19 families (14 genera) in Orellana between the months August—December; and 10 families (8 genera) in Loja in September, in the year 2018. *Miconia* (Melastomataceae) was presented as the main source of pollen as it was found in the three study areas, followed by the families Asteraceae, Alismataceae, Piperaceae, Fabaceae, Bursecaceae and Molluginaceae.

This survey is the first to shows the importance of morphometric analysis as a support in the differentiation of taxa between provinces and meliponaries for pollen grains of the Melastomataceae and Asteraceae. These results suggest the existence of more than one species, belonging to the same genus, to both *Miconia* and *Bidens*.

The diversity indexes showed high richness but low uniformity in the abundance of each family identified. Calculations was made using each pot as the calculation unit. This suggest that inside of each pot, stingless bees storage just one type of pollen whose classification depend on floral origin. This last sentence could be a theorical explanation.

Stingless bees could be used as a sentinel, to show the health of the environment, specifically in tropical zones. In Ecuador study areas were close to oil exploitation, cattle farms and crops, therefore the possibility of finding traces of chemicals substances is a potential indicator of contamination.

As a recommendation, the usefulness of developing software (artificial intelligence) as a computer tool that allows the classification of pollen grains from microscopy photos is highlighted. It is also recommended to: i) use molecular biology techniques to support certain concepts about the biology and ecological role of stingless bees; ii) carry out the identification of plant species, using pollen, through a phenological study in the study areas of the nests, in order to compare findings by pollen and botanical identification.

Supporting information

S1 Table. Botanical families review chart. Plant families features used as pollen and nectar resources by stingless bees [41–91, 126].
(DOCX)

S2 Table. Morphological description, and their parameters, of pollen grains with the highest representation in the three study areas [43, 47, 52, 84, 86, 99, 100, 118–129].
(DOCX)

S3 Table. Indexes explaining chart. Comparison between the diversity, dominance and similarity indices in each study area.
(DOCX)

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Author Contributions

Conceptualization: Joseline Sofía Ocaña-Cabrera, Claude Saegerman, Sarah Martin-Solano, Alexis Debut, Jorge Ron-Román.

Data curation: Joseline Sofía Ocaña-Cabrera.

Formal analysis: Joseline Sofía Ocaña-Cabrera, Jonathan Liria, Karla Vizuete, Cristina Cholota-Iza, Sarah Martin-Solano, Alexis Debut, Jorge Ron-Román.

Funding acquisition: Claude Saegerman, Jorge Ron-Román.

Investigation: Joseline Sofía Ocaña-Cabrera, Fernando Espinoza-Zurita.

Methodology: Joseline Sofía Ocaña-Cabrera, Claude Saegerman.

Project administration: Cristina Cholota-Iza, Claude Saegerman, Jorge Ron-Román.

Software: Joseline Sofía Ocaña-Cabrera.

Supervision: Claude Saegerman.

Validation: Joseline Sofía Ocaña-Cabrera, Claude Saegerman.

Visualization: Joseline Sofía Ocaña-Cabrera.

Writing – original draft: Joseline Sofía Ocaña-Cabrera, Jonathan Liria.

Writing – review & editing: Fernando Espinoza-Zurita, Claude Saegerman, Sarah Martin-Solano, Alexis Debut, Jorge Ron-Román.

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Experimental section

Study 3:

Pot-pollen DNA barcoding as a tool to determine the diversity of plant species visited by Ecuadorian stingless bees

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Joseline Sofía Ocaña-Cabrera, Sarah Martin-Solano, Jorge Ron-Román, Jose Rivas, Mutien-Marie Garigliany, Claude Saegerman

Preamble

The application of the ITS2 region and the rbcL gene in combination, as pot-pollen barcodes, has been demonstrated to enhance the scope of taxonomic identification at the species level of Ecuadorian flora used by stingless bees. Furthermore, barcodes facilitate enhanced comprehension of the plant-pollinator relationship by elucidating the origins and dispersal patterns of the pollinators. In Ecuador, the pollen sources available in tropical dry forests and tropical rainforests vary according to the season.

In the Orellana province (Amazon region), a total of 24 taxonomic identifications were made. The Melastomataceae family leads the list of pollen source plants for the dry months, followed by the *Artocarpus*, *Croton,* and *Euphorbia* genera, and the *Prockia crucis* species. For the rainy months, 19 identifications indicated the genera *Theobroma, Miconia,* and *Artocarpus,* the family Anacardiaceae, and the species *P. crucis* as the main sources of flora. For Loja province (dry tropical forest), six identifications in total mentioned *Coffea canephora*, *P*. *crucis*, *Miconia nervosa*, *Laurus nobilis,* and the *Theobroma* genus, as the main pollen sources during the dry months. During the rainy months, the main five of 23 taxonomic identifications were *Cecropia ficifolia*, *C*. *canephora, Coffea* sp.*, Makania* sp.*,* and *Ophryosporus* sp*.*

The pot-pollen richness included introduced flora, like *Coffea canephora, Laurus nobilis, Brassica napus, Secale cereale,* and the *Artocarpus* genus, while the preferred vegetation type ranged from shrubs to weeds. Trees were also present. A comprehensive understanding of the available sources of pollen is imperative for the effective management of stingless bees.

As all the sampling sites were private, these sites can be recommended as suitable for the development of meliponiculture, as well as for the cultivation and conservation of beneficial plant species for honey-producing stingless bees in Ecuador.

PLOS One

RESEARCH ARTICLE

Pot-pollen DNA barcoding as a tool to determine the diversity of plant species visited by Ecuadorian stingless bees

Joseline Sofía Ocaña-Cabrera[1], Sarah Martin-Solano[2], Jorge Ron-Román[3], Jose Rivas[4], Mutien-Marie Garigliany[4], Claude Saegerman[1]*

1 Research Unit of Epidemiology and Risk analysis applied to Veterinary sciences (UREAR-ULiège), Fundamental and Applied Research for Animal and Health (FARAH) Center, Faculty of Veterinary Medicine, University of Liège, Liège (Sart-Tilman), Belgium, **2** Departamento de Ciencias de la Vida y de la Agricultura, Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería en Biotecnología, Universidad de las Fuerzas Armadas ESPE, Sangolquí, Ecuador, **3** Departamento de Ciencias de la Vida y de la Agricultura, Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera Agropecuaria, Universidad de las Fuerzas Armadas ESPE, Campus Politécnico Hacienda el Prado Selva Alegre, Sangolquí, Ecuador, **4** Department of Pathology, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, University of Liège, Liège (Sart-Tilman), Belgium

* claude.saegerman@uliege.be

Abstract

Identifying the main species of plants from where Ecuadorian stingless bees collect pollen is one of the key objectives of management and conservation improvement for these insects. This study aims to determine the botanical origin of pot-pollen using two barcodes, comparing two methodologies (DNA barcoding versus electron microscopy and morphometric tools) and determine the genus and species of pollen source plants of the main honey-producing stingless bees in Ecuador. As main results, *Prockia crucis*, *Coffea canephora*, *Miconia nervosa*, *Miconia notabilis, Laurus nobilis*, *Cecropia ficifolia*, *Theobroma* sp., *Artocarpus* sp., *Croton* sp., *Euphorbia* sp., *Mikania* sp., and *Ophryosporus* sp., were the genera and species with the highest presence in the nests (n = 35) of three genera of stingless bees of two provinces located in different climatic regions inside the continental Ecuador. Plant species richness in both areas was statistically similar (p-value = 0.21). We concluded that floral sources' molecular identification with the ITS2 region had a higher number of genera and species detected, than the rbcL gene and microscopy tools, for the Ecuadorian landscapes. We confirmed that the foraging behavior of *Melipona* sp., *Scaptotrigona* sp., and *Tetragonisca* sp., could include non-native flora (27%, 12/44 identifications) that provide a rich source of pollen. Stingless beekeepers could use this information to create flower calendars and establish a schedule for better management of stingless bees in secondary and modified environments.

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Introduction

Pollination services are closely linked to ecosystem stability and biodiversity conservation [1]. Pollinators play a pivotal role in the successful reproduction of flowering plants, thereby promoting genetic diversity and resilience within plant populations [2,3]. Moreover, they ensure the production of seeds and fruits that are vital for human nutrition and food security [4]. The pollination of angiosperms has been found to be beneficial to the health of wildlife, thereby supporting entire food chains (consisting of herbivores, predators, and decomposers) while providing shelter [5]. The fertilisation of plant life by pollinators has been demonstrated to enhance the resilience of these organisms, a factor which is crucial in the context of adapting to environmental changes. Plant diversity helps to sequester carbon, reducing atmospheric CO_2 (carbon dioxide) and aiding in climate change mitigation [6]. The diversity of plant life is also known to stabilise soil, prevent erosion, and regulate water cycles [7].

Since the late 20th century, there has been an ongoing decline in pollinators, a phenomenon that has serious consequences for biodiversity, food security and ecosystem stability [8]. The percentage of pollinating insects, including bees and butterflies, at risk of extinction approached 40% [9]. The decline of bees was attributed to a combination of factors, including habitat destruction, climate change, pesticides, and disease [10–12]. This loss has been recognised as a global crisis by scientists, governments and international organisations. In light of the critical status of insect pollinators, several initiatives have been established on a global scale to ensure their conservation. These initiatives include the International Pollinator Initiative (IPI), the Global Action on Pollination Services for Sustainable Agriculture (FAO), and the Coalition of the Willing on Pollinators. The primary objective of these initiatives is to protect pollinator populations by promoting conservation strategies that integrate agricultural policies and best practices, while also enhancing public awareness [13,14]. The ongoing decline in plant-pollinator interactions is a consequence of the ongoing decline in species of pollinators [15]. The study of these interactions is crucial for preventing the loss of biodiversity in plant communities, as many species are dependent on specific pollinators. A drop in pollination network vitality could limit food sources for other wildlife, affecting their well-being and potentially impacting human food security. A comprehensive understanding of these interactions can help reduce economic losses from pollination services and industries that depend on effective pollination [2,16].

The floral richness of the tropics is particularly affected by the decline of pollinator populations, as the maintenance of their biodiversity is highly dependent on native pollinators, and these are the invertebrates least able to adapt rapidly to changing climates and thus most vulnerable to extinction [17,18]. The Amazon rainforest is characterized by its biodiversity and ecological significance. It plays a crucial role in regulating the climate, storing carbon, and providing essential ecosystem goods and services. These include oxygen, fresh water, medicinal and economic benefits for indigenous communities, to the existence of human life, and future generations [19]. Animals pollinate around 94% of native tropical plants [20], and in the Amazon rainforest, 54% of these animal pollinators are bees [21].

Stingless bees are the main pollinators inside tropical ecosystems, moving from flower to flower until they find the most suitable food [22–24]. Daily-ranging patterns of stingless bees depend on each species' foraging behaviour, which may differ in space use, detection, and foraging distance. For example, the Asian stingless bee (*Tetragonula biroi*) has a short flight range, 250–500 meters (m), as do Australian stingless bees (*T. carbonaria*) have (333–712 m) [25], while American stingless bee genera (*Melipona* sp. and *Trigona* sp.) have longer flight ranges of 1.5 and 2.1 km respectively [26]. The role of native fauna in stabilizing ecosystem services is to buffer the effects of climate change [27]. However, there is evidence of high thermal thresholds that may mark weak selection processes or strong evolutionary constraints [28]. An abnormal accumulation of polyols (mannitol and sorbitol), which act as preventive molecules against protein denaturation or cell inactivation, has been found in insects [29,30]. To mitigate the effect of all these changes it is necessary to understand the basic natural functioning of bees and their relationship, especially with floral sources, directly concerned with the upkeep of crops for human consumption [31].

Foraging of floral sources by stingless bees does not follow a described pattern. In general, it has been concluded that foraging occurs according to the need of the nest and the availability of sources [32], i.e., temporal specialization intervals [33,34].

To obtain a better understanding of the pollen sources of bees, different techniques have been developed, such as light microscopy and pattern recognition carried out by a visual expert [35], phase contrast and dark field microscopy [36], and vibrational spectroscopy [37,38]. Moreover, even software and artificial intelligence development now allow for automated pollen recognition [39]. Due to continuous scientific improvements, molecular biology techniques have been included in pollen research [40,41]. Indeed, barcoding is a technique that allows species recognition through the characterization of standard genes [42]. In the case of land plant barcoding, selected DNA regions must satisfy the following criteria: (i) be routinely amplifiable; (ii) to have a sufficient variation to differentiate closely related species and yet also show sufficient sequence consistency to ensure that intraspecific variation does not confound species assignation; (iii) to have specific amplification unsusceptible to the amplification of other DNA regions [43]. An effective DNA barcode region possesses conserved flanking sites for developing universal PCR primers for wide taxonomic applications. Nuclear regions, such as the Internal transcribed spacer 2 (ITS2) provide more information than barcoding based on the organellar gene [44,45]. Nuclear DNA (nDNA) exhibits faster rates of evolution in comparison to chloroplast DNA (cpDNA), resulting in the accumulation of a greater number of mutations over time. The genetic variation that results from this process facilitates the distinction between closely related species [46,47]. Multiple gene copies from nuclear genome increase the species resolution. The biparental inheritance in angiosperms capture the recombination and hybridization, thereby contributing to the augmentation of diversity identification [48–50].

The ribulose 1,5-biphosphate carboxylase oxygenase (rbcL) gene is a constituent of chloroplast DNA and represents a valuable marker due to its documented ease of amplification when using primers that apply to all land plants. Additionally, it has been demonstrated that this gene is capable of identifying taxa at the genus and family levels, and has also been shown to be an effective species-level identifier in comparative data mining tests. Furthermore, it is the most extensively characterized plastid coding region in GenBank [51,52]. The low mutation rate of this gene, when used in conjunction with other markers, enhances analysis at both ecological and evolutionary levels [53]. Some organellar genomes, like those in organelles like mitochondria and chloroplasts, change very slowly. This can make closely related but distinct species appear genetically identical. This can mask true species diversity and lead to an underestimation of richness [54,55]. Conversely, an overestimation may occur due to species interbreeding. In such instances, organellar DNA from one species may be retained in the hybrid, while nuclear DNA remains distinct. This phenomenon can result in the erroneous classification of hybrids as new species. Nevertheless, a combination of nuclear and chloroplast markers is frequently employed for the purpose of robust species identification [56,57]. The ITS2 region and the rbcL gene were selected as markers due to their high universality, sequence variability, and ease of amplification. The combination of these markers is further enhanced by the following factors, the ITS2 region has been shown to provide high resolution at the species level, but can be difficult to amplify in some plant groups. In contrast, the rbcL gene is easy to sequence and works in all land plants, but has lower species discrimination power. The utilization of these two markers serves to mitigate the occurrence of erroneous identifications and taxonomic misclassifications [58–60].

South America has a low level of scientific data production with metagenomics tools and microbiome studies [61]. Ecuador is not an active player in biodata generation often due to the technology gap, but its unique biodiversity has great potential to contribute to global projects related to this field [62], especially for the knowledge of Ecuadorian biodiversity. To contribute to the knowledge of biodiversity through the identification of plants using pollen collected by stingless bees in two provinces of Ecuador, this study aims to (i) determine the taxonomy of plants using pot-pollen from stingless bee nests, using two the ITS2 region and rbcL gene for barcoding, to (ii) make a comparison between the scope of taxonomic identification obtained by microscopy and by DNA, and to (iii) evidence the difference of the main pollen sources for stingless bee genera in two environmentally different areas of Ecuador (Fig 1).

Materials and methods

Ethical aspects

The applicable legislation was applied during the manipulation of specimens and habitat involved in this study. Habitat was not disturbed or altered in any significant way, and no animals were harmed in the process of obtaining the samples.

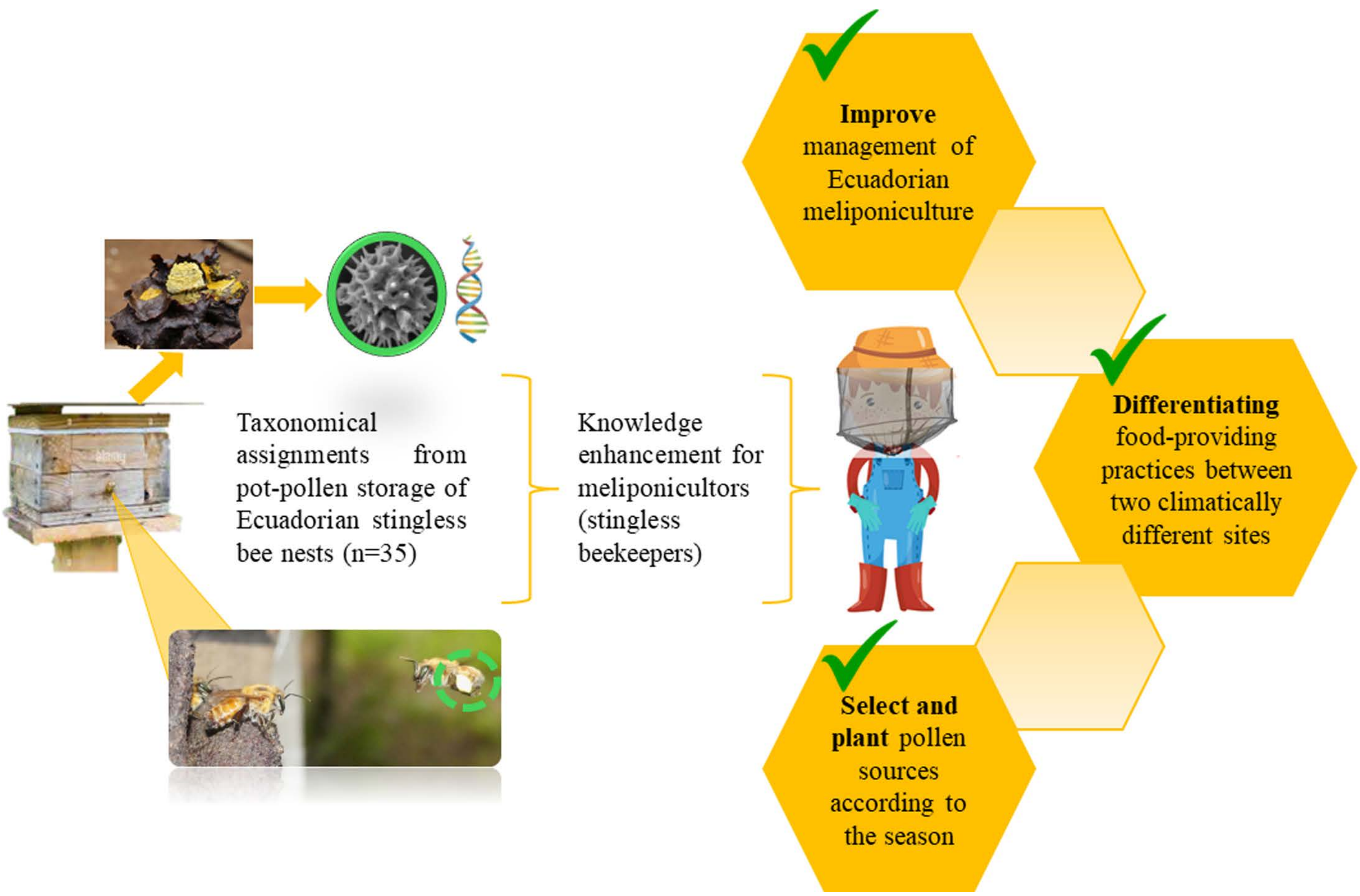

Fig 1. Graphical abstract of the study.

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Pollen as a product of a stingless bee nest is classified as a food supplement and/or natural medicinal product. The objective of the collection was explained to each owner, and field site access was obtained by each meliponicultor (from Orellana and Loja province) under the terms of informed consent as part of the Synergy Project, which was approved under the number CVGP-0025–2017 by the Universidad de las Fuerzas Armadas ESPE, Ecuador. Meliponicultors signed a consent for the collection of pollen samples from their stingless bees' nests in August-September, December 2018, and March 2019.

Study areas

The sampling areas were the Amazon rainforest (Orellana province) and the southern highland region with dry tropical forests (Loja province) (Table 1). In Ecuador, the summer lasts from September to February. For Orellana province, the average temperatures and precipitations are 25 °C and 127 mm, respectively. The average temperature and precipitation in Loja province are 21 °C and 36 mm.

Pollen sampling

This cross-sectional survey randomly selected 35 pot-pollen samples from technical stingless bee nests (**S1 Table**) belonging to 4 meliponaries in Orellana and 11 meliponaries in Loja, with several samples from the same nest set ($n = 21$ and $n = 14$, respectively).

We collected pollen samples only from sealed pots of nests that were sampled once during the four months.

Pollen wall lysis and DNA isolation

Fifty milligrams of pollen were weighed into a 2 mL Eppendorf tube. 500 uL of buffer lysis of Macherey-Nagel NucleoSpin Food kit (Macherey-Nagel, Düren, North Rhine-Westphalia, Germany) and 500 uL of ceramic beads (1mm) were added. We vortexing samples to achieve a homogeneous mixture. Each tube was placed into a Tissuelyser II instrument (QIAGEN®) for 3 min, 30 Hz. The tubes were centrifuged for 2 min at 5000 g. 10 uL of proteinase K (20 mg/mL) was added and incubated for 30 min, 65 °C.

Total genomic DNA was extracted using the Macherey-Nagel NucleoSpin® Food kit (Macherey-Nagel, Bethlehem, Pennsylvania, USA), following the "Isolation of genomic DNA from honey or pollen" supplementary protocol. Negative control was included in the experiment, consisting of sterilized water instead of pollen. Finally, the DNA purity of each sample was measured using NanoDrop ® Spectrophotometer ND-1000, ISOGEN Life Science.

Real-time PCR

Temperature and time conditions [hold stage 95 °C 180 sec (95 °C 30 sec, 60 °C 30 sec, 72 °C 45 sec) x 40 cycles, melting stage 95 °C 15 sec] were established for the amplification of both regions (Table 2). The amplification of each region was carried out separately and in duplicate per sample (Table 3) using Luna® Universal Probe One-Step RT-qPCR Kit.

Table 1. Geographical localization of sampling zones.

Province	Locality	Geographical localization
Orellana	Dayuma	0°40'16"S, 76°52'54"W
Loja	Celica	0°40'10"S, 80°04'90"W
	Pindal	0°30'57"S, 79°59'04"W
	Puyango	0°30'57"S, 79°58'27"W

S: south. W: west

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Commercial pollen from Belgium was used as a positive control and sterile water as a negative control. The PCR assembly was conducted in two chambers: one for the preparation of the master mix and one for the addition of the DNA. We used this endpoint PCR modality to assess the quality of pollen DNA through C_T (cycle threshold) values.

Illumina sequencing

Amplicon libraries were prepared according to the Illumina 16s metagenomic workflow protocol [66], with adaptations. PCR1 was done for both amplicons separately (ITS/rbcL) and then mixed before cleaning up with Ampure beads (cf 16s-metagenomic-library-prep-guide-15044223-b, page 8). For this PCR1, we used 40 cycles instead of 25, and Q5® High-Fidelity DNA Polymerase (M0491), but PCR2 was processed with Kapa HiFi polymerase like in the 16s Illumina protocol. PCR2 was done with 5 µl of purified PCR1 product. At the end of PCR2, all libraries were dually indexed. Different combinations of indexes (Nextera Index Kit - Index 1 (i7) Adapters, from N708 to N712 and Index 2 (i5) Adapters, from N501 to N508. Oligonucleotide sequences © 2015 Illumina, Inc. All rights reserved) were used for each sample.

PCR2 products were then purified with AMpure beads (cf 16s-metagenomic-library-prep-guide-15044223-b, page 13), and amplicon QC was done on QIAxel (size profile) (QIAGEN®, Germany).

PCR2 products were quantified and normalized at 7 ng/µl using Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific). We generated an equimolar pool at 5 ng/µl. Before proceeding to Illumina MiSeq paired-end 300 bp, the final pool was quantified by qPCR using KAPA SYBR® FAST qPCR Kits (Sopachem) with Library Quantification DNA Standards Illumina from Roche. 8.5 PM of the denatured final pool was loaded on a Miseq 600 cy v3 kit. As a control step, we added 10%pf Phix (PhiX Control v3), a ready-to-use control library for Illumina sequencing runs.

Bioinformatic analysis

The process started with 2 213,285 forward sequences and the same number of reverse sequences for ITS2 and rbcL. We adapted steps 1–3 of Quantitative Insights Into Microbial Ecology (QIIME 2) workflow for metabarcoding analysis

Table 2. Primers sequence information.

Gen	Sequence (5' ◊ 3')	Reference
rbcLaF (forward)	ATGTCACCACAAACAGAGACTAAAGC	[63]
rbclr506 (reverse)	AGGGGACGACCATACTTGTTCA	[64]
ITS-3p62plF1 (forward)	ACBTRGTGTGAATTGCAGRATC	[65]
ITS-4unR1 (reverse)	TCCTCCGCTTATTKATATGC	

Note: for ITS2 uncommon letter the interpretation is: *B is C, T or G, R is A or G, K is G or T.*

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Table 3. PCR preparation by microtube.

Product	Quantity
Luna ® Universal Probe qPCR Master Mix	10 µL
Nuclease-free water	6 µL
Primer forward (ITS2 or rbcL)	2 µL
Primer reverse (ITS2 or rbcL)	2 µL
Sample DNA	5 µL
Total volume	**25 µL**

µL: microlitres

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(18S/16S rRNA) with already-demultiplexed fastq files (https://github.com/BikLab/BITMaB2-Tutorials/blob/master/QIIME2-metabarcoding-tutorial-already-demultiplexed-fastqs.md), to specific information of ITS2 and rbcL reads. To illustrate, we avoided the demultiplexed command in step 1 "Importing data, summarize the results, and examining quality of the reads". The values for truncated bases of sequences in step 2 "Quality controlling sequences and building Feature Table and Feature Data" were modified. The DADA2 plugin in Qiime 2 employs a default filtering process that excludes any PhiX reads from the sequencing data and filters out chimeric sequences. A quality plot was consulted to eliminate noise and establish the requisite parameters. It was noted that the mean quality score was 34, along the initial bases, which led to the decision to set --p-trim-left = 0 for both, forward and reverse reads. The quality plot, in turn, informed the decision to set the parameter --p-trunc-len = 250 for forward reads and --p-trunc-len = 200 for reverse reads, sites after which the quality drops significantly. After the last step, four samples were removed. This decision was made due to the limited number of sequences available for each sample, and the substandard quality of the sequences. Due to the sampling site's biodiversity, we dereplicate the sequences in Amplicon Sequence Variants (ASVs). Following the filtration and denoising processes, 541,174 paired-end reads were obtained for ITS2, and 301,612 were received for rbcL.

The DUBOIS curated NCBI_ITS2_Viridiplantae and NCBI_rbcL_Viridiplantae [67] database (both dereplicated-restricted) was employed to assign the taxonomy of ITS2 and rbcL unknown sequences in step 3 "Assigning Taxonomy". The percentage of the similarity threshold for assignment to the species level was 95%. The training classifier was configured using the classify-consensus-blast algorithm, with a max accept value of 1. The final assigned reads were 1,007. However, the reverse rbcL reads demonstrated a consistent lack of quality (step 2), which complicated the pairing and analysis of pairs of reads. For the taxonomic assignment, the focus was exclusively on the rbcL forward reads. However, the classifiers (Blast and vSearch) were unable to identify reliable assignments. Manual mapping against specific reference databases found in NCBI was then performed for rbcL barcode, although this process was more laborious and time-consuming.

Finally, step 4 "Summarizing Feature Table and Feature Data" of the workflow for metabarcoding analysis was followed similarly.

Scanning electron microscopy (SEM) and morphometry method

We used the method developed in our previous work [33], in which high-quality 2D SEM images and morphological anatomical points were used to identify plant families and genera, to compare the results obtained in the present study.

Statistical analysis

Alpha diversity was used to compare samples from the two provinces. To measure the branching length between the taxonomic assignments of the ASVs, Faith's Phylogenetic Diversity (PD) was used. The Alpha diversity calculation used the total number of samples ($n = 35$).

The significance of the difference between biodiversity values was measured using the pairwise Kruskal-Wallis Test.

Results

A total of 35 samples were obtained for inclusion in the study. The ratio absorbance 260/280 of DNA extracted from the samples was 1.64–2.21 ng/μL. The C_T for ITS2 region was 13.696–34.794 (22.24 ± 0.27) while the C_T for rbcL gene was 15.901–39.336 (25.78 ± 0.33).

Following the quality control step in the bioinformatics analysis, samples 9, 10, 27, and 35 were excluded due to the poor quality of the reads generated after sequencing. The total number of taxonomic assignments made for the ITS2 sequences at the family level was 26, at the genus level was 51, and at the species level was 204. In the case of the rbcL sequences, no taxonomic assignments were made at the family level; however, a single assignment was identified at the genus level, and 61 taxonomic assignments were made at the species level. Although 31 samples successfully passed the quality filtration process, obtaining the aforementioned number of taxonomic assignments was only possible from 28 pollen samples (GenBank accession numbers from SAMN46265110 to SAMN46265137). No valid taxonomic assignments were obtained from any DNA barcode for samples 11, 28, and 32.

The taxonomic assignments obtained from the two DNA markers represented families, genera, and species that were repeated. Consequently, a total of 64 ASVs were identified as unique and distinct taxa, of which 4 (6%) were classified at the family level, 34 (53%) at the genus level, and 26 (41%) at the species level.

We identified 58% (37/64) of the ASVs through the ITS2 region, and 42% (27/64) through the rbcL gene. Taxonomy identification scope was superior using pollen DNA analysis than morphology and geometric morphometry (SEM identification) analysis, since it was not possible to reach species with the last methodology (Fig 2).

The SEM methodology enabled the identification of up to five families within the same sample. Using the ITS2 region we were able to identify at least four different families (six species) per sample, whereas using the rbcL gene, it was possible to identify only four families (four species) within the same sample.

Regarding the alpha diversity of the samples based on the phylogenetic distribution (Fig 3), there was no significant difference between species richness in the two sampling sites (p-value = 0.21).

We were able to differentiate the pollen sources according to the seasons, August and September were dry months for both study sites (Amazonian regions and southern highlands), while December and March were rainy months (Fig 4).

