Trace element and isotopic analyses of raw and commercial beehive products

Xiaoteng Zhou

Department of Environmental Sciences

Faculty of Science and Engineering

Macquarie University

Sydney, Australia

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MACQUARIE University

SYDNEY·AUSTRALIA

Cover photo: European honey bees *(Apis millifera*), the most common bee species, feeding larvae and capping their brood.

Photo is provided by Mr. Doug Purdie, The Urban Beehive, Sydney, Australia.

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I also feel very privileged to have had the opportunity to experience these 3.5 years of overseas study. Before I came here, I was so worried about living by myself in a completely strange country without friends or family. Looking back on the past years, I have gained so much. Now, I can cook several delicious dishes even though I still burn them sometimes, and I did get my Australian driving license even if I did have a minor accident and put several scratches on the car. Studying abroad is similar to skydiving. You are so excited at first and then cowardly and hesitant when you have to start to jump from 15,000 feet, but after the heart-stopping and life-changing freefall, finally you would wholeheartedly appreciate this extreme thrilling adventure which makes you step outside your comfort zone and push personal boundaries to enjoy breathtaking rainforests.

Declaration

I certify that the work in this thesis has not been submitted previously, in whole or in part, for a degree at this or any other university. Nor does it contain, to the best of my knowledge, any material published or written by another person, expect acknowledged. This thesis is comprised solely of my own work.

Xiaoteng Zhou

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Abstract

Bees have existed on the planet for more than 100 million years and their relationship with early humans dates to the Pliocene period (5.3 million to 2.6 million years ago). As a result of recent human industrial activity, bees' foraging environments have been subject to variable levels of contamination. Consequently, bees have been used as bio-indicators to assess anthropogenic contamination. The primary product of bees is honey, a valued and popular human food. However, honey can be subject to adulteration via the addition of sugars and misleading labelling in relation to its geographic origin. Some international studies have evaluated beehive products for their authenticity and use in environmental monitoring. This study builds on existing approaches by applying multiple geochemical analytical techniques to link environmental sources to the concentrations and compositions of both raw and commercial beehive products.

This thesis uses trace element analysis and isotopic techniques to: (a) evaluate the use of bees as environmental bio-indicators; (b) investigate the quality of Australian and global honey; and (c) explore a valid method to authenticate the geographic origin of honey. Trace element analysis of beehive products demonstrates that measurements of As, Pb, Mn and Zn in two bee species (*Apis mellifera*, European honey bees and *Tetragonula carbonaria*, an Australian native bee species) correlates to co-located soils and dusts. Furthermore, Pb isotopic composition analysis verifies that the contamination found in bees can be attributed to a range of local sources, specifically, current mining activities in the city of Broken Hill (NSW), former leaded petrol depositions and geogenic background according to their locations.

Carbon isotopic ratios ($^{13}C/^{12}C$) and trace elements are used in this thesis to identify if commercial honey samples (from Africa, Asia, Europe, North America and Oceania) have been

adulterated by synthetic sugars derived from C-4 plants. These techniques are also used to test whether the stated geographic information on the honey samples is correct. The carbon isotopic analysis demonstrates that the adulteration practice of adding C-4 sugars remains a common problem in the Australian and global market, with a 17 % and 27 % adulteration rate, respectively. Manganese, P, K and Sr concentrations are shown to be the most important trace elements for distinguishing Australian from international honey.

Overall, this thesis contributes to the growing body of research that evaluates the effectiveness of bees and their respective products for use as bio-indicators of current and legacy trace element contamination. The research findings also demonstrate that isotopic composition and trace element concentrations can be used to authenticate and establish the geographic origin of honey, which has benefits for *bona fide* honey producers and consumers alike.

Table of Contents

A note for the reader

This thesis investigates the relationship between trace elements in the environment and those in beehive products (including bees, honey and wax) and includes discussion of relevant themes ranging from environmental science to food science. Three research articles comprise the main thesis data chapters (Chapters 3, 4, 5). Paper One identifies positive linear relationships between trace elements in European honey bee hive products and in adjacent soil and dust deposition samples, indicating their potential application as bio-indicators of contamination. This study shows that Pb isotopic composition analyses are an effective tool for tracing current and legacy contamination sources in European honey bees and their products.

Paper Two applies a similar sampling and analytical approach to that used in Paper One but examines a common Australian native bee, *Tetragonula carbonaria*, and its products as an alternative bio-indicator to European honey bees. The results from the native bee study are also compared to those from European honey bees. This latter study reveals that native bees are found to be a suitable bio-indicator as the more widely used European honey bee. Trace element concentrations in native bees and their products had stronger correlations with those in ambient soil and dust. Hence, they are more sensitive for monitoring trace element contamination across relatively smaller geographic areas, i.e. those at the neighbourhood and suburban levels.

Paper Three investigates the quality and authenticity of global commercial honeys. This study uses a combination of carbon isotopic analysis and trace elements to determine if samples were genuine pure honey and to authenticate their declared geographical origin. This study demonstrates that detailed high quality geochemical approaches can be used to (a) discriminate genuine honey products from those that are adulterated; (b) confirm the claimed geographic origin of honey.

While the initial thesis idea to examine bees and their products came from Professor Mark P Taylor, fieldwork, laboratory analysis, data processing and manuscript writing were performed and conducted by Xiaoteng Zhou. The three research articles included in this thesis along with author contributions are detailed below.

Paper One

Xiaoteng Zhou (70 %), Mark Patrick Taylor (20 %), Peter J. Davies (5 %) & Shiva Prasad (5 %) (2018). Identifying sources of environmental contamination in European honey bees (*Apis mellifera*) using trace elements and lead isotopic compositions. *Environmental Science & Technology*, **52** (3), pp 991-1001.

This study was developed by myself and Professor Taylor. Fieldwork and laboratory analysis were conducted by myself. The manuscript was written by myself and Professor Taylor with editing and review by Associate Professor Davies and Dr Prasad.

Paper Two

Xiaoteng Zhou (80 %), Mark Patrick Taylor (15 %) & Peter J. Davies (5 %). Tracing natural and industrial contamination and lead isotopic compositions in an Australian native bee species. *Environmental Pollution*, **242**, pp 54-62.

The concept for using native bees for comparison purposes came from Associate Professor Davies, while I was responsible for the project design. I conducted all fieldwork and laboratory analysis and authored the manuscript, while Professor Taylor and Associate Professor Davies provided editorial input.

Paper Three

Xiaoteng Zhou (80 %), Mark Patrick Taylor (10 %), Helen Salouros (5 %), Shiva Prasad (5 %). The authenticity and geographical origin of global honeys determined using carbon isotope ratios and trace element analysis. Submitted to *Scientific Reports*.

The concept for this study came from Dr Prasad and I designed the project. I conducted the laboratory analysis in conjunction with Dr Salouros. I authored the manuscript and Professor Taylor, Dr Prasad and Dr Salouros provided editorial input

Acronyms and Abbreviations

A. mellifera	*Apis mellifera*
ABS	Australian Bureau of Statistics
ACCC	Australian Competition and Consumer Commission
Al	Aluminium
ANOVA	Analysis of Variance
ANSTO	Australian Nuclear Science and Technology Organisation
AOAC	Association of Analytical Communities
As	Arsenic
B	Boron
Ba	Barium
Ca	Calcium
CDA	Canonical Discriminant Analysis
CHAID	Chi-square Automatic Interaction Detector
CRM	Certified Reference Materials
Cu	Copper
EA-IRMS	Elemental Analyser – Isotope Ratio Mass Spectrometry
EPA	Environment Protection Authority
FAO	Food and Agriculture Organization
Fe	Iron
hrs	Hours
kg	Kilogram
km	Kilometre
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IQ	Intelligent Quotient
K	Potassium
Mg	Magnesium
mg/kg	Milligrams per kilogram
mm	Millimetre
Mn	Manganese
Na	Sodium
NATA	National Association of Testing Authorities
NEPC	National Environment Protection Council
Ni	Nickel
NMI	National Measurement Institute
NSW	New South Wales
NPI	National Pollutant Inventory
P	Phosphorus
PCA	Principal Component Analysis
Pb	Lead
RIRDC	Rural Industries Research and Development Corporation
RSD	Relative Standard Deviation
Sn	Tin
Sr	Strontium
QLD	Queensland
Rb	Rubidium
SA	South Australia
TAS	Tasmania
T. carbonaria	*Tetragonula carbonaria*
µg/mg	Micrograms per kilogram
US	United States
VIC	Victoria
WA	West Australia
WHO	World Health Organisation
Zn	Zinc

Chapter 1: Introduction

The interaction between bees and the ancestors of modern day humans may go back millions of years when hominids, *Ardipithecus* and *Australopithecus*, likely collected honey from wild bees in Africa during the Pliocene era (Crane 1999). This custom was doubtlessly continued by *Homo habilis* and *Homo erectus* during the Pleistocene, and by *Homo sapiens* from the Palaeolithic era, 250,000 years BP, to current times (Crane 1999). Rock paintings from the Upper Palaeolithic, 13,000 BC, appear to provide the earliest known evidence for honey collecting (Crane 1999) and Spanish Levant rock art depicts honey harvesting dated from the end of the Palaeolithic to early Mesolithic, approximately 8,000 BC (Dams and Dams 1997). The use of beeswax was recorded on early pottery shards from Turkey, dating back from 6,500 to 6,000 BC (Roffet-Salque et al. 2016).

Bees are an invaluable agricultural resource for the vital role they play in pollination (Torchio 1985, Allen-Wardell et al. 1998, Klein et al. 2003, Calderone 2012, Alemberhe and Gebremeskel 2016). Bees also play an important role in ecosystem conservation and stability, genetic variation, floral diversity, speciation and evolution (Heard 1994, Heard 1999, FAO 2008, Murray et al. 2009, Minja and Nkumilwa 2016). In addition, bees provide a plethora of products such as honey, beeswax, propolis and royal jelly (European Commission 2013, FAO 2015).

Honey is the most important end product of beekeeping, and was probably the first bee product harvested by humans (Krell 1996). It is produced by bees collecting nectar and pollen during their foraging, with majority of collected nectar and pollen destined for regurgitation, enzymatic activity, water evaporation, and storage as honey (FAO 2001).

During the nectar and pollen collection, bees prefer to forage plants that use a C-3 pathway to fix carbon in photosynthesis, a process that approximately 85 % of the plants on earth use (Kumar et al. 2017). Bees also visit occasionally C-4 and CAM plants that account for only 5 % and 10 % of the total plant biomass, respectively (Kumar et al. 2017). Hence, sugars in honey are derived primarily from C-3 plants (White and Winters 1989, Padovan et al. 2007, Tosun 2013).

In addition to natural sugars, honey is also comprised of small quantities of proteins, enzymes, amino acids, minerals, trace elements, vitamins, phenolic and volatile aroma compounds (da Silva et al. 2016). Given its nutritional components and sweet flavour, honey is a common and popular food, which is also considered to have medicinal value due to its antibacterial and antifungal properties (Weston 2000, Combarros-Fuertes et al. 2019).

Beeswax is a natural material produced from abdominal glands of worker bees and is used to build cells in hives for both honey storage and for the development of larvae and pupae. The total production of beeswax from any given hive is estimated to be 8–10 % of that of honey (Girma et al. 2008). Beeswax is edible, and also has multiple uses. For example, in the cosmetic industry, it is used in the manufacture of hand creams, lip balm, eye shadow and moustache wax. Beeswax is also used for candle making, furniture varnish, shoe polish and surfboard wax (Bogdanov 2016).

1.1 Overview of beekeeping

The European honey bee (*Apis mellifera*) is the best known and most widespread of the estimated 25,000 bee species (Gould 2015). Between 1961 to 2014, world beehive stocks increased from 50 million to 83 million, with average annual growth of 1.3 % (Figure 1-1).

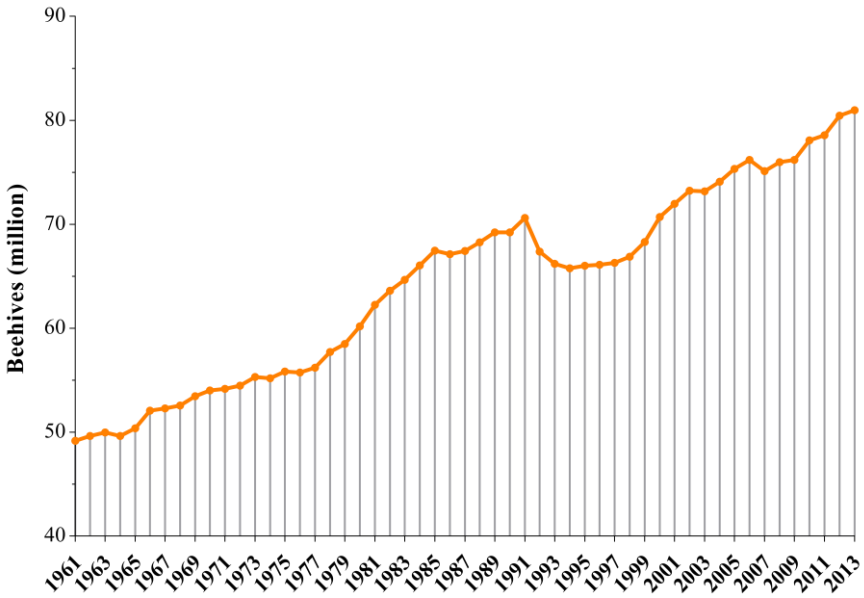

Figure 1-1. World beehive stocks from 1961 to 2013 (FAO 2017).

The practice of beekeeping *A. mellifera* has significant economic benefits, which are primarily associated with pollination and honey production. *A. mellifera* is estimated to pollinate approximately two thirds of all agricultural crops, which provides 90 % of global food (Kluser et al. 2010). In 2005, the economic value of insect pollination services, that are dominated by *A. mellifera*, was estimated to be worth €153 billion (US$187 billion) (Gallai et al. 2009). In 2015, global honey production, again largely from the *A. mellifera*, had an estimated value of US$6.6 billion (Andreeva 2017).

In Australia, the honey bee was introduced in the early 1800s by European settlers (Hopkins 1911, Western Australian Museum 2015) and managed honey bee hives are now found in all Australian mainland states and territories (RIRDC 2007, Granger and Woodburn 2010) (Figure 1-2). Around 65 % of Australia's agricultural production is dependent on pollination by European honey bees (RIRDC 2009), contributing $4 billion per annum to the Australian economy (Commonwealth of Australia 2014, Parliament of Australia 2016, RIRDC 2017).

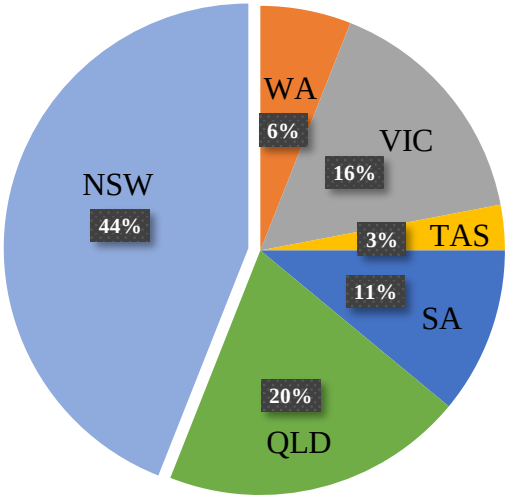

Figure 1-2. The distribution and percentage of registered hives of European honey bees (*A. mellifera*) across Australian states, including New South Wales (NSW), Queensland (QLD), Western Australia (WA), South Australia (SA), Victoria (VIC) and Tasmania (TAS) (RIRDC 2007, Granger and Woodburn 2010). Registration of beehives is not compulsory in the Australian Capital Territory (ACT) and Northern Territory (NT), so there is no available data from these regions.

Although European honey bees are the most common source of honey, there are over 2000 species of native bees in Australia (Houston 2011, Honan 2016). The majority of Australian native bee species are solitary and live in tree hollows, underground burrows or inside plant stems, with few species being social and producing honey in small volumes.

Tetragonula carbonaria, is a stingless, honey producing bee that is found mainly in the eastern coastal regions of Australia (Figure 1-3), and is the native bee species most commonly maintained by beekeepers. *T. carbonaria* has been studied more extensively than any other native bee species and is an important pollinator of crops in Australia and in other tropical and subtropical regions of the world where it resides (Heard 1999, Dyer et al. 2016). Unlike many other native bee species in Australia, *T. carbonaria* is integrated within the urban landscape and

its habitat overlaps with that of almost half of Australia's population (Figure 1-3) (Atlas of Living Australia 2018, Dollin and Heard 2016).

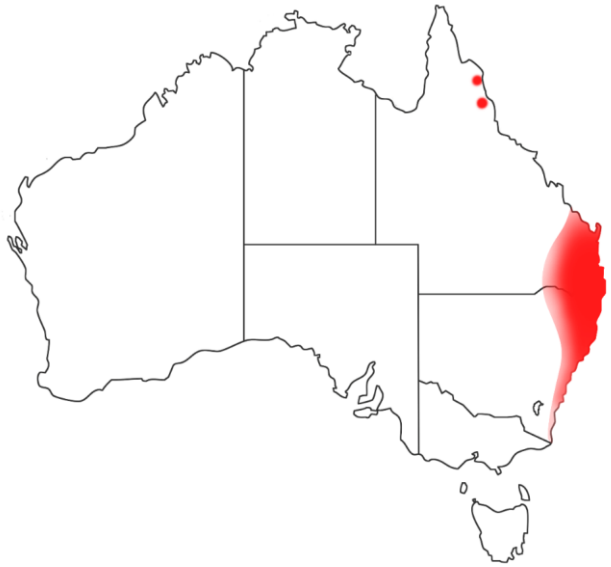

Figure 1-3. The distribution of the Australian native bee *Tetragonula carbonaria* (Atlas of Living Australia 2018, Dollin and Heard 2016).

European honey bees (*A. mellifera*) and Australian native bees (*T. carbonaria*) have a number of important biological and behavioural differences (Table 1-2, Figure 1-4). The *T. carbonaria*, has a much smaller body (4 mm) (Smith et al. 2017), a considerably shorter foraging distance of 0.3–0.7 km (Beekman and Ratnieks 2000, Smith et al. 2017); forages for shorter periods of time each day (7 hrs vs. 10 hrs) (Heard 1999); produces less honey per year (1 kg vs. 50 kg) (Heard 2016a), but has a longer lifespan (100 days vs. 40 days) (Heard 2016b).

European honey bees are comparatively large (15 mm) and live in colonies with maximum populations of 30,000–80,000 individuals and of these, 98 % are workers (Adjare 1990). Worker honey bees have abundant interactions with the environment during their pollen and nectar foraging. For example, in order to collect 20 kg pollen and 125 kg nectar, worker bees need to visit 1,125,000 and 4,000,000 flowers, respectively, across a maximum foraging area of 314 km^2, 5–9 km from the hive (Van der Steen 2015).

Table 1-2. Summary of the comparative data of the two bee species studied in this thesis.

	Apis mellifera	***Tetrogonula carbonaria***
Name used in this thesis	European honey bees	Australian native bees
Hive size (litres)	40	8
Numbers in colony	Strong colony: 30000 – 80000	Strong colony: 5000
Body length (mm)	15	4
Travel distance (km)	5 – 9	0.3 – 0.7
Forage time (hours per day)	10	7
Honey production per hive (kg)	50	1
Lifespan (days)	40	100

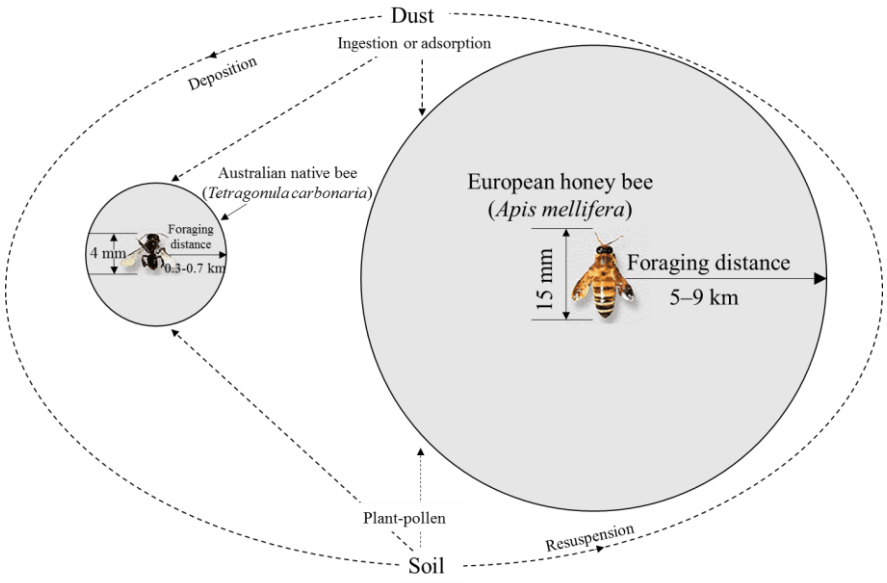

Figure 1-4. Comparison of body size and foraging distance of the European honey bee, *A. mellifera*, and the Australian native bee, *T. carbonaria*, as well as foraging pathways potentially exposed to contaminants (source: Zhou et al. 2018a).

1.2 Beekeeping and environmental contamination

This section summarizes anthropogenic environmental contamination in urban environments, and its impacts on human and ecological systems.

1.2.1 Environmental contamination

Environmental pollution is a global problem and is considered to be one of the single most important risks to human health (Landrigan et al. 2018). It was estimated that nine million people died in 2015 as a result of air, water and soil pollution (Landrigan et al. 2018). Environmental contamination is considered to be present when chemical substances or compounds are present in water, air or soil above natural background values. High levels of contamination in the environment that can cause harm to organisms and ecosystems is defined as pollution (Chapman 2007).

Air pollution is one of the major environmental risk to health, with an estimated one in nine deaths caused by poor air quality (World Health Organization 2016). Indeed, the World Health Organization (WHO) estimates that nine out of ten people worldwide breath polluted air causing seven million deaths annually (World Health Organization 2018). Poor air quality affects not only low- to middle-income countries but it also presents a risk in developed nations (Burnett et al. 2018), including the USA (Cascio 2018, McGuinn et al. 2019), Canada (Villeneuve et al. 2003, Stieb et al. 2016), France (Grattan et al. 2005, Kulhanova et al. 2018), UK (Royal College of Physicians 2016) and New Zealand (Barnett et al. 2006, Ministry for the Environment and Statistics New Zealand 2018). Australian cities are also subject to poor quality (Department of the Environment and Heritage 2005, World Health Organization 2008). It has been estimated that in urban locations poor air quality is responsible for more than 3,000 premature deaths per annum (Environmental Justice Australia 2014). For example, elevated NO_2 levels in cities which are caused predominantly by vehicle-related emissions, adversely affect children's respiratory health by impairing lung function and causing asthma and other

related respiratory symptoms (Williams et al. 2012). In addition, significant hotspots of poor air quality are also associated with emissions from industrial activities, such as smelting. For example, the city of Port Pirie in South Australia has extremely poor air quality periodically due to elevated levels of lead (Pb) and SO_2 caused by metal-ore smelting emissions (Taylor et al. 2019 in review). Contaminants resulting from industrial activity (including the combustion of fossil fuels) are typically incorporated with fine particulate matter ($PM_{2.5}$), which are persistent and readily transported in the atmosphere (World Health Organization 2006, 2013). Further, epidemiological research has established that increased ambient $PM_{2.5}$ concentrations are causally linked to cardio-vascular disease (Pope et al. 2004). Other studies have shown that acute exposures lead to an increase in childhood hospital admissions and the daily total number of deaths across Australian capital cities (Barnett et al. 2005, Simpson et al. 2005).

Atmospheric emissions from anthropogenic activities ultimately form depositions in dust and soil, with soil being the most significant reservoir of environmental contaminants (Mohammed et al. 2011). Environmental contamination of soil has the potential to degrade the quality of food, water and air (Rodríguez-Eugenio et al. 2018). According to the Pure Earth database (Sim 2017), up to 200 million people across 50 countries are estimated to live in locations affected by contaminated soils, including agricultural lands (Rodríguez-Eugenio et al. 2018). In Australia, approximately 160,000 sites are considered to be contaminated, which have a current market value of more than AUD$3 billion (Naidu et al. 2015, Taylor and Cosenza 2016).

Amongst all the contaminants in the environment, heavy metals, particularly lead (Pb) are recognized one of the most pervasive toxicants, because they cannot be degraded or destroyed (Mohammed et al. 2011). Consequently, they accumulate and persist in the environment, posing a long-term hazard and significant potential adverse health risks to both natural and human systems.

Lead depositions into soils and dusts from Australian leaded petrol consumption during its 7-decades of use in motor vehicles resulted in 240,510 tonnes of Pb emitted from petrol being emitted to the atmosphere between 1932-2002 (Kristensen 2015). The consequent deposition of lead have caused widespread contamination of natural environments across Australia (Kristensen et al. 2017) and beyond, including Antarctica (e.g. Vallelonga et al. 2002, McConnell et al. 2014, Ndungu et al. 2016). Recent Australian studies have revealed the prevalence of anthropogenic contamination in residential home environments (Gulson et al. 2014, Laidlaw et al. 2014). Garden soils in its most two populous cities of Sydney (Rouillon et al. 2017) and Melbourne (Laidlaw et al. 2017) have been significantly contaminated as a result of leaded petrol depositions along with the former use of Pb-based paints on exterior household surfaces.