During dry months for Orellana province, we found 24 miscellaneous sources, mainly Melastomataceae, *Artocarpus* sp., *Croton* sp., *Euphorbia* sp., *Prockia crucis*, while during a rainy month (December) 19 different pollinic plants were identified, such as *Theobroma* sp., *Prockia crucis*, *Miconia* sp2., Anacardiaceae, *Artocarpus* sp. (Table 4).

In March, the rainiest month for Loja province, we identified 23 plant sources mostly *Cecropia ficifolia*, *Coffea canephora*, *Coffea* sp., *Mikania* sp1., *Ophryosporus* sp., compared to only six in the dry month (September), *Coffea canephora*, *Prockia crucis, Miconia nervosa*, *Theobroma* sp., *Laurus nobilis*, and *Cecropia ficifolia* (Table 5).

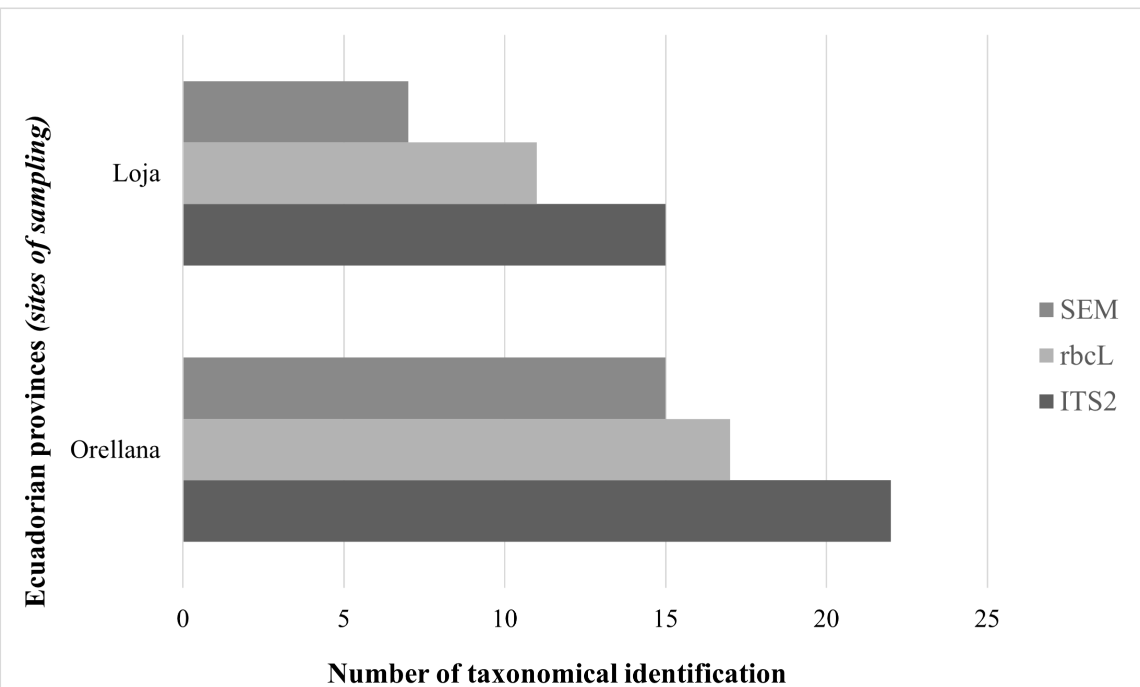

Fig 2. Scope comparison of plants identifications using three different methods: barcode with ITS2 region, barcode with rbcL gene, scanning electron microscopy and morphometry identification (SEM).

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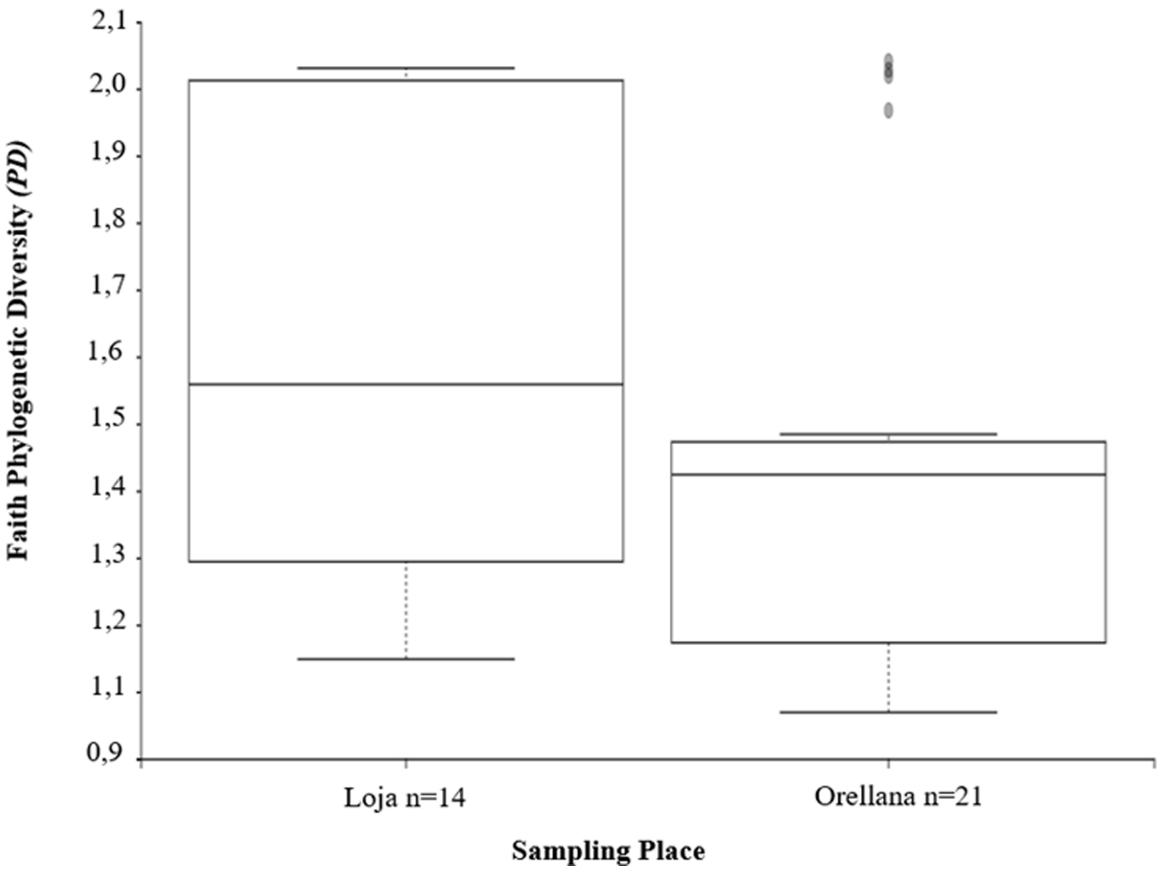

Fig 3. Alpha diversity boxplot by sampling place, tropical dry forest (Loja), Amazonian rainforest (Orellana).

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Once, we identified 64 ASVs, we found that 19% (12/64) of the taxonomic identifications were considered as introduced flora (Table 6) for Ecuadorian territory (Fig 5), with a variety of weeds (33%), shrubs (50%) and trees (17%).

Discussion

We were able to identify 64 plants as pollen sources at different taxonomic levels, 6% at the family level, 41% at the species level, and 53% at the genus level, using DNA barcode analysis. We differentiate the seasonal pollen sources for two climatological distinct regions in continental Ecuador, the southern highland with dry tropical forest (Loja province) and the Amazonian rainforest (Orellana province). For both sites, the main identifications based on the highest abundance (number of reads per ASV) of plants present per sample were *Prockia crucis*, *Coffea canephora*, *Miconia nervosa*, *Laurus nobilis*, *Theobroma* sp., *Miconia notabilis*, *Artocarpus* sp., *Croton* sp., *Euphorbia* sp., *Cecropia ficifolia*, *Mikania* sp., and *Ophryosporus* sp.

The ITS2 region obtained the major scope for taxonomic assignments, because its variability allows for distinguishing closely related species. Additionally, its conserved regions make it valuable for designing universal primers [44]. The internal transcribed spacer 2 length is 180–390 bp for plants [68]. Meanwhile, the rbcL gene has a full length of 1400 bp [69]. RbcL gene is a good region for phylogenetic studies due to a low mutation rate that maintains sequence stability over generations, allowing evolutionary relationships between plants to be mapped. Its highly conserved sequence also enables broad applicability across different taxa [63].

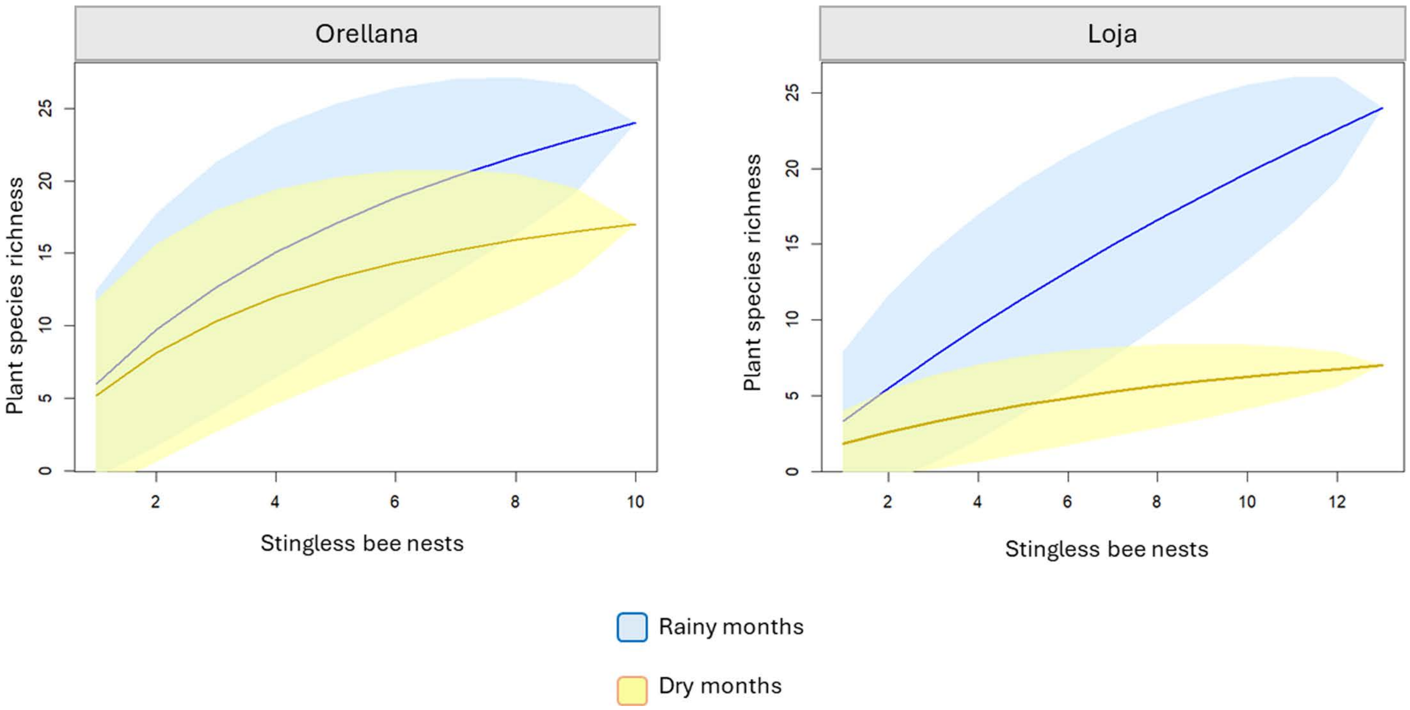

Fig 4. Seasonal acumulation curve (rarefaction) for plant taxonomical assignment according to the stingless bee nests sampled. Rainy months (blue line) December 2018, and March 2019. Dry months (yellow line) August, and September 2018. The 95% of confidence interval (blue and yellow transparency) is also indicated.

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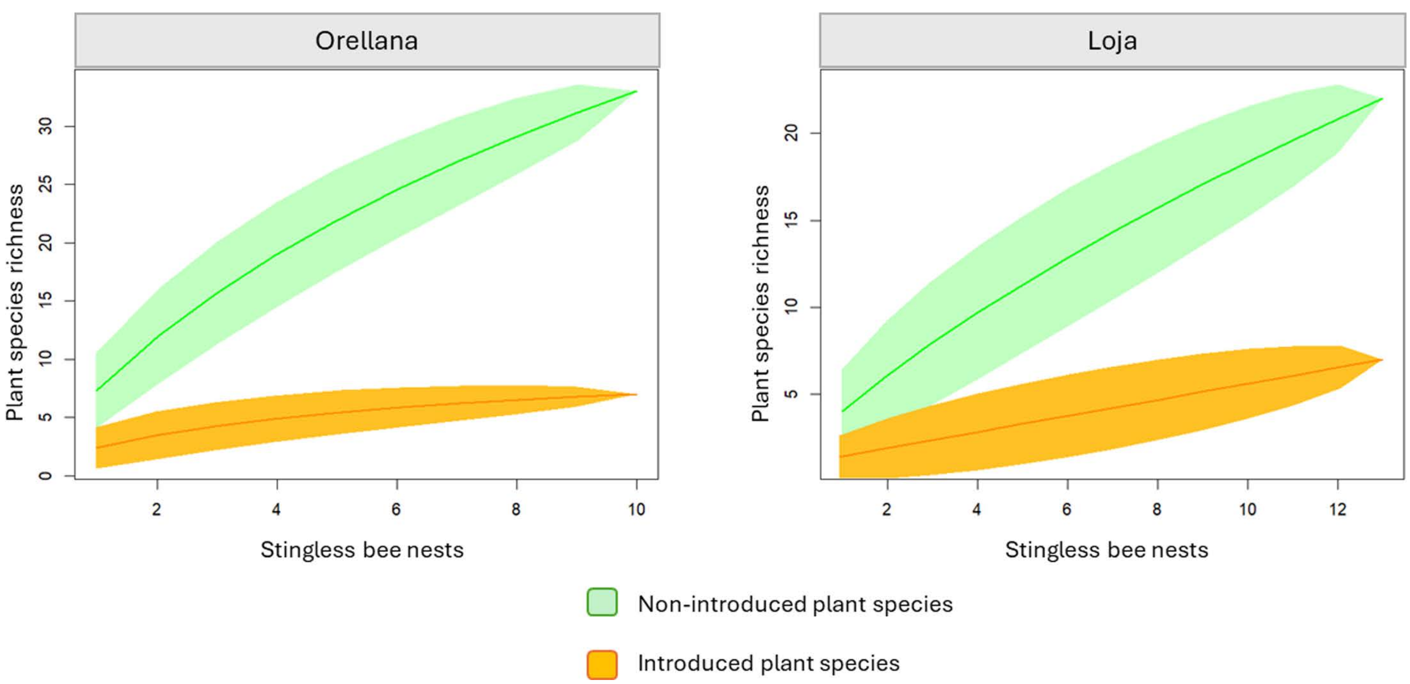

Fig 5. Type of flora acumulation curve (rarefaction) for plant taxonomical assignment according to the stingless bee nests sampled. Non-introduced plants (green line), and introduced plants (orange line) for Ecuador. 95% of confidence interval (green and orange transparency).

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The rbcL barcode as a gene in the plastid DNA began to be less recommended for the analysis of pollen DNA since it is not present in all pollen grains. However, it is essential to maintain it for reliable plant identification with close taxa [70] and the quantitative data that produce at least at the family level. The majority of the families identified in this study fall into this category, including Asteraceae, Brassicaceae, Fabaceae, Moraceae, Rosaceae, and Salicaceae [71]. The elevated number of species-level identifications in this study may appear surprising, but the efficacy of rbcL as a DNA marker for species-level identification has been demonstrated in specific groups of plants that show greater interspecific variation, i.e., greater sequence divergence, which allows species resolution. This feature has been identified in species belonging to the families Asteraceae, Fabaceae, Poaceae, and Orchidaceae [72–75]. However, it is important to recommend the use of the matK gene in conjunction with the rbcL gene to improve species resolution in similar studies.

The combination of ITS2 and rbcL markers was found to facilitate more precise species-level identification [76] than that achievable with either marker in isolation [77–82]. The optimisation of the methods employed was also a factor that enriched our results. Increasing the number of PCR cycles from 25–35 to 40 has a small impact on species-level identification [83]. However, this increase allows the amplification of ITS2 sequences from other plants, which would not be possible with fewer cycles. During our study, the use of 40 cycles for the PCR amplification may have made the ITS2 region the best identification marker. The next-generation sequencing method has been a popular way to analyse pollen [84]. In this study, the MiSeq system worked, as is usual, with 300 pb paired-end reads, which is a key tool in the case of ITS region analysis because this specific length recovers the informative sequence ITS2 and ITS1 [85]. However, MiSeq is a short-read NGS sequencing platform. Consequently, we would recommend HiFi-based platforms such as PacBio, which

Table 4. Seasonal pollen references for Orellana province (amazon region).

		August – September 2018 (dry months)		December 2018 (rainy month)
	1	Melastomataceae	1	*Theobroma* sp. (Malvaceae)
More	2	*Artocarpus* sp. (Moraceae)	2	*Prockia crucis* (Salicaceae)
↓	3	*Croton sp* (Euphorbiaceae)	3	*Miconia* sp2. (Melastomataceae)
	4	*Euphorbia* sp. (Euphorbiaceae)	4	Anacardiaceae
	5	*Prockia crucis* (Salicaceae)	5	*Artocarpus* sp. (Moraceae)
	6	*Schefflera* sp. (Araliaceae)	6	*Choerospondias axillaris* (Anacardiaceae)
	7	*Miconia notabilis* (Melastomataceae)	7	*Coffea canephora* (Rubiaceae)
	8	*Theobroma* sp. (Malvaceae)	8	Melastomataceae
Less	9	*Dendropanax* sp. (Araliaceae)	9	*Miconia notabilis* (Melastomataceae)
	10	*Bellucia grossularioides* (Melastomataceae)	10	*Schefflera* sp. (Araliaceae)
	11	*Solidago* sp. (Asteraceae)	11	*Eugenia* sp. (Myrtaceae)
	12	*Triolena amazonica (*Melastomataceae)	12	*Baccharis* sp. (Asteraceae)
	13	*Calyptranthes* sp. (Myrtaceae)	13	*Mikania cordifolia* (Asteraceae)
	14	*Miconia affinis* (Melastomataceae)	14	*Brassica napus* (Brassicaceae)
	15	*Psidium* sp1. (Myrtaceae)	15	*Acmella* sp. (Asteraceae)
	16	*Brassica napus* (Brassicaceae)	16	*Erigeron sumatrensis* (Asteraceae)
	17	*Coffea canephora* (Rubiaceae)	17	*Mauria* sp. (Anacardiaceae)
	18	*Trophis caucana* (Moraceae)	18	*Aster* sp. (Asteraceae)
	19	*Miconia tococoidea* (Melastomataceae)	19	*Muntingia calabura* (Muntingiaceae)
	20	*Baccharis* sp. (Asteraceae)		
	21	*Ficus andicola* (Moraceae)		
	22	*Triplaris melaenodendron* (Polygonaceae)		
	23	*Aster* sp. (Asteraceae)		
	24	*Ficus* sp. (Moraceae)		

https://doi.org/10.1371/journal.pone.0323306.t004

Table 5. Seasonal pollen references for Loja province (southern highland region).

		September 2018 (dry month)		March 2019 (rainy month)
	1	*Coffea canephora* (Rubiaceae)	1	*Cecropia ficifolia* (Urticaceae)
More	2	*Prockia crucis* (Salicaceae)	2	*Coffea canephora* (Rubiaceae)
↓	3	*Miconia nervosa* (Melastomataceae)	3	*Coffea* sp. (Rubiaceae)
	4	*Theobroma* sp. (Malvaceae)	4	*Mikania* sp1. (Asteraceae)
	5	*Laurus nobilis* (Lauraceae)	5	*Ophryosporus* sp. (Asteraceae)
	6	*Cecropia ficifolia* (Urticaceae)	6	*Withania* sp. (Solanaceae)
			7	*Leucaena* sp. (Fabaceae)
			8	*Psidium* sp2. (Myrtaceae)
Less			9	*Trophis caucana* (Moraceae)
			10	*Dillenia* sp. (Dilleniaceae)
			11	*Schefflera* sp. (Araliaceae)
			12	*Tapirira guianensis* (Anacardiaceae)
			13	*Theobroma* sp. (Malvaceae)
			14	Myrtaceae
			15	*Swartzia polyphylla* (Fabaceae)
			16	*Brassica napus* (Brassicaceae)
			17	*Secale cereale* (Poaceae)
			18	*Triticum turgidum* (Poaceae)
			19	*Baccharis* sp. (Poaceae)
			20	*Alternanthera* sp. (Amaranthaceae)
			21	*Pisonia* sp. (Nyctaginaceae)
			22	*Ageratina adenophora* (Asteraceae)
			23	*Bougainvillea praecox* (Nyctaginaceae)

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provide long-read sequencing of fragments ranging in size from 1000 to 20,000 bases or more. Such platforms would be more appropriate for barcodes such as rbcL

Genus or species misidentification is often attributed to missing plant sequences in reference databases [51]. Ecuador is one of the 20 megadiverse countries in the world, with two biodiversity hotspots [86]. Therefore, it is common to record new species in this tropical country, which may explain the under-representation of our sequences at the species level, in general databases. Plant taxonomy is important, especially in biodiversity hotspots, for several reasons: the contribution to knowledge by identifying and classifying species that are unknown or believed to be extinct, thus contributing to the advancement of knowledge and, consequently, to the establishment of conservation programmes for those species that require protection [87–89]. Taxonomic studies are also established as a baseline against which to work with programmes to monitor, track and make decisions on changes in species diversity due to habitat destruction and climate change. Furthermore, they facilitate the establishment of measures aimed at preventing the spread of invasive species that have the capacity to alter the integrity of ecosystems [90–92].

Although we demonstrated that the DNA methodology was superior for species-level identification to the SEM method, the latter allowed us to detect almost the same number of families within a sample [93]. The method of morphology and morphometric geometry, which uses high-quality 2D images (SEM) was applied a year before the current barcoding method to the same samples. We can attribute the low DNA quality of the samples in this study to the long period and conditions of storage, 3 years at 4 °C. In addition, the pollen samples did not undergo any prior washing or preservation

Table 6. Introduced flora identified as pollen sources for Ecuadorian stingless bees.

Plant	Origin	Vegetation type	Spanish common name
Coffea canephora (Rubiaceae)	W. Tropical Africa to S. Sudan and N. Angola	Shrub or tree up to 10 m	Café robusta
Brassica napus (Brassicaceae)	Europe to Mongolia and Pakistan, Canary Islands, N. Africa to Somalia and Arabian Peninsula	Weed	Canola, colza
Artocarpus sp. (Moraceae)	Tropical & Subtropical Asia to W. Pacific	Tree	Árbol de pan, frutipán
Choerospondias axillaris (Anacardaceae)	Nepal to S. China and Indo-China, Taiwan	Tree	NA
Solidago sp. (Asteraceae)	N. & Central America, Caribbean, Bolivia to Brazil and S. South America, Azores, Temp. Eurasia, NW. Africa.	Weed	Plumero Amarillo
Aster sp. (Asteraceae)	Eurasia to Jawa, NW. Africa, Subarctic America to NW. U.S.A.	Weed or small shrubs	NA
Dillenia sp. (Dilleniaceae)	W. Indian Ocean to SW. Pacific	Shrubs or trees up to 30 m	Falsa magnolia, manzana de elefante
Withania sp. (Solanaceae)	Tropical & S. Africa, Medit to Temp. Asia	Shrubs or weeds	Ginseng indio, hierba mora mayor
Secale cereale (Poaceae)	S. Türkiye	Weed	Centeno
Triticum turgidum (Poaceae)	E. Medit. To Iran and Xinjiang	Weed	Trigo
Ageratina adenophora (Asteraceae)	Mexico	Shrubs	Flor de la espuma
Laurus nobilis (Lauraceae)	Medit.	Shrubs or tree	Laurel, lauro

N: north. S: south. W: western. E: east. Medit: mediterranean. NA: not assigned

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method specific to DNA [94]. The ratio absorbance 260/280 of ≤ 1.6 may indicate the presence of proteins in samples [95]. The MiSeq recommendation is that a minimum of 50 ng to 500 ng of good-quality DNA should be utilized. It is imperative to note that DNA of substandard quality may contain traces of ethylenediaminetetraacetic acid (EDTA), organic contaminants such as ethanol, or other inhibitors that may interfere with library preparation (the case of this study) or DNA sequencing.

Twelve flora identified in this study are considered as introduced (non-native) in Ecuador [96]. Asia and Africa were the main origin sites which reflects the ecologically modified environment where the stingless bee sets are located. Forest trees were found as the main sources in a mixed native and exotic environment for Brazilian stingless bees [23] even when obtaining pollen and/or nectar, had higher energy costs than other shrubs and weeds. Stingless bees have been observed to follow a feeding pattern across all regions of their pantropical distribution. They visit both native plants and exotic species, including crops, ornamental plants, and weeds [19].

Plants in Melastomataceae, Myrtaceae, Asteraceae, Anacardiaceae, Euphorbiaceae, and Sapindaceae families are commonly reported to provide pollinic sources for stingless bees [97]. While reports of Polygonaceae, Solanaceae, Poaceae, Amaranthaceae, Dilleniaceae, and Araliaceae are frequent in other studies [98–100]. And as rarely reported we found Muntingiaceae and Nyctaginaceae families [22,101]. Some plants of Anacardiaceae family, genera *Croton* and *Cecropia,* and species such as *Trophis caucana, Secale cereale* include wind pollination (anemophilous) in their pollination [102–104]. The presence of the pollen in question in the nests of the stingless bees under study may be attributable to an indirect entry of pollen into the nests through wind currents or electromagnetic attraction to pollen charges that the bees carry in their corbiculae. It is recommended that the pollination mechanisms of these particular plants be studied to ascertain whether their presence in the pollen pots was due to indirect contamination or whether it was attributable to the pollination activity of stingless bees.

Our results from Loja province, a tropical dry forest, indicated a greater diversity of pollen species during the rainy season. In the case of Orellana province, tropical rainforest, a greater diversity of pollen species was detected during the dry season [105]. Therefore we support the assertion that food stored is positively correlated with field food availability, which is greater when temperature and rainfall increase in the tropics [106]. Pollen richness and diversity inside the nests are positively related to environmental plant richness and the distance between nests and pollen sources [107].

In species of the genus *Melipona* sp., it has been observed that the flight distance may vary from 2 to 10 km when the resource reward is high, but it is surprising to observe this pattern in species such as *Scaptotrigona* sp., or *Tetragonisca* sp., for which there is no record of flight distances greater than one kilometer [97]. Thus, it is extremely important to maintain enough diverse floral sources around stingless bee nests, especially for those that are intended purely for the production of honey.

In Ecuadorian stingless bee keeping, we suggest differentiating the management of stingless bees according to the area in which the producer is located. Because when you generalize the bee keeping practices from one region to another without taking into consideration the months of local flowering, actions such as honey harvesting or nest division, can drastically affect the survival of the nests.

Conclusion

ITS2 region and rbcL gene increase the scope of taxonomical identification at the species level, by using them together. They also contribute to the understanding of the plant-pollinator relationship by revealing the origin and dispersal patterns of pollen as well as the specialisation of pollinators.

In Ecuador, tropical dry forests and tropical rainforests offer different pollen sources according to the season, an important factor to consider in the management of stingless bees, which must be differentiated for each region.

The pot-pollen richness included introduced flora, while the preferred vegetation type ranged from shrubs, weeds, and trees.

Understanding the available pollen sources is crucial for the effective management of stingless bees, identifying appropriate locations for meliponiculture, and cultivating or preserving plant species that are beneficial for honey-productive species in Ecuador.

Supporting information

S1 Table. Detailed list of Stingless bee species per sampled nest. The species were identified using two distinct methods: molecular biology and morphometric analysis. These methods were developed by two undergraduate students, Esteban Palacios and Ransey Pachacama in 2021 (unpublished information). ID code meaning: *H* meliponary or nest set, *N* nest, *P* pollen sample.
(DOCX)

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Author contributions

Conceptualization: Joseline Sofía Ocaña-Cabrera, Sarah Martin-Solano, Jorge Ron-Román, Claude Saegerman.

Data curation: Joseline Sofía Ocaña-Cabrera.

Formal analysis: Joseline Sofía Ocaña-Cabrera, Jose Rivas, Mutien-Marie Garigliany.

Funding acquisition: Sarah Martin-Solano, Jorge Ron-Román, Claude Saegerman.

Investigation: Joseline Sofía Ocaña-Cabrera.

Methodology: Joseline Sofía Ocaña-Cabrera, Sarah Martin-Solano, Claude Saegerman.

Project administration: Sarah Martin-Solano, Jorge Ron-Román, Claude Saegerman.

Resources: Sarah Martin-Solano, Claude Saegerman.

Software: Joseline Sofía Ocaña-Cabrera, Claude Saegerman.

Supervision: Sarah Martin-Solano, Mutien-Marie Garigliany, Claude Saegerman.

Validation: Joseline Sofía Ocaña-Cabrera, Claude Saegerman.

Visualization: Joseline Sofía Ocaña-Cabrera.

Writing – original draft: Joseline Sofía Ocaña-Cabrera.

Writing – review & editing: Sarah Martin-Solano, Jorge Ron-Román, Jose Rivas, Mutien-Marie Garigliany, Claude Saegerman.

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Experimental section

Study 4:

Chemical contaminants in cerumen samples from Ecuadorian stingless bees: reporting glyphosate, aminomethylphosphonic acid, and the presence of metals and metalloids

Insects: accepted

Joseline Sofía Ocaña-Cabrera, Jorge Ron-Román, Sarah Martin-Solano, Claude Saegerman

Preamble

Given that stingless bees produce cerumen by mixing wax they produce with plant resins they collect, it seems reasonable to conclude that external material brought into the nest by foragers is the main source of agrochemical contamination of this product. As they do not produce large amounts of wax like honey bees do, stingless bees use plant resins as both a building material and a means of defending their nest.

Glyphosate (GLY) and its metabolite AMPA are widely used herbicides in Ecuador. Although legislation restricts their use in protected and urban areas, it does not prohibit their use nationwide. Certain metals are naturally present in the Earth's crust as a result of erosion and volcanic activity. Ecuador is located within the Pacific Ring of Fire and has 27 potentially active volcanoes out of a total of 80 distributed across its four natural regions. Conversely, human activities such as the extraction and use of fuels, mining, metallurgy, the use of fertilisers and pesticides, and waste disposal can increase the presence of metals on Earth's crust.