Australian environments have also been adversely affected by other forms of industrial emissions particularly those associated with mining and smelting (Csavina et al. 2012). For example, Pb emissions and depositions have severely contaminated the Pb and Zn mineral processing cities of Broken Hill (NSW), Mount Isa (Queensland) and Port Pirie (South Australia), which support relatively large (by Australian standards) inland populations of around ~20,000. Lead production from Australian mining and smelting operations is in the order of 40 million tonnes (Mudd 2013), with half of the Pb produced coming from Broken Hill, NSW (Mudd 2007), which continues to have significantly contaminated air (Dong et al. 2015, Zhou et al. 2018b), soils (Yang and Cattle 2016) and dusts (Dong and Taylor 2017). Lead contamination of the environment from mining emissions in Broken Hill is considered to the primary cause of elevated blood Pb levels in young children (Western NSW Health Intelligence Unit 2018, Dong et al. 2019).

1.2.2 The use of beehive products as environmental bio-indicators of contamination

The costs of using standards methods for measuring and monitoring environmental contamination in air, dusts and soils is labour intensive, expensive and typically spatially limited. The use of proxy indicators such as bio-indicators of environmental contamination to overcome these limitations have been have been examined elsewhere (South Africa: Mandiwana et al. 2006, Italy: Paoletti et al. 2009, US: Flegal et al. 2010, France: Cresson et al. 2015), with mixed success (Yusof et al. 2004, Kardel et al. 2012). The application of bio-indicators in Australia for measuring and monitoring anthropogenic contamination in air, dust and soil has received limited attention (leaves: Halliwell 2000, mosses: Huang and Gulson 2002, lichens and fungi: Wu et al. 2016a, Wu et al. 2016b, birds:Andrew et al. 2019), even though the evidence suggests they pose great promise (Holt and Miller 2011). Interestingly prior to this PhD, the use of bees in Australia as a bio-indicator of environmental contamination has never been attempted, even though international studies have demonstrated that beehive products can have distinctive contaminant characteristics related adjoining land uses. For example, the chemical composition of beehive products collected in agricultural areas have been shown to be related to the use of specific pesticides (Porrini et al. 2003). Other studies have identified that other anthropogenic emissions such as those from thermal power plants and vehicles generate specific element elemental signatures that are also found in co-located beehive products (Zarić et al. 2016). Consequently, given that the environments of Sydney and Broken Hill have well-established contamination issues caused by inorganic trace elements (e.g. Davis and Birch 2010, Gulson et al. 2013, Kristensen and Taylor 2016, Yang and Cattle 2016, Rouillon et al. 2017), one of the primary aims of this PhD study was to evaluate the efficacy of using bees as bio-indicators of contamination in Australian environments.

The use of European honey bees and their products as bio-indicators for environmental contamination was first attempted by Svoboda (1962). Worker honey bees were studied as they have thousands of interactions with soil, air, water and vegetation as part of their foraging

(Erbilir and Erdoğrul 2005). Foraging brings the bees into contact with soil and dust that may contain contaminants that adhere not only to the hairs on their body (Figure 1-4, Figure 1-5) (Negri et al. 2015, Pellecchia and Negri 2018), but may be subsequently incorporated into the honey and wax they produce (Figure 1-5).

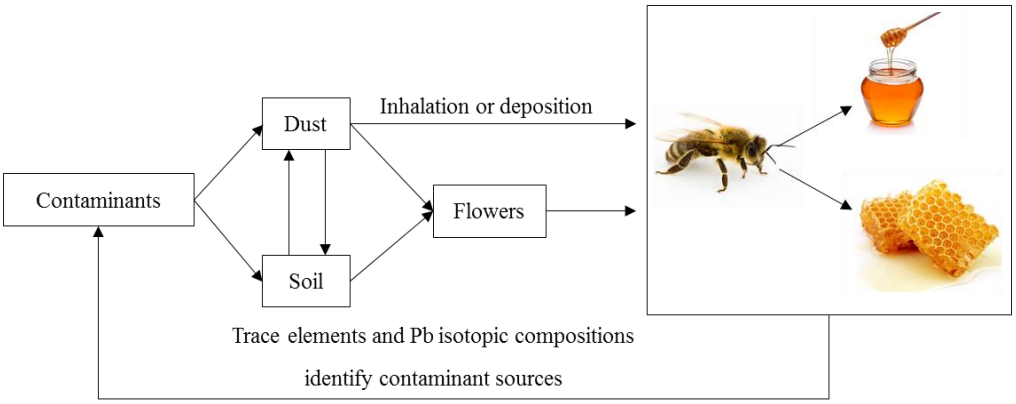

Figure 1-5. Pathways by which European honey bees make contact with contaminants from soil and dust (source: Zhou et al. 2018b).

A number of more recent international studies have examined the use of European honey bees and their products as bio-indicators to detect environmental contamination by trace elements (see references in Supplementary Table S1-1, Appendix A). However, the use of European honey bees, or their products, for use as reliable bio-indicators has been questioned by Balestra et al. (1992), and Conti and Botre (2001) (see references in Supplementary Table S1-2, Appendix A) primarily due to the absence of quantitative measurements linking European honey bees to contaminants in co-located soils (Saunier et al. 2013, Herrero-Latorre et al. 2017) and dusts (Van der Steen et al. 2015). In addition, highlighting the role of other bees, including endemic species, as bio-indicators can add more value to their already important ecological and economic service.

1.3 Honey adulteration

According to European Commission (2002) and Codex Alimentarius Commission Standards (2010), genuine pure honey is formally defined as:

> 'The natural sweet substance, produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature.' (European Commission 2002, Codex Alimentarius Commission Standards 2010)

As the primary food produced by bees, honey is a highly valued natural product, not only for its taste and nutritional value, but also for its perceived health benefits. Hence, it is a popular and an important global commodity, being valued at US$6.6 billion in 2015 (Andreeva 2017).

However, commercial or retail honey is not always the genuine pure product derived entirely from a beehive. Given that honey is a more expensive sweetener than other products, such as sugar (derived from sugar cane), the addition of cheap sugar syrup to increase honey volume is one of the most common fraudulent practices in the honey market (Soares et al. 2017). For example, an investigation from the European Union showed that 14 % (127 of 893) of honey samples contained unacceptable levels of added sugar (Aries et al. 2016). Compared to sugar, honey is less processed and contains significantly more nutrients, such as amino acids, antioxidants, enzymes, minerals and vitamins. Thus, honey with added sugar decreases its nutritional and medical benefits.

Another common fraudulent practice in honey marketing is mislabelling the origin, geographical and/or botanical source (Soares et al. 2017). This issue likely arises due to the fact that the cost of collecting and distributing honey is relatively cheap in developing countries, but

the final saleable product is not necessarily of an equivalent standard (Kamruzzaman 2016, Zhang and Xue 2016).

Mislabelling of the geographical origin of honey occurs in international honey markets and can have health and safety concerns (Strayer et al. 2014). For example, blended honey of unknown origins may contain antibiotics, toxins, irradiated pollen, alkaloids or pesticides, which pose potential health concerns (Edgar et al. 2002, Kempf et al. 2010, Al-Waili et al. 2012), damaging the reputation of the global honey industry (Garcia and Phipps 2017).

Australia is one of the top ten honey producing countries in the world, with annual production between 20,000–30,000 tonnes (Granger and Woodburn 2010). Both bulk and retail shipments of Australian honey are exported to a variety of countries (Benecke 2007). Australian honey producers target high-value markets, achieving premium prices by trading internationally on Australia's reputation as a 'clean and green' country with one of the world's most rigorous apicultural management systems (Centre for International Economics 2005). Consumers rely on Australia's reputation for safe, quality honey and, increasingly, for its sustainable production.

However, as Australia has a reputation for providing high quality honey (Centre for International Economics 2007), this has also meant its products are particularly subject to fraudulent practices. For example, an importer of food products based in the Mediterranean-Turkey region was fined $30,600 AUD for a product labelled '*Victoria Honey*' (Figure 1-6). The product was mainly composed of sugars from C-4 plants including corn and sugar cane (ACCC 2014, Robb 2014). In 2014, another food importer was fined $10,200 AUD for selling honey labelled '*Hi Honey*' (Figure 1-7). The label included a map of Australia, but the product was largely composed of C-4 plants sugars and produced in Turkey (Macgrath 2014, Clemons 2016).

Figure 1-6. The claimed 'Victoria Honey' produced by Basfoods (Aust) Pty Ltd (Basfoods) was tested to contain significant C-4 sugars (primarily corn or cane) (image source: ACCC 2014).

Figure 1-7. The product of 'Hi Honey' included an Australian map on its label and was revealed to be imported from Turkey and made up of mostly C-4 sugars (image source: Macgrath 2014).

1.4 Research questions

This PhD thesis proposed three research questions involving environmental contamination and honey adulteration.

Trace element contamination is related to not only current anthropogenic activities, such as road traffic, industrial activities, coal burning, mining and smelting, but also historical depositions, particularly from leaded petrol and paint, that continue to be an important source of trace

elements in the environment that can have significant adverse health impacts (Landrigan 2002, Kristensen et al. 2017). The development of simple, straightforward and cost-effective contamination-measuring proxies, such as bees, honey and wax, are valuable additional resources for scientists to identify and assess contemporary and historical contamination in the environment.

In this regard, this study firstly aims to quantify the relationship between trace elements in beehive products (bees, honey and wax) and the environment (soil and dust), using beehive products of the European honey bee (*A. mellifera*). Secondly, this PhD study compares the utility of using different bee species (*A. mellifera* vs. *T. carbonaria*) as bio-indicators of historical and ongoing contamination in populous areas of Australia.

The third aim is to develop a method using both trace elements in, and carbon ratios of, honey to authenticate honey and determine its geographical origin. This aspect of the thesis addresses questions pertaining to the reputational value of honey as a product and its importance to countries such as Australia.

Drawing on these thesis research aims, this PhD study further develops an understanding of the use of beehive products as bio-indicators to trace current and historical contamination in order to utilise this information as a representative and more cost-effective, non-traditional approach to assess environmental contamination, particularly in more densely populated city areas. As part of the study research outcomes, a dataset of trace elements and carbon isotopic compositions were developed that can be used to accurately determine the geographical origin of honey.

1.5 Structures of the thesis

This thesis is structured into six chapters, comprising of an Introduction, Research Method and Approach, three research papers – Chapters 3, 4 and 5, as well as a Discussion and Conclusion. Chapter 3 identifies the use of European honey bee hive products as bio-indicators to trace contemporary and historical Pb emissions in the environment. Chapter 4 evaluates the Australian native bee hive products as bio-indicators and compares their application with European honey bee hive products. Chapter 5 evaluates Australian and international commercial honeys (n = 100), using a suite of statistical methods to distinguish the geographic origin of honey at regional and continental scales.

Supporting material to this thesis is included in the six appendices. Appendix A contains a summary of references cited in the entire thesis introduction. Appendix B includes sample information, such as collection date and locations of raw beehive samples as well as contributors of commercial honey samples. Appendix C, Appendix D and Appendix E are supplementary materials corresponding to each main research paper. Appendix F includes distribution maps of both bee species, *A. mellifera* and *T. carbonaria*, studied in this thesis to support the Discussion chapter.

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Chapter 2: Research Method and Approach

As identified in Chapter 1, humans and bees have had a long association, stretching back 2.5 million years. However, impacts arising from the use and dispersal of pesticides (e.g. neonicotinoids) (Tong et al. 2018) and pollutants from industrial emissions, including lead from vehicle emissions (Lambert et al. 2012, Ruschioni et al. 2013), has raised questions about the deleterious effects of environmental contamination on bees and their products. This chapter provides an overview and rationale for the different methods used to address two key questions (link back to Chapter 1): (a) can bees and their products be used as a reliable bio-indicator; and (b) can geochemical techniques be used to verify the authenticity and geographic origin of honey. The detailed methods used to address these questions are provided in Chapters 3, 4 and 5.

2.1 Method and rationale for the use of beehive products as bio-indicators

2.1.1 Study area

European honey bees are considered effective 'environmental samplers' since the hairs on their bodies can readily retain non-floral particles (including contaminants) originating from atmospheric sources (Negri et al. 2015, Pellecchia and Negri 2018). The amount of contaminated particles carried by honey bees is related to the background levels of contamination within their foraging landscapes (e.g. Conti and Botre 2001, Rodríguez García et al. 2006, Stankovska et al. 2008, Perugini et al. 2011). A number of previous studies have shown that higher trace element concentrations were found in honey bees collected from airports (Perugini et al. 2011), urban areas (Wannaz et al. 2012) and mining locations (Saunier et al. 2013, Losfeld et al. 2014) compared to those found in rural and control sites. However, a statistical difference was not identified ($p > 0.05$) in the trace element concentrations in beehive products collected from polluted and unpolluted areas in studies of Aghamirlou et al. (2015) Perugini et al. (2011) Iwegbue et al. (2015) and Roman (2010). This has contributed to the set-up of small scale investigations (Van der Steen et al. 2012) and the measurement of

homogeneous contamination across studied areas (Zarić et al. 2015, Alvarez-Ayuso and Abad-Valle 2017).

In order to further examine the utility of European honey bees and their products as bio-indicators, Chapter 3 reports the results from a spatially wide sampling study in New South Wales, Australia. The sample sites included a range of land use types (national park areas, coastal residential suburbs, high density inner city, airport and light industrial areas) with co-located beehives covering the greater Sydney metropolitan area and the Pb-Zn-Ag mining town at Broken Hill, approximately 1100 km west of Sydney. The sampling sites across the greater Sydney metropolitan area incorporated locations across different residential densities, central business district (CBD), inner city, middle ring and peri-urban locations. Previous studies have reported marked differences in contamination related to trace elements As, Pb, Mn and Zn (e.g. Kachenko and Singh 2006, Davis and Birch 2010, Birch 2011, Birch et al. 2011, Johnson et al. 2017, and Rouillon et al. 2017). The city of Broken Hill was added to the study as an outlier, because it is known to have significantly elevated concentrations of trace elements present in its soil and dust due to mining operations in the region (Figure 2-1) (Taylor et al. 2014, Kristensen et al. 2015, Kristensen and Taylor 2016, Yang and Cattle 2016, Dong and Taylor 2017).

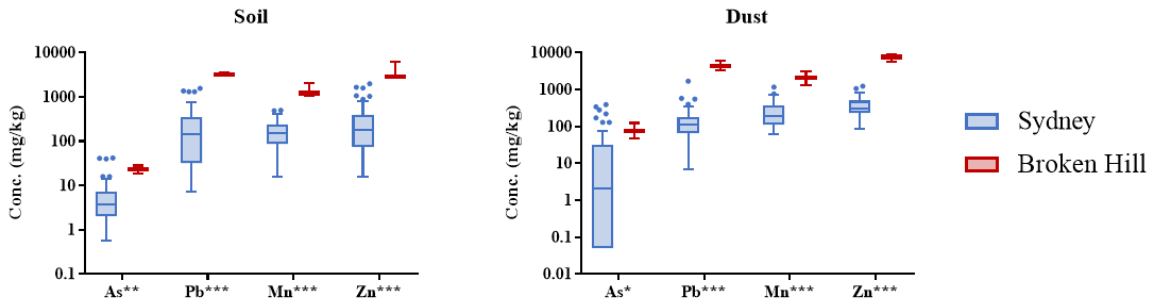

Figure 2-1. Concentrations (mg/kg) of As, Pb, Mn and Zn in soil and dust samples across Sydney and Broken Hill. Data for As, Pb, Mn and Zn were sourced from Zhou et al. (2018) (Sydney: n = 46 for soil, n = 61 for dust; Broken Hill: n = 3 for soil), and Broken Hill dust (n =

3) were sourced from Dong and Taylor (2017). All samples were collected in the vicinity of beehives sampled in this study. Diagram of box with Turkey whiskers was plotted and significant differences were determined using the Mann-Whitney test at p = 0.001*** and 0.01** levels.

2.1.2 Raw beehive sample collection

The European honey bee (*A. mellifera*) is the most studied bee species used as a bio-indicator as part of trace element monitoring. This is largely due to the fact that products from this species dominate the global honey supply market and are present across all continents, except Antarctica. However, with 25,000 bee species identified across the world (Gould 2015), it is pertinent to question whether other bee species, particularly those that are local and native, can also be used as bio-indicators. As introduced in Chapter 1, *Tetragonula carbonaria* is a native and popular domesticated bee species in Australia. Furthermore, their distribution corresponds to a number of major centres of population on the east coast of Australia (Figure1-3, Chapter 1), suggesting they could potentially be used as an alternate species to assess trace element contamination originating from anthropogenic emissions.

In order to firstly verify the viability of using European honey bees (*A. mellifera*) as a bio-indicators in Australia and, secondly, compare the results to the *T. carbonaria,* beehive samples from both bee species coupled with corresponding soil and dust were sampled across Sydney and Broken Hill. Sampling protocols included one European honey bee hive in Broken Hill proximate to Pb-Zn-Ag ore mining (Figure 2-2) and nine hives across the Sydney metropolitan area (Figure 2-3). No *T. carbonaria* hives are present in Broken Hill as this is outside their ecological habitat (Figure1-3, Chapter 1). In Sydney, eighteen *T. carbonaria* bee hives were sampled across an equivalent area to that of the European honey bee hives (Figure 2-3). A similar study design was used in Sydney to enable a comparison between the two species.

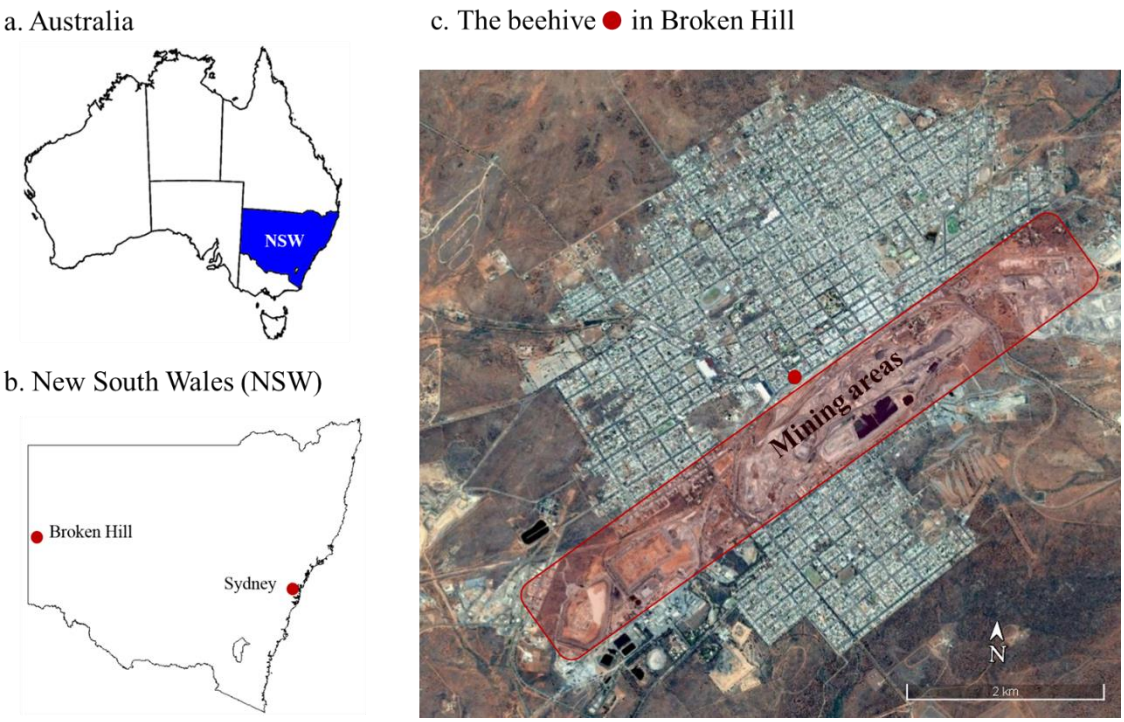

Figure 2-2. Location of the beehive in Broken Hill. Mining areas lie within the red box in.

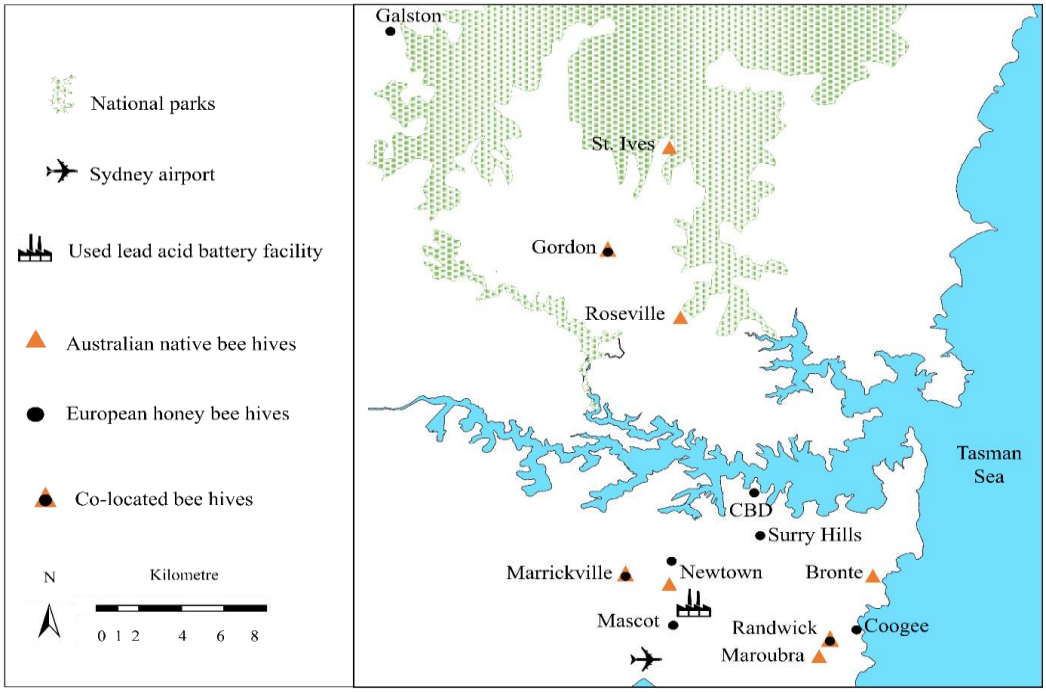

Figure 2-3. European bee hives (n = 9) and Australian native bee hives (n = 18 at eight locations) sampled across Sydney metropolitan areas.

Beehive samples, including bees[1], honey[2] and wax[3], from both bee species of *A. mellifera* and *T. carbonaria* were collected between November 2015 – November 2016 (Supplementary Table S2-1, Appendix B). Over this period, there was no major shift in industrial emissions (NPI 2017) or other sources, largely as a result of successful regulations eliminating Pb in petrol and paint across Sydney areas (e.g. Gulson et al. 1995, Kristensen 2015). The Mascot (Sydney) European honey sample site was located approximately 2 km from a used lead acid battery (ULAB) facility (Figure 2-3). This facility is the largest emitter of Pb in Sydney. Atmospheric Pb emissions from the ULAB site have been estimated at 400 kg between the years of 2015 and 2016 (NPI, 2017). Also, located in Mascot is the Australian Nuclear Science and Technology Organisation's PM2.5 air monitoring system, which recorded average concentrations of 4.65 ng/m^3 of airborne Pb and a maximum of 14.18 ng/m^3 over the study sampling period (Figure 2-4) (ANSTO, 2017).

By comparison, the total Pb emissions from Broken Hill mining operations across the study period was estimated at 28,095 kg (NPI, 2017) with atmospheric Pb concentrations ranging from 114 ng/m^3 to 527 ng/m^3 (Figure 2-4). The total suspended Pb particles across the two major study locations would likely influence trace element concentrations in bees and their products since they are exposed directly to atmospheric contamination (Lambert et al. 2012, Ruschioni et al. 2013).