In the highland region, levels of GLY and AMPA between 0.02 – 0.2 [µg/kg] and 0.028 [µg/kg], respectively, were detected in cerumen samples mainly belonging to stingless bees of the genera *Scaptotrigona* and *Partamona*. Meanwhile, metals and metalloids such as cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), tin (Sn), arsenic (As), antimony (Sb), and selenium (Se) were detected in cerumen samples from the highland and low Amazon regions, primarily in samples belonging to the genera *Melipona*, *Tetragonisca*, *Trigona*, and *Scaptotrigona*.

To estimate the health risk of stingless bees being exposed to this herbicide, we assessed a worst-case scenario. To achieve this, we used data from published literature and from this study. This resulted in a possible case of acute exposure in *T. angustula* workers (risk quotient = 60.8).

The adoption of good management practices (GMPs) and ecological criteria is strongly recommended for the sustainability of meliponiculture. Agrochemicals pose a serious threat to bees worldwide, and stingless bees are no exception. Research into the impact of chemical contaminants on the health of stingless bees must be species-specific.

insects

Article

Chemical Contaminants in Cerumen Samples from Ecuadorian Stingless Bees: Reporting Glyphosate, Aminomethylphosphonic Acid, and the Presence of Metals and Metalloids

Joseline Sofía Ocaña-Cabrera [1], Jorge Ron-Román [2], Sarah Martin-Solano [3] and Claude Saegerman [1,*]

[1] Research Unit of Epidemiology and Risk Analysis Applied to Veterinary Sciences (UREAR-ULiège), Fundamental and Applied Research for Animal and Health (FARAH) Centre, Faculty of Veterinary Medicine, University of Liège, Quartier Vallée 2, Avenue de Cureghem 6, B43a, 4000 Liège, Belgium; jocana@doct.uliege.be

[2] Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería Agropecuaria, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Campus Politécnico Hacienda el Prado Selva Alegre, Sangolquí 171103, Ecuador; jwron@espe.edu.ec

[3] Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, Av. Gral. Rumiñahui S/N, Sangolquí 171103, Ecuador; ssmartin@espe.edu.ec

* Correspondence: claude.saegerman@uliege.be; Tel.: +32-4-366-45-79

Simple Summary

Stingless bee cerumen is made of wax and plant resins. While collecting materials, these Meliponine bees may pick up chemical contaminants, which can enter their nests. This study investigated chemical pollutants in Ecuadorian cerumen, focusing on glyphosate (GLY), aminomethylphosphonic acid (AMPA), pesticides, metals, and metalloids. Researchers used advanced chromatography techniques to detect contaminants. Glyphosate and AMPA were found in samples from the highlands, while metals and metalloids were detected in both the Amazon and highland regions. No other pesticides were found. The risks to humans are minimal, though one stingless bee species (*Tetragonisca angustula*) may be more affected. Cerumen could be useful for monitoring environmental pollution. Clear guidelines are needed for its safe use and production.

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Abstract

Stingless bee cerumen is a mixture of wax and plant resins. Foragers of stingless bees are exposed to various chemical contaminants during their plant visits and collection activities. These contaminants have the potential to be transferred into the nest. This study aimed to elucidate the existence of chemical contaminants in Ecuadorian cerumen. To this end, the following aims were established: (i) to determine and quantify glyphosate (GLY), aminomethylphosphonic acid (AMPA), some other pesticides, metals and metalloids in cerumen and (ii) to establish possible risks associated with the presence of these chemical contaminants to the health of stingless bees and humans. The quantification of chemical contaminants was conducted using gas chromatography (GC), liquid chromatography (LC), and ion chromatography (IC) coupled to mass spectrometry (MS). Glyphosate (0.02–0.2 mg/kg) and AMPA (0.028 mg/kg) were detected in four of the pooled samples (n = 14) from the northern and southern highland regions. Other pesticide traces were not detected in any cerumen samples. Metals (Cd, Cr, Pb, Ni, Sn) and metalloids (As, Sb, Se) were found in all samples, including highlands and the lower Amazon. The potential risks of exposure to glyphosate and AMPA for stingless bees and humans appear to be minimal (except for the specific conditions given for *Tetragonisca angustula*) and safe, respectively. It seems that cerumen may serve as an effective biomonitoring matrix for assessing the environmental health of stingless bee nests. Establishing guidelines and regulations for the safe use and handling of products derived from the stingless bee consumption is therefore imperative.

Keywords: cerumen; Meliponini; environmental pollution; health risks; hazard quotient

1. Introduction

The term "cerumen" is used to describe a malleable structural material that is used by stingless bees to build, repair, adapt [1], and protect [2] their nests. The cerumen is composed of wax and plant resins [3]. According to the stingless bee species, cerumen is part of the involucrum, pillars, storage pots [4], brood cells [5], and the imprisonment chamber [6], which are located at the inner level of the nest. In addition, in certain stingless bee species, the cerumen is part of the entrance refinements at the outer level of the nest.

From a phytochemical perspective, resins are plant-derived secondary metabolites—comprising terpenoids, phenolics, and fatty substances [7]—that either form on plant surfaces or internally; in stingless bee colonies, these resins play a crucial protective role by deterring predators and intruders [8], while also contributing to the stingless bees' cuticular chemical profiles [9] aiding in colony member recognition. The proportion of the workforce engaged in resin collection has been estimated to be less than 10% of the total workforce in the *Trigona* genus [10], and up to 50–90% in other genera [11,12]. In the Neotropics, these figures are less than 20% of the total workforce in the *Melipona* genus [13,14]. The intensity of resin collection is influenced by several factors, including specific stingless bee species, weather conditions, seasonal variations, internal colony needs, external resource availability [15–17].

The foraging activity of stingless bees can transfer contaminants from the surrounding environment to the nest and subsequently to pot-honey and other stingless bee by-products. In areas where foraging grounds for stingless bees were polluted, several undesirable chemicals may enter stingless bee products via the nectar, pollen, or sugary exudates of plants that were grown there [18]. Plants can accumulate chemical pollutants in their tissues through various processes, including soil and water absorption and atmospheric adsorption. The bioaccumulation of these chemical pollutants is a gradual process, and as a result, they become biomagnified within the food chain [19].

The indiscriminate use of agrochemicals represents a growing threat to the sensory and cognitive abilities of foraging honey and wild bees [20–23]. Exposure to sub-lethal doses of pesticides has been demonstrated to compromise the neuronal plasticity of stingless bees during ontogenesis [24]. This has been shown to reduce the brain volume of worker stingless bees, particularly in the mushroom bodies and optic lobes, which has a detrimental effect on their foraging performance [25].

Due to the exposure to multiple agrochemicals through various routes in the field, stingless bees may be more susceptible to toxic effects, suggesting a need for more comprehensive toxicological experiments. A study conducted in Brazil evaluated the lethal and sublethal toxicity of various agrochemicals on an endangered native Brazilian bee species, *Melipona* (Michmelia) *capixaba* Moure & Camargo, 1994 [24]. Thiamethoxam induced a high mortality rate in this species of stingless bee, irrespective of the exposure route or dosage. Furthermore, a shift in the flight capacity was detected in response to the lowest observed dose via contact exposure. The administration of a sub-lethal dose of glyphosate resulted in elevated mortality rates following oral exposure, in addition to a significant impairment in the flight capacity of *M. capixaba* following contact exposure [22].

Despite the superiority of research conducted on honey bees compared to stingless bees regarding pesticide toxicity, most studies on the lethal effects of pesticides on stingless bees have focused on adults. However, experimental evidence suggests that ingestion of pollen and nectar contaminated with neonicotinoids and organophosphates can affect the health of larval stingless bees. The effects of exposure of the larvae of *Scaptotrigona bipunctata* to different doses of chlorpyrifos (an organophosphate compound) resulted in the production of lighter, smaller, and deformed adult workers [26]. The exposure to neonicotinoid products during the larval stage results in alterations to the brain [27]. On the other hand, the risks associated with exposure and contact with chemical pollutants, such as parathion, benomyl, and arsenic pesticides, can affect human health [28], and others, such as DDT, dieldrin, heptachlor [29,30], and neonicotinoids, can affect animal and environmental health [31,32].

Chemical compounds, including pesticides, herbicides, volatile organic compounds (VOCs), metals, and metalloids, can be defined as chemical pollutants [33]. However, metals and metalloids differ from other contaminants in terms of their environmental impact. Metals and metalloids are continuously emitted from both natural sources and anthropogenic activities. As they do not degrade, they enter into physical and biological cycles [34]. On the other hand, pesticides are dispersed in time and space by human action and, depending on the main chemical compound, are degraded by various environmental factors over a longer or shorter period [35].

Pesticides can become persistent environmental pollutants [36]. Among the most widely studied of these is glyphosate (GLY), due to its widespread use and the perceived threats to the ecosystem, human [37], and animal health [38]. Glyphosate, N-(phosphonomethyl) glycine, a broad-spectrum herbicide, is primarily used for weed control and canopy clearing before seeding [39]. Glyphosate can be degraded via bond cleavage. The C-N bond cleavage forms aminomethylphosphonic acid (AMPA), a persistent metabolite in the environment [40]. The C-P bond cleavage of glyphosate forms sarcosine and glycine, safer metabolites [41]. The photodegradation of amino-polyphosphonates [42] and the textile and paper industries [42] are other sources of AMPA. Aminomethylphosphonic acid accumulation has been reported in a variety of species, including plants [43], chickens [44], fish [45], and honey bees' wax [46].

Fungicides, pesticides, and herbicides contain a range of metals and metalloids, including iron (Fe), manganese (Mn), and arsenic (As). The recurrent utilization of these chemical compounds fortified with metals and metalloids, fertilizers, and organic matter, including sludge and sewage, can potentially result in extensive contamination, exemplified by elevated copper (Cu) and zinc (Zn) concentrations in soils employed for the cultivation of citrus and other fruit crops [47]. Some trace metals, such as cadmium (Cd) and lead (Pb), enter the soil as fertilizer impurities [48]. Several anthropogenic activities contribute to the release of nickel (Ni) and chromium (Cr) into the environment, including iron and steel production, food processing, waste incineration, fertilizers, mining and metallurgy, the textile industry, paints and pigments, tanning, the chemical industry, and the leather industry [49,50]. Metals such as Pb [51], Zn, Cr, Cd, and metalloids such as As [52] have been reported in pot-honey, propolis [53], geopropolis [54], pot-pollen, beebread, and wax [55] from stingless bees. Studies of metals, metalloids, and pesticides in cerumen (a mixture of wax and plant resins) in stingless bees are, to the best of the authors' knowledge, scarce or non-existent. The present study included the detection of seven metals: cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), tin (Sn), nickel (Ni), and lead (Pb), and three metalloids: antimony (Sb), arsenic (As), and selenium (Se).

The accumulation of pollutants in bee products can be attributed to the prevalence and persistence of these chemical compounds in the environment [56]. Additionally, bees collect chemical contaminants from plants (nectar, pollen, and resins) that have been contaminated with pesticides, metals, metalloids, and persistent organic pollutants (POPs) [57]. Meliponiculture, an economic activity that consists mainly of collecting products derived from stingless bees for human use, is a topic that continues to gain recognition worldwide. However, there is currently no regulation in place, for example, within the Codex Alimentarius, regarding the quality or safety of these types of products. Brazil has developed national regulations, but these only apply to pot-honey from the *Melipona* genus. Ecuador has no such regulations. For this reason, Brazilian regulations will be used for comparison wherever possible, alongside references to European honey bee products and World Health Organization (WHO) regulations on the risk of human contaminant intake.

The present study was thus designed to elucidate the existence of chemical contaminants in stingless bee nest products. The specific objectives were as follows: (a) to determine the presence of glyphosate, its metabolite aminomethylphosphonic acid (AMPA), other pesticides, metals, and metalloids in cerumen; and (b) to establish possible risks associated with the presence of these chemical contaminants to the health of stingless bees and humans.

2. Materials and Methods

2.1. Sample Geographical Description

Cerumen samples were obtained from ten localities in two distinct geographical regions: the Amazon and the highlands. Cerumen samples (n = 41) were collected in March 2019 in two highlands provinces, Loja province (seven localities), in August, September, October, and December 2023, and in July 2023 in Imbabura province (one locality) (Figure 1). Cerumen samples were also taken in January 2024 from Napo province (two localities) in the Amazon region. Cerumen samples were taken from storage pots and involucrum during the transfer of natural nests to wooden boxes for easier handling, and from nests in wooden boxes that had been established for a long time. The amount taken varied according to each nest, and care was always taken not to deprive the colony of this important material.

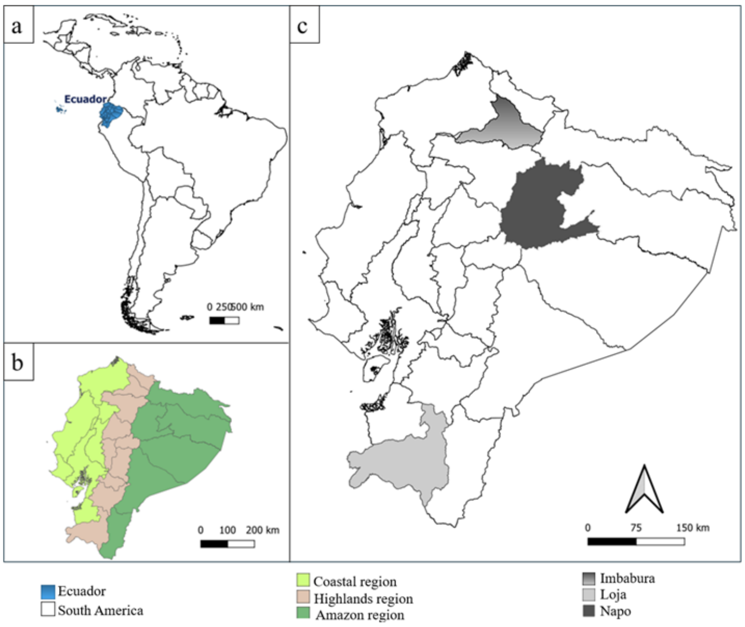

Figure 1. (**a**) Geographical location of Ecuador in South America. (**b**) Natural regions of continental Ecuador. (**c**) Sampled provinces of Ecuador.

To fulfil the requisite minimum weight for laboratory analysis of cerumen and reduce costs, a method of pooling the cerumen was employed. The composition of the pools was primarily determined by the proximity of the samples (Table 1).

Table 1. Sampling sites and the organization of cerumen pools.

Ecuadorian Region	**Province**	**Localities**	**#n.°. Pool**	**# n.°. Samples Within the Pool**	**Stingless Bee Genera ***
Southern Highlands	Loja (mostly crop environment)	Naranjo, Huertas, Faique, Algarrobillo	1	10	*Scaptotrigona* (58%) *Melipona* (42%)
		Caminuma, Panecillo	2	5	*Scaptotrigona* (81%) *Melipona* (19%)
		Arenal	3	7	*Scaptotrigona* (74%) *Nanotrigona* (14%) *Paratrigona* (12%)
			4	3	*Scaptotrigona* (65%) *Melipona* (35%)
Northern Highlands	Imbabura (crop area and secondary forest)	Intag	5	2	*Partamona* (50%) *Trigona* (50%)
			6	3	*Partamona* (100%)
Low Amazon	Napo (urban environment-Archidona) (primary and secondary forest-Agua Santa)	Archidona	7	2	*Melipona* (50%) *Nanotrigona* (50%)
			8	1	*Melipona* (100%)
		Agua Santa	9	4	*Tetragonisca* (83%) *Nanotrigona* (17%)
			10	1	*Scaptotrigona* (100%)
			11	1	*Melipona* (100%)
			12	1	*Trigona* (100%)
			13	1	*Scaptotrigona* (100%)
			14	1	*Melipona* (100%)

* Images of genera of stingless bees from Ecuador were included in Appendix A.

2.2. Chemical Contaminants Selected for Analysis

A total of seventeen pesticides (Table 2) were selected for detection and quantification in cerumen samples, including GLY and AMPA, as well as seven trace metals: Cd, Cr, Cu, Hg, Sn, Ni, and Pb, and three trace metalloids: Sb, As, and Se. Note that Se is one non-metal but sometimes considered as a metalloid. We encapsulated Se as a metalloid in this paper. The rationale behind this selection was twofold: firstly, the recurrent use and reporting of these pesticides in the country, and secondly, the importance of stingless bees as pollinators in the region [58–60].

Table 2. Description of pesticides used in Ecuador and selected for analysis.

Name	**Type**	**Group**	*** CAS Number**	**Main Crop Use in Ecuador**
Acetamiprid	Insecticide	Neonicotinoid	135410-20-7	Vegetable, fruit, and ornamental crops

Carbendazim (benomyl)	Fungicide	Benzimidazole	10605-21-7	Roses, rice, bananas, coffee crops
Chlorantraniliprole	Insecticide	Ryanoids	500008-45-7	Corn crops
Clothianidin	Insecticide	Neonicotinoid	210880-92-5	Tomato, broccoli, roses
Cypermethrin	Insecticide	Pyrethroids	52645-53-1	Corn and broccoli crops
Deltamethrin	Herbicide, Acaricide	Pyrethroids	52918-63-5	Potatoes, grapes, and rose cultivation
Diafenthiuron	Herbicide	Sulfonylurea compound	80060-09-9	Tomatoes, beans, roses
Diazinon	Insecticide	Organophosphorus	333-41-5	Roses, rice, fruits
Flonicamid	Insecticide	Pyridine carboxamides	158062-67-0	Roses, tomatoes
Glyphosate + AMPA (its metabolite)	Herbicide and crop desiccant	Organophosphorus	1071-83-6, 1066-51-9	Weeds, perennial shrubs
Imidacloprid	Insecticide	Neonicotinoid	138261-41-3	Potatoes, corn, fruits, and vegetable crops
Malathion (malaoxon)	Insecticide	Organophosphorus	121-75-5	Rice and corn crops
Methomyl	Insecticide	Carbamates	16752-77-5	Rice and corn crops
Pyrethrin I	Insecticide	Pyrethrins	8003-34-7	Avocado, blueberry, potatoes
Thiacloprid	Insecticide	Neonicotinoid	111988-49-9	Roses crops
Thiamethoxam	Insecticide	Neonicotinoid	153719-23-4	Potatoes, African palm, and cocoa crops
Trichlorfon	Insecticide, Anthelmintic	Organophosphorus	52-68-6	Human and animal drugs

* CAS number: unique identification number, assigned by the Chemical Abstracts Service (CAS).

2.3. Chemical Extraction and Detection Process

The analysis was carried out in July 2024 by the GIRPA laboratory (France), COFRAC ESSAIS accredited under the number N° 1-6951 for laboratory analysis by the French competent authority.

2.3.1. Sample Preparation for Multi-Residue Analysis

Cerumen samples (0.25 g) were homogenized using ceramic homogenizers and transferred into a 7 mL polypropylene (PP) tube. A 5 mL solution of isopropanol/hexane (50/50, *v*/*v*) was added and shaken for one minute. After freezing for an hour, the tube was centrifuged (9000× *g*, 5 °C), and the supernatant was evaporated to form an oily residue. The extract was reconstituted with acetonitrile, then dispersed, and $MgSO_4$ + PSA was added and shaken. After centrifugation, an aliquot of the supernatant was diluted with water and filtered before LC-MS/MS analysis (LC Exion system and API 7500 triple quadrupole mass detector (Sciex, Framingham, MA, USA), column C18 Synergi Hydro-RP 100 mm × 3 mm; 2.5 µm (Phenomenex, Torrance, CA, USA)). An additional aliquot of the supernatant was concentrated (nitrogen stream) and reconstituted with ethyl acetate before GC-MS/MS analysis (Chromatograph 8890 and 7010 quadrupole mass detectors (Agilent Technologies, Santa Clara, CA, USA), columns 1 and 2 HP-5 MS (15 m × 0.25 mm ID; 0.25 µm) (Agilent Technologies)).

The limit of quantification (LOQ) was 20 µg/kg for cypermethrin, flonicamid, malaoxon, thiacloprid, 50 µg/kg for diafenthiuron, imidacloprid, pyrethrin I, and 10 µg/kg for the other ten pesticides.

The validation method was based on five recoveries at LOQ plus five recoveries at 10xLOQ. Appendixes B and C show LC-MS/MS (acetamiprid, benomyl, carbendazim, methomyl, flonicamid, thiacloprid, thiamethoxam, malaoxon, malathion, chlorantraniliprole, trichlorfon, pyrethrin I, imidacloprid, diafenthiuron), GC-MS/MS (diazinon, cypermethrine, deltamethrine), detection parameters, and detailed validation data.

2.3.2. Sample Preparation for Glyphosate (GLY) and AMPA Detection

A 0.25 g sample was weighed in a 7 mL PP tube with ceramic homogenizers. A radiolabelled glyphosate 13C-15N standard was added. 1.25 mL of methanol/formic acid (100/1, *v*/*v*) was added and shaken for 30 s. Another 1.25 mL of ultrapure water was added, and the tube was agitated for 30 s. The tube was centrifuged (3000× *g*), and 2 mL of the supernatant was collected and washed with 1 mL of hexane. The hexane layer was discarded, and an aliquot of the extract was filtered before LC-MS/MS analysis.

For both compounds, the limit of quantification (LOQ) was 10 μg/kg. The validation method was based on five recoveries at LOQ plus five recoveries at 10xLOQ. For LC-MS/MS (LC Exion system (Sciex) and API 6500+ triple quadrupole mass detector (Sciex), column Metrosep A, Supp 5 (150 mm × 4 mm ID; 5 μm) (Metrohm, Herisau, Switzerland)), detection parameters, and detailed validation data, see Appendix D.

2.3.3. Sample Preparation for Metals and Metalloid Trace Detection

A microwave-assisted acid digestion process was conducted on 0.25 g of the homogenized sample with 10 mL of concentrated ultrapure nitric acid ($\omega \geq 67\%$). A microwave-assisted heating program was applied, utilizing a ramp time of 25 min and a holding time of 10 min at 240 °C. Upon cooling, the extract was diluted to 50 mL with ultrapure water. IC-MS was then used to trace inorganic analytes. We detail the LOQ limits along with the concentrations found in the results section.Table 4.

The validation method was based on five recoveries at LOQ plus five recoveries at 10xLOQ. See Appendix E for details about the limit of quantification, IC-MS (Model 7850 (Agilent Technologies), automatic sample changer model SPS 4 (Agilent Technologies), and micromist Nebulizer (Agilent Technologies)), detection parameters, and detailed validation data.

2.4. Statistical Analysis

The Dunn–Kruskal–Wallis multiple comparison test and *p*-values adjusted with the Bonferroni correction were employed to identify significant differences between the mean ranks of each chemical contaminant concentration according to the sampling location (Ecuadorian region). The univariate analysis (General Linear Model) was used to ascertain a correlation between the detection of chemical contaminants and the geographical locations from which they were sampled.

2.5. Risk Assessment of Glyphosate Exposure in Stingless Bees

The cerumen "pools" sampled in this study indicate the environmental health surrounding the meliponaries (nest set) in the localities under investigation. It is interesting to provide a preliminary estimation of the possible impact of glyphosate, as detected in cerumen samples in this study.

To estimate the risk for stingless bees of exposure to glyphosate we used the Risk Quotient (RQ) (Equation (1)) as the ratio between an exposure estimate, in this study defined as the concentration of the residue (mg/kg) and a point estimate of effect, in this study defined as the acute oral LD_{50} (expressed as μg/bee or μg/L) Table 3.

$$\text{Risk Quotient (RQ)} = \text{Exposure/Toxicity} = \text{(Estimated concentration of GLY in cerumen)/(}LD_{50}\text{ of GLY for stingless bees)}, \quad (1)$$

Table 3. The concentration of exposure to glyphosate in the stingless bee *T. angustula*.

Stingless Bee Species	Glyphosate (GLY) Concentration (LD_{50})	Time of Exposure Until 100% of Death	Type of Exposure	Stingless Bee Stage	Reference
Tetragonisca angustula	0.015 µg a.i./bee (95% CI: 0.005–0.04) (GLY mixed with honey/water)	48 h	Orally	Adult bees (foragers)	[61]

a.i., active ingredient; CI: confidence interval; µg: micrograms; h: hours.

For this study's screening level assessment of GLY, the Limit of Concern (LCO) was defined as follows: LCO = 0.4 for acute exposure and LCO = 1 for chronic exposure [62]. If the obtained RQ values were below their respective level of concern, it can be presumed that the risks to stingless bees were minimal.

We set 4 g of cerumen as the amount handled/chewed by stingless bees [63]. The body weight of stingless bees was set at 4.68 mg for *Tetragonisca angustula* [64].

2.6. Glyphosate Human Exposure Risk Assessment

According to the reports of Paredes (2022) [65], based on 14 personal interviews and the personal communication conducted by the authors of this study with 16 Ecuadorian stingless bee keepers and their families, cerumen has the following main uses: cleansing rituals involving burning of cerumen within the domestic environment, eating honey-pots [66,67], the application of cerumen slides on the scalp of neonates, and direct application to the skin in regions where an injury has occurred. The potential for adverse human health effects can arise through exposure by inhalation, ingestion, and dermal contact [68].

To estimate the human health risk of exposure (Equation (2)) to GLY, we used two toxicological safety thresholds established by EFSA in 2015 and currently applicable [69], and adjusted to the reality of meliponiculture in Ecuador. In this study for healers [65], we set 1.5 mg/kg human body weight per day for the acute reference dose (ARfD), and for consumers [70,71], we set 0.5 mg/kg human body weight per day for the acceptable daily intake (ADI).

To estimate the amount of cerumen daily handled/chewed, we used 0.80 g for the weight of a cerumen pot as the minimum unit of handled/chewed (the pot weight is closely related to the stingless bee species) [72,73]. The average weight of the Ecuadorian population that may be exposed to these risks is exemplified as follows: 67.9 kg for men, 62 kg for women [74], and 3.2 kg for newborns [75].

We have posited a worst-case scenario to assess the risks of the daily consumption/skin contact of cerumen pots for human health. We multiplied the highest levels of consumption/skin contact of cerumen by the highest GLY and AMPA residues quantified in pooled samples and added a factor for the dilution required to prepare the pools (i.e., for pool 1, the factor was 10).

$$\text{Human health risk assessment} = \left(\text{GLY \& AMPA}_{\text{concentration}} \frac{mg}{kg_{cerumen}}\right) \times \text{consumption/skin contact rate} \frac{kg_{cerumen}}{kg_{body\ weight}\ per\ day} \times \text{dilution factor} \quad (2)$$

Ultimately, the levels of glyphosate exposure were compared with the toxicological reference values previously mentioned to describe the potential risk.

3. Results

Fourteen "pools" of cerumen samples were analyzed. Samples were obtained from two regions, 3 provinces, 10 localities, 17 meliponaries, and 41 stingless bee nests. A comprehensive account of the residues of contaminants quantified in cerumen pools is presented in Table 4.

The results of the multi-residue analysis did not identify any of the 16 agrochemicals analyzed.

3.1. Metal and/Metalloids Trace Detection

The minimum number of chemical compounds (metals, metalloids, glyphosate, and AMPA) detected in each sample was 3/10, with a maximum of 9/10. The number of compounds identified in each region was as follows: southern highlands 6/10, northern highlands 9/10, and low Amazon 5/10.

Each compound's mean concentration ± standard error (minimum and maximum values) was calculated and presented in mg/kg. The main results for arsenic (As) were 0.133 ± 0.087 (0.028–0.27), chromium (Cr) 1.528 ± 2.304 (0.11–7.1), and lead (Pb) 0.247 ± 0.393 (0.031–1.5).

A single pool was identified as having tin (Sn) and selenium (Se). The positive pools were five and three, with respective concentrations of 0.17 mg/kg and 0.16 mg/kg (see Table 4).

3.2. Glyphosate (GLY) and AMPA Detection

The mean concentration of glyphosate ± standard error (minimum and maximum values) was 0.096 ± 0.094 mg/kg (0.014–0.2) in pools 1, 2, 3, and 5 from northern and southern highlands, while the mean concentration of AMPA was 0.028 mg/kg in pool number five (northern highlands). It was noted that this pool contains both GLY and AMPA.

Significant differences were observed in the mean rank concentrations of arsenic, chromium, and lead (Dunn Test with Bonferroni correction, p-value = 0.04, 0.03, and 0.02, respectively) between the low Amazon (Napo) and northern highlands (Imbabura) regions. The detection of glyphosate in cerumen samples from north and southern highlands did not yield statistically significant differences (Table 5). The latter compound was not detected in the low Amazon region.

Table 4. Chemical contaminants detected in cerumen pools.

	Highlands							Amazon							Basic Sample Statistics					Permitted Levels		
Pool / ChC	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Av ± SD**	**Min**	**Max**	**No**	**%**	**Brazil. Legis. for SB**	**EU. Guid. for HB**	**WHO for Humans**
Sb		0.04	0.063	0.063	0.038		0.028								0.046 ± 0.016	0.028	0.063	5	36			
As	0.083	0.14	0.14	0.087	0.25	0.27			0.064		0.028				0.133 ± 0.087	0.028	0.27	8	57	0.30	1	
Cd	0.025	0.64	4.4	1.4	0.1	0.048	0.018	0.009	0.1	0.068	0.093	0.034	0.056	0.051	0.503 ± 1.184	0.009	4.4	14	100	0.10		0.008
Cr	0.34	1.7	0.74	0.32	7.1	6	0.11	0.25	3.3	0.29	0.66	0.22	0.15	0.21	1.528 ± 2.304	0.11	7.1	14	100	0.10		0.0003-0.00013
Sn					0.17										0.170	0.17	0.17	1	7			
Ni	0.13	0.52	0.63	0.3	1.8	1.4	0.093	0.08	0.95	0.13	0.37	0.71	0.48	0.19	0.556 ± 0.518	0.08	1.8	14	100	5		0.5
Pb	0.16	0.26	0.32	0.13	1.5	0.35	0.044	0.031	0.23	0.036	0.082	0.034		0.035	0.247 ± 0.393	0.031	1.5	13	93	0.30	0.10	0.05
Se			0.16												0.160	0.16	0.16	1	7			
GLY	0.02	0.014	0.15		0.2										0.096 ± 0.094	0.014	0.2	4	29	0.05	0.05	0.5
AMPA					0.028										0.028	0.028	0.028	1	7		0.2	

All values are concentrations in mg/kg. Blank spaces represent a numerical value under the Limit of Quantification (LOQ) or absence of limit values. Sb = Antimony (LOQ = 0.02 mg/kg); As = Arsenic (LOQ = 0.02 mg/kg); Cd = Cadmium (LOQ = 0.008 mg/kg); Cr = Chromium (LOQ = 0.02 mg/kg); Sn = Tin (LOQ = 0.1 mg/kg); Ni = Nickel (LOQ = 0.02 mg/kg); Pb = Lead (LOQ = 0.02 mg/kg); Se = Selenium (LOQ = 0.1 mg/kg); AMPA = Aminomethylphosphonic acid (LOQ = 0.01 mg/kg); GLY = Glyphosate (LOQ = 0.01 mg/kg). ChC = Chemical contaminant, Av = average; SD = standard deviation; Min = minimum value; Max = maximum value; No. = number; % = percentage. Permitted levels of contaminants: Brazilian legislation applicable to honey produced by stingless bees (Brazil Legis. for SB) [76,77], European guidelines for honey of *Apis mellifera* (EU. Guid. for HB) [78,79], World Health Organization's maximum level of daily ingestion of contaminants for humans (WHO for humans) [80].

Table 5. Summary of the *p*-value adjusted with the Bonferroni correction for multiple comparison tests between chemical contaminants' mean ranks and sampling locations.

	Low Amazon—Northern Highlands	Low Amazon—Southern Highlands	Northern—Southern Highlands
Glyphosate			0.1797
Antimony	1	0.2754	0.7831
Arsenic	0.0411 [a]	0.4544	0.4644
Cadmium	1	0.2237	1
Chromium	0.0378 [b]	0.4297	0.6425
Nickel	0.0805	1	0.2344
Lead	0.0255 [c]	0.1603	0.8981

[a,b,c] significant differences ($p < 0.05$).

3.3. Risk Assessment of the Presence of Agrochemicals and on the Health of Stingless Bees and Humans

Metals such as cadmium and metalloids such as antimony and arsenic appear to be associated with the geographical region of Ecuadorian habitats of stingless bees (Table 6).

Table 6. Univariate analysis. Location (independent variable) versus each chemical contaminant detected (dependent variable).

Variable	Estimated Coefficient	Standard Error	Z	*p*-Value	95% Confidence	
					Lower Limit	Upper Limit
Glyphosate	−5.59	3.82	−1.462	0.169	−13.07	1.90
Antimony	−25.79	7.97	−3.36	0.005 [a]	−40.82	−10.76
Arsenic	−5.83	2.27	−2.57	0.024 [b]	−10.28	−1.39
Cadmium	−0.44	0.18	−2.42	0.033 [c]	−0.79	−0.08
Chromium	−0.06	0.11	−0.52	0.616	−0.28	0.16
Nickel	−0.23	0.50	−0.46	0.658	−1.22	0.76
Lead	−0.68	0.66	−1.03	0.325	−1.97	0.62

[a,b,c] significant differences ($p < 0.05$).

In our study, the stingless bee genera represented in the glyphosate and AMPA-positive cerumen (pools 1, 2, 3, and 5) were the genera *Melipona*, *Scaptotrigona*, *Partamona*, and *Trigona*. To illustrate the RQ calculation, we developed a *T. angustula* worst-case scenario. The last means a cumulative exposure of GLY (the highest concentration of this study) and AMPA (the only concentration identified in this study) over *T. angustula* foragers. The toxicity was set at 0.015 μg a.i./bee (see Table 3 and the methodology for handling and chewing). Exposure calculation included (0.02 + 0.028) mg GLY+AMPA/kg cerumen * 0.004 kg cerumen = 0.000912 mg GLY+AMPA. The toxicity calculation included 0.000015 mg a.i. for GLY/*T. angustula*. The estimation of RQ was equal to 60.8. Considering the 95% CI for the 48 h oral LD_{50}, the RQ is between 22.8 and 182.4. This RQ represents a case of acute exposure [60].

Using the highest concentration of glyphosate found in this study, 0.2 mg/kg, and the highest cerumen handled and chewed, based on 0.80 g of cerumen and the average weight of Ecuadorian neonates, women, and men, none of the three toxicological safety thresholds exceeded the ADI and ARfD thresholds established by EFSA [70].

4. Discussion

Stingless bee cerumen is sometimes compared to honey bee propolis, but cerumen serves as the primary building material in the nest and is continuously reworked and recycled. A more accurate comparison is with honey bee wax, part of a permanent honeycomb structure [81]. In addition, in some Latin American communities, cerumen, as a by-product of stingless bees, has been used in folk medicine to improve human health, typically in the form of infusions added to beverages [71].

4.1. Glyphosate (GLY) and AMPA in Stingless Bees' Cerumen

Glyphosate is a globally used herbicide [39], and as a pesticide, it is a pollutant that harms non-target organisms. There is no consensus on its toxicity among specialists around the world. Glyphosate is absorbed across the leaves and stems and is translocated throughout the plant [82]. The foraging activities of stingless bees may result in the inadvertent introduction of agrochemicals applied to plants into nests [83].

AMPA, the major degradation metabolite of glyphosate, along with glyphosate itself, persists in the environment due to its binding to soil particles; thus, they are accumulated in surface layers and dispersed via various means, including surface runoff, windborne dust, and vertical transport in the soil to groundwater and aquatic sediments [84,85]. There is evidence to suggest that the presence of glyphosate in soil can enhance microbial activity [86], including bacteria involved in its degradation process (*Bacillus* and *Proteus*) [87]. These bacteria are phylogenetically related to bacteria found inside the nests of four stingless bee species in Brazil, which are associated with larval development [88]. It can thus be postulated that the presence of these bacteria in the stingless bees' nest, in conjunction with specific environmental conditions, contributed to the degradation of glyphosate to AMPA in cerumen as a matrix. However, further research is required to ascertain the potential role of cerumen bacteria in the degradation of this herbicide.

Glyphosate and AMPA residues were found in beeswax samples, reporting that those maximum concentrations may cause sublethal effects in honey bees [46]. Wax is primarily composed of hydrocarbons and can act as a lipid sink for lipophilic pesticides. In contrast, glyphosate and AMPA are polar pesticides. This herbicide gains access to a matrix such as cerumen in two ways: indirectly, via stingless bees' foraging activities, and directly, due to its solubility in compatible compounds, including terpenoids and hydrophilic phenols, which may be present in plant resins.

The toxicity of a pesticide may vary depending on the bee species [89,90]. However, in the case of *Partamona helleri*, a stingless bee species belonging to one of the genera represented in this study, acute oral exposure to commercial formulations of glyphosate resulted in increased glutathione S-transferase (GST) activity and 100% survival after exposure to 7400 μg/mL of herbicide. Although its potential to alter feeding behaviour, resulting in a decrease in food intake, is known [91]. Two of the samples in this study, in which glyphosate was detected, 0.15–0.2 mg/kg, exceeded the permitted limit for European honey from honey bees, 0.05 mg/kg.

Adult bees of the *Tetragonisca angustula* exposed to concentrations of 400 μg/L, which were found in water bodies close to soybean crops, showed no short-term mortality effect. However, when the stingless bee *T. angustula* directly consumed 178 g/L of the product, they died rapidly [92]. A recent study estimated a 48 h oral LD_{50} for *T. angustula* foragers to equal 0.015 μg a.i./stingless bee, with a 95% CI: 0.005–0.040 [61]. This has been observed to affect the worker's locomotion, behaviour, and biology. Further research is required on the various stingless bee species to elucidate their potential impact on survival. Furthermore, additional research is essential to examine lethal and sublethal glyphosate effects in conjunction with other pesticides. For example, studies should investigate the impact of glyphosate on larval mortality in *Melipona scutellaris* [93]. This may provide a more comprehensive explanation of the acute risk quotient (RQ) values found in this study and why they exceed the Level of Concern according to the Guidance for Assessing Pesticide Risks to Bees [62].

Although the estimated thresholds of human health risks from glyphosate exposure in this study did not appear to be cause for concern, further research is recommended. Humans can be exposed to glyphosate and AMPA particles daily. This exposure can occur through the inhalation of dust particles present in domestic environments. The concentration of these particles is influenced by several factors, including the geographical location of the residence (rural or urban), the proximity of crops, and the use of herbicides on driveways or lawns [94]. Glyphosate and its metabolites have been linked to a slight increase in the incidence of non-Hodgkin lymphoma, as well as to the induction of genetic damage, increased oxidative stress, interference with the estrogenic pathway, and altered brain function. It also has mutagenic/carcinogenic potential and endocrine-disrupting effects (EDEs) on the human reproductive system [68].

4.2. Metals, Metalloids, and Micronutrient Elements in Cerumen

Arsenic (As) concentrations are strictly related to geographical locations. The most toxic forms of arsenic are inorganic arsenic (As III) and arsenic (As V). The latter is the form of arsenic associated with bioaccumulation in plant pollen or nectar, which subsequently enters stingless bee nests [95]. The concentration of arsenic in European honey bee propolis was found to be in the range of 0.075 to 0.66 μg/g [96,97], a reliable arsenic indicator, especially compared to honey from honey bees. The concentration of arsenic found in this study falls within the range above, suggesting the usefulness of stingless bee cerumen as a good indicator of metalloids in the environment. *Apis mellifera* honey samples from Chile showed total arsenic concentrations between 0.0022 and 0.1719 mg/kg, and up to 0.0246 mg/kg of inorganic arsenic, with honey being a good indicator of environmental pollution [98]. Volcanoes and their emissions, mineralised zones, fluid migration, gas emissions (particularly in regions of geographic faulting), and hydro(bio)geochemical processes are common sources and routes of arsenic in Latin America. These sources and routes are directly responsible for the discharge of arsenic into near-surface ecosystems, where it contaminates soil, water, dust, and aerosols, as well as living organisms, plants, and humans [99]. Forager bees exposed to the highest concentrations of arsenic have severe growth defects and deficits in learning and memory [100]. The human health effects of chronic arsenic poisoning include cardiovascular disease and neuropathy. In women, they include miscarriage, premature birth, and low birth weight, even at low levels of exposure [101].

A high concentration of cadmium (Cd) in soil results in increased plant uptake, leading to elevated human exposure through the consumption of fruits and vegetables [102]. Cocoa plantations in coastal and Amazonian regions of Ecuador clearly illustrate it [103], which are endemic to areas with stingless bee populations. In these areas, cocoa beans were labelled to exceed European Cd guidelines. The findings of Barraza et al. (2017) [104] indicate that agricultural practices, rather than oil activities, are the primary source of Cd in Ecuadorian soils. This could explain the higher concentrations of Cd observed in cerumen, 0.64, 1.4, 4.4 mg/kg, from areas of intensive agriculture in the country's southern highlands, which also exceed the limit, 0.10 mg/kg, set by Brazilian legislation for stingless bee honey.

Lead (Pb) is a naturally occurring metal. Humans are exposed to it through inhalation, ingestion, and dermal contact [105]. In the Amazonian region of Ecuador, lead human contamination through food and water is associated with oil exploitation [106] and mining in southern provinces. The lead concentrations found in the cerumen of the stingless bee genera *Scaptotrigona* and *Partamona* from the Ecuadorian highlands exceeded the European safety guideline of 0.10 mg/kg for honey produced by honey bees [79] and also those set by Brazilian legislation for stingless bee honey [76,77]. In Brazil, samples of "samburá" (fermented pollen) and wax from *Melipona subnitida* had high Pb concentrations (7.4–1.3 mg/kg, respectively) [55].

The U.S. Food and Drug Administration (FDA) prioritizes the assessment of cadmium (Cd) and lead (Pb) to ensure food safety, given the potential harm that these metals can cause to human health, particularly during human brain development [107]. According to the latter guidelines, the Pb and Cd concentrations in the cerumen samples from the highlands of this study also exceed the established limits for *A. mellifera* honey (0.30 and 0.10 mg/kg, respectively). After 10 days of exposure to Cd and Pb, a constant bioaccumulation was observed in the bodies of honey bees, along with an association with higher levels of α-tocopherol (the most active form of vitamin E, with antioxidant function) [108].

The levels of cadmium found in different honey bee and stingless bee products in different Latin American countries are as follows: 0.070–2.875 mg/kg in Peru [109]; 2.1–3.4 mg/kg in Mexico [110]; 0.008–0.009 mg/kg in the Amazon region and southern Brazil [111]; and 0.044 mg/kg in Chile [112]. Our results are comparable with those for the lowest concentrations, such as in the Amazon, and the highest concentrations, such as in the Highlands. This confirms that the detection of this metal depends heavily on the region and the anthropogenic activities that may be the source of these metals in each area. Lead concentrations in bee products in Peru: 0.001–2.478 mg/kg [108], 0.015–0.148 mg/kg in Brazil, and 0.339 mg/kg in Chile [111] show that concentrations of this heavy metal in the samples studied remain relatively similar to those reported.

Chromium (Cr III) is essential for all living organisms, including humans [113]. The contamination of land and waterways with hexavalent chromium has increased as a consequence of human activity, including the processing of metals and metalloids and leather, the textile industry, steel welding, and the use of pigments [114]. Furthermore, it is a relatively soluble anion that is highly active and mobile in soil matrices [115]. The contamination of the environment with chromium is a significant threat, particularly to water and soil [116], which are two of the main resources for stingless bee nests. Our analysis revealed that nine of fourteen cerumen samples exceeded the Cr limits set forth by Brazilian legislation of maximum limits for inorganic contaminants in foodstuffs, which also applies to pot-honey from stingless bees [18]. The chromium concentration in honey bees in Mexico was found to vary between 0.39 and 1.84 mg/kg [109] while the concentration in bee products from the same country varied between 0.001 and 4.52 mg/kg [117]. Concentrations in honey from stingless bees in Brazil range from 0.28 to 0.51 mg/kg [18]. The reported concentrations are similar to, and vary as much as, the concentrations observed in our study, depending on the geographical area.

The levels of chromium (Cr) that are acutely lethal are significantly higher than those normally found in bee matrices and the environment. This suggests a moderate risk of chromium in real-world scenarios for wild pollinators. However, chronic effects include impacts on larval development and cognitive impairment, similar to those observed with Pb [118]. Chromium (III) is an essential nutrient for humans as it plays a role in carbohydrate and fat metabolism. In contrast, Cr (VI) can lead to respiratory irritation, lung cancer, skin ulcers, and kidney and liver damage. Exposure to Cr (VI) can damage DNA and cellular repair mechanisms [119].

In Ecuador, contamination of crops by non-essential metals and metalloids has been a subject of growing concern over the past three decades, particularly in the Andean highland region, where several potentially contaminating elements from volcanic and subterranean sources, such as Tin (Sn) and Selenium (Se), were mentioned [120]. Nickel (Ni) and

Antimony (Sb) are both present in the environment and dispersed due to anthropogenic activities, for example, in sulphur compound mine drainage waters [121], and in human wastewater due to their use in treatments against human parasites [122], and the discharge of metals and metalloids in the form of gunshot residues (barium, antimony, and lead) due to military activities [123] or to hunting.

The foraging behaviour of stingless bees is responsive to environmental changes, particularly those related to pollutants such as metals and metalloids. Stingless bees serve as reliable indicators of environmental quality, a method known as biomonitoring [18]. However, the absorption of metals and metalloids alters the magnetic particles in stingless bees, affecting their orientation and food-foraging behaviour [124,125].

For humans, the primary routes of exposure to particles of substances such as arsenic, cadmium, lead, thallium, and mercury are ingestion, skin contact, or inhalation [126,127]. Excessive exposure to metals and metalloids in human internal tissues (absorption) can impact the central nervous system and act as a pseudo-cofactor or promoter of several illnesses [33].

The study areas in the northern and southern highlands of the country showed the highest levels of glyphosate/AMPA, metals, and metalloids, which is evidence of the unhealthy environment for stingless bees in these areas, where agriculture and mining are the main sources of pollution. Detecting glyphosate in cerumen samples inside stingless bee nests may represent a risk to nest health.

4.3. Absence of Several Other Pesticides in Stingless Bee Cerumen

Toxic contaminants in both managed and wild colonies of stingless bees prove that agrochemicals are a significant factor in their decline. Soil-applied chemicals are absorbed and transported by the plant to reproductive organs. Aerially applied chemicals are absorbed through the foliage. Both applications have the potential to permit the chemical to reach pollen and/or nectar [125].

The application of pesticides has been linked to a decline in resin collection visits, as it results in alterations to the odour (a combination of terpenes) of a resin [8,128]. Stingless bees utilize specific combinations of volatile mono- and sesquiterpenes to identify and differentiate resin sources, and thus, exposure to pesticides may disrupt this process [129].

Tetragonisca angustula, *Scaptotrigona postica*, and *Melipona scutellaris* are particularly susceptible to the effects of oral exposition of thiamethoxam, a neonicotinoid pesticide [130]. A study investigating the prevalence of organochlorine pesticides in honey and pollen of *Scaptotrigona mexicana* revealed that 88.44% of honey samples tested positive for at least one organochlorine pesticide. In comparison, only 22.22% of pollen samples showed evidence of contamination [131]. The most prevalent pesticides were heptachlor, γ-HCH, DDT, endrin, and DDE [131].

Few studies have reported the presence of pesticides in other stingless bee products [24,132,133], but there are almost none in matrices such as cerumen or geopropolis. This study's lack of detection of other pesticides in cerumen samples may be attributed to the prolonged cold storage period (maximum 4 years), which may have rendered the molecules undetectable. However, this does not explain the negative results observed in the fresher samples, which also exhibited no evidence of other pesticide contamination than glyphosate and its AMPA metabolite. Thus, in light of the absence of other pesticides than glyphosate and its AMPA metabolite in cerumen in the present study, it can be postulated that cerumen from Ecuadorian stingless bee species from the genera *Melipona*, *Scaptotrigona*, *Partamona*, *Nanotrigona*, *Paratrigona*, *Trigona*, and *Tetragonisca* from the ten specified Ecuadorian localities do not function as a matrix for the bioaccumulation of the 16 other agrochemicals selected for quantification in Table 2.

It was necessary to incorporate an additional factor to account for the glyphosate risk due to the dilution of detected chemical contaminants resulting from the mixing of cerumen samples in the methodology. However, there are two potential limitations to this approach. Firstly, there is a possibility that a single contaminated sample may have had sufficient chemical concentration to contaminate the potentially negative samples. Secondly, an overestimation of chemical contaminant concentration may have occurred when more than one contaminated sample was present within the same pool.

The susceptibility of different stingless bee species to a chemical contaminant is subject to interspecific variability [134]. The latter was supported by the concentrations of glyphosate, as documented in the literature, which demonstrated lethal effects on three species of stingless bees. In addition to the limited information on LD50 and LC50 of the herbicide in other species of stingless bees and the lack of information for equatorial species, another limitation was noted in the concentrations of glyphosate used in the literature studies.

In a study conducted by Seide et al. (2018) [135], 100% mortality was observed in stingless bee larvae reared in vitro with 3 µL of glyphosate. Given that the acceptable mortality level for stingless bees is 15% [136], the impact of glyphosate on *M. quadrafasciata* larvae appears to be considerable. Further information is required to refine the RQ estimation. This could be achieved by utilizing a range of doses and decreasing concentrations of glyphosate, commencing with the Seide et al. (2018) [135] dose and extending to subsequent dilutions.

In considering the potential impact on humans, it is essential to acknowledge the variability between individuals in terms of their consumption and/or skin contact with cerumen that may be contaminated [137].

To mitigate the potential risks and long-term effects associated with the presence of glyphosate, AMPA, trace metals, and metalloids on stingless bee larvae and adult bees, we propose the implementation of environmentally sustainable agricultural practices among meliponicultors and farmers in surrounding areas where native stingless bees are managed. This approach could facilitate the establishment of mutually beneficial agreements, aligning with the One Health vision.

The reduction in pesticide application, both individually and in cocktails, can be achieved through the establishment of agreements between meliponicultors and farmers for pesticide application, and the implementation of measures to prevent stingless bees from receiving pesticides. These include the relocation of nests or the utilization of quarantine with nests as a preventive measure, which contributes to improving overall management.

Further study of pesticide residues in other stingless bee products is recommended, as well as species-specific studies on the effects of both direct and indirect short- and long-term exposure.

Further studies on lethal doses in each stingless bee species of different pesticides, including glyphosate, are also recommended.

The estimation of consumption and/or skin contact of stingless bee products by humans is also important, and further studies are recommended on the possibility that contaminated matrices such as honey, pollen, or cerumen may be pathways for human health risks.

It is urgent to establish guidelines and safe values that regulate the production and commercialization of stingless bee products in Ecuador and the rest of the countries where Meliponiculture is rapidly entering the market for human consumption and/or skin contact.

5. Conclusions

It appears that cerumen may be an effective matrix for monitoring environmental contaminants, including glyphosate (GLY), AMPA, metals, and metalloids. However, it is not the optimal stingless bee product for pesticide monitoring.

Certain levels of glyphosate, cadmium (Cd), chromium (Cr), and lead (Pb) in the cerumen of stingless bees have been identified as exceeding the safe guidelines set out in European and Brazilian legislation for honey bee and stingless bee honey, respectively. Nevertheless, determining their potential hazards remains challenging without Ecuador establishing guidelines specific to this type of matrix.

Given the sampling environment of the stingless bee nests in the northern and southern highlands of the country, it is reasonable to conclude that agriculture and the intensive use of pesticides are the main sources of contamination in products such as cerumen.

The degradation of the environments in which stingless bees naturally live poses an emerging threat to the survival of these species, as well as to food security in relation to the use of their products for human health purposes.

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Appendix A

The following images of stingless bees from Ecuador were included in the cerumen study.

(**A**) (**B**)

(**C**) (**D**)

Figure A1. (**A**) *Melipona grandis*; (**B**) *Nanotrigona* sp.; (**C**) *Scaptotrigona* sp.; (**D**) *Tetragonisca angustula*. Fundación Yachana. Napo, Ecuador. Author: Alfonso Jiménez.

Appendix B

LC-MSMS detection parameters, and detailed validation data for multi-residue analysis

Appendix B.1. Chromatographic Parameters

Eluent A: ultrapure water + 0.1% acetic acid + 5 mM ammonium acetate
Eluent B: LC-MS grade methanol + 0.1% acetic acid + 5 mM ammonium acetate
Flow rate: 0.7 mL/min
Oven temperature: 60 °C
Injection volume: 10 µL
Gradient:

Table A1. Time and eluent conditions for the LC-MSMS Please add Caption.

Time (min)	% Eluent A	% Eluent B
0	80	20
0.1	80	20
1	50	50
9	20	80
12	0	100
13	0	100
13.5	80	20
15	80	20

Appendix B.2. Detection Parameters

Table A2. Summary of parameters obtained from the mass spectrometer.

Group ID	Compound ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (ms)	EP (V)	CE (V)	CXP (V)
		ESI positive					
Acetamiprid	acetamiprid_1	223.2	126	20	10	27	15
Acetamiprid	acetamiprid_2	223.2	90.1	20	10	45	15
Benomyl	benomyl_1	291	192	20	10	17	10
Benomyl	benomyl_2	291	160	20	10	39	8
Carbendazim	carbendazim_1	192.1	160.1	20	10	25	15
Carbendazim	carbendazim_2	192.1	132.1	20	10	41	15

Methomyl	methomyl_1	163	88	20	10	13	14
Methomyl	methomyl_2	163	106.1	20	10	15	8
Flonicamid	flonicamid_1	230.1	203.1	20	10	25	12
Flonicamid	flonicamid_2	230.1	174.05	20	10	27	8
Thiacloprid	thiacloprid_1	253	126	20	10	29	15
Thiacloprid	thiacloprid_2	253	186	20	10	19	15
Thiamethoxam	thiamethoxam_1	292	211	20	10	17	10
Thiamethoxam	thiamethoxam_2	292	181	20	10	33	8
Malaoxon	malaoxon_1	315	127.1	20	10	17	15
Malaoxon	malaoxon_2	315	99.2	20	10	31	15
Malathion	malathion_1	331	127	20	10	17	29
Malathion	malathion_2	331	284.9	20	10	11	16
Chlorantraniliprole	chlorantraniliprole_1	484	453	20	10	23	12
Chlorantraniliprole	chlorantraniliprole_2	484	286	20	10	19	18
Trichlorfon	trichlorfon_1	257	108.9	20	10	27	18
Trichlorfon	trichlorfon_2	257	220.8	20	10	17	14
Pyrethrin I	pyrethrin I_1	329.179	161.2	20	10	15	14
Pyrethrin I	pyrethrin I_2	329.179	143.2	20	10	25	10
Pyrethrin I	pyrethrin I_3	329.179	133.1	20	10	25	14
Imidacloprid	imidacloprid_1	256.007	209.1	20	10	23	12
Imidacloprid	imidacloprid_2	256.007	175.2	20	10	29	10
Diafenthiuron	diafenthiuron_1	385.238	329.148	20	10	26	7
Diafenthiuron	diafenthiuron_2	385.238	287.05	20	10	36	6
Diafenthiuron	diafenthiuron_3	385.238	262.045	20	10	64	8
Diafenthiuron	diafenthiuron_6	385.2	278.2	20	10	45	8
ESI negative							
Clothianidin	clothianidin_1	247.873	57.9	20	−10	−16	−9
Clothianidin	clothianidin_2	249.966	58.1	20	−10	−16	−7

Appendix C

GC-MSMS detection parameters, and detailed validation data for multi-residue analysis

Appendix C.1. Chromatographic Parameters

Table A3. Main conditions used in GC-MSMS.

Carrier Gas	Helium
Constant flow	1 mL/min
Oven temperature	60 °C
Injection mode	Solvent vent
Injection volume	1 µL

Table A4. Injector program.

Temperature (°C)	Ramp (°C/min)	Hold (min)	Total (min)
45	-	0.02	0.02
325	600	5	18.497

Table A5. Oven program.

Temperature (°C)	Ramp (°C/min)	Hold (min)	Total (min)
45	-	1.0	1.0
170	45.5	0.0	3.7473
310	10	0.75	18.497
310 (backflush)	2.0 (backflush)	2.0 (backflush)	20.497 (backflush)

Appendix C.2. Detection Parameters

Table A6. Parametres of the tandem mass spectrometry experiment

Molecule	N° Transition	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CE (V)
Diazinon	2	199.1	135.1	10
Diazinon	1	137.1	84	10
Cypermethrine	2	164.9	127	5
Cypermethrine	1	163	127	5
Deltamethrine	1	252.9	174	5
Deltamethrine	2	250.7	172	5

Appendix C.3. Validation Data

Validation 5 recoveries at LOQ + 5 recoveries at 10xLOQ.

Table A7. Main statistics during the validation data process.

Molecule	LOQ (µg/kg)	Mean Recovery (%)	RSD (%)
Acetamiprid	10	75	8
Benomyl	10	-	-
Carbendazim	10	64	14
Chlorantraniliprole	10	67	7
Clothianidin	10	65	5
Cypermethrin	20	123	10
Deltamethrin	10	73	14
Diafenthiuron	50	-	-
Diazinon	10	65	17
Flonicamid	20	63	3
Imidacloprid	50	59	12
Malaoxon	20	76	7
Malathion	10	70	4
Methomyl	10	64	5
Pyrethrin I	50	63	13
Thiacloprid	20	69	5
Thiamethoxam	10	56	8
Trichlorfon	10	88	16

Appendix D

LC-MSMS detection parameters and detailed validation data for glyphosate and AMPA analysis.

Appendix D.1. Chromatographic Parameters

Table A8. Eluent parameters in LC-MSMS.

Eluent A	0.1 mol/l ammonium bicarbonate in ultrapure water/ammonium hydroxide (100/0.1; *v*/*v*)
Eluent B	Ultrapure water/formic acid (100/0.02; *v*/*v*)
Flow rate	0.7 mL/min
Oven temperature	40 °C
Injection volume	40 µL

Appendix D.2. Gradient

Table A9. Time and eluent conditions for the LC-MSMS .

Time (min)	% Eluent A	% Eluent B
0	10	90
0.5	10	90
0.6	20	80
2	20	80
7	50	50
10	50	50
10.5	100	0
11.5	100	0
11.6	10	90
14	10	90

Appendix D.3. Detection Parameters

Table A10. Summary of parameters obtained from the mass spectrometer.

Q1	Q3	Dwell Time	Pesticide	DP	CE	CXP
110	63	80	AMPA_1	−20	−25	−8.5
110	79	80	AMPA_2	−20	−35	−10
168	63	40	glyphosate_1	−25	−28	−6.7
168	81	40	glyphosate_2	−25	−21	−8
168	150	40	glyphosate_3	−25	−14	−7

Appendix D.4. Validation Data

Validation 5 recoveries at LOQ + 5 recoveries at 10xLOQ

Table A11. Main statistics during the validation data process..

Molecule	LOQ (µg/kg)	Mean Recovery (%)	RSD (%)
AMPA	10	105	8
Glyphosate	10	106	8

Appendix E

IC-MS detection parameters and detailed validation data for metals and metalloids trace analysis.

Appendix E.1. General Detection Parameters

Table A12. Gas injection parametres.

Nebulising gas	Argon at 1.08 L/min
Auxiliary gas	Argon at 0.90 L/min
Plasma gas	Argon at 15 L/min
Collision gas	Helium

Table A13. Isotope daltons used for each metals and metalloid.

												ISTD		
	Metalloids						Metals							
	Sb	As	Sc	Cd	Cr	Cu	Sn	Hg	Ni	Pb	Se	Sc	Ir	Rh
Isotope (Da)	121	75	45	111	52	63	118	201 + 202	60	206,207,208	78	45	193	103

Appendix E.2. Validation Data

5 recoveries at LOQ + 5 recoveries at 10xLOQ

Table A14. Main statistics during the validation data process.