[1] Bees were captured by bug vacuum at the entrance of each beehive and then euthanised by freezing (Tomasik 2018).
[2] Honey collected in this thesis was nectar honey, not honeydew honey. Further information was provided in the Supplementary Table S1, Appendix B.
[3] The wax sampled for this study was that recently produced by the bees excluding the wax sheets used on the combs. Native bee hives do not include wax sheets. The sampling information was provided in the Supplementary Table S1, Appendix B

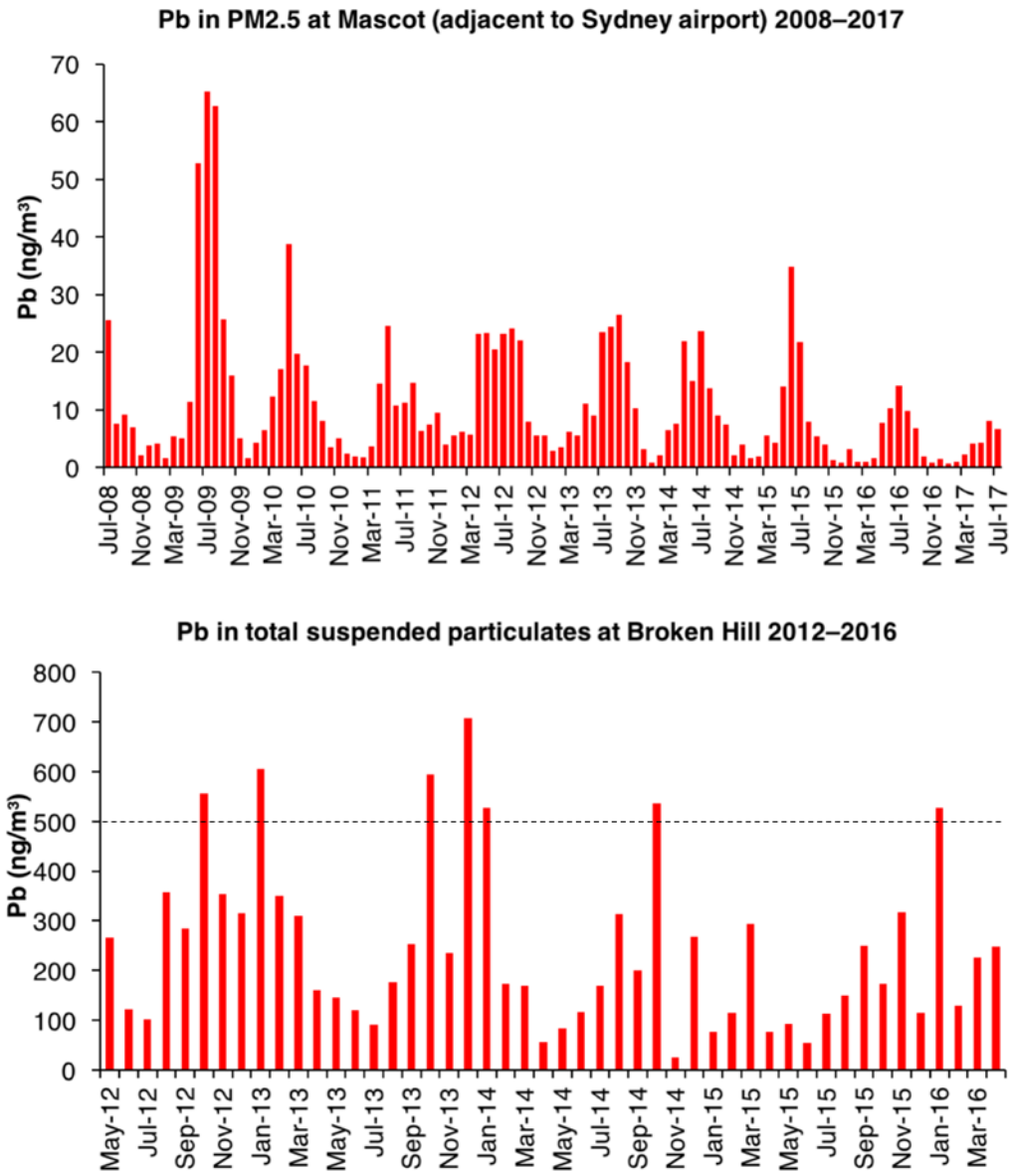

Figure 2-4. Recent monthly average air Pb concentrations within Sydney (Mascot) (ANSTO, 2017) and Broken Hill (Perilya Limited 2017) (Data for PM2.5 graphs provided by Dr Armand J Atanacio, Australian Nuclear Science and Technology Organisation—more limited data is available on line) (source: Zhou et al. 2018). The Australian national standard of air-borne lead is 500 ng/m^3 (NEPC 1998), as indicated by the horizontal dotted line.

2.1.3 Sample analysis

Trace element analysis using an Agilent 7900 ICP-MS instrument at the National Measurement Institute was used to evaluate beehive samples and environmental samples. The analysis sought to test if a correlation existed between beehive products (bee, honey and wax) and co-located and temporarily-matched samples of environmental (soil and dust) concentrations. It was hypothesised that a linear correlation would support the use of beehive products as bio-indicators of contamination (Roman 2010, Perugini et al. 2011, Aghamirlou et al. 2015, Iwegbue et al. 2015). Statistical differences between environmental concentrations and those between the two bee species were also undertaken to compare the utility of using each species as a bio-indicator of trace element contamination.

The measurement of Pb isotopic compositions ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$) has been used to identify various ore body and trace geogenic and anthropogenic inputs of Pb contamination in the environment (e.g. Erel et al. 1997, Gulson 2008, Cheng and Hu 2010). Multiple environmental media have been subject to Pb isotopic composition analysis for source identification, including soil (Emmanuel and Erel 2002, Li et al. 2011), dust (Gulson and Taylor 2014, Dong and Taylor 2017) and water (Caurant et al. 2006), along with bio-indicators including lichens (Cloquet et al. 2006, Flegal et al. 2010), mosses (Farmer et al. 2002), snails (Notten et al. 2008), birds (Scheuhammer and Templeton 1998, Tsuji et al. 2008) and marine mammals (Outridge et al. 1997). However, until now, there has been no published study examining Pb isotopic compositions in any bee species or their products to apportion sources of Pb contamination in the environment. Following the publication of Zhou et al. (2018), Zaric et al. (2018) published a honey bee study from Belgrave that included the use of Pb isotopic compositions.

According to previous studies (e.g. Kristensen and Taylor 2016, Dong and Taylor 2017, Kristensen et al. 2017), the likely emission sources over the areas sampled are related to current

and legacy contamination. Current mining operations in Broken Hill are a key factor in driving Pb contamination across the local environment (soil and dust) (e.g. Gulson et al. 1985, Kristensen and Taylor 2016). Leaded petrol that was phased out in 2002, has been a significant source of historic Pb emissions in Australia (240,000 tonnes over its 70-years of use), with the vast proportion being emitted across major urban areas, such as Sydney (Laidlaw et al. 2014, Wu et al. 2016b), Melbourne (Wu et al. 2016a) and Adelaide (Kristensen et al. 2016).

The application of Pb isotopic composition analysis to environmental samples and beehive products from both species (*A. mellifera* and *T. carbonaria*; Chapters 3 and 4) was designed to apportion sources of contamination and verify their effectiveness as bio-indicators. The approach also presented an opportunity to establish if legacy Pb emissions continued to be remobilised in dust and soil and were contributing to the contamination of both food and ecological systems.

2.2 Method and approach for determining the authenticity of honey

Chapter 1 introduced the market value of honey for Australian and international industries, and also revealed the ongoing fraudulent practices of honey adulteration by the addition of artificial sugars and the deliberate mislabelling of produce sources. There has been limited research investigating global honey quality with a particular focus on Australian honey, as well as the development of a method to identify whether honey has a unique signature that can separate it from different regions and continents.

In order to address these issues, the research in Chapter 5 outlines the collection and analysis of five raw honey (directly from beehives) samples, defined as pure ‘benchmark’ samples, together with 95 commercially produced honey samples from Australian (n = 38) and 18 from other countries (n = 54) covering the five continents of Africa, Asia, Europe, North America and Oceania. In addition, there were also three samples of a blended origin included in the

analysis (Supplementary Table S2-2, Appendix B). The analytical process uses carbon isotopic analysis, following the methods outlined by the Association of Official Analytical Chemists (AOAC 991.41, AOAC 978.17, AOAC 998.12) and a in recent study of Dong et al. (2016), to detect the most common adulterant of C-4 sugars (derived from corn or cane) in honey. This analysis was able to evaluate the purity of commercial honeys. Samples that passed C-4 sugar detection levels were then subject to additional trace element analysis to provide a geographic identification or signature for the honey's origin.

Various statistical analyses are conducted in Chapter 5. These include a one-way ANOVA test, principal components analysis (PCA), canonical discriminant analysis (CDA) and a C5.0 classification model. The one-way ANOVA test was used to determine differences in significance (set at $p < 0.05$) in trace element concentrations according to honey geographic origin. Statistical analysis of PCA and CDA was utilised to provide a visible clustering for geographic groups by reducing the dimensionality of the number of variables. The decision tree of C5.0 was applied to build equations to separate honey groups and predict unknown samples. All the statistical analyses serve to verify the potential and utility of trace elements in honey as unique signatures to distinguish its geographic origin at regional and continental scales.

Overall, in order to investigate the correlation between trace elements in beehive products and in the environment, this thesis collected 128 raw beehive samples, 110 environmental samples of soil and dust, and 100 commercial honey samples. Specifically, trace element analysis and Pb isotopic compositions in raw beehive products and environmental samples were used to verify the use of bees and their products as suitable bio-indicators to assess contamination levels and identify contamination sources. Carbon isotopic analysis of honey and protein ($\delta^{13}C_{honey}$ and $\delta^{13}C_{protein}$) in commercial honey samples (n = 100) were used to test whether honey is adulterated by C-4 sugars. The honey samples (n = 69) that passed the purity C-4 sugar test were then used to investigate whether the trace elements in the environment entering honey can

generate a unique signature to distinguish different honey products according to their geographic origin (Table 2-1).

Table 2-1. Sample numbers and their analysis applied in this thesis.

Samples		**Trace elements**	**Pb isotopic compositions**	**$\delta^{13}C_{honey}$[a]**	**$\delta^{13}C_{protein}$[a]**
European honey bee hives	Bees	38	26	/	/
	Honey	17	6	/	/
	Wax	16	6	/	/
Australian native bee hives	Bees	16	5	/	/
	Honey	20	0	/	/
	Wax	21	19	/	/
Soil		49	47	/	/
Dust		61	57	/	/
Commercial honey		69[b]	/	100	100
Total analysis No.		***307***	***166***	***300***	***300***

a. Every sample was prepared and analysed in triple.
b. Only 69 commercial honey samples (n = 100 in total) passed purity tests and were therefore used for later trace element analysis.

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Chapter 3: Trace elements in the environment and European honey bees (*Apis mellifera*)

Chapter 3 presents the following study:

Xiaoteng Zhou (70 %), Mark Patrick Taylor (20 %), Peter J. Davies (5 %) & Shiva Prasad (5 %) (2018). Identifying sources of environmental contamination in European honey bees (*Apis mellifera*) using trace elements and lead isotopic compositions. *Environmental Science & Technology*, **52** (3), pp 991-1001.

European honey bees (*Apis mellifera*) were first examined in 1962 for strontium concentration in the both the bees and their products (Svoboda 1962). However, since this time various studies have reported mixed results with respect to the use of honey bees and their products as bio-indicators of environmental contamination. This is likely related to investigations having a limited spatial coverage (Van der Steen et al. 2012) along with the fact that trace elements are often homogeneous (Zarić et al. 2015, Alvarez-Ayuso and Abad-Valle 2017). The study presented in this chapter was designed to evaluate the relationship between trace elements in the environment and beehive products at a city level (Sydney) where land use types are markedly different. In addition, samples from the Pb-Zn-Ag mining town of Broken Hill were included in the study to ascertain the response of honey bees to contemporary dust emission sources (Dong and Taylor 2017). This chapter documents the full cycle of trace elements from the environment to beehive products of European honey bees (*A. mellifera*) in Australia. In addition, Pb isotopic composition analysis was used to identify the source of Pb in beehive products versus those in the ambient environment. These analyses show that Pb contamination is not only related to current emissions but also legacy depositions. This chapter is the first stage of the PhD investigation into the link between trace elements in the environment and those in the food web via honey and wax. The results presented in this Chapter were of significant

interest to the general public, being covered by national media in Australia as well as overseas (Appendix C).

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Identifying Sources of Environmental Contamination in European Honey Bees (*Apis mellifera*) Using Trace Elements and Lead Isotopic Compositions

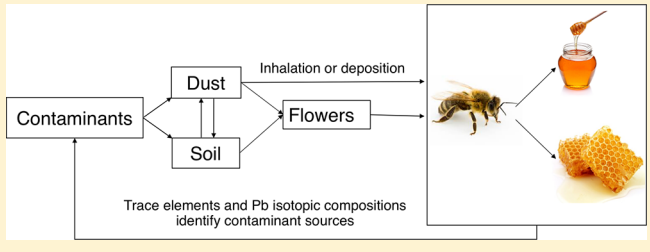
Xiaoteng Zhou,[†] Mark Patrick Taylor,[*,†,‡] Peter J. Davies,[†,‡] and Shiva Prasad[§]

[†]Department of Environmental Sciences, Faculty of Science and Engineering and [‡]Energy and Environmental Contaminants Research Centre, Macquarie University, North Ryde, Sydney, NSW 2109, Australia

[§]Analytical Service Branch, National Measurement Institute, Department of Industry, Innovation and Science, Australian Government, Riverside Corporate Park, North Ryde, Sydney, NSW 2113, Australia

Supporting Information

ABSTRACT: Trace element concentrations (As, Mn, Pb, and Zn) and Pb isotopic compositions were analyzed in honey bees, wax, and honey along with co-located soil and dust samples from Sydney metropolitan and Broken Hill, Australia. Compared with the other trace elements, Pearson correlations show that Pb concentrations in soil and dust had the strongest relationship to corresponding values in honey bees and their products. Dust Pb was not only highly correlated to corresponding soil values ($r = 0.806$, $p = 0.005$), it was the strongest predictor of Pb concentrations in honey bees, wax, and honey ($p = 0.001$, 0.007, 0.017, respectively). Lead isotopic compositions ($^{206}Pb/^{207}Pb$ and $^{208}Pb/^{207}Pb$) showed that honey bees and their products from Broken Hill were nearly identical (95−98%) to the composition of the local ore body. Samples of honey bees and their products collected from background sites adjacent to national parks in Sydney had Pb isotopic compositions ($^{206}Pb/^{207}Pb$ = 1.138−1.159, $^{208}Pb/^{207}Pb$ = 2.417−2.435) corresponding to local geogenic values ($^{206}Pb/^{207}Pb$ = 1.123−1.176, $^{208}Pb/^{207}Pb$ = 2.413−2.500). By contrast, honey bees and their products from Sydney metropolitan ($^{206}Pb/^{207}Pb$ = 1.081−1.126, $^{208}Pb/^{207}Pb$ = 2.352−2.408) were similar to aerosols measured during the period of leaded petrol use ($^{206}Pb/^{207}Pb$ = 1.067−1.148, $^{208}Pb/^{207}Pb$ = 2.341−2.410). These measurements show Pb concentrations and its isotopic compositions of honey bees, and their products can be used to trace both legacy and contemporary environmental contamination, particularly where sources are well documented. Moreover, this study demonstrates that legacy Pb emissions continue to be remobilized in dust, contaminating both food and ecological systems.