	LOQ (µg/kg)	Mean Recovery (%)	RSD (%)
Metals			
Cd	8	102	5
Cr	20	101	3
Cu	125	113	8
Hg	20	102	3
Ni	20	104	5
Pb	20	101	5
Se	100	107	9
Sn	100	103	4
Metalloids			
Sb	20	95	12
As	20	105	7

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Experimental section

Study 5:

Development of Tools to Understand the Relationship between Good Management Practices and Nest Losses in Meliponiculture: A Pilot Study in Latin American Countries

Insects 2024, 15, 715

Joseline Sofía Ocaña-Cabrera, Sarah Martin-Solano, Claude Saegerman

Preamble

The practice of stingless beekeeping (Meliponiculture) in Latin America, notably in Ecuador, is experiencing accelerated growth. Fortunately, guidelines on biosecurity demonstrate that acceptable practices for managing stingless bee nests are in place. However, several issues must be addressed to ensure optimal nest health. These include the implementation of biosecurity measures, the protection of the environment in which stingless bees reside, the quality and efficiency of technology used for the handling and management of nests, and the diagnosis and monitoring of the health status of stingless bees.

The results of 92 online surveys conducted among various stakeholders in 14 Latin American countries revealed that hand-washing and sterilisation during management constituted a good basis of good management practices (GMP) in meliponiculture. Several risk factors have been identified as being pertinent to the conservation of stingless bees. These include the potential for disease dispersal resulting from the introduction of species such as the European honey bee (57%), pollution (61%), and plague attacks (71%). In addition to other threats such as anthropogenic activities, including the relocation of colonies outside their natural distribution area, and feeding colonies with synthetic food or food of honey bee origin.

The calculated death rate in this study (15%) does not exceed the natural rate (13%) to a significant degree. This is a clue that the human practice is being carried out appropriately. However, implementing the practices identified as weak or in need of improvement in this study (GMP in biosecurity practices and environmental protection) could reduce this percentage to an acceptable level and improve the quality of life of stingless bees.

The barometer-traffic light tool indicated an overall GMP compliance rate of 32.6%, signifying the necessity for the implementation of comprehensive action plans and corrective measures in the practice of Meliponiculture. The employment of graphical instruments, including the spider web and the barometer, has been demonstrated to facilitate the identification of a more effective theoretical meliponicultor and can be considered a beneficial instrument in the field. These tools facilitate the identification of deficiencies in meliponiculture and allow them to be rectified after the implementation of improvement plans and projects. One limitation of this study was that the analysis of the results did not take into account differences based on environmental type (rural, urban, or forested area) or season.

insects

Article

Development of Tools to Understand the Relationship between Good Management Practices and Nest Losses in Meliponiculture: A Pilot Study in Latin American Countries

Joseline Sofía Ocaña-Cabrera [1], Sarah Martin-Solano [2] and Claude Saegerman [1,*]

[1] Research Unit of Epidemiology and Risk Analysis Applied to Veterinary Sciences (UREAR-ULiège), Fundamental and Applied Research for Animal and Health (FARAH) Center, Department of Infections and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liege, 4000 Liège, Belgium; jocana@doct.uliege.be

[2] Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y de la Agricultura, Universidad de las Fuerzas Armadas ESPE, P.O. Box 171-5-231, Sangolquí 171103, Ecuador; ssmartin@espe.edu.ec

* Correspondence: claude.saegerman@uliege.be; Tel.: +32-4-366-45-79

Simple Summary: The overall decline of bees may be exacerbated by the simultaneous presence and interaction of multiple causal factors. To elucidate how these factors interact and their collective impact, it is of the utmost importance to develop effective analytical tools. We collected data through an online questionnaire. We started estimating the annual mortality of stingless bee nests at 15%. Four risks to stingless bee survival were identified: invasive species (73%), the proximity of nests to sources of environmental pollution (61%), the presence of honey bees as potential transmitters of diseases (57%), and unusual behavior reports (44%). The biosecurity practices with the highest compliance rates were hand washing (79%), sterilization (75%), storage conditions for product quality (66%), and the use of protective equipment (40%). The spider web and barometer tools facilitate a unified observation of the status of implementation or non-implementation of biosecurity measures, actions to care for the environment in which stingless bees live, the quality and efficiency of nest management techniques, and the monitoring of the health status of stingless bees. The comprehensive evaluation of these factors within best management practices (BMPs) facilitates immediate decision-making and the implementation of enhancements, as well as individual and collective feedback.

Abstract: Insect pollination services amount to USD 235–577 billion. Seventy five percent of agricultural production for human consumption depends on pollination, mainly by bees. A decline in pollinators, including Meliponini tribe bees, will impact the economy, food security, human health, and ecosystem stability, especially in tropical forests where stingless bees are the main pollinators. The objective of this survey was to understand the relationship between good management practices and nest losses in meliponiculture, encompassing biosecurity and conservation criteria. A 36-question survey was organized and spread. We received 92 responses, representing 4548 managed nests. The primary motivation for engaging in meliponiculture was biodiversity conservation (92%). More than 50% of the questions on biosecurity were answered as "applied". Hand washing before any activity with bees was the main rule, followed by material sterilization and personal protective equipment use. The annual mortality rate of stingless bee nests was estimated at 15%. Nest invaders (72%) and nearby sources of pollution (60%) were identified as the main potential causes of nest losses. From a general perspective, meliponiculture practices continue to expand remarkably. The implementation of effective nest management strategies is associated with a reduction in nest losses. It is important to consider One Health's perspective to ensure optimal management practices.

Keywords: stingless bees; management; practices; biosecurity; nest loss; Latin America; evaluation tools

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1. Introduction

The global economic value of pollination services amounted to USD 235–577 billion, representing 10% of the total value of agricultural production for human consumption in 2021. Around 75% [1] of this agricultural production depends on pollinators, especially bees [2]. It is evident that the decline of main pollinators, including stingless bee species [3], will have a great economic impact on food security, human health, and ecosystem stability.

The available data indicate that the Neotropics are home to more than 15,150 species of bees [4], and it is only a third of the total animal species richness in that region. Worldwide, the number of stingless bee species exceeds 500 [5]. There is the possibility of finding subspecies or cryptic species due to the complexity of certain genera such as *Melipona beecheii* [6] or the taxonomic updating of stingless bees [7]. In Ecuador, a great contribution showed the presence of >200 [8,9], consolidating the megadiverse label despite the small size of the country with other neighbors.

There are multiple approaches to practicing meliponiculture, and they are contingent upon the motivations, needs, and objectives of the practitioner [10]. Meliponiculture represents a fusion of ecological (from the academy) and cultural (empirical local) knowledge, and both, along with stingless bees, serve as interesting fusion that facilitates the transition to sustainable practices within complex farming systems [11].

The five major threats for native tropical bees are deforestation, agriculture intensification, the spread of exotic species [12], climate change, and resource–habitat loss [13]. The introduction of non-native pollinators modifies socioecological interactions between insects and environmental health, i.e., by competing with native insects for floral resources or due to the spread of new diseases [14] for which the native insects have no immune defense [15]. The effects of deforestation include habitat loss and fragmentation [16], which are mainly caused by the expansion of crops such as potatoes in the Colombian and Ecuadorian Andes [17], soybeans in the Brazilian Amazon rainforest [18], or the expansion of areas focused on cattle breeding [12].

Meliponiculture practices that include harvesting honey and pollen, dividing nests, and selling nest products have faced several other menaces, such as the loss of numerous daughter colonies from a single mother, inbreeding, and queen succession problems in *Scaptotrigona* and *Cephalotrigona* species [19]. There are mainly two stingless bee nest invasive insect problems. The first, *Lestrimelitta* sp., is a kleptobiotic stingless bee, considered a resource thief that uses a chemical trickery mechanism based on its cuticular characteristics [20]. The other major invasive insect problem is Phoridae flies (*Pseudohypocera kerteszi*), which, avoiding the guardians at the nest entrance, lay eggs in pollen pots, containers, and near the brood, which will develop into white larvae that feed on the bee bread [21].

A study of the population dynamics of stingless bees in seasonal dry lowlands in Costa Rica reveals that they invest more efforts in colony survival rather than in increasing their reproductive rates, which means that, under better life conditions, these stingless bees can survive around 23 years [22], but the most recent study of colony loss in Latin America indicated a 39.6% loss of stingless bee colonies per year across the region. Furthermore, the study found that losses were highest in summer and increased with farm size [23]. These findings suggest that maintaining the overall health of bee colonies is challenging, which could have significant implications for the economic survival of stingless bee keepers. The role of stingless bee keepers is an option to care for intangible heritage and the conservation of natural resources [9], as well as their training and adoption of best practices to preserve the life of stingless bees and thus the environment.

The FAO, the WHO, and the European Commission have recognized good farming practices in beekeeping and describe their advantages, such as improved bee colony health, decreased medicinal costs, increased hive production, and the yield of healthier and higher quality honey [24]. In this sense, stingless beekeeping also needs the application of good management practices, since it has been recognized as an informal activity with poor management [25] which continues to grow and expand, especially in Latin America, at an accelerated rate [26–29]. Good practices in the management of stingless bees are a means to reduce risks associated with human error that impact human public health due to the consumption of nest products, such as honey, contaminated with agrochemicals [30]. In addition, the same risks can affect bee health, as pesticide residues can bioaccumulate in bees' bodies, in their food, and in nest structures, affecting their health, condition, and ability to survive.

Ecuadorian meliponiculture has developed depending on the climatic region. The southern highlands region, especially the province of Loja, has the highest development at the national level in dry tropical forest meliponiculture, followed by Amazon rainforest meliponiculture, urban tourist-productive meliponiculture in the coastal region, and conservations projects in protected areas.

In terms of regulations on stingless bee products, the Ecuadorian Service of Normalization (INEN) does not contemplate quality standards for pot honey or pot pollen [31]. Regarding good management practices, the Agency for Regulation and Phytosanitary—Zoo Sanitary Control (AGROCALIDAD) has only issued beekeeping guidelines [32]. In terms of bee health, the capital of the country, Quito, recently issued an ordinance banning some herbicides and pesticides [33].

A more comprehensive approach to the assessment of the impact of stingless bee breeding and management is required, encompassing social, ecological, and cultural dimensions. This approach will facilitate the development of more effective pollinator-friendly strategies and diversified agricultural systems [34].

Thus, in response to the need to develop tools to improve decision-making and provide guidance for practical actions to reduce and prevent pollinator decline, this survey aims to (i) collect stingless bee keepers' knowledge about the management of stingless bee nests (from the origin of the nest to the harvesting of products); (ii) estimate the nest death rate; (iii) identify specific health risk factors for stingless bee nests; and (iv) develop tools to correlate the application of good management practices with nest losses.

2. Materials and Methods

2.1. Online Survey Development

The free software KoboToolbox (v2022 1.2.) was used to prepare an online questionnaire with 36 questions (Table S1). All questions were configured as mandatory to ensure that all were answered. The anonymity of respondents was maintained. The survey was organized into 4 sections: (i) socio-demographic variables, (ii) biosecurity and product management, (iii) nest management and infrastructure of the farm, and (iv) sanitary and environmental aspects. The questions used for nest death rate estimation were not included in any of the previous groups since the data obtained were directly processed with the formula in Section 2.3 (namely, "Statistical Analysis"). The types of questions included in the questionnaire were single-choice, multiple-choice, and open-ended questions. The survey was available from 23 March 2022 to 31 December 2022, in two languages: Spanish and Portuguese. The target audience was meliponicultors (stingless bee keepers) with experience in managing at least one (1) nest of any stingless bee species in any country of Latin America.

Before the public launch, the questionnaire was reviewed by three experienced stingless bee keepers. They gave points for improvement and suggestions for the survey, for a better understanding of the target audience. After adding these modifications, the survey was officially launched online. The survey link (https://ee.kobotoolbox.org/x/HVbthWiD, accessed on 31 July 2023) was disseminated through social networks (meliponicultors' groups on WhatsApp and Facebook) as well as through e-mails sent to local meliponiculture organizations (when available) and to the authors of scientific articles related to stingless bees. The rationale behind selecting this particular methodology for the survey spread is twofold. Firstly, this is a pilot study designed to test the operationality of a data relation–visualization tool. Secondly, according to the Ecuadorian Observatory of Information and Communication Technologies (TIC), 82.88% of citizens in rural areas with access to a phone use social networks as their primary source of information. Together with

Brazil, Colombia, Costa Rica, and México are included in the medium- and high-Significant Rural Connectivity Index countries [35]. Third, without a national official registry of meliponicultors, we used social networks as a census tool.

2.2. Scoring System Development

The questions in section (i), socio-demographic variables, and other open-ended questions of the inquiry were not included in the subsequent phase of the study.

All answer options, from single-choice and multiple-choice questions, were numerically scored by the authors. The lowest score represented the "worst situation" and the highest score represented the "best situation". The criteria for this scoring considered those answers that were based on scientific evidence and focused on the conservation and guarantee of the best living conditions for stingless bees as a priority and of greater weight. In addition, a consensus was reached among a panel of four experts in biology, epidemiology, meliponiculture, and biosecurity. The panel agreed on the options for each question, from "worst situation" to "best situation".

Each question had different maximum scores. Each section—(ii) biosecurity and product management, (iii) nest management and infrastructure of the farm, and (iv) sanitary and environmental aspects—had a different number of questions. To ensure the fairness, consistency, and accuracy of the weighting of each section on the results, the maximum score was normalized and the minimum difference in the number of questions within each section was targeted.

2.3. Statistical Analysis

Questions were classified into five groups, one including socio-demographic information (INF) and four explaining the application of good management practices (GMP) in meliponiculture: (i) environment and conservation (ENV PROTEC), (ii) producer training and modern techniques (TECHN), (iii) the use of personal protective equipment and biosecurity measures (BIOSEC), and (iv) health care (HEALTH).

The scoring of the questions was applied to those from which quantitative information could be obtained. The maximum was calculated for each question based on the response options and we categorized these options as "best" if they adhered to conservation criteria and "worst" if they were far from it (called "theoretical best score"). To verify the analyses, the same procedure was performed, except that the maximum this time was taken according to the "best" answer given by the respondents (called "best meliponicultor score").

An overall score for each respondent was calculated using the sum of scores obtained for all their responses and the sum of the "best" scores for each question (for explanation, see Table S1).

The calculation of the nest death rate (NDR) of stingless bees was calculated as follows according to the formula modified from [36]:

$$\text{Nest death rate (NDR)} = \frac{\text{\#nest dead}}{\text{\#nest until 2021} + \text{\#nest IN} + \text{\#nest OUT}} \quad (1)$$

The terms inside the numerator and denominator are explained as follows:

#nests dead—the number of nests of stingless bees that died the last year (question (Q) 28);

#nests until 2021—the number of nests of stingless bees that existed until 2021 (Q 27);

#nests IN—the number of nests of stingless bees that were added during the last year (Q 20);

#nests OUT—the number of nests of stingless bees that were sold, donated, or given away during the last year (Q 21).

To determine any relation between the NDR (independent variable) and the overall score (dependent variable), we made a linear correlation test to obtain the Pearson's coefficient. To check the normality of the data (both overall score and NDR), a Kernel density estimation and a Shapiro–Wilk test were performed. A two-sample Wilcoxon rank sum test (Mann–Whitney) was used to test whether melipolicultors who had an NDR of less than 15% and an NDR equal or above 15% belonged to the same population or not.

2.4. Spider Web and Barometer Tools

For a general visualization of the status of meliponiculture, as an activity that must include minimum standards of compliance with GMPs in each group of questions, two tools were developed. The first one, the spider web tool, contrasts the status of each area: information sources, the application of basic biosecurity standards and the use of personal protective equipment, monitoring in health care, and conservation actions. For this purpose, we used the total score obtained per respondent and an average obtained per question group (see Table S2). The result (percentage) given for each group of questions indicates how closely the practices are aligned with what is expected according to scientifically based theoretical criteria. The closer the result is to 100%, the better the practices are considered, and the closer the result is to 0%, the more there is an opportunity for intervention and improvement in that area.

The second one, the barometer tool, ranks the overall status using the average of the above values. It means that from a global perspective, meliponiculture is evaluated and qualified. To determine the status, we divided the barometer bar into three zones, using quartiles (Q1 and Q3) of the overall score. Each zone has an action proposal, i.e., red zone: to write an action plan, implement it, and audit again within a month; orange zone: to take corrective actions and check their implementation; green zone: the management and practices are the best.

3. Results

We collected a total of 94 surveys, of which only 92 were used because two were eliminated during data cleaning and validation. Surveys were collected from 14 Latin American countries (Figure 1). In terms of academic level, a university degree was obtained by the largest percentage of respondents (38%). The mean age of the respondents was 43 years. Experience as a stingless bee keeper ranged from 5 months to 52 years. An average of 48 nests per meliponicultor was calculated. The total number of nests among all respondents amounted to 4548 (by nest, the median = 17, min = 1, and max = 700). Most respondents spent part of their time (about 8 h per week) on the care and management of stingless bees. The individual product with the highest percentage of harvest was honey (16%), followed by a combined harvest that included honey, cerumen, pollen, and geopropolis (63%) (Table 1).

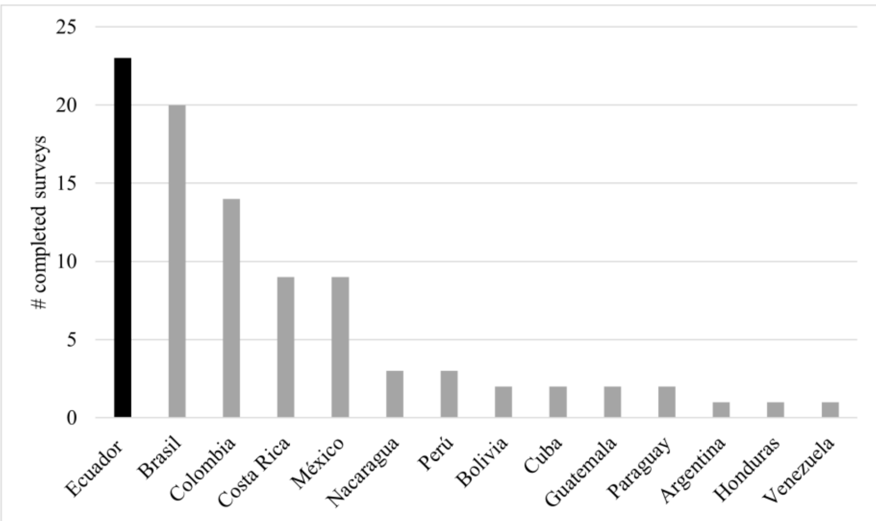

Figure 1. Survey participation by country. The bars represent the number of completed surveys (y-axis) per country (x-axis).

Table 1. Summary of the main socio-demographic variables.

Variable	Range		Percentage
Age (years)	Young	≤28	25
	Adult	>28 and ≤60	63
	Old adult	>60	12
Stingless beekeeping experience (years)	Beginner	≤5	54
	Upper beginner	>5 and ≤10	18
	Intermediate expert	>10 and ≤20	16
	Expert	>20	11
Full academic level	Elementary		1
	High School		29
	Technology		12
	University		38
	Post grade		20
Spending time	Full Time (≥8 h/day)		9
	Part-time (<8 h/day)		23
	Hobby (~8 h/week)		68
Amount of nests (quantity)	≤10		34
	>10 and ≤50		47
	>50 and ≤100		8
	>100		12
Main product harvested from nests	Honey		16
	Geopropolis		4
	Cerumen		3
	Honey, cerumen, geopropolis, pollen		63
	Other reason for nest keeping *		13

* Among other reasons for keeping nests of stingless bees were (i) nest multiplication for sale, (ii) stingless bee conservation, and (iii) protection.

3.1. Environment and Conservation (GMP–CONSERV)

A total of 61% of stingless bee keepers consider that there are one or more sources of pollution around their nests. From the highest to lowest number of reports, there were plantations using agrochemical products for pest control, companies extracting oil and oil derivates (plastics), mining, city pollution (urban meliponiculture), and polluted rivers. In addition, 96% of respondents consider that climate change affects or will affect the life of bees. The same percentage of respondents take climate-friendly actions such as recycling, saving energy, not using agrochemicals for pest control, and planting more plants, and a small percentage of producers (n = 4/92, 4%) mention "agroecology" as a new climate-friendly practice.

The main reason for keeping stingless bees was the conservation of land (93%), pollinators, or biodiversity in general and the conservation of ancestral agricultural heritage in particular. Respondents (n = 21/92, representing 22%) purchased whole nests or brood disks to obtain more stingless bee nests. In general, those who buy nests try to get them from nearby areas (n = 10/21, representing 48%), same region (n = 6/21, representing 28%), or same country (n = 2/21, representing 9%), except in one case (n = 1/21, representing 4%) (international purchase).

A total of 60% of stingless bee keepers feed their managed stingless bees with water, *Apis mellifera* honey, honey from other stingless bee species, commercial food, and processed substances such as sugar, flour, or lemon juice. They do it according to stingless bees' necessity, i.e., breeding seasons, winter/non-flowering, new splits, weak nests/no reserves, and also for the maintenance and stimulation of nests.

3.2. Producer Training and Modern Techniques (GMP–TECHN)

To obtain their first nest, 76% of respondents practiced trapping in the wild. It is important to notice that some other meliponicultors (8%) obtained their first nest by rescuing stingless bee nests that were in significant danger. Respondents (37%) mentioned that they received expert support or some previous training for the transfer of natural nests to wooden boxes for technical nest management. However, a percentage of respondents (n = 12/92, representing 13%) keep nests in natural structures (i.e., hollowed tree trunks).

During nest division, stingless bee keepers confirmed that they ensure the following conditions: the existence of a viable virgin queen and old virgin, the health of and sufficient food for the old nest and the new nest, the seasonal flowering of plants (summer), positioning the new nest and scheduling the time of bees' work that avoids damages or loss of workers, the existence of mature–viable brood discs, an abundant population, and a strong and disease-free nest of origin. Excluding urban meliponiculture, 92% of the producers maintain their nests in open spaces with plants.

The organization of stingless bee nests (meliponaries) was attributed to being specific to the species managed, the size of the bees, their behavior, and the ease with which the nests can be harvested. The most reported conditions are described as follows: at least 1 m above the ground, one nest next to the other, minimum separation between nests of 0.40 to 3 m, nests stacked one on top of the other (condominium or tower blocks), and nests directly on the ground. This survey did not ask species-specific questions about nest organization in a meliponary; thus, the conditions detailed above are a general guide.

Among the places where respondents located their meliponaries were their own land (n = 61/92, representing 67%), common land (n = 21/92, representing 23%), association land (n = 4/92, representing 4%), natural tourist spaces (n = 4/92, representing 4%), and land belonging to academic institutions (n = 2/92, representing 2%).

Academia is the main source of producer training or teaching (n = 57/92, representing 62%). Knowledge sharing among producers is strong (around 28%), with social networks being the main channel of information transfer, where experienced meliponicultors share their knowledge with those who are new to the activity.

3.3. Use of Personal Protective Equipment and Biosecurity Practices (GMP–BIOSEC)

One person manages the meliponary in 73% of the cases, while 27% of respondents stated that they do not carry out meliponiculture alone. The accompaniment for activities in the meliponary ranged from 2 to associations of 25 people (Ecuadorian example).

The application of biosecurity practices and the use of appropriate materials are summarized in Figure 2. It is important to mention that hand washing and the use of personal protective equipment (PPE) during regular nest checks had the same behavior in both management cases (one person or more than one person). The main PPE and instruments used for different activities at the surveyed meliponaries are summarized in Table 2. The use of a sterilized material for product storage (n = 75/92, representing 82%) as a biosafety measure was also emphasized in the survey. The main storage conditions for products were as follows: refrigeration (4 °C) (n = 34/75, representing 45%), protection from humidity (n = 25/75, representing 33%), protection from light (n = 12/75, representing 16%), and environmental temperature and freezing (−20 °C) (n = 4/75, representing 5%).

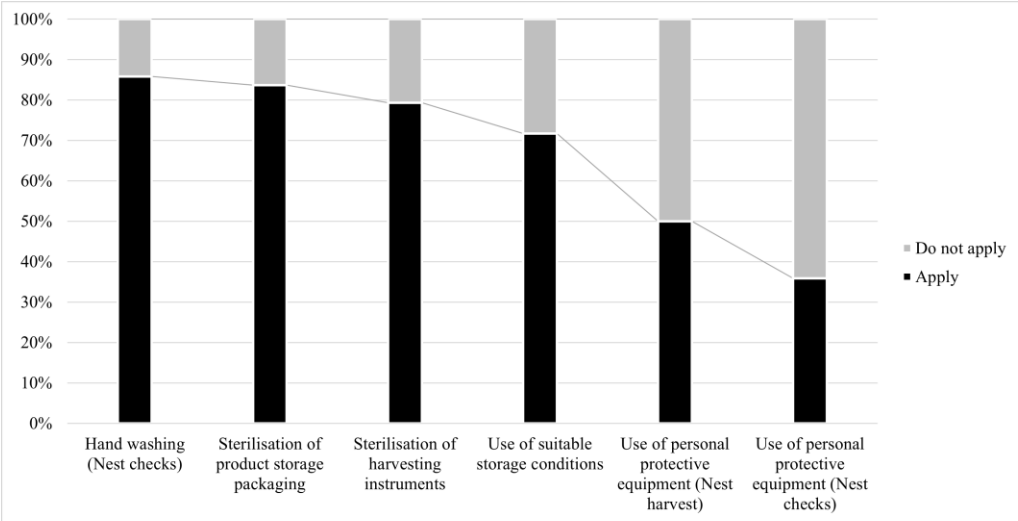

Figure 2. Application of basic biosecurity standards in stingless bee nests. Percentage of compliance (y-axis) with specific biosecurity standards in stingless bee nests (x-axis). Ordered from highest to lowest and differentiated by stage during nest management.

Table 2. Summary of the main biosecurity measures complied with in the key stages of meliponiculture (regular check, harvesting, product storage).

Item	**Activity in the Nest Set (Meliponary)**	
	Regularly Check (n = 33)	Harvesting (n = 45)
(a) Personal Protective Equipment		
Head coverings	30	18
Sterile gloves	15	28
Face mask	9	31
Clean boots	9	
Clothing cover	10	
Protective glasses	5	
Tent for creating a sterile environment		5
	Harvesting (n = 71)	Product storage (n = 75)
(b) Instruments		
Food-grade containers	45	
Spoons or paddles	31	
Syringes	51	
Filters	41	
Palette, knife, scrapers	7	
Vacuum pumps	3	
Glass bottles with lids		66
Plastic bottles with lids		24
Plastic bags with hermetic seals		8
Glass bottles with gas release		1

3.4. Health Care (GMP–HEALTH)

Meliponicultors (n = 62/92, representing 57%) kept a record of activities carried out in their meliponaries. In these records, they have been able to observe aspects such as insects/organisms invading stingless bee nests (73%) and unusual behavior (44%), detailed from the highest to lowest rates of sighting in Figures 3 and 4.

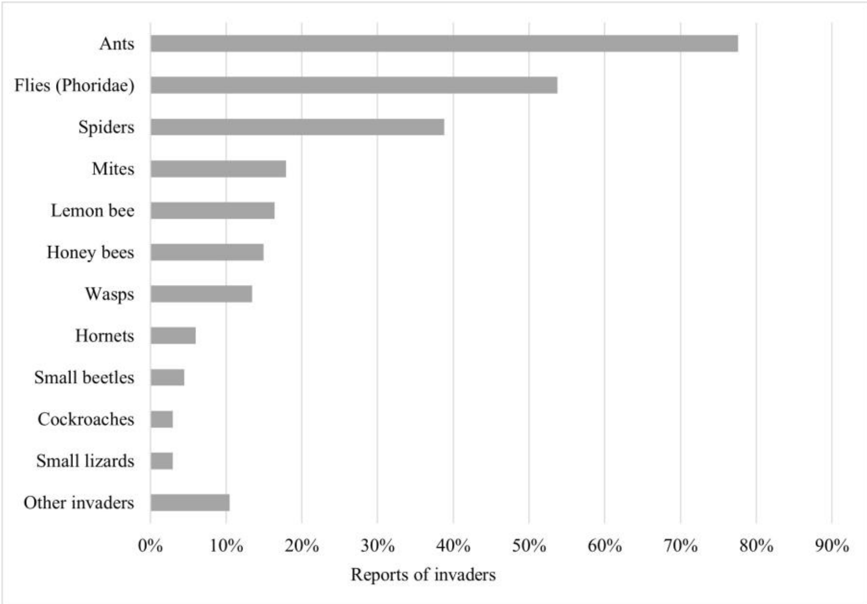

Figure 3. Presence of invaders in stingless bee nests. List (y-axis) and percentage of stingless bee nest invaders reported (x-axis). Sorted from highest to lowest number of reports. Other invaders include just one report of Euglossini and Bombini bees, crickets, mammals, blank soldier fly (*Hermetia illucens*), termites, and arapuá bee (*Trigona spinipes*).

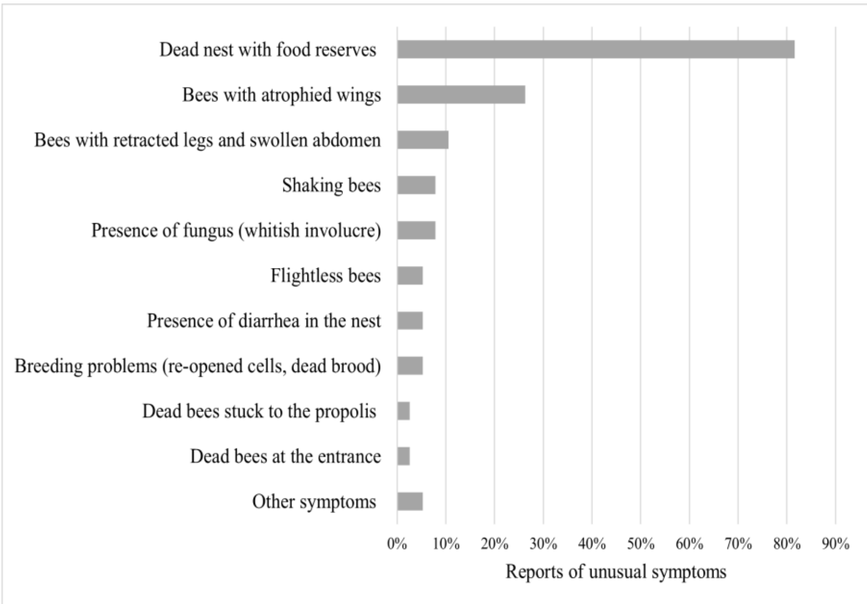

Figure 4. Presence of unusual symptoms in stingless bees. List (y-axis) and percentage of reported unusual clinical symptoms in stingless bees (x-axis). Sorted from most to least severe. Other symptoms include just one report of death by pesticides and invasion by the same species.