INTRODUCTION

Urban agriculture is growing rapidly in popularity.[1,2] As part of the urban agricultural revolution, European honey bees (*Apis mellifera*) are increasingly being kept in cities,[3] which are known to be polluted by a range of environmental contaminants.[4] Honey bees are a critical part of the agricultural system and pollinate approximately two-thirds of crops that provide 90% of global food.[5] The global economic value of insect pollination services, which are dominated by honey bees, was estimated to be worth €153 billion (US$187 billion) in 2005,[6] with recent estimates indicating that annual honey bee pollination is worth >USD$15 billion in the USA[7] and around AUD$4−6 billion in Australia.[8]

In a single beehive of *Apis mellifera*, there is one queen bee, and up to a few thousand drones, and tens of thousands of worker honey bees.[9] Drones are relatively inactive, stay in the beehive most of the time, and their only role is to mate with the queen.[10] By contrast, worker honey bees spend a significant amount of their adult life outside of the hive foraging over an area of 7 km^2 or more for nectar, pollen, and water.[11,12] Consequently, honey bees have been considered as a potential sentinel species for monitoring trace element contamination in the environment.[12,13]

Trace elements are ubiquitous in the environment, occurring either from natural processes (forest fires, volcanic emissions, sea spray, and biogenic sources) or anthropogenic activities such as agrochemicals, industry, mining and mineral processing, and traffic emissions.[14−16] The combustion of coal (e.g., Hg, Mn, Se, Sn) and oil (e.g., Ni, V) are major sources of trace metal emissions to the environment along with those from nonferrous metal production (e.g., As, Cd, Cu, Pb, Zn).[16] Emissions of Pb along with other co-contaminants from vehicle brakes and tires (As, Cd, Cr, Ni) was dominated by the combustion of leaded petrol, which emitted millions of tonnes to the atmosphere.[17−20] The accumulation of certain trace elements in the environment can have a detrimental impact on ecosystems and living organisms where there are exposure pathways.[21]

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Ingestion and inhalation of elevated concentrations of trace elements including As, Pb, Mn, and Zn have been associated with a variety human health problems arising from chronic or acute exposures.[22] In particular, As and Pb are considered toxic, even in minute amounts.[23] Although the global withdrawal of leaded petrol is almost complete[24] and its significance as a global atmospheric contaminant has declined rapidly in the last 20 years,[25] it continues to remain an environmental health challenge. Not only does Pb persist in the environment, but it has contaminated urban environments leading to adverse human health outcomes even from low exposures.[26,27]

The use of standard methods to characterize large-area spatial variations of trace elements requires evenly distributed sample collection sites, which are costly.[28] In order to overcome the cost and challenge of setting up a well-distributed sampling regime, the use of indicator species such as honey bees has been suggested as a more efficient way of gathering data to monitor environmental quality and the concentrations of contaminants.[13,29,30] On the basis of worker honey bees' extensive foraging behavior, it is more likely that they have greater potential as a environmental bioindicator[31,32] than drones from the same hive. During their short life cycle in summer (2−4 weeks), the worker honey bee completes thousands of interactions with soil, air, water, and vegetation.[33] During foraging, worker honey bees make contact and interact with contaminant particles derived from soil and dust, which adhere to their body parts and hairs.[13] Adhered contaminants are returned to the beehive, influencing the trace element composition of its honey and wax.[4] Environmental contaminants can also accumulate in and on the bodies of worker honey bees.[13]

The use of honey bees and their products as bioindicators for environmental contamination was first attempted by Svoboda in 1962 using strontium (^{90}Sr).[34] A number of recent international studies have examined the use of honey bees and their products as biomarkers to detect environmental contamination by trace elements.[13,35−39] However, the utility of honey bees or their products for use as a reliable bioindicator has had mixed success in previous studies,[40−42] often because of the absence of quantitative assessment of the relationship between honey bees and co-located soils[43] and dusts.[44] This is the first Australian study to measure and analyze the relationships between trace element concentrations (As, Mn, Pb, Zn) in European honey bees, honey, and wax and corresponding temporal soil and dust samples. Samples for this study were collected from Australia's largest city, the Sydney metropolitan area, and the >130-year old Pb−Zn−Ag mining city of Broken Hill, New South Wales, approximately 1160 km west of Sydney (Figure 1). Unlike other investigations of trace elements in honey bees and their products, this study uses Pb isotopic compositions to discriminate potential sources of contamination such as former petrol Pb and mining emissions compared with background geogenic materials.

MATERIALS AND METHODS

Study Area. Sample locations in Sydney included sites close to national parks (Galston and Gordon), low to medium density coastal residential suburbs (Coogee and Randwick), high density city areas of Sydney (central business district (CBD), Marrickville, Newtown, Surry Hills), and a mixed land use suburb containing light industrial, commercial, and residential uses (Mascot). The inner city areas of Sydney have been most affected by former lead petrol emissions, with diminishing effects away from the CBD.[28,45] Mascot is adjacent to Sydney Airport, which is the oldest and busiest continually operating commercial airport in Australia. Samples from the Pb−Zn−Ag mining city of Broken Hill were included in the study because it is still subject to ongoing atmospheric contamination that significantly impacts trace element concentrations in the immediate local environment (especially Pb).[46−48] Contamination from Broken Hill Pb and other Australian ores with a similar Pb isotopic signature (e.g., Mount Isa ore) from leaded petrol and mine-related emissions can be identified in global environmental archives,[49−52] with it still being persistent in contemporary Antarctic ice[25] and ocean waters.[53]

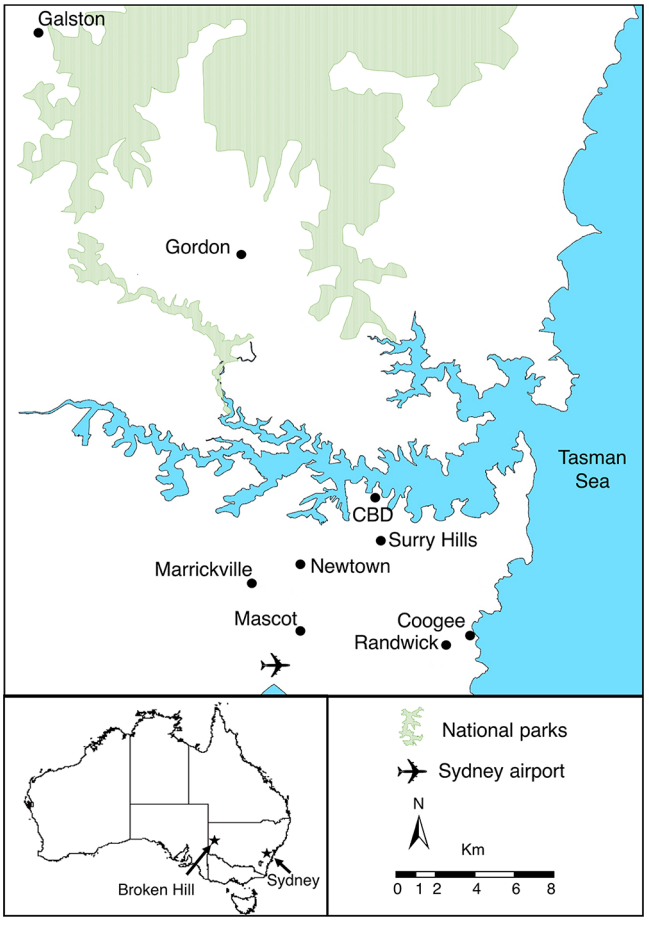

Figure 1. Location of beehive sites sampled in Sydney metropolitan area (n = 9) and Broken Hill (n = 1). Sample locations are adjacent to the following land uses: national park (Galston, Gordon), coastal residential suburbs (Coogee, Randwick), high density inner city (Sydney CBD, Surry Hills, and Newtown), residential−industrial (Marrickville), airport and light industrial, commercial, and residential (Mascot) and Pb−Zn−Ag mining (Broken Hill).

Sample Collection. Ten European honey bee hives (*Apis mellifera*) (nine across Sydney metropolitan and one from Broken Hill) were sampled in the warmer Australian months between spring and late summer (November 2015 to April 2016), the most intensive foraging period for honey bees. Thirty live worker honey bees were collected at monthly intervals by use of a bug vacuum at the entrance of each beehive. In addition, 60 live drones were collected from the beehives at Coogee (n = 30) and Randwick (n = 30) in November 2015. Thirty dead worker honey bees were collected on two separate occasions (November 2015 and April 2016) from the area in front of the beehives at the Surry Hills city site.

Raw, unprocessed samples of honey (n = 17) and wax (n = 16) produced during the study period were collected from all

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Environ. Sci. Technol. 2018, 52, 991−1001

10 beehives (Figure 1). These were supplemented with additional honey samples from Marrickville (two samples) and Paddington (one sample), which is an older residential area some 3.3 km southeast of the CBD.

Trace metal clean techniques were employed for sample collection and processing using established procedures as detailed below. Dust deposition samples were collected using published methods.[47] Dust sample collection equipment was constructed in accordance with the Australian Standard 3580.10.1−2003[54] and consisted of a 150 mm diameter glass funnel inserted inside a 2.75 L glass bottle, secured in a plastic bucket affixed to a ∼ 2 m high tripod. Funnels were rinsed with Milli-Q (Milli-Q) water during each replacement. Sample bottles were sealed in the field and transported to the National Measurement Institute (NMI), North Ryde, Sydney, for analysis. Dust samples (n = 41) were collected monthly at the same time as honey bee collection over the sampling period. Established methods[55] were used to collect surface soils (0−2 cm) (n = 44) around each beehive at the beginning of the study in November 2015. Soil samples were oven-dried at 40 °C for 48 h and sieved to <2 mm using a stainless steel mesh prior to trace element analysis at the NMI.

Sample and Data Analysis. Samples of bees, honey, wax, soil, and dust were analyzed at the NMI, using National Association of Testing Authorities (NATA) accredited in-house reference methods for food, soil and dust. Raw, unprocessed honey bees and honey samples were used for analysis. Wax samples were rinsed with Milli-Q water until all honey residues were removed and then oven-dried at 60 °C. One gram of honey bees, honey, and wax was digested with 3 mL of HNO_3 before heating at 100 °C for 2 h.

Soil samples were digested by adding HCl and HNO_3 (1:1 respectively, 6 mL) followed by heating at 100 °C for 1 h. After cooling, 10 mL of Milli-Q water was added and the sample reheated to 100 °C for another 1 h. Dust deposition samples were collected on glass microfibre filters (Whatman 934-AH, 47 mm diameter), and after weighing, digested using HCl and HNO_3 (1:3, respectively, 8 mL) for 2 h. Each digested sample of honey bees, honey, wax, soil, and dust was topped up to 40 mL with Milli-Q. Samples were diluted prior to analysis for their As, Mn, Pb, and Zn concentrations on an inductively coupled plasma mass spectrometer (Agilent 7900 equipped with an ISIS sample introduction system). Each sample batch (n = 20) contained a laboratory reagent blank and duplicate, blank spike, blank matrix, duplicate sample, and matrix spikes.

Procedural blanks were below NMI's Limit of Reporting (LOR) of 10 μg/kg for As, Mn, Pb, and Zn in honey bees, honey, and wax. Dust sample blanks were < LOR of 0.1 mg/kg and 0.05 mg/kg for As and Zn, Pb and Mn, respectively. Procedural blanks for the soil samples were < LOR of 0.05 mg/kg for As and 0.01 mg/kg for Pb, Mn, and Zn. The NMI's internal reference materials AGAL-10 (Hawkesbury River Sediment, n = 4) and AGAL-12 (biosoil, n = 4) were processed with soil and dust samples. Mean recovery rates of As, Mn, Pb, and Zn in AGAL-10 and AGAL-12 were 101% and 108%, respectively. Recovery rates for As, Mn, Pb, and Zn ranged between 96−99% for soil and 98−100% for dust. Matrix spike recovery rates for As, Mn, Pb, and Zn for honey bees, honey and wax were 99−106%. Analytical uncertainties for all elements were 14−24%.