The first place in terms of the most commonly reported invaders of stingless bee nests is occupied by ants, followed by Phoridae flies and spiders. The two best-known problem insects for meliponiculture are the phorid fly and the lemon bee (ranked fifth in this study as an invader).

Respondents (n = 28/92, representing 30%) know about nosemosis (no statistically significant effect on NDR, Mann–Whitney test, p-value = 0.262). More than half of the total respondents (n = 52/92, representing 57%) confirmed the existence of apiaries near their meliponaries (no statistically significant effect on NDR, Mann–Whitney test, p-value = 0.733).

Knowledge of nosemosis was not associated with the existence of honey bees near stingless bee nests (no statistically significant correlation between the variables in question, Pearson product–moment correlation test, p-value = 0.219).

Only one meliponicultor replied that he treated his bees with veterinary medicine and did not store this medicine after it was opened (this survey did not collect data regarding the specific type of medicine employed by stingless bee keepers for the treatment of their bees). Among the sources of reference to face and solve unusual health concerns in nests, the meliponicultors answered that 69% prefer to ask other stingless bee keepers, 13% consult an expert (veterinarian), another 13% prefer to experiment by themselves, 9% treat the bees by themselves since they have previous knowledge, and a small 1% go to academic bibliographic sources or theses.

3.5. Relationship between the NDR and the Application of Good Practices in Meliponiculture

Normality was verified for the overall score (dependent variable) (Shapiro–Wilk test, p-value =0.614 for theoretical best score, p-value =0.617 for best meliponicultor score) but not for the NDR (independent variable), giving us a cut-off point = 0.15 (i.e., 15%), which divides the population into two groups based on nest losses (Figure 5).

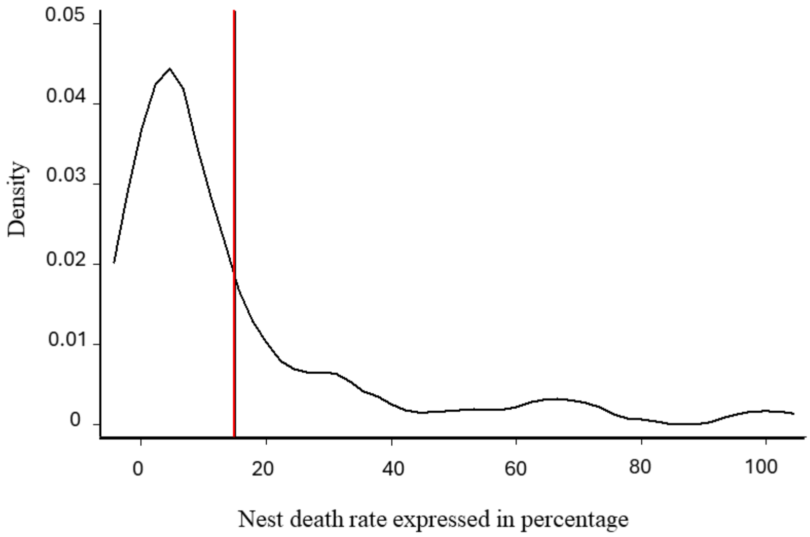

Figure 5. The kernel density estimate of the nest death rate. X-axis: probability density. Y-axis: nest death rate calculated and expressed as a percentage (scale between 0 and 100%). The red vertical line at 15% represents the observed cut-off point to separate the population into two parts.

An inverse relationship was observed between compliance with GMPs and NDR (Figure 6). The linear correlation between variables explained 8% of the NDR concerning the overall score (p-value = 0.005).

The overall scores are significantly different in the two sub-groups of meliponicultors depending on the NDR and considering the cut-off point of 15% (Mann–Whitney test, p-value = 0.001) (Figure 7). The last three calculations were verified by both methods using the best theoretical and best meliponicultor scores.

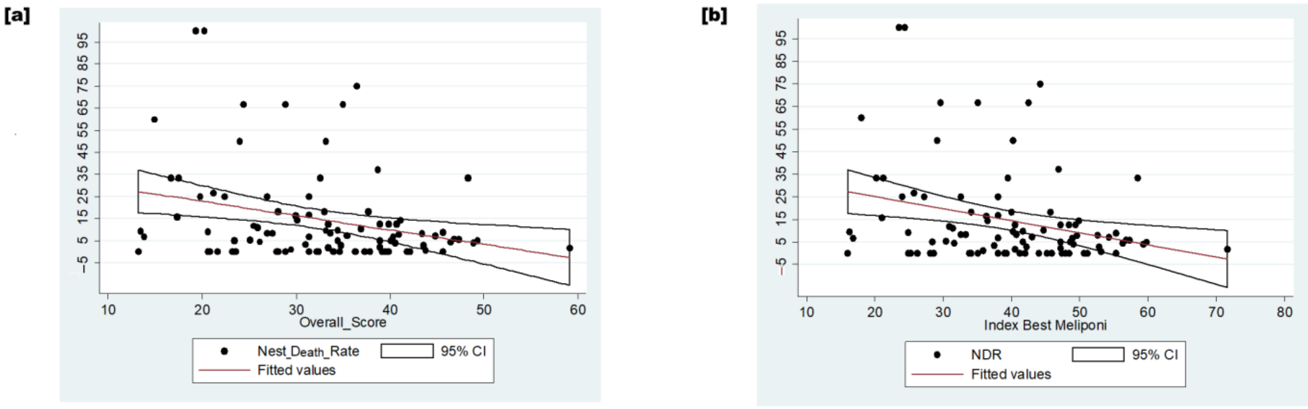

Figure 6. The relation between dependent and independent variables. (**a**) The inverse relation between the overall score and nest death rate. (**b**) The inverse relation between the index of the best meliponicultor and the nest death rate. Legend: NDR—nest death rate.

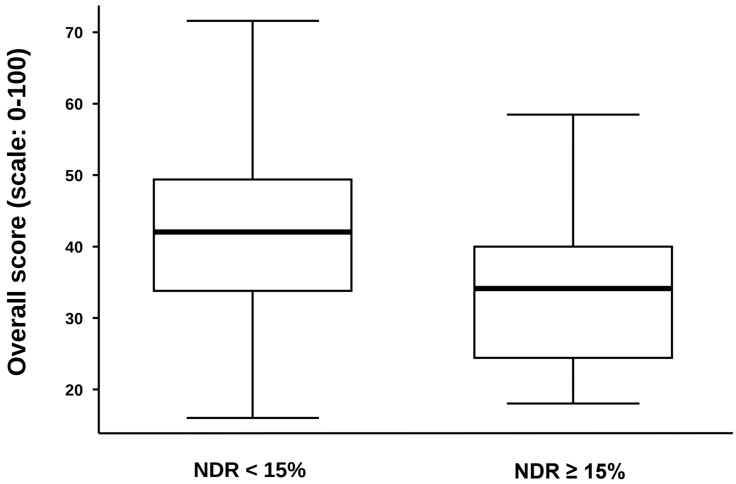

Figure 7. A boxplot of the overall score (y-axis) vs. the estimated nest death rate (x-axis). Population division is visualized considering the estimated mortality rate. NDR: nest death rate. Legend: The horizontal bold line in the rectangle represents the median of the overall score; the solid lines at the top and bottom of each rectangle represent, respectively, the first and third quartiles; adjacent lines to the whiskers represent the limits of the 95% confidence interval.

3.6. Spider Web and Barometer Tools

The spider web tool showed a great socio-demographic status (65.4% of compliance). Items better aligned with scientific theoretical criteria, from the highest to lowest percentage of compliance were as follows: GMPs applied to training and modern techniques, GMPs in healthy controls, GMPs in biosecurity practices, and environmental protection actions (Figure 8a). However, when it is differentiated by the best meliponicultor score, GMP—HEALTH comes in second place, followed by GMP—TECHN, GMP—BIOSEC, and GMP- ENV PROTEC (Figure 8b).

The barometer tool gave a result of 32.6% for the theoretical best score (Figure 9a) and 39.5% for the best meliponicultor (Figure 9b), both right in the middle of the orange zone, which asks respondents to take corrective actions and check their implementation.

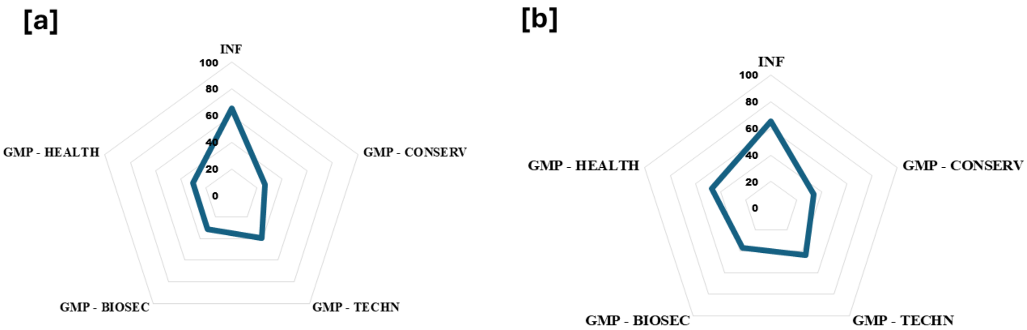

Figure 8. Spider tool. Overview of compliance in each analyzed area: social aspects, modern techniques, health, biosafety, and conservation. (**a**) The percentage of compliance based on the theoretical best score. (**b**) The percentage of compliance based on the score obtained by the best meliponicultor. INF: socio-demographic information. GMP: good management practices. CONSERV: environment and conservation. BIOSEC: biosecurity measures. TECHN: producer training and modern techniques.

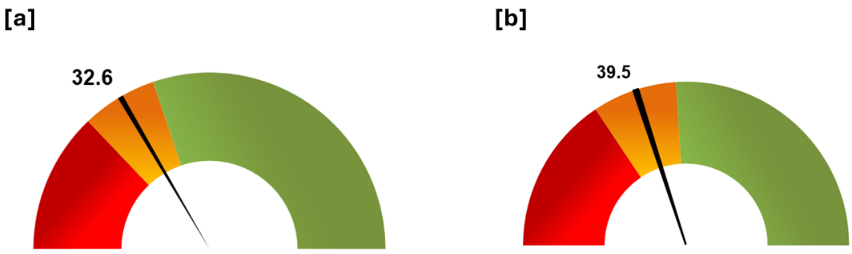

Figure 9. Barometer tool. Summary of the general status of the stingless bee keeper respondent population in terms of good management practice compliance. (**a**) Status based on the theoretical best score (Q1 = 25.8, Q3 = 39.6). (**b**) Status based on the score obtained by the best meliponicultor (Q1 = 31.3, Q3 = 48.03). Legend: The barometer was divided in three zones, using quartiles (Q1 and Q3) of the overall score. Each zone has an action proposal, i.e., red zone: to write an action plan, implement it, and audit again within a month; orange zone: to take corrective actions and check their implementation; green zone: the management and practices are the best.

4. Discussion

This pilot study mainly reached a "sector" of the stingless bee keepers population with access to the internet, a cell phone, or a computer, as well as to studies, which is reflected in the highest percentages of respondents with university and high school education, which may be surprising given the rural reality of the world. In Ecuador, a 2019 study showed a shift in university enrollment among rural youth in a coastal province, largely due to the confidence parents now have in university education [37]. The rise in student demand for distance education has reached 10% per semester, an alternative modality to solve the problem of remote locations, through a system of grants for the implementation of technology at home.

It is important to note that the current statistics about education enrollment do not reflect the reality of the entire rural youth population of Ecuador, let alone Latin America. However, they do provide an approximation of meliponiculture and the potential loss of its 'rurality' in the context of a globalized world. This could potentially result in the loss of ancestral knowledge on meliponiculture, which has been practiced for a considerable length of time [38,39], more than 2000 years [11].

Furthermore, the utilization of technologies, such as these online surveys, facilitated the gathering of data and insights into the contemporary practices and management of meliponiculture. A significant approach was to gain an understanding of the processes involved in the care of stingless bee nests, which is predominantly a collective endeavor involving family members or associations. Thus, knowledge is still inherited, and teamwork [25] helps to reduce errors, since each person assumes a single task.

The survey also shows the participation of the academy with the provision of institutional lands as a strategy for mutual benefit between producers and research. This community work extends knowledge among stingless bee keepers [40]. The hybridization between traditional knowledge and modern stingless beekeeping improves local practices, thus increasing production. If this were the case, above all, it would reduce the chance of colony losses [41].

This study highlights the role of more experienced meliponicultors, since they become sources of new knowledge and promoters of stingless beekeeping. While these examples of collaborative behavior and knowledge transfer are commendable, there is a need to recognize the continued risk associated with the perpetuation of less ethical practices in this field, especially risks associated with the introduction of animal or plant species (nectiferous) that may facilitate the spread of diseases or new predators/competitors. This is exemplified by the case of African tulips [42].

The mean age of stingless bee keepers as well as the variability in years of experience in this study compares with another Ecuadorian study [43], with ages from 22 to 72 years old, and with the average age of Brazilian meliponicultors being 44.1 $\pm$ 2.14 for women and 43.4 $\pm$ 0.78 for men, including 5.9 $\pm$ 0.5 years of experience in stingless beekeeping [44].

As a field activity, stingless beekeeping is a side job in families that practice it, even though the marketing value of honey is around USD 133–200/Kg [45]. As it is a secondary activity, people invest 8 h per week on average. Taking time between revisions helps to keep nests free of pests. Even in critical periods, such as the time after the split, experts recommend checking the new nest every three days for three weeks, and then once a week [46], but above all, meliponicultors should not over-manipulate the brood comb [47].

4.1. Environment and Conservation (GMP–CONSERV)

Regarding stingless bee conservation aspects, a low percentage of respondents purchase nests from outlying areas from meliponaries. However, interregional and one international sale were reported in this survey, making it imperative to create awareness programs on the impact of colony displacement. The consequences of anthropogenic nest displacement have been widely reported [28,48,49].

Feeding stingless bees is appropriate at specific times, i.e., after honey harvest (low nutritional reserves) [50], during non-flowering seasons or harsh winters [51], to strengthen colonies after a split [52], and under pollination greenhouses [53], as well as the cases of urban meliponiculture found in this study. Feeding may include nectar (energy source) or pollen (protein source) replacement, such as the protein substitute in the diet of *Melipona flavolineata* that was tested and accepted under laboratory conditions [54].

It is our contention that the utilization of flour as a pollen substitute in stingless bees is a matter of concern. A study was conducted to evaluate the acceptance of four types of flours in a mixture of honey and water by honey bees. The results demonstrated that all mixtures were accepted, with soybean meal being the most accepted [55]. The quality of nutrition is associated with alterations in the gut microbiota of honey bees, which in turn impact their immune system and susceptibility to pathogens [56]. The impact of flour as a protein substitute in stingless bees remains largely unstudied.

4.2. Producer Training and Modern Techniques (GMP–TECHN)

Producers who followed training courses in meliponiculture were able to make nest divisions and provide adequate supplementary feeding according to the nests' needs [52]. Good nest management depends mainly on the practice and continuity with which it is practiced and the support that can be provided by the academy [57] or field technicians.

A disadvantage of maintaining nests in their natural structures, i.e., tree logs, is difficulty during honey harvesting and the possibility of contamination, as it passes through waste areas [50]. In addition, shaking and turning the nest upside down to let the honey fall by gravity induces the loss of eggs that sink in the larval food, causing nest collapse [57]. Thus, the management suggestion is the use of technical boxes with vertical divisions and separate cavities for the brood chamber as well as for honey and pollen pots so that during the honey harvest, only the storage modules are removed and it would be possible to continue using the gravity honey harvesting technique.

In the case of Mexican "jobones", whose structures are horizontal, single-story structures for brood chambers and food storage, the technique of gravity honey harvesting has been used since the pre-Hispanic Mayas [38] with no major reports of brood collapse. It is therefore possible to attribute this to the density of larval food and suggest that the bee larvae do not 'drown' but remain afloat for a certain time during the gyrus downwards from the nest for harvesting. This last topic merits further in-depth study, as well as the application of vacuum pumps or automated suction devices for honey extraction reported in this study.

The artificial division of colonies is recommended once a year [52]. Among the precautions to be taken during the division of nests are that the nest of origin must have abundant brood discs, a large population, and reserves of honey and pollen [46]. It should be performed at night or in an enclosed space with a mosquito mesh to avoid fly (Phoridae) infestation [58].

A 50/50 method for nest multiplication is being practiced [59]. Thanks to this study, it is possible to add the following suggestions: First, 4–6 brood disks should be transferred to the new nest. In species that build a queen cell, it is recommended that this queen cell should be included in one of the brood disks. It is preferred to feed the new nest 24 h after being transferred and to check it at least twice a week. It is not recommended to transfer pots of honey or pollen in poor conditions [60,61]. All these considerations contribute to making the propagation techniques sustainable and self-sufficient because they will always have new queens available [62].

Trap nests are considered a viable tool to study stingless bee colonies for meliponicultors, researchers, and conservationists [54]. Traps are used to identify species and differentiate their distribution in primary and degraded forests [63]. The use of traps should not be for the over-exploitation of natural resources, as this may generate a disturbance in the ecological balance [64].

The primary motivation for engaging in meliponiculture was conservation, while the primary source of meliponicultors' initial nests was through trapping. This does not necessarily indicate a contradiction but rather a potential deficiency in understanding the true nature of conservation. Trapping may potentially contribute to the unnecessary extraction of stingless bee nests from the wild. The removal of nests from their natural habitat should only occur when stingless bees are at risk, e.g., due to deforestation.

4.3. Use of Personal Protective Equipment and Biosecurity Practices (GMP–BIOSEC)

The implementation of biosecurity measures on a farm prevents the introduction and spread of infectious agents and diseases [65]. For example, the use of personal protection equipment and hygiene were considered protective factors against colony loss in Belgian beekeeping [66]. The use of personal protective equipment as well as sterilized instruments are keys to improving nest management because stingless bee keepers can focus their attention on an activity free of bites or any discomfort that these species can cause [67].

The maintenance of colony hygiene is directly correlated with the safeguarding of bee health and the protection of bee products. Disinfection represents a hygienic measure that is designed to prevent and eliminate agents that are capable of causing infectious diseases in bees. Furthermore, it serves to avoid the contamination of honey and other bee products with harmful microorganisms [68]. Given the toxicity and other negative effects of chemical disinfectants, it is recommended that physical methods of disinfection be employed wherever feasible.

In regard to physical methods of disinfection, the following is recommended for implementation in the field: boiling the instruments in water at normal (atmospheric) pressure for a period of 30 min. It is recommended that instruments be washed with hot water at a temperature of 90 °C or use hot air (110 °C and 150 °C) [69].

Stingless bee honey is characterized by having high moisture in comparison with *A. mellifera* honey, causing a natural fermentation process [70]. This fermentation process made by symbiont microorganisms contributes to the preservation of honey and the transformation of pollen into bee bread [71]. The findings of this study allow us to propose storage conditions for honey: refrigeration (4 °C) and containers that protect from humidity and light.

4.4. Health Care (GMP–HEALTH)

Local experts in Mexico reported attacks on stingless bee nets by different predators [72]. Indeed, the list of predators includes skunks (*Mephitis* sp), *Canis latrans*, *Dasypus novemcinctus*, ants, wasp rams, kleptobiotic stingless bees (*Lestrimelitta chamelensis*), and *A. mellifera*. Some of those predators were reported in this study in Brazil, Costa Rica, Colombia, Ecuador, and Perú.

Both Phoridae flies (*Pseudohypocera kerteszi*) and *Lestrimelitta* sp. can cause the complete loss of stingless bee nests, but the Phoridae fly is considered the most representative risk as far as stingless bee plagues are concerned. At least, *Lestrimelitta* sp is considered a biological population controller of stingless bees. Therefore, the recommendation that is under the control of the stingless bee keepers is the maintenance of hygiene in the nests, especially at the beginning of a transfer from a natural nest to a technical one.

For Phoridae flies, a useful recommendation is to collect all honey and pollen from the nest pots to prevent fly eggs from hatching and to constantly check these three areas of the nest which are the favorite places to start an invasion, and the use of white or red vinegar traps inside the nests [73].

Unusual signs in stingless bees such as extended proboscis, expanded or unhooked wings, wrinkled bodies, and defecation on cage covers are visible signs of poisoning with some agrochemicals (e.g.,): fipronil, cypermethrin, dimethoate, imidacloprid, and indoxacarb [74]. Crippled wings and a contracted abdomen are visible indicators of a possible infection with deformed wing virus (DWV), Israeli acute paralysis virus, and Kashmir bee virus (KBV) [75]. Trembling movements in bees and the inability to fly are reported as signs of acute bee paralysis virus (ABPV) infestation [76]. These unusual behaviors raise alarm bells regarding the health of stingless bees since their signs are similar to those described in *A. mellifera*. However, there are no reports in native bees, except for *Vairimorpha ceranae (Nosema ceranae)* [77].

In this study, the reported proximity of *Apis mellifera* to meliponid sets may present a risk to the health of stingless bees, given the potential for their interaction in the same floral resource during foraging [14]. It has been demonstrated that honeybee pollen loads frequently contain pathogenic protozoa and microsporidia [78]. The utilization of this pollen as a food source for stingless bee nests suggests a heightened probability of the transmission of infectious agents. Nevertheless, research has demonstrated that propolis derived from stingless bees can effectively mitigate the progression of *Nosema* infections in honey bees [79]. It is possible that propolis, a resinous substance used by stingless bees in the construction of their nests, may offer protection against *Nosema* infection.

It is therefore recommended that the use of honey bee products in stingless bee nests be avoided. In cases where the use of such products is unavoidable and within the reach of stingless bee keepers, it is advised that they verify that the products do not contain any agents or substances that could prove harmful to the stingless bees.

The natural ecology of stingless bees includes natural biological controllers such as lemon bees and phorids [80], as well as their natural competitive relationships, such as fights with solitary bees for resources [81]. These examples also cause morphological damage and even death to stingless bees. It is recommended to examine this symptomatology in depth and make accurate diagnoses of possible viruses or bacteria that are pathogenic to native bees.

Registering activities such as unusual behaviors, invasions, death, and other aspects in the meliponary [25] can be used as a basis for creating or providing records that can be submitted to or socialized with legal entities for regularization and health surveillance purposes.

4.5. Developed Tools

The annual calculation of the death rate under technical management conditions and without considering the difference in calculation between forage and non-forage stingless bees compares with the natural nest death rate reported at 13% for stingless bees [82] and 10% for honey bees [83]. Therefore, this value of the death rate in stingless bees should be considered an acceptable level of colony loss rates under domestic management. It was also verified that the better the compliance with good management practices, the lower the loss or mortality (inverse relationship).

The three main groups of causes associated with an increase in nest loss, namely GMP-CONSERV, can be attributed to two key factors: the high prevalence of polluting sources in close proximity to the meliponaries and the growing consensus regarding the adverse impact of climate change. Additionally, the GMP-BIOSEC group is included due to the dearth of adherence to fundamental biosecurity standards during nest inspection and product harvesting. This is a significant concern for the preservation of nest health and the quality of the products obtained. Finally, the GMP-HEALTH group is of note for the high number of reports of nest-invading insects causing nest collapse, as well as the observation of unusual behaviors in bees. These observations are comparable to those made in honey bees, but it is unclear whether the same causal and effect relationships can be applied to stingless bees.

The spider web and barometer tools are pedagogic instruments to interact with meliponicultors and identify margins of improvement. The interpretation of the spider tool means that the sources of information, experience, and management practices of meliponicultors are alienated to extend stingless bees' life, as well as environmental protection, according to scientific theoretical criteria. At the same time, the barometer tool confirms the widely discussed need for the implementation of good management practices.

The benchmarking made for score assignment showed that meliponiculture should have its guidelines, and even within meliponiculture, management should be separated according to the stingless bee species being managed, according to the region where the activity is developed, and according to the scientific information that each country generates.

The limitations of the present pilot study can be attributed to the continuous growth of meliponiculture and therefore research, since we only have three examples of developing tools for the evaluation of stingless beekeeping, in Mexico, Brazil, and Costa Rica. People dedicated to this activity are located mainly in rural zones, and the lack of access to internet sources (the main medium of dispersion of this pilot survey), is a limitation. The reliability that researchers can create with producers must be considered.

Despite evidence of the positive influence of the training and education of stingless bee keepers, more programs of this kind should be created or research results should be disseminated in the language of stingless bee keepers and on freely accessible platforms, as a large percentage base their management practices on the advice of others meliponicultors. Improved management and risk control in meliponiculture should be addressed using this economic activity as a tool inside agroecological systems. A loss/death rate calculation will improve long-term nest management conditions. Finally, we recommend the application and socialization of spider and barometer tools with meliponicultors in the field through an app.

5. Conclusions

Stingless beekeeping in Latin America, especially in Ecuador, is growing rapidly. Fortunately, guidelines related to biosecurity show acceptable nest management. However, some items need to be addressed to ensure better health: global compliance with biosecurity measures, actions for the care of the environment in which stingless bees live, the quality and efficiency of technology in the handling and management of nests, and the diagnosis/monitoring of the health status of stingless bees.

Hand washing and sterilization are applied during management and constitute a very good basis for turning meliponiculture into a sustainable practice.

Risk factors for the conservation of stingless bees include the effect of the introduction of species such as the European honey bee as a potential disease disperser, the use of agrochemicals, the pollution that bees face, and the effect of anthropogenic activities such as colony movement that are not aligned with good management practices.

Honey, as the main product harvested, must have an adequately good management procedure from harvesting to storage, due to its unique physical and chemical characteristics. However, it can become complex as the number of nests increases.

The nest death rate calculated here does not exceed the naturally calculated rate by far. It is a good indicator that the human practice is performed in a good way. However, the application of practices that were found to be missing in this study could reduce this percentage to a more acceptable number.

Graphic tools such as the spider and the barometer are instruments for the empowerment of each meliponicultor, as they help in the field and instantly help detect shortcomings to be corrected after entering some parameters.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects15090715/s1, Table S1: Dispersed online questionnaire 'Good management practices for stingless bees'. Table S2: Example of calculation of the score by group of questions and the overall score.

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General discussion, perspectives, and recommendations

- **Steps towards improving Meliponiculture in Neotropics**

Meliponiculture (stingless beekeeping) must be practised according to the ecological characteristics of each region and each stingless bee species, without neglecting the cultural aspects of the population in the tropics (Cortopassi-Laurino et al. 2006). Meliponiculture is considered a powerful tool for the sustainable development of communities that raise and protect these meliponine bees, given the growing global interest in their products, including pot-honey, pot-pollen, plant resin compounds (propolis or cerumen), as well as their role as efficient pollinators (Jaffé et al. 2015a). As was the case in Mayan culture, the evolution of meliponiculture in general progressed from harvesting wild nests, resulting in their loss, to managing them in wooden boxes. This change occurred when people mastered the process of splitting (dividing) colonies (Quezada-Euán 2018). Subsequent knowledge gained about stingless bee nests and trapping in the wild also highlighted the need for meliponicultors to adapt more wooden boxes for colonies (Oliveira et al. 2013). However, this practice is under discussion regarding its sustainability and ethics.

The species of stingless bee bred depends on the location. For example, in Mesoamerica, only two species of stingless bees have been systematically cultivated, *Melipona beecheii* and *Scaptotrigona mexicana* (Quezada-Euán and Alves 2020). In communities in the Peruvian Amazon, *Melipona eburnea*, *M. illota*, and *M. grandis* have been bred (Delgado et al. 2023). In Bolivia (Chuquisaca department), *Tetragonisca angustula*, *Scaptotrigona* (totally black bee negro), and *M. rufiventris* have been cultivated (Adler et al. 2023). In addition to Melipona quadrifasciata, M. capixaba, M. seminigra, and M. scutellaris are also raised and managed in Brazil (Carvalho 2022). In Ecuador, *Scaptotrigona vitorum*, *S. polysticta*, *T. angustula*, *M. mimetica*, *M. eburnea*, and *Geotrigona* sp. have been cultivated as the main honey-producing stingless bees (Vit et al. 2023). Enhancing the practice of meliponiculture requires a thorough understanding of the distinct requirements of each stingless bee species and the specific type of meliponiculture practised (under the concept proposed by Aldasoro Maya et al., (2023). An illustration of this advancement was the design of species-specific wooden hives. These technified hives consider three essential aspects for optimal development and conservation of stingless bee nests: the biology of the species colony, the improvement of a section in the hive suitable for the separation of the brood during splits, and the least invasive and destructive method of harvesting products, especially pot-honey (Nogueira-Neto 1997; Quezada-Euán 2018; Quezada-Euán and Alves 2020). An important issue when developing rational hives is the volume and the thickness of the walls. The thickness should be at least 2.5–3 cm, or up to 4 cm in some areas of the Ecuadorian Amazon region (personal observation). Both features, volume and thickness, should resemble the species' natural features to help them due to their inefficient thermoregulation capacity (Macías-Macías et al. 2011).

Estimates suggest that meliponiculture gained popularity at the beginning of the 21st century, evolving into both a commercial enterprise and a reliable economic source (Cortopassi-Laurino et al. 2006; Rattanawannee et al. 2019). This development attracted a growing number of inexperienced practitioners and transformed the perspective of meliponicultors who had previously engaged in the practice with limited colonies. Consequently, some of these people transitioned into the collection of stingless bee species as a gallery art. The endeavour to manage novel stingless bee species has the potential to engender an elevated risk of colony loss (Quezada-Euán et al. 2022).

- **Management and estimated loss rate of stingless bee colonies**

The development of rational hives, alongside colony feeding and pest control, has enabled an increase in the practice of meliponiculture in terms of both area and time (Quezada-Euán 2018), for example, areas where the flora does not flourish all year round. This also enables the preservation of stingless bee populations whose numbers have decreased (Requier et al. 2024). These losses varied considerably between countries and the years in which the study was conducted.

Between 2016 and 2018, a large-scale study in Latin American countries demonstrated a proportional relationship between summer losses of stingless bee colonies and exploitation (meliponary) size; winter losses exhibited a decline (Requier et al. 2024). The annual colony loss in the aforementioned study ranged from 14.7% (95% CI: 0.0–98.6%) in Peru in 2016–2017 to 65.0% (95% CI: 16.8–94.4%) in Bolivia in 2016–2017. For those countries where a comparison between years (2016-2017 and 2017-2018) was possible, such as Brazil, the loss in the first year was 42.8% (95% CI: 26.3-61.2%), but was lower in the second year at 30.4% (95% CI: 12-58.5%). Conversely, for Colombia, the average loss was 22.3% (95% CI: 6.5-54%) in the first year, and this average increased in the second year to 31.7% (95% CI: 7.7-72.1%). The study conducted by the present author revealed an average annual loss rate of 15% for various Latin American countries in 2022, with Ecuador (n=25) being the most participative country (Ocaña-Cabrera et al. 2024). The survey audience had a small sample size, representing only one-eighth of the estimated number of meliponaries in the country (N=209, according to AGROCALIDAD, unpublished). The estimated death rate is comparable to the lower outcome of the large-scale study conducted between 2016 and 2018, since the participants were mainly experienced meliponicultors. Additionally, this value, which is representative of most of Ecuador, indicates the preliminary stage of meliponiculture development and the limited number of participants. At this stage, the survey application process needs improvement. In Ecuador, the most effective method for achieving a representative sample size is for researchers to conduct face-to-face surveys in the field.

To explain why losses were higher in summer, we will refer to the behaviour of certain predatory and parasitic species, which have been shown to increase as temperatures rise (Pepi et al. 2018). Some of these insects are in the order Lepidoptera, which are known as leaf miners of coffee and citrus peel, while insects in the order Coleoptera feed on fruit, i.e., the coffee borer (Subedi, Poudel, and Aryal 2023). This increase in activity may be related to the increased use of pesticides on surrounding crops of meliponaries, to control pests, or to fumigations taking place in the meliponaries themselves to control disease-carrying insects (Castilhos et al. 2019), whose effects on stingless bees can be lethal (Bogo et al. 2025). Concerning the exploitation size (the extent of the meliponary) and the increase in losses, *Lestrimelitta* sp. attacks, followed by invasion of the Phoridae fly, may occur more frequently in areas with a greater number of stingless bee colonies. Under their natural attraction to these populations, *Lestrimelitta* act as biological controllers.

In nature, the annual loss rate for stingless bees was estimated to be between 12% and 15% in studies conducted in Africa (Kajobe and Roubik 2006) and Asia (Eltz et al. 2002), respectively. In Costa Rica (Latin America), an estimation of 11% was proposed for natural mortality, including events of predation by humans and/or animals. In this case, natural nest survival was affected by the species under study, the habitat, and the season (Slaa 2006). These factors could also explain the losses in managed stingless bees, as well as the risks associated with the human handling of stingless bees' nests. Feeding is a common beekeeping practice that has also been adopted in Meliponiculture. However, this practice poses a risk to stingless bees' health due to the transmission of pathogens through honey (Teixeira et al., 2025), pollen (Fleites-Ayil et al., 2023), resource-sharing (Guimarães-Cestaro et al., 2020), or honey bee wax contaminants (El Agrebi et al., 2020), the latter used to make “artificial” pots to stimulate pot-honey production in stingless bees.

The inexperience and ambition of new practitioners, coupled with inadequate or absent guidance from an experienced meliponicultor, can pose unnecessary risks to the lives of stingless bees (Jaffé et al. 2015a). Furthermore, certain practices requiring experience and knowledge can result in the death of entire colonies. The division of strong, well-managed nests is a common method for increasing the number of stingless bee nests. This practice has well-known guidelines that are followed by practitioners of meliponiculture (Ocaña-Cabrera 2025a). However, other aspects, such as the recovery of the “mom” and “daughter” nests, as well as the impact on foraging habits after division (Quezada-Euán 2018), have received little or no attention. A 31-day study of the splitting of a *Tetragonula carbonaria* nest found that the number of foraging stingless bees decreased to less than a third of those in the control (undivided) nest, and the proportion of pollen and resin foragers dropped to less than a quarter of those in the control group. Nectar foraging workers also decreased significantly until day 16, and increased significantly from day 29 onwards (Newis et al. 2023). In conclusion, dividing stingless bee colonies requires an experienced meliponicultor, as well as a period of post-division care to ensure satisfactory recovery and prevent nest loss.

Both climate change and human-induced relocation of stingless bee nests outside their natural distribution areas can cause thermal stress in colonies (Bender de Souza et al. 2025). Among the sublethal effects observed, larvae exposed to higher temperatures than normal had smaller body sizes and reduced symmetry in adulthood. The immune response of males was reduced, and the workers began foraging earlier than expected, resulting in a shorter lifespan (Quezada-Euán et al. 2024). These consequences demonstrate how even slight temperature changes can compromise the colony's fitness. A Brazilian study monitored stingless bee nest listings on product buying and selling platforms for one month and reported 159 transactions involving different species of stingless bees. On average, these nests were transported 365 km between the sellers and buyers (dos Santos et al., 2022). The main areas of shipment were exotic, meaning that previous nest translations had occurred, and the new translations represented the introduction of species to the environment. For the Amazonian species *Melipona flavolineta* and *M. seminigra*, the areas in which demand was the highest were, on average, colder and had lower precipitation than their natural habitat (dos Santos et al., 2022). The latter is a risk factor for the survival of stingless bees due to their poor thermoregulation, as mentioned previously.

Displacing nests outside of their natural environment could also affect their genetic structure or genetic expression due to anthropogenic stress, as is the case with *Melipona mandacaia* (Bender de Souza et al. 2025). A study of four partially sequenced genes in 70 colonies (both wild and managed) of the common Asian stingless bee, *Heterotrigona itama*, revealed lower nuclear genetic variability and higher mitochondrial genetic variability (Wongsa et al. 2024). Conversely, the mitochondrial genetic variability of managed nests of *T. angustula*, a commonly kept stingless bee in South America, was lower than in wild populations (Santiago et al. 2016). To ensure a successful displacement of nests, in exceptional cases, careful consideration must be given to the local adaptations of stingless bee species that are revealed by their high genetic differentiation according to the region. Small-scale male dispersal, together with the practice of nest splitting, seems to be enough to maintain a good nuclear genetic variability in a stingless bee population.

- **Improvement of Meliponiculture in Ecuador**

Meliponiculture in Ecuador has probably existed for centuries, but unfortunately, there are no extensive historical records. Kichwa communities in the Amazon region have a long history of using stingless bees' pot-honey in ancestral medicinal practices (Vit et al. 2017). In the coastal region of the country, Silvio Loayza, one of the most respected Ecuadorian meliponicultors, has practised this activity with his family since 1941 (personal communication). In the Highlands region, the distribution of stingless bees does not extend beyond the sub-Andean regions or pre-Andean zones (up until 2100 m.a.s.l) (Vit et al. 2018).

The practice of meliponiculture gained significant momentum during the COVID-19 pandemic due to the high demand for products to treat respiratory disease and the high economic value of pot-honey and other stingless bee products, as well as the lack of work opportunities. This has brought to light the various methods of raising and managing stingless bees, as well as the need for regulation to mitigate losses and risky practices. Table 3 summarises projects developed in Ecuador.

Table 3. Initiatives on the conservation of stingless bees and the improvement of meliponiculture in Ecuador.

Initiative	Objective	Beneficiates	Funding entities
Improvement of meliponiculture in Ecuador	Improve the meliponiculture in Ecuador through the technology transfer and training. Propose a micro-business model for the commercialisation of pot-honey and pot-pollen	Small stakeholders of Orellana (Amazon region) and Loja provinces	ARES from Belgium. Université de Liège, BOS+, and ESPE University as a partner
Meliponiculture: a sustainable tool in agroecological systems for rural communities in Ecuador	To study the health of stingless bee nests that form part of agroecological systems in rural areas of the Ecuadorian Amazon by identifying pesticides, conducting microbiome	Large, medium, and small-scale stakeholders in the Amazon region (Orellano, Napo, and	ARES from Belgium. Université de Liège, Eclosio NGO, and ESPE University as a partner

	studies, and promoting good management practices.	Pastaza provinces)	
Mancomunidad "Las Meliponas"	Organisation of the local value chain of meliponiculture through technical strengthening, collective management, marketing, and conservation strategies.	Rural communities of Ciano, El Arenal, and Vicentino, in the Loja province	Municipal (local) funds, regional rural development projects, contributions from NGOs, and technical cooperation from universities
Universidad particular de Loja (UTPL) research programs	Socioeconomic and technical research on meliponiculture, training, promotion of sustainable enterprises, and university-community links	Rural communities in the southern area of Loja province	University funds, international cooperation projects, regional funding (local governments), and collaborating NGOs
Conservation and meliponiculture initiatives in the Ecuadorian Amazon	To promote the conservation of native pollinators through the transfer of local knowledge and the development of meliponiculture as an economic source of income for Amazonian families, as well as to encourage communication and sustainable management	Rural and indigenous families in the Ecuadorian Amazon	Environmental NGOs, international cooperation programmes, and multi-sector financing (funds aimed at conservation and livelihoods)
Food and Agriculture	Strengthen the production, health certification, and	Community initiatives working	Forest and Farm Facility (FFF)

Organization (FAO)	marketing of honey in the Podocarpus region	with bees and landscape management in Ecuador (i.e., Abejita Lonjeva, TSAPAU agricultural association)	funding for bio enterprises, local governments, technical cooperation, and non-reimbursable funds

These supportive projects added valuable scientific knowledge and skills, improving the abilities of meliponicultors in Ecuador. However, unethical practices were also employed to achieve ambitious objectives. The cruel extraction of stingless bees from their natural nests, followed by the relocation of colonies to create a set (meliponary), without considering climatic or floral conditions, resulted in colony losses and unnecessary deforestation. As is the case with Mexican meliponiculture, a critical paper about it mentioned the ecological requirements, such as breeding, in addition to population movement, as their main concerns with this dramatic shift in meliponiculture from a neglected to a spotlighted field (Quezada-Euán et al. 2022).

As demonstrated in Table 3, the data clearly show that projects are predominantly developed within designated preference areas. This creates a growing discrepancy with other provinces where meliponiculture is also practised, but with lesser support. This allows meliponicultors to experiment independently or adopt poor practices based on unverified information from sources such as social media (Quezada-Euán et al. 2022). In Brazil, a study revealed a significant influence of years of experience, acquired technical skills, and adequate property as predictive factors of successful colony multiplication and the sale of honey or nests. The cultural relevance of specific species was the main reason to prefer the management of some species above others, followed by the quality of products, the facilities of the management, and the behaviour of the species (Campos et al., 2025). Thus, on one hand, to conserve stingless bees, areas with less intervention by unethical initiatives could act as sanctuaries or protected areas for this species (Adams et al. 2023). On the other hand, training provided to meliponicultors by academia or experienced entities, as well as specific local research, would significantly improve meliponiculture practices. This would help to maintain equilibrium in the face of increased exploitation and losses of stingless bee colonies (Requier et al. 2024).

Last but not least, the lack of knowledge about stingless bees is a topic that should be improved. The loss of stingless bee species due to several causes, as well as the interaction of various other factors, poses a problem concerning the genetics of species that have gone extinct without even being recognised by the community (Martins et al., 2025). Emphasis should be placed on species of bees deemed to be at risk of extinction due to the lack of nest reports in the wild. A salient example is *Melipona illota*, whose nesting habits are intricately linked to long-lived trees, which are frequently sought after for their pot-honey high commercial value (Marconi et al., 2020). *Melipona titania* and *M. fallax* are also at risk of extinction because their nests are frequently destroyed when they are erroneously identified as those of robbers or aggressive bees (Camargo & Pedro, 2008).

In addition to deforestation, the destruction of nests by pot-honey collectors has been identified as a contributing factor to the decline in populations of stingless bees, particularly those of the genus *Melipona*, which are renowned for their high pot-honey production. Concerted efforts must be made to promote greater awareness of *Melipona* species and others, along with their respective biological characteristics. These efforts should also include the improvement of meliponiculture through best management practices and community awareness programs to curb nest destruction (Carvalho-Zilse & Nunes-Silva, 2012).

- **Data representativeness and bias**

In a significant contribution to the diversity of stingless bees in Ecuador, Vit et al. (2018) reported the presence of Meliponines in 23 provinces of the continental Ecuador. The Galapagos Islands are the only province of Ecuador where these bees are not found or have not been reported to date. Given the latter study, one can reasonably assume that there are meliponicultors in every province. However, the most recent census conducted by AGROCALIDAD in 2024 revealed a total of 209 meliponaries across 14 provinces, with 2162 managed colonies (unpublished information). The present research focused on three provinces: two in the Amazon region (Orellana and Sucumbios) and one in the highlands (Loja). According to the census conducted by the National Health Regulatory agency, Loja has 54 meliponaries (second largest), Orellana has 29 (third largest), and Sucumbios has two. This highlights the discrepancy between the collected data and the current situation in the field. However, the study areas were strategically selected to encompass two of the three provinces with the highest number of registered meliponaries, and the resulting information can serve as a reference for other provinces in the country.

One reason for the discrepancy between the current data and the recorded data on the total number of meliponicultors is that much of the meliponiculture in Ecuador occurs in rural areas that are inaccessible to researchers, universities, and national agencies. According to the latest census, Napo and Loja are the provinces with the highest number of registered meliponaries. Both provinces have extensive NGO activity thanks to the strong relationships and trust that these organisations have built with local communities. These organisations are usually funded from abroad and typically collaborate with universities to promote research rather than with national agencies (Coreau 2020).

One reason why there are no up-to-date censuses in meliponiculture is that it is not recognised as a farming activity in Ecuador, unlike agriculture, livestock farming, poultry farming, and aquaculture. It only refers to wildlife in Article 247 of the Organic Environmental Code Regulations, the Manual for the Sustainable Management of Wildlife in Ex-Situ Conservation and Management Facilities for Commercial Purposes, and the Wildlife Use and Exploitation Regulation (MAATE and PNUD 2024). This causes meliponiculture to be practiced informally, as a hobby, and creates a lack of participation and interest among meliponicultors in engaging with regulatory institutions. In this sense, Mexican researchers emphasise the importance of local and national entities maintaining records that include details such as project numbers, colony numbers, species numbers, products, and commercialisation numbers. They also recommend implementing processes for evaluating the outcomes of stingless bee conservation initiatives by cultural and biological authorities (Quezada-Euán et al. 2022). The first country to enact federal and state laws and regulations on meliponiculture and its products was Brazil (dos Santos et al., 2022). Colombia and Bolivia have specific subnational-level regulations. Argentina has approved regulations relating to specific aspects, such as registration and activity management systems. In Mexico, Costa Rica, and Bolivia, national permits for meliponiculture address the sanitary aspects of honey production and marketing, as applied by the Secretaría de Medio Ambiente y Recursos Naturales. Peru has published Law No. 32235, which declares apiculture to be of national interest. The objective is to protect native stingless bees, as well as *Apis mellifera*, and to promote their rearing as a sustainable, productive activity (Agencia Agraria de Noticias 2025).

Despite this attempt to strengthen regulations, Brazil and other countries, such as Colombia and Bolivia, have faced challenges due to a lack of official data and low compliance with environmental standards among meliponiculors. These factors have hindered the development of public policies to improve and promote the sustainability of the sector (Santos et al. 2021). A similar situation arises between the meliponiculture community and academia in Ecuador. Given that these bee species are considered wildlife in almost all countries, their management may be subject to different regulations, permits, authorisations, or licenses from the competent authority (Gutiérrez-Chacón et al. 2025).

In Ecuador, meliponiculture is practised in a variety of forms, including artisanal and subsistence models, as well as commercial, scientific, educational, conservation, and entomotourism activities (Aldasoro Maya et al. 2023). Meliponicultors have organised themselves into legally constituted or informal associations in provinces such as Loja, Imbabura, Esmeraldas, Manabi, El Oro, Pastaza, Orellana, Napo, and Morona Santiago to access certifications and funding that will enable them to continue training and advancing in the rearing, management, and technical modernisation of stingless bees. Recent years have seen the emergence of civil society initiatives, such as the "Colectivo en Defensa de los Polinizadores", which focuses on political activism on behalf of these insects (Colectivo en Defensa de los Polinizadores 2025).

According to the pot-honey analysis, the main exploited species along the three natural regions in Ecuador are *Cephalotrigona capitata*, *Melipona indecisa*, *M. mimetica*, *M. grandis*, *M. eburnea*, *M. cramptoni*, *Scaptotrigona polysticta*, *S vitorium*, *T. angustula*, *Geotrigona* sp., *Nannotrigona chapadana*, *Oxytrigona mellaria*, *Paratrigona* sp., and *Trigona silvestriana* (Villacrés-Granda et al. 2021; Vit et al. 2023). In the present study, some species were analysed, with a particular focus on the pollen sources (Ocaña-Cabrera, Martin-Solano, Ron-Román, et al. 2025). This enabled the creation of interaction networks (Ocaña-Cabrera et al. 2022). We were also able to identify the genera and areas affected by pesticides such as glyphosate.

- **Challenges for stingless bees in Ecuador**

Plant Protection Products and metals concerning aspects for stingless bees

Despite the extensive distribution of meliponines, the impact of pesticides on stingless bees remains largely unknown. A systematic review conducted through October 2024 identified only 144 research articles on the effects of pesticides on Meliponini, 80% of which had been carried out in Brazil. The main genera studied were *Melipona*, *Tetragonisca*, and *Scaptotrigona,* and 72% of the individuals analysed were sampled from managed colonies (Bogo et al. 2025). No studies have yet been conducted in Ecuador. Given the country's intensive use of pesticides and poor regulatory enforcement, pesticides are among the leading causes of loss of stingless bee nests.

In terms of experimental methodologies, there is a significant lack of studies focusing on chronic exposure and field trials, as well as a scarcity of studies examining sublethal effects in stingless bees and effects on larvae. *Apis mellifera* is generally used as a model species in assays conducted in the temperate zones of the Northern Hemisphere, where Meliponini bees do not exist (Raine and Rundlöf 2024). Therefore, specific assays for stingless bees, and in particular for each species, are also recommended (Bartling et al. 2024). The risk posed by pesticides depends on the specific biological characteristics of each bee species, including their nesting and foraging habits and substrates, level of sociality, population dynamics, reproductive behaviour, dispersal, use of resources (Cham et al. 2019), body size, overwintering strategies, and ratios of pollen and nectar in larval and adult diets (Boyle et al. 2019).

In areas where agriculture and beekeeping or meliponiculture are practised, bees are commonly exposed, either directly or indirectly, to chemical contamination, including pesticide cocktails. The application of pesticides in fields is neither stationary nor localised; they contaminate all surrounding surfaces and can spread, polluting soil (Brühl et al. 2024), bodies of water (Didoné et al. 2021), the air (Zaller et al. 2022), and plants (Fan et al. 2021). This can result in the contamination of nectar, pollen, and plant resins, which then become potential sources of internal contamination within the colony where they can accumulate (El Agrebi et al., 2020).

Both honey bees and stingless bees actively seek water for various functions within the nest, including regulating the internal temperature, diluting larval food, and meeting metabolic needs (Schmaranzer 2000; Vollet-Neto et al. 2015). Therefore, water sources contaminated by agricultural activities or other industries can expose them to pesticide residues. Most fungicides are moderately lipophilic and can leach into the surface waters (Zubrod et al. 2019). Similarly, glyphosate, a hydrophilic herbicide, moves easily around the environment and can accumulate in plant tissues (Singh et al., 2020). This risk is exacerbated in stingless bees due to their habit of collecting mud and their intricate link with plant resin collection for defence (Shanahan and Spivak 2021). Stingless bees use this mud either to build the entrance to their nests or to maintain the batumen, an internal structure (Vossler 2024). In that sense, the non-*Apis* bee workshop's pesticide exposure paradigm proposed likelihood values of pesticide exposure, by route, ranging from 0 (marginal or no exposure) to 4 (high exposure), for both the adult and larval stages. For bees of the Meliponini tribe, the designated values for pollen (oral), mud/soil (contact), wax (contact), and propolis/resin (contact) routes were all 4. For water (oral) and nectar (oral) routes, the values were both 4 for adults and 2 - 3 for larvae, respectively (Boyle et al. 2019). This demonstrates the highest vulnerability to risk and the greatest number of exposure routes for stingless bees to agrochemical substances compared to other bees (Lourencetti et al. 2023b).

Cerumen, a substance made by stingless bees from wax and plant resins, is a valuable indicator of ecosystem health in terms of glyphosate, heavy metals, and metalloids presence. This allows for a broader perspective by simultaneously evaluating three pesticide exposure routes for both adults and larvae. In addition to assessing human risk associated with the honey storage function, this material serves within the nest. The concentrations found in this study were among 0.014 – 0.2 mg/kg, restricted to the zones in the Highlands region in Ecuador. Regarding *A. mellifera* products such as honey, the range of glyphosate contamination in 20 studies was from 0.002 mg/kg to 5.5 mg/kg. The maximum residue levels (MRLs) for glyphosate in honey bee products have not yet been established in Ecuador or Latin America. However, when compared with the limits set by various other countries (0.05 – 0.2 mg/kg), the concentrations in the present study do not exceed them, whereas certain illustrative values do so by a factor of 20 or 40. We can compare the composition of cerumen to that of beeswax from honey bees, for which a Belgian study determined an average of 0.062 mg/kg of glyphosate (El Agrebi et al., 2020).

The investigation into the presence of essential and non-essential trace elements in products such as honey from stingless bees remains limited; this issue gives rise to public health concerns (Demaku et al. 2023; Rai et al. 2019). While foraging for resources, bees come into contact with plants, water, air, and soil. They pick up suspended particles from these sources and carry them on their bodies (de Oliveira et al., 2025). Trace elements are essential for the human body because they act as catalysts in a variety of biochemical processes. However, problems can arise when concentrations exceed safe levels, leading to serious health issues. This increase in the levels of metals, metalloids, or heavy metals can occur through the direct ingestion of contaminated food (Neisi et al. 2024). Stingless bee pot-honey is widely consumed (Vit et al., 2024). The main factors explaining the higher presence of heavy metals in *Heterotrigona itama* honeys in Malaysia were the distance of nests from vegetation types and pollutant sources such as power plants, petrochemical centres, highways, and cities/towns, as well as relative humidity and temperature (Salman et al. 2022). Similarly, a study of raw honeys revealed a strong correlation between place of origin and variation in mineral content in pot-honey of stingless bees (Binjamin et al. 2024). The study in the present work of heavy metals and metalloids found that the maximum values for Sb, As, Cd, Cr, Sn, Ni, Pb, and Se were all found in the provinces of the Highlands region. The origin of the samples was also linked to the observed concentrations. Regarding the effects on bees, the expression levels of CYP9Q1, CYP9Q2, and CYP9Q3, as well as the genes encoding catalase and superoxide dismutase, were notably higher in urban honey bees due to a detoxification process in response to contaminants such as mercury and lead (Gizaw et al. 2020). In the interest of this work, conducting studies on the detoxification processes of stingless bees exposed to pesticides or heavy metals is recommended. In this regard, functional orthologues of CYP6AQ1 could be used, as these enzymes have been documented to hydroxylate flupyradifurone (Xiao, Haas, and Nauen 2023).

Lack of guarantees for the protection of stingless bees in Ecuador

Using *Apis mellifera* wax to stimulate honey production in meliponines and feeding them honey or pollen poses a health risk due to the origin and quality of this material. Several studies have detected residues of contaminants, such as pesticides (Bischoff, Baert, and McArt 2023), PAHs (Schaeffer et al. 2024), heavy metals (Aljedani 2020), veterinary drug residues (El Agrebi et al., 2020), as well as pathogens (Colwell, Pernal, and Currie 2025; Flores, Spivak, and Gutiérrez 2005) in honey bee wax. In Ecuador, there is no consolidated public policy to promote and regulate beekeeping, let alone meliponiculture. The few existing regulations aim to ensure the sanitary control of beekeeping products, guarantee food safety, and prevent bee diseases (Rosero 2016). These regulations, primarily issued by the Ecuadorian Ministry of Agriculture and Livestock (MAG) and AGROCALIDAD, include the registration of apiaries, the acquisition of animal health certificates (Programa Nacional Sanitario Apícola 2024), adherence to good production practices, and the implementation of biosecurity measures (Vizcaíno and Betancourt 2015b). Requirements have also been established for the import and export of beekeeping products, such as honey and beeswax, with a particular focus on traceability (Comité de Comercio Exterior 2021). In 2024, a bill to encourage and regulate beekeeping was presented to the Food Security Committee of the National Assembly of Ecuador. The project aimed to establish a legal framework that supports the sustainability of the industry, guarantees the traceability of apiculture products, and protects bee-supporting ecosystems. However, the success of the project will depend on the State's capacity to improve interinstitutional cooperation (Figure 15), provide sufficient funding, and guarantee the active involvement of beekeepers in policymaking (Meza 2025).

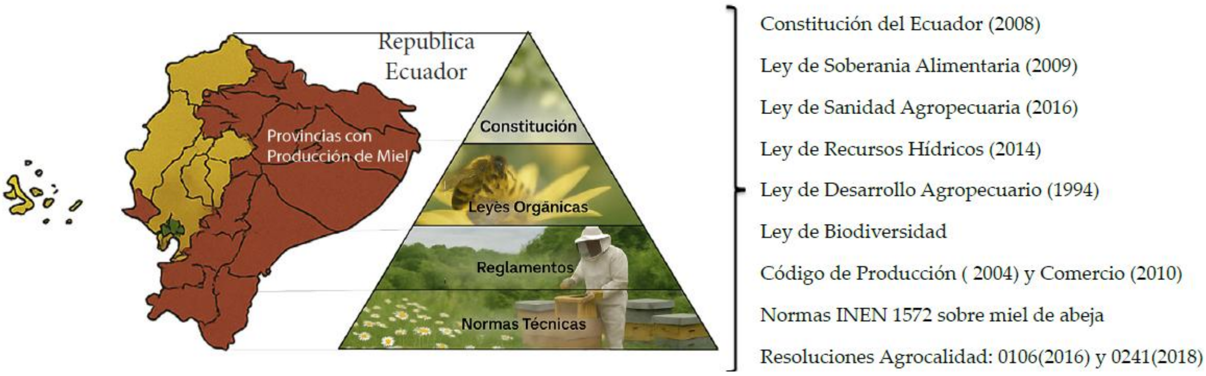

Figure 15. Application of Kelsen's Pyramid to the Legal Framework of Apiculture in Ecuador (Meza 2025). The translation in English of Ecuadorian laws, from top to down, the Constitution of Ecuador (2008), the Food Sovereignty Law (2009), Agricultural Health Law (2016), Water Resources Law (2014),

Agricultural Development Law (1994), Biodiversity Law, Production (2004) and Trade (2010) Code, INEN 1572 Standards on honey, AGROCALIDAD resolutions: 0106 (2016) and 0241 (2018).

Beekeeping is not Meliponiculture (stingless beekeeping)

As mentioned previously, despite the efforts made to do so, beekeeping in Ecuador is not fully regulated. This legal vacuum, coupled with the authorities' lack of commitment to enforcing the limited regulations that do exist, has prevented beekeeping from being practised in the best possible way. Beekeepers have primarily relied on trial and error, as well as experiences shared among stakeholders or on social media, to improve their practices. Due to the growing popularity of meliponiculture, several beekeepers have become involved in stingless beekeeping, believing that the same techniques and 'tricks' can be used for both. This mindset has spread among inexperienced meliponicultors who base their training on practices in *A. mellifera* management, thereby increasing the risk of death for stingless bees beyond that imposed by management alone.

The joint practice of beekeeping and meliponiculture must be carried out with great caution and a vast knowledge of the species being managed. Poor practices in both, in addition to being unsustainable, lead to the loss of stingless bee nests on many fronts. Certain species of stingless bees, such as *Trigona corvina*, compete fiercely for food resources such as pollen and flower nectar (Johnson and Hubbell 1974). Battles can last up to two days and result in the death of 1,800 stingless bees. Initially, the reason for this behaviour was explained by describing a bait with a high sucrose concentration as the resource that triggered a fight, as well as a maximum number of individuals — between 50 and 60 — at the same resource, triggering a battle between workers from different colonies. However, in the field, the stingless bee species showed no particular preference for any floral resource (Hubbell and Johnson 1978; Toledo-Hernández et al. 2022). In addition to this behaviour among the stingless bees themselves, human activity has introduced other competitors into their habitat, putting them at a disadvantage. Pioneering studies have shown that introducing *Apis mellifera* hives near flower patches leads to a decline in *Melipona* bee populations and a decrease in their pollen-collection frequency (Roubik, 1980). This results in reduced brood production (Maia-Silva et al. 2016). In response to this pressure, changes in the behaviour and foraging times of stingless bees have been documented, thereby reducing competition (Hung et al., 2019; Roubik, 2009).

The production and selection of queens in stingless bees is a natural phenomenon. However, human intervention through artificial queen selection and breeding raises ethical and sustainability considerations regarding animal welfare and the potential exploitation of these species. Acceptance tests of in vitro-reared *Plebeia droryana* queens demonstrated an 80% acceptance rate when the colony's older workers were absent (dos Santos et al., 2016). The stingless bee caste differentiation is predominantly governed by the quantity of sustenance absorbed by the larva (Baptistella et al. 2012). However, a remarkable phenomenon occurs within the genus *Melipona* during the process of queen production: self-determination. The brood cells of workers and queens are similar in size, and it is estimated that up to 16% of larvae not destined to become queens undergo a form of selfish development, resulting in the emergence of virgin queens. In *M. beecheii*, there is a rapid and efficient elimination of excess virgin queens, resulting from a caste-fate conflict (Wenseleers et al. 2004). In addition to the existing gap in the study of queen development and its implications within the nest for a greater number of stingless bee species, the limited knowledge available indicates that after excessive queen production, worker bees focus their efforts on killing virgin queens. This process takes them between 27 and 47 hours (Wenseleers et al. 2004), and as a result, they neglect other important tasks within the nest, such as resource collection or nest defence. For a successful queen introduction, acknowledging the necessity of excluding older workers is crucial due to the low acceptance rate (10%) (dos Santos et al., 2016), so if this practice is followed, the nests would be left with insufficient workers, making them vulnerable to attacks and resource scarcity. Even if the meliponicultors carry out all the activities that the stingless bee workers would not perform due to the stressful process of introducing and selecting queens, this disrupts the natural development of stingless bee nests. This practice is something that was learned from beekeeping, where an entirely different species and biology are involved. The justifications for adopting this practice range from increasing the number of pollinator nests to boosting pot-honey production; in both cases, the economic benefit to humans is clear. Further studies should be conducted on this topic to determine the best approach or to list the irreversible consequences that could occur.