Sample heterogeneity was assessed by replicate analysis (n = 4) of the different study sample matrices along with NMI's internal reference materials AGAL-10 and AGAL-12. The relative standard deviations (RSDs) for all study matrices (honey bees, honey, wax, dust. and soil) were less than 5%. The exceptions were As in honey bees and wax at 7.5% and 6.4%, respectively, and Pb concentration in honey bees, which was 22.7% in samples from Sydney CBD. The elevated sample RSD in a bulk 1 g sample of honey bees prompted a more detailed investigation of sample heterogeneity involving analysis of Pb concentrations in individual live worker honey bees from the Sydney CBD (n = 12) and Surry Hills sites (n = 14). These analyses returned Pb concentration RSDs of 26% and 31.5%, respectively. The implications of this are considered below. The RSDs for the NMI's internal reference materials ranged between 0.3% and 1.4%.

Samples of honey bees (n = 26), honey (n = 6), wax (n = 6), soil (n = 42), and dust (n = 35) were subjected to Pb isotopic composition analysis ($^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$) after sample volumes were optimized on the basis of their Pb concentrations. The certified values of $^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, and $^{208}Pb/^{207}Pb$ for National Institute of Standards and Technology (NIST) SRM981 (natural Pb isotope composition standard) are 0.0646 ± 0.000047, 1.0933 ± 0.00039, and 2.3704 ± 0.0012, respectively. The measured values (n = 164) were 0.066 ± 0.001, 1.103 ± 0.005, and 2.389 ± 0.010. The overall limits of error for NIST SRM981 certified values and measured values are based on 95% confidence limits for the mean of the ratios measured. The NIST SRM981 was used to correct for mass discrimination. Analytical uncertainties for Pb isotopic compositions (expressed for the NIST SRM981) were $^{204}Pb/^{207}Pb$ = 0.065 ± 0.0005, $^{206}Pb/^{207}Pb$ = 1.093 ± 0.005, $^{208}Pb/^{207}Pb$ = 2.370 ± 0.01, respectively. The mean RSDs for NIST981 $^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$ were 0.55%, 0.23%, 0.22%, respectively. The mean RSDs for sample analysis $^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$ were 0.20%, 0.13%, and 0.23%.

Data were analyzed using SPSS 22.0 statistics software for arithmetic mean, standard deviation, and minimum and maximum values, and Minitab Express v 1.5.1 was used for generating linear regressions and tests of significance (Pearson correlations and t tests). Concentrations of As, Mn, Pb, and Zn in honey bees, honey, and wax with <LOR (10 μg/kg) are considered as 5 μg/kg for statistical analysis. Trace element concentrations in various beehive products and environmental samples of soil and dust were non-normally distributed and were $\log_{10}$ transformed for correlation analysis. Pearson correlation coefficients and two-tailed tests of significance were applied to assess the relationships between beehive products (honey bees, honey, wax) and the environment (soil, dust) across the sampling sites. Two sample t tests were used to compare concentrations of As, Mn, Pb, and Zn in worker honey bees and drones, live worker honey bees, and dead worker honey bees.

RESULTS

Trace Elements in Worker and Drone Honey Bees. Given that worker and drone honey bees have different life cycles and roles in a hive, with the latter being less active in the outside environment, their trace element concentrations were compared to ascertain if there were discernible differences in exposures. Higher, but nonsignificant, levels of As, Mn, Pb, and Zn were found in worker honey bees compared to drones (Figure 2) (p = 0.284−0.404, two-sample t tests). Concentrations of As and Zn in worker honey bees were found to be double that in drones, with Pb being four and Mn eight times greater (Table S1). Dead worker honey bees contained higher

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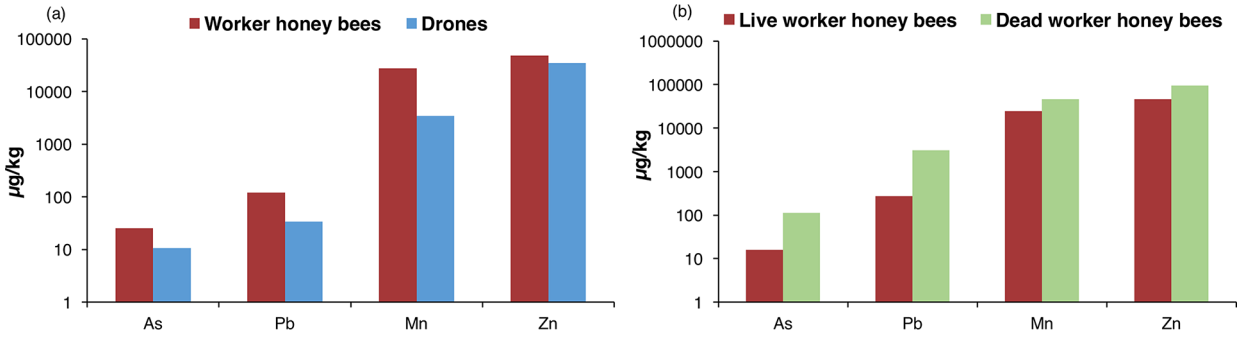

Figure 2. (a) Mean concentrations of trace elements As ((min−max) 24−26 µg/kg), Mn (15000−40000 µg/kg), Pb (79−160 µg/kg) and Zn (39000−58000) in worker honey bees (n = 60) and drones (As ((min−max) <10−21 µg/kg), Mn (2100−4800 µg/kg), Pb (26−41 µg/kg), and Zn (31000−38000)) (n = 60) collected from the coastal residential areas of Coogee (n = 30) and Randwick (n = 30) in November 2015. Trace element concentrations between the two honey bee types were not significantly different (p = 0.284−0.404, two sample t tests). (b) Mean concentrations in live worker honey bees (n = 60) of trace elements As ((min−max) 14−18 µg/kg), Mn (22000−27000 µg/kg), Pb (250−290 µg/kg), and Zn (39000−54000 µg/kg) concentrations and in dead worker honey bees (n = 60). As ((min−max) 66−160 µg/kg), Mn (19000−73000 µg/kg), Pb (3100−3100 µg/kg), and Zn (41000−150000) collected from the city area of Surry Hills in November 2015 (n = 30) and April 2016 (n = 30). Only the Pb concentrations were significantly different between live and dead worker bees (p < 0.005, two sample t test). Full data set is available in Table S1.

levels (p = 0.005−0.572, two-sample t tests) of all trace elemental concentrations than live worker honey bees, indicating contamination from the surrounding environment (Table S1 and Figure 2). However, only lead concentrations were significantly higher (p < 0.005, two-sample t test) in dead compared with live worker honey bees (3100 µg/kg vs 270 µg/kg) (Figure 2). A separate analysis of individual live worker bees from a single sample collected from Sydney CBD and Surry Hills sites showed that the mean Pb concentrations were 188 µg/kg with RSD 26% (range from 140−260 µg/kg) and 251 µg/kg with RSD 31% (range from 110−430 µg/kg) at each site, respectively.

Trace Element Concentrations in Beehive Products, Soil, and Dust. Mean concentration data for As, Mn, Pb, and Zn in honey bees (µg/kg), honey (µg/kg), wax (µg/kg), soil (mg/kg), and dust (mg/kg) are provided in full in Table S2 and are summarized below.

The highest mean concentrations of Pb in honey bees, honey, and wax were recorded in Broken Hill samples (2570, 295, and 11600 µg/kg, respectively). By comparison, Pb in honey from the nine Sydney sites did not exceed 22 µg/kg (Table S2). Concentrations of As in honey bees (160 µg/kg) and wax (77 µg/kg) were higher in Broken Hill compared with Sydney. Across Sydney, mean concentrations of Pb in honey bees were lowest (50 and 56 µg/kg) in the locations adjacent to national parks (Galston and Gordon) followed by the coastal residential areas of Coogee and Randwick (125 and 146 µg/kg). Honey bees sampled in city and industrial areas of Sydney contained higher Pb concentrations: Sydney CBD/Surry Hills/Newtown 230−440 µg/kg; inner west residential and industrial area of Marrickville 150−350 µg/kg and the mixed (industrial, commercial and residential) land use area of Mascot 418 µg/kg. Concentrations of Mn and Zn in beehive products (wax) were higher proximal to industrial locations, with the highest Mn and Zn values recorded in Broken Hill honey at 6280 µg/kg and 2180 µg/kg, some five and three times greater than at sites adjacent to national parks (Gordon: Mn 1320 µg/kg, Zn 800 µg/kg; Galston: Mn 4580 µg/kg, Zn 465 µg/kg).

Trace element concentrations in soil and dust were typically lower at background Sydney sites compared to Sydney metropolitan sites, with the highest values recorded at Broken Hill (Table S2). Elevated trace element concentrations in the Broken Hill beehive samples is consistent with high levels of contaminants found in the city's soils and dusts.[46,47] Soil Pb at the sample site in Broken Hill was 3290 mg/kg compared with 20−810 mg/kg at the Sydney sample sites (Table S2). Soil Pb from the city and inner west areas of Surry Hills, Newtown, and Marrickville exceeded the Australian Health Investigation Level (HIL-A) of 300 mg/kg applicable to residential dwellings,[56] corresponding with other studies of soil contamination in Sydney.[28,57−61] Maximum concentrations of As (23 mg/kg), Mn (1450 mg/kg), and Zn (3890 mg/kg) were found in Broken Hill soils but were below the Australian HIL guidelines for residential properties of 100, 3800, and 7400 mg/kg, respectively.[56]

Maximum dust Pb concentration was 4360 mg/kg in Broken Hill, 115 times greater than at the Galston site (38 mg/kg), which is adjacent to a national park. Similarly, the highest mean values of dust Mn and Zn were from Broken Hill: Mn 3130 mg/kg and Zn 7570 mg/kg. Sydney metropolitan soil trace element values were much lower at 104−700 mg/kg for Mn, and 307−590 mg/kg for Zn. Dust As concentrations were markedly greater at Surry Hills (230 mg/kg) and Broken Hill (124 mg/kg) than at other sampling sites across Sydney (2−77 mg/kg).

Pearson correlations were analyzed between trace element concentrations ($\log_{10}$ transformed) in beehive products and co-located samples of soil and dust (Table S3a−d). Of the trace elements analyzed, Pb had the most robust relationships among the trace elements and variables examined. Dust Pb was highly correlated to corresponding soil values (r = 0.806, p = 0.005, Figure 3a), with it being the most reliable predictor of outcomes variables. For example, only Pb concentrations in honey bees were significantly related to all corresponding concentrations in soil (r = 0.674, p = 0.033) and dust (r = 0.882, p = 0.001) (Figures 3b, c) as well as honey (r = 0.882, p = 0.003) and wax (r = 0.789, p = 0.007). Further, Pb in honey was significantly correlated (Pearson's r) to co-located soil (r = 0.716, p = 0.020) and dust Pb (r = 0.786, 0.007, Figure 3d,e). Wax Pb concentrations were correlated to dust Pb (r = 0.730, p < 0.017; Figure 3f) but not soil (Table S3a).