Pathogen spillover affecting stingless bees

The directionality of pollinator disease networks is dynamic and context-dependent. Nevertheless, transmission between species could cause stress to both managed and wild pollinator species, which are already experiencing population decline (Deutsch et al. 2023). The first case of *Melissococcus plutonius* in stingless bees was found in honey bee pollen used to feed them, showing the risk posed to susceptible species by the use and trade of contaminated apicultural products (Teixeira et al., 2020). The main natural route of pathogen transmission is pollen, also. In this regard, a study concluded that there is continuous spillover from domesticated to wild bee species due to the high prevalence of identical viral haplotypes in honey bees (80% BQCV and 14% DWV-A), compared to *M. beecheii* stingless bee, where the prevalence was much lower (15% BQCV and 1% DWV-A) (Fleites-Ayil et al., 2023).

A study in Ecuador found a prevalence of 4.88% of *Nosema apis* in stingless bees, suggesting that the main cause was the presence of honey bees in the same meliponary. Other risk factors included temperature and the genera studied: *Tetragonisca* and *Scaptotrigona* (Guaita Gavilanes 2019). Individuals of the species *Melipona colimana* infected with *N. ceranae* spores developed infections exceeding 467,000 spores per stingless bee. This resulted in a significant decrease in haemocyte counts. In a separate group of individuals within the same study, infection with *N. ceranae* spores and exposure to thiamethoxam (4.2×10^{-3} ng/µL) resulted in increased mortality and an inhibitory effect of thiamethoxam on the microsporidium (Macías-Macías et al. 2020). These results demonstrate that *N. ceranae* can infect and proliferate in stingless bees, causing cellular immunosuppression, and that exposure to sublethal concentrations of thiamethoxam in infected stingless bee colonies can lead to nest loss. A comprehensive study of the health consequences of *N. apis* infection in stingless bees is strongly recommended, as is the expansion of *N. ceranae* infection studies to a greater number of species. Meliponicultors must also exercise the highest level of caution and commitment to managing bees of different species responsibly and differentiating their practices. Adopting biosecurity measures could be an effective way of combating cross-infections.

- **New threats facing stingless bees**

Microplastics

Polypropylene particles were detected in honey from the Brazilian bee species *Melipona quadrifasciata.* The particles were predominantly transparent fibres measuring less than 299 µm in size, with concentrations ranging from 0.1 to 2.6 particles per millilitre of pot-honey (Rani-Borges et al. 2024). An average concentration of 8.18 ± 2.57 microplastics per gram (MPs/g) was detected in *Heterotrigona itama* pot-honey in Malaysia, predominantly in the form of transparent fibres and fragments ranging in size from 0.7 to 1.8 mm (Ibrahim et al. 2025). Pot-honey can become contaminated with microplastics at various stages of production, from the collection of nectar by stingless bees from contaminated floral sources to processes carried out by meliponicultors, such as harvesting and packaging.

The ingestion of 500 ng of PET (polyethylene terephthalate) microparticles by *Partamona helleri* larvae increased body weight. Alterations in behaviour and total haemocyte count were also observed in adult *P. helleri* that ingested these microparticles (Viana et al. 2023). Plastic nano- and microparticles can enter the human body through the respiratory system when inhaled (Gaylarde, Baptista Neto, and da Fonseca 2024), through the digestive tract when consumed in contaminated food or water (Mamun et al. 2023), or through the skin when in contact with cosmetics or clothing (Allen et al. 2019; Enyoh et al. 2020). Bioaccumulation of plastics in the human body can lead to respiratory disorders such as lung cancer, asthma, and pneumonitis (Saha and Saha 2024), neurological symptoms such as fatigue and dizziness, inflammatory bowel disease, and alterations to the gut microbiota (Sofield, Anderton, and Gorecki 2024). In vitro studies have shown that the cytotoxic and apoptotic effects of MPs, as well as cell damage, are influenced by particle characteristics such as type, size, and charge (Winiarska, Jutel, and Zemelka-Wiacek 2024).

In Ecuador, the practice of using acetate sheets to divide modernised hives has spread rapidly. There is growing concern among meliponicultors about the impact of microplastics on stingless bees and the quality of the pot-honey they produce. However, no microparticles originating from cellulose acetate were detected in the aforementioned studies. Nevertheless, there is a strong recommendation to study the impact of this widely used material on the health of stingless bees and the effect of its bioaccumulation in pot-honey packaged for human consumption.

Mining as an exacerbating factor in the habitat loss of stingless bees

Mining has become an important source of income for Amazonian countries. However, restoration projects intended to reduce the environmental impact of mining activity suffer from a lack of supplies, poor planning, and technical and legal issues that delay them (Martins et al., 2022). In the Brazilian Amazon, mining has a significant impact on tropical deforestation, with an increase of up to 70 km beyond the boundaries of mining concessions, resulting in 11,670 km² of deforestation in a decade. This increase in area losses is attributable to processes not incorporated within environmental licences, including the establishment of mining infrastructure, urban expansion to accommodate a growing workforce, and the development of mineral product supply chains (Sonter et al. 2017). The failure to address this impact in the plans submitted by mining companies caused significant and often underestimated damage to biodiversity and communities. This phenomenon is not exclusive to Brazil and also occurs in countries like Ecuador (Mena-Quintana et al. 2025). The gold rush has had a significant impact on the Ecuadorian Amazon, with deforestation related to mining increasing by up to 300% over the last decade. This has had a severe impact on the country's water and land resources (Mestanza-Ramón et al. 2023; Passarelli et al. 2024).

In 2022, Ecuador experienced a significant loss of natural forest, amounting to 51,700 hectares. This deforestation led to an estimated emission of 36.9 million tons of CO_2 into the atmosphere. The primary drivers of deforestation were identified as open-pit mining, oil extraction, and intensive agriculture (Herrera-Feijoo 2024). Degradation of soil and water quality due to a reduction in forest cover is associated with the extinction of species, a reduction in ecosystem services, and an increase in greenhouse gas emissions, notably CO_2 (Mena-Quintana et al. 2025). Sucumbios, Napo, and Morona Santiago provinces of the Amazonian region in Ecuador account for the largest share of land affected by open-pit mining (Monitoring of the Andes Amazon Program 2023). The activity under discussion introduces toxic heavy metals into the environment. These include mercury (Hg), lead (Pb), cadmium (Cd), zinc (Zn), and arsenic (As). All of these can have harmful effects on living organisms and ecosystems, even at low concentrations (Balali-Mood et al., 2021; de Oliveira et al., 2025).

As elucidated in this study, stingless bees naturally inhabit both hollow logs and subterranean nests throughout the Ecuadorian Amazon. These insects are not considered in mining concession implementation plans, and restoration plans are also ineffective for the survival of these hymenopterans. Other animals with migratory tendencies may have a chance of survival by fleeing to areas that have been destroyed; however, the perennial nests of stingless bees condemn them to perish when their habitat is deforested.

- **Opportunities: nutritional improvement, biosecurity, and good management practices**

Understanding the plants that stingless bees use for trophic or nesting resources is essential for conserving them and maintaining plant species that are useful for pot-honey production in their distribution areas (Absy et al. 2018). *Melipona seminigra* and *M. interrupta* tend to rely on a few continuous pollen sources throughout the year; these sources are key to maintaining pot-honey production over time (Ferreira & Absy, 2015). Identifying the pollen sources of the managed stingless bees is key to promoting colony development and health, enabling stingless bees to produce high-quality pot-honey. Effective management of stingless bees involves observing and learning about each species, as they differ in terms of honey production volume, resilience to changing conditions, seasonal production, and the types and flavours of pot-honey produced (Absy et al. 2018). Knowledge of pollen resources can also be used to justify forest preservation plans and sustainable production systems (Nicholls and Altieri 2013). Landscapes that are primarily focused on agriculture tend to eliminate or fragment natural areas. This results in a scarcity of food resources and nesting sites for stingless bees (Slaa et al. 2006). This is why agroecological systems are preferable when using these insects for pollination.

The natural diet of most stingless bees is based on nectar and pollen. While pollen contains proteins, lipids, vitamins, and minerals as well as carbohydrates (Vit et al. 2016), nectar is the main source of sugars. When stored in pots, pollen undergoes vigorous fermentation by yeasts and bacteria; this process seems to impact preservation quality and a preference factor by stingless bees (Menezes, Vollet-Neto, Contrera, et al. 2013). In a meliponiculture with the aim of commercialization, pot-honey and pot-pollen substitutes are useful, especially during periods of scarcity and after colony division or artificial multiplication, to enhance the colony. Replacing pollen through intentional feeding products proves difficult (Vollet-Neto et al. 2010).

Insects are unable to synthesize sterols; as a consequence, they must obtain them from their food. Bees incorporate sterols via pollen, utilising them as cholesterol precursors to accumulate fat reserves that are advantageous during periods of food scarcity. Proteins are vital for the sustenance of larvae and the general development of young bees, as well as for the repair of cells and organs in older bees (Pang et al. 2022; Schwarz et al. 2024). However, the nutritional value, diversity, and composition of floral resources for bees vary depending on the surrounding environment and can be significantly changed in environments affected by human activities. When the surrounding environment has a larger species richness of plants, the quality of the larval diet (pollen, nectar, and saliva) for *Tetragonula carbonaria* bulk provisioning was likewise higher. Higher levels of the omega-3 fatty acid linolenic acid and a 40% rise in the protein: fat ratio—which is known to have an impact on bee foraging (Vaudo et al. 2020)—were indicative of this. A decline in the omega-6:3 ratio, which is known to impair bees' cognitive function, was linked to plant species richness (Trinkl et al. 2020). The number of males in stingless bees is lower when the colony's food reserves are depleted. Males that were reared with 300 grammes of pot-pollen reserves had significantly smaller bodies and lower sperm counts than those that were reared with 700 grammes of pot-pollen reserves. The production of male offspring in *M. beecheii* colonies appears to be strongly influenced by the season and the quantity of pollen available (Pech-May et al. 2012).

The direct correlation between pollen reserves and the capacity of stingless bees to develop an immunological response against infections or stresses makes it clear that this ability is dependent on the bees' nutritional status (Fonte-Carballo et al. 2021). Large volumes of pollen make up the larval feeding of stingless bees; in *Melipona marginata*, pollen makes up 50% of the larval food's volume (Rensi 2006). Both adults and immature individuals are impacted by protein consumption, and a low protein diet might impair colony health (Wu et al. 2024). The nutritional deficiencies in *M. quadrifasciata* foragers following a monofloral diet of eucalyptus pollen, which is characterised by a low lipid content, can result in alterations to lipid metabolism and an increased susceptibility to disease in bees. The microbiome may be affected, with a decrease in symbionts that have been shown to have direct antimicrobial activity against bee pathogens (Haag et al. 2022; Miller, Smith, and Newton 2021).

The implementation of biosecurity measures in beekeeping aims to mitigate the movement of microbes and pests within the apiary, prevent their introduction, and minimize their impact. To maintain bee health and honey quality, biosecurity principles must be followed, with appropriate beekeeping practices employed to prevent the spread of infectious agents. Such agents can be spread through bees, feed, and technological systems (Borum 2022). Given reports of pathogen transmission from *A. mellifera* to stingless bees, it is possible to adopt this biosecurity concept in meliponiculture. One lesson we can adopt is applying a combination of the best disease management and biosecurity practices to keep bees healthy and vigorous. This will improve their disease resistance (Obbink and Roth 2021). An international survey was conducted to ascertain the most efficacious biosecurity measures capable of preventing and controlling the main infectious diseases of the honey bee. The respondents, predominantly European, expressed a high level of acceptance towards sustainable practices, including the removal of honeycombs exhibiting signs of dysentery and the feeding of colonies to combat nosemosis. They also expressed a high level of acceptance towards the rapid identification and management of hives affected by American foulbrood and European foulbrood. To a considerable extent, these responses can be attributed to the substantial knowledge base that beekeepers in this region possess, as well as their heightened awareness of the health status of their hives, a consequence of advanced, practice-oriented training (De Carolis et al. 2024).

The Australian Native Bee Association acknowledges the paucity of knowledge surrounding biosecurity practices for native bees. However, the Association recommends a series of steps to initiate improvements in this area. Firstly, it is imperative to gain an understanding of the pests and diseases that have the potential to impact native stingless bees (Ocaña-Cabrera 2025b), and to disseminate this knowledge to other meliponicultors. Colonies must be familiarised with through meticulous observation and care to recognise new or unusual symptoms or behaviours. Secondly, it is imperative to ascertain the provenance of a stingless bee colony or log before its acquisition. Equipment must be acquired from reliable sources that are free of pests. Thirdly, once in the meliponary, ensure that the tools to use on the colonies are clean and free from residue or materials before and after each use. Recommended cleaning solutions include a bleach solution diluted in water (1:100) or hot-soap water. Pests and diseases can be transmitted between managed and wild colonies through pot-honey robbing. Ensure that any colonies that have died suddenly or show signs of fly and/or beetle infestations or brood diseases are never left outside for stingless bees to rob. If a colony has completely perished and there are no live stingless bees, it is possible to clean and reuse the wooden box. This should consist of removing the nest contents, scrubbing with water and a stiff brush, disinfecting, and allowing the wooden box to dry completely. Take care when opening hives in conditions conducive to robbing (i.e., when floral resources are scarce), as stingless bees may raid other hives and spread pests or diseases to other colonies (Plant Health Australia 2022). In this regard, adapting the biosecurity measures proposed by Saegerman et al. (2012) for animal facilities in meliponiculture is recommended (Figure 16).

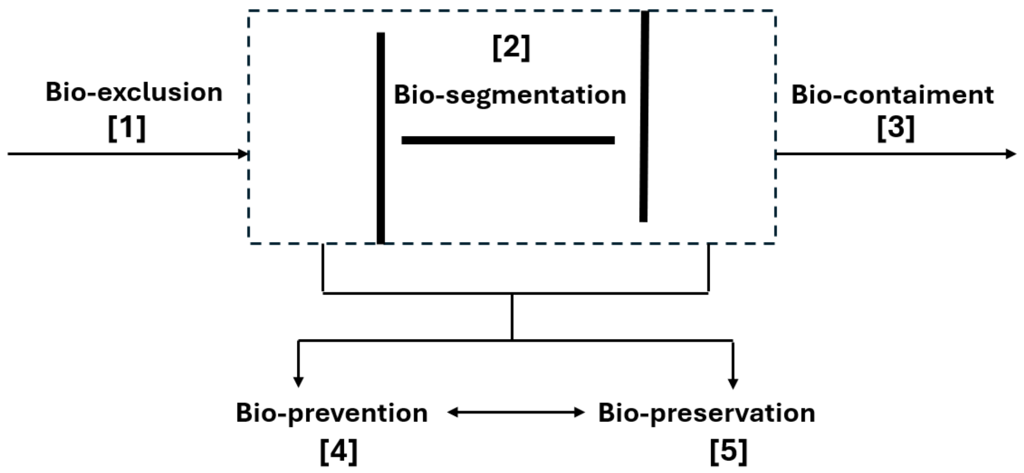

Figure 16. Biosecurity principles (5 Bio's) in the stingless bees' facilities. Modified from (Saegerman et al. 2012).

1. Bio-exclusion: Biosecurity measures to prevent the introduction of a pathogen/contaminant into a meliponary.
2. Bio-segmentation: Biosecurity measures to prevent the spread of a pathogen within a meliponary.
3. Bio-containment: Biosecurity measures to prevent the pathogen from spreading to other meliponaries or facilities (i.e., the pot-honey storage room)
4. Bio-prevention: Biosecurity measures to prevent the spread of pathogens/contaminants to humans through products intended for consumption.
5. Bio-preservation: Biosecurity measures to prevent environmental contamination.

Adherence to biosecurity measures would have a direct and significant impact on the quality of pot-honey produced by commercial meliponaries. The quality standards for meliponine pot-honey are characterised by a higher moisture content compared to honey produced by honey bees. This attribute is conducive to the process of microbial fermentation, which represents a distinctive adaptation exhibited by stingless bees in their endeavour to preserve their watery pot-honey through the active metabolites that are produced by their microbial symbionts (Menezes, Vollet-Neto, Contrera, et al. 2013). The process of fermentation results in the generation of a mixture of organic acids and alcohols, thereby increasing the free acidity and decreasing the pH level (Mokaya et al. 2022). Following harvesting, the acidity levels in pot-honey are subject to change over time due to the ongoing process of fermentation. In addition to fructose and glucose, stingless bee pot-honey contains trehalulose, a reducing disaccharide sugar that serves as an indicator of the authenticity and quality of a natural product (Zhang et al., 2022). The implementation of biosecurity measures and the adoption of good management practices in meliponiculture would ensure the health of the stingless bees and the quality of pot-honey. Furthermore, it would improve the handling of other derived products, such as pot-pollen, propolis, and cerumen. These products have the potential to become important supplementary income sources, enhancing the resilience and profitability of meliponiculture (Mduda et al. 2025). In humans, stingless bee pot-pollen is highly nutritious and constitutes a healthy “alternative” food source, as pot-pollen extracts are known to inhibit oxidising agents and free radicals — an important property in the prevention of various diseases (Silva et al., 2009). As the pollen from the flowers that stingless bees feed on is typically acidic, it is recommended that it be consumed as a honey-and-pollen jelly or a creamy milk-and-pollen smoothie to neutralise the acidic taste of the pot-pollen (Menezes, Vollet-Neto, Contrera, et al. 2013).

Valorising cultural heritage as a conservation and sustainability strategy

The ancestral knowledge and values of indigenous peoples and local communities in Ecuador, Peru, and Colombia, which challenge the status quo, have been used to inform various proposals for addressing the current environmental crisis. However, indigenous knowledge and practices are still not widely recognised as agents of transformative change. These initiatives include strategies to empower people, reconnect them with nature, and provide intercultural and ancestral education (I-Seeds), as well as knowledge co-development processes led by indigenous peoples and local communities with the greatest potential to promote sustainability (Jiménez-Aceituno et al. 2025).

The indigenous Kichwa people of the Ecuadorian Amazon employ a cultivation technique called the Chakra, which is known as a sustainable agricultural method that guarantees food production without interfering with the ecosystem's ability to function. To establish and manage the Chakra system, primary forests that are easily accessible (near rivers and roads) are selectively logged to create mixed cropping systems (chakras/ajas), after which the land is left fallow to allow the soil to recover (Álava-Núñez et al. 2025; Luna and Barcellos-Paula 2024).To exemplify these mixed crops, a total of 740 trees and palms were surveyed in 18 Chakra systems in Napo province; these and other plant species were actively managed to prevent competition and safeguard saplings. Food and construction are examples of utilitarian objectives that appear to influence the choice of trees and palms in the Chakra (Bredero zur Lage, Peña-Claros, and Rios 2023). Typically, a chakra system is between 0.2 and 4 hectares in size (Vera-Vélez, Cota-Sánchez, and Grijalva Olmedo 2019), and with 100 species of stingless bees described in the Yasuní Biosphere Reserve (Roubik, 2018), Orellana and Pastaza provinces, a Chakra system is a great solution for managing stingless bees.

A study in Ecuador identified 12 species of stingless bees in the Pastaza province, 11 of which had Kichwa names. The bees' honey, cerumen, entrance resin, and nests were found to have a diverse range of uses in ancestral ceremonies, rituals, natural medicine, and food (Paredes-Bracho 2022). Thus, indigenous communities in Ecuador possess valuable knowledge of agroecological systems and stingless bees. Together, these offer an excellent approach for anyone practising any form of meliponiculture. In this regard, science and research should support this knowledge, rather than imposing practices that do not benefit the community.

Midgut microbiome of stingless bees as an indicator of health status

In social bees, anthropogenic influences, including pollution, pesticides, and habitat fragmentation, drastically disrupt the makeup and function of the microbiota, which impacts the bees' capacity to absorb nutrients, fend off illnesses, and adapt to changing environmental conditions (Botina et al. 2019). The season, the stingless bees' age, colony development, and the study area or region can all affect the bacteria in stingless bees' guts. The great variability of Meliponini's microbiota may be a reflection of the evolutionary diversity of their morphologies, behaviours, and life histories (Kwong et al. 2017). Because stingless bees feed on pollen and nectar, they offer bacteria a nutrient-rich habitat that has been linked to their metabolic capacity (Li et al. 2015).

The valorisation project initiated by the author of this thesis, for which she obtained funding from ARES for its execution between 2024 and 2026, has yielded preliminary results on the bacteria inhabiting the gut of three genera of stingless bees in Ecuador. The identification of bacteria such as *Bifidobacterium asteroides*, *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus mellifer* Firm-4, and *Lactobacillus helsingborgensis* Frim-5 was facilitated by the utilisation of restriction enzymes (AluI, HpaII, EcoRI). In general, the functions of these bacteria are beneficial to the immune protection, nutrition, metabolism, digestion, and synthesis of bioactive compounds in stingless bees (Ramírez-Ahuja et al. 2025). The analysis of bacterial diversity by province revealed that El Oro, a coastal province, was ranked as the most diverse, followed by Pastaza, Orellana, and Napo (Amazonian provinces). The predominant bacterial strain in the gut of the stingless bee genus was identified as *Lactobacillus mellifer* Firm-4 (Bradford et al. 2022). *Melipona illota* demonstrated the highest bacterial alpha diversity, and concurrently exhibited a close correlation with the bacterial diversity of *Melipona eburnea*, both in the Ecuadorian Amazon.

- **One health approach**

A key element of managing stingless bees should be a One Health strategy that unifies the domains of environmental health, animal health, and human health (Figure 17). Because these fields are interrelated, this technique guarantees sustainable meliponiculture practices. In addition to increasing crop yields and generating pot-honey with a variety of uses in food, medicine, and cosmetics, stingless bees are essential to the preservation of biodiversity in tropical regions. As a result, food security and ecological resilience depend on their conservation. To enhance colony health, sustainable management techniques for these bees include preserving natural habitats, reducing chemical exposure, and fostering a variety of native floral supplies.

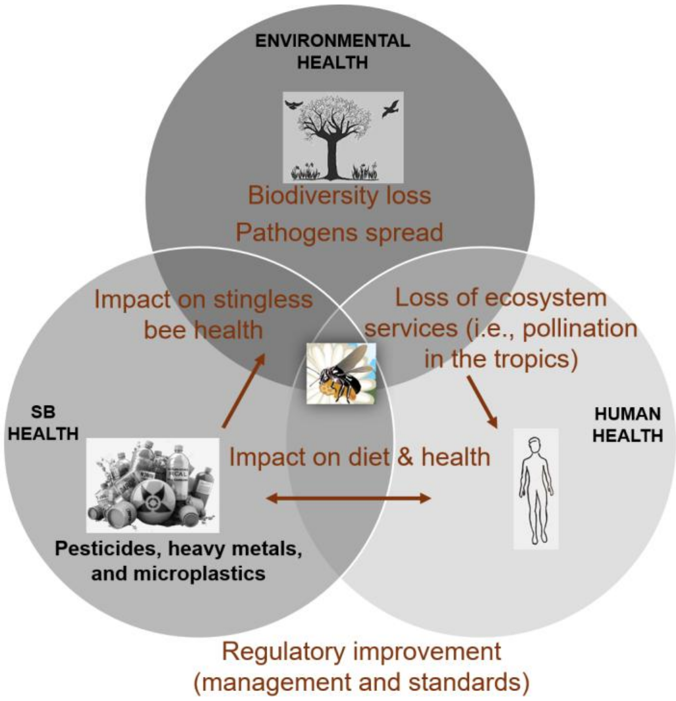

Figure 17. Stingless bees (SB) and the one health approach. Modified from (Mahefarisoa et al. 2021).

Land management determines which plant species are available to stingless bees. The political authorities are in charge of this management. Working directly with communities can be accomplished through environmental education programs, reforestation, propagation, and seed rescue. Involving local people in conservation and education initiatives ensures the long-term sustainability of stingless beekeeping by fostering resilience against habitat loss and climate change.

For the benefit of stingless bees and those who consume their nest byproduct, biosecurity measures (5Bio's), ethical harvesting methods, and clean nest maintenance have been associated with a decreased risk of pathogen spread. Additional preventive efforts could be beneficial, such as tracking and monitoring the effects of environmental or anthropogenic factors on stingless bees. Meliponicultors and stingless bees can both act as early warning systems for environmental deterioration and/or the existence of hazards to human health.

In addition to encouraging local food production and a deeper comprehension of ecosystems, the care and management of stingless bees' support sustainability and community living. Additionally, the human consumption of pot-pollen or pot-honey, or even the use of propolis and its derivatives in traditional medicine, completes this One Health cycle. Taking care of nutrition and the environment that are beneficial to stingless bees should be the first step in ensuring the quality of these items.

- **Recommendations**

The enhancement of meliponiculture and the management of risk factors for stingless bee health necessitate the concerted efforts and involvement of relevant stakeholders, comprising meliponicultors, veterinarians/apicultural technicians, researchers, regulatory bodies, and NGOs. The following recommendations (Table 4) are proposed for each of the stakeholders, with a short, medium, and long-term timeframe.

Table 4. Short-, medium-, and long-term recommendations for stakeholders in Ecuadorian meliponiculture.

	Short-term	Medium-term	Long-term
Meliponicultors	• Adopt good management practices and biosecurity measures • Practice meliponiculture with an ecological basis, considering appropriate environmental conditions and reducing the impact of activities such as colony relocation • Keep a meliponary logbook where abnormal incidents, harvest dates, and floral calendars are recorded for organized and effective management	• Improve good management practices and biosecurity measures • Stay in continuous training and keep up-to-date with new information about stingless bees from reliable sources, while filtering information obtained on social media • Report and share strange incidents with technicians, competent authorities, and researchers to seek solutions. • Create associations that benefit meliponiculturists as farmers and the conservation of stingless bees	• Maintain good management practices and biosecurity measures • Share the knowledge gained (be the reliable source) and train new meliponicultors as the next guardians and protectors of stingless bees • Share past experiences with researchers and regulatory bodies to actively collaborate in the creation or updating of regulations governing meliponiculture
Wildlife veterinarians/ apicultural technicians	• Train meliponicultors most appropriately, considering the ecosystem's carrying capacity based on the number of nests	• Update knowledge on meliponiculture, working closely with researchers to create ongoing training programs	• Encourage the creation or inclusion of meliponiculture in veterinary curricula

	and the availability of resources necessary for effective management • Promote sustainable meliponiculture by valuing the ancestral knowledge of the communities where it is practiced	• for meliponicultors • Learn basic taxonomy of stingless bees, aspects of healthy nests, and laboratory techniques to become the best guides for meliponicultors and liaisons with control agencies and researchers	• Promote local and national pest and contaminant control programs for stingless bees within the institutions to which they belong
Researches	• Disseminate the results of research on stingless bees through talks or educational materials, and leverage social media or reliable media outlets • Adopt the One Health concept in research studies	• Work on studies and research that fill existing gaps in stingless bee knowledge in the country, starting with the identification of stingless bees • Conduct ongoing, interdisciplinary research to enrich the results	• Propose projects that involve indigenous and rural communities, respecting their knowledge and recovering useful ancestral information • Actively participate in decision-making regarding regulations on stingless bees and their products as consultants or experts on technical committees
Regulatory bodies	• Establish regional guidelines for meliponaries and for stingless bee by-products to	• Efforts to comply with established regulations to prevent the indiscriminate relocation of	• Establish national regulations for stingless bees, working jointly with the Ministry of the

	ensure food safety and product quality for both human consumption and other uses, such as nutraceuticals • Approve the law promoting beekeeping based on scientific evidence and sustainable, non-extractive approaches	stingless bee nests and the emerging problems of pot-honey and pot-pollen counterfeiting	Environment, the Ministry of Agriculture and Livestock, Agrocalidad, researchers, and meliponicultors to maintain environmentally and economically sustainable meliponiculture.
NGOs	• Provide ongoing support to small and medium-sized meliponicultors in the local area. • Give regular environmental education talks about stingless bees to the general public.	• In partnership with universities and local governments, promote training or certification programmes in meliponiculture. • Set up citizen science programmes in which the community actively participates in caring for stingless bees.	• Become mediators between meliponicultors and potential buyers of stingless bee products. • Promote local fairs to raise awareness of stingless bees, conservation efforts, and product sales.
General population	• Respect the lives of stingless bees and help to protect them from harm.	• Take part in environmental education programmes to find out more about stingless bees.	• Keep in touch with NGOs, universities, and local organisations so that you can report any data or incidents involving stingless bees.

- **Perspectives and conclusions**

The studies presented in this thesis have identified risk factors for Ecuador's stingless bees and have highlighted the need to implement good management practices and biosecurity measures, both to ensure colony health and to obtain the highest-quality products for human use. Stingless bee cerumen has emerged as a material of choice for environmental health monitoring, both within and outside of colonies. The evaluation tools proposed in this study function as mechanisms to facilitate the implementation of training and learning programs, with the capacity to assess alterations in real time. The annual mortality rate of stingless bees under management was estimated and compared with that of one of the longest studies on the same topic in Latin America. Furthermore, the documentation of plant species as pollen resources for stingless bees was conducted, and an initial reference database was created for scanning electron microscopy images and DNA analysis. The dissemination of our research has been achieved through the publication of scientific and outreach articles in Spanish, English, and French, thereby ensuring its accessibility to meliponicultors and beekeepers worldwide, particularly in Ecuador, Latin America, and other regions.

The importance of research in the context of preventing the decline of stingless bees should be addressed from a One Health perspective, incorporating the ancestral knowledge of the communities that first managed them. Nevertheless, the decline of stingless bees remains undetermined with greater precision due to the incomplete description of species on a global scale, particularly in Ecuador. Research on this subject should guarantee human food security through the pollination services these stingless bees provide in tropical regions. However, it is also imperative to investigate their role in a distinct food chain and wildlife ecosystem that contribute to greenhouse gas mitigation and the regulation of the planet's climate.

This thesis was initiated in the context of a meliponiculture improvement project in Ecuador. During its development, funding was secured for a new project on meliponiculture in agroecological systems. In the future, long-term projects are planned that will include the identification of stingless bee species in the country, as well as the implementation of good practices and a biosecurity programme in collaboration with regulatory bodies such as AGROCALIDAD.

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