Zinc soil and dust values were correlated at lower levels compared to those in Pb (r = 0.634, p = 0.049), while soil and dust concentrations in As (r = 0.138, p = 0.704) and Mn (r = 0.547, p = 0.102) were not statistically significant. As per

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Environ. Sci. Technol. 2018, 52, 991−1001

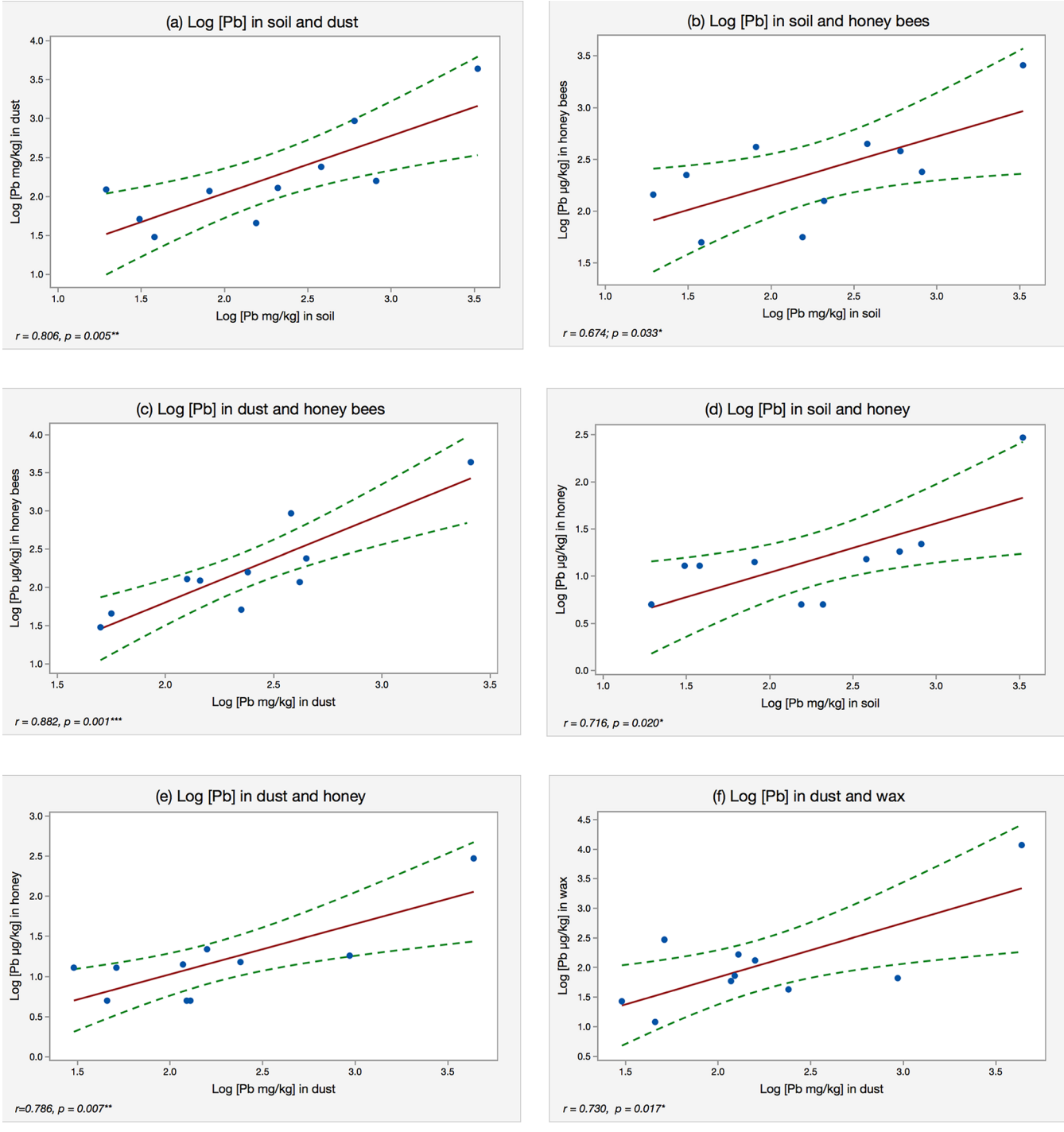

Figure 3. (a–f) Plots showing the relationship (with 95% confidence intervals) between $\log_{10}$ transformed mean Pb concentrations in honey bees (µg/kg, n = 39), honey (µg/kg, n = 17), wax (µg/kg, n = 16), and co-located soil (mg/kg, n = 44) and dust (mg/kg, n = 41), as well as the correlation between co-located soil Pb and dust Pb samples. Pearson correlation significant at the *0.05, **0.01, and ***0.001 level.

Pb, Zn in honey and wax were significantly correlated with co-located soil and dust concentrations (r = 0.725, p = 0.018; r = 0.725, p = 0.018), respectively. The statistical relationships for the remaining trace elements analyzed (As, Mn, and Zn) and their corresponding soil or dust values are provided in Tables S3a–d. Arsenic concentrations in honey and wax were below the NMI's LOR of 10 µg/kg, limiting statistical analysis of its relationship to the corresponding variables.

Lead Isotopic Compositions. Mean values of Pb isotopic compositions ($^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$) in honey bees, honey, wax, soil, and dust were compared to published values of the Broken Hill ore body, Sydney background values (subsurface soils and rocks representing geogenic values), and aerosols collected during the period when leaded petrol was consumed (Figure 4).[46,62−65]

The data show that Broken Hill samples are distinct from Sydney samples. Source apportionment modeling[66] showed that beehive samples from Broken Hill and corresponding environmental sample Pb isotopic compositions were within 95–98% of the local ore body (Figure 4; Table S5). The mean

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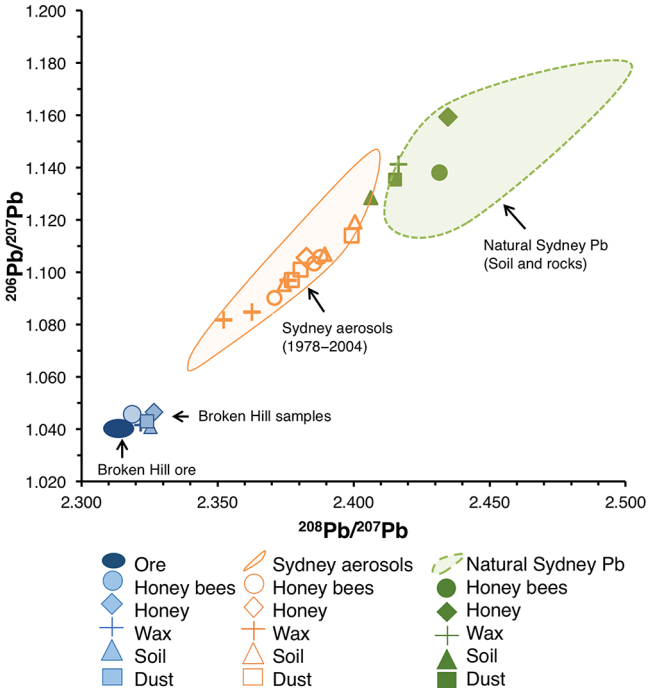

Figure 4. Mean Pb isotopic compositions for honey bees (n = 26), honey (n = 6), wax (n = 6), soil (n = 42), and dust (n = 35). Lead isotopic compositions for Sydney background soils, rocks, and aerosols were obtained from Wu et al.[62] Broken Hill ore body data were obtained from Kristensen and Taylor[46] and Gulson.[64]

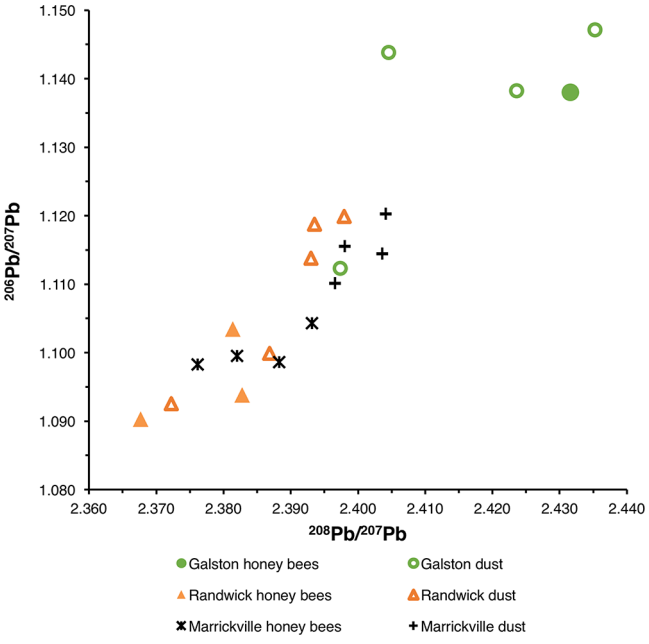

Figure 5. Lead isotopic compositions for individual site samples of honey bees (n = 8) and dust (n = 13) from Galston (Sydney background area), Randwick (coastal residential area), and Marrickville (residential–industrial area).

Pb isotopic compositions of honey bees, wax, and honey ($^{206}Pb/^{207}Pb$ 1.138–1.159; $^{208}Pb/^{207}Pb$ 2.417–2.435) and co-located dust from sites adjacent to Sydney national parks had Pb isotopic compositions ($^{206}Pb/^{207}Pb$ = 1.123–1.176, $^{208}Pb/^{207}Pb$ = 2.413–2.500) corresponding closely to those in background geogenic soil and rocks ($^{206}Pb/^{207}Pb$ 1.098–1.176; $^{208}Pb/^{207}Pb$ 2.388–2.500) (Table S5, Figure 4). Surface soils from the background sites were close to the "leaded petrol" aerosol envelope indicating adulteration of natural values by depositions from former petrol emissions, consistent with other Australian Pb isotopic composition analysis of soils distant from cities.[67,68] The mean Pb isotopic compositions of Sydney metropolitan samples (honey bees, honey, wax, soil, and dust) correspond to the Pb isotopic composition of Sydney aerosols (1978–2004) collected during a time period dominated by leaded petrol consumption, which ended in 2002.[17,62] However, the distribution of Pb isotopic composition values available for each site reveals there is some variation in Pb sources (Figure 5; Table S5). This is not surprising given the inherent variability of likely Pb sources in a large city such as Sydney (i.e., petrol, paint, industrial emissions and related sources), coupled to the fact that the honey bees forage over an area greater than 7 km^2.

DISCUSSION

Marginally higher concentrations of trace elements in worker honey bees compared to drones in this study are consistent with those in Mihaly Cozmuta et al.'s Romanian study[69] that found worker honey bees had approximately twice the Pb concentrations of drones. Exposure to environmental contaminants in worker honey bees is anticipated to occur during foraging activity outside of the hive, where atmospheric and substrate particles can attach to their body hair.[13] Drones usually have three or less flights per day, totaling about 30 min, around the beehive.[70] By contrast, a worker honey bee has about 10 flights per day, totaling up to 90 min and traveling up to 13 km, foraging for nectar and pollen.[71] Increased lifetime exposure to environmental contaminants in worker honey bees is corroborated by Figure 2, which shows statistically higher levels of Pb in dead worker honey bees than those alive (two-sample t test, p = 0.005) at the time of sampling (Table S1, Figure 2). Analysis of individual live honey bees from the CBD and Surry Hill in Sydney city showed that contamination is highly variable between individual insects. Given it is not possible to determine the age of the honey bees, we suggest that differing levels of Pb contamination may be a suitable proxy for their age. This inference is supported by the fact that worker honey bees are more contaminated than drones, which can only occur from their greater activity outside the hive where contamination is more prevalent. Thus, the data implies that worker honey bees are either accessing places consisting of variable Pb levels and/or are being contaminated as they age. This latter conclusion is not only consistent with other studies[72] but is further evidenced by the fact that dead bees at the Surry Hills site had statistically higher levels of Pb than live specimens from the same hive (Figure 2b). The issue of honey bee Pb contamination warrants further study to ascertain if it results in impairment of their function as they age.

Arsenic was below the LOR (<10 μg/kg) in all honey samples collected and analyzed in this study (Table S2). Although the highest As concentration in honey bees was found in Broken Hill (160 μg/kg) where elevated levels of atmospherically deposited dust trace elements are prevalent,[47,73,74] it was not detected in the corresponding honey sample (Table S2). This is consistent with the study by Alvarez-Ayuso and Abad-Valle[40] who showed there was limited transfer of As from honey bees to their honey. The physiological mechanism for the reduced transfer of As and other trace element contaminants in honey is not well-established but may be related to the honey bees'

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Environ. Sci. Technol. 2018, 52, 991–1001

internal biological filtering[42,72,75−78] with some of the contaminants being excreted through its fecal mass[79] or its wax.[80]

Measurable concentrations of As in wax from Sydney CBD (14 μg/kg) and Broken Hill (77 μg/kg) are likely to reflect environmental contamination from dense traffic movements and mining activities, respectively.[72,81] Higher As concentrations in wax compared with honey[75,82] may be a result of the honey bees' process of producing and secreting wax[80] and its longevity in the hive versus that of honey. Typically, the wax comb is retained for reuse after seasonal honey production has been harvested.

Honey bees and their products have previously been shown to be linked to sources of environmental Pb, but the specifics of the spatial and temporal relationships remain poorly explained.[83−85] In our study, the following Pearson correlations were found between Pb in honey bees ($r = 0.882$, $p = 0.001$), honey ($r = 0.786$, $p = 0.007$), wax ($r = 0.730$, $p = 0.017$), and co-located dust concentrations (Figure 3c,e,f; Table S3a−d). Given that dust Pb is partly derived from surface soils (Figure 3a),[86] this indicates that honey bees and honey have potential application for monitoring the resuspension of soil Pb in the environment (Figure 3b,d).

The absence of a significant correlation between Mn and Zn in honey bees and co-located soil ($r = 0.511$, -0.587, $p = 0.131$, 0.074, respectively) and dust ($r = 0.137$, -0.731, $p = 0.705$, 0.016) (Table S3a−d) could be because Mn and Zn are essential elements for honey bee development and are more readily absorbed and secreted.[87,88] The honey bee's physiological system is likely to be better adapted to processing these elements compared to As and Pb, for which there are no biological uses due to their toxicity.[89,90] This interpretation is reinforced by the data in Table S2, which shows Mn and Zn concentrations are two times higher in dead honey bees versus live ones, whereas As and Pb concentrations are seven and 11 times, respectively, higher in dead specimens than those alive at the time of sampling.

The use of honey bees, honey, and wax as environmental proxies for discriminating sources at a larger scale appears to have some validity, particularly where land use types are markedly different. For example, the study data indicates that Pb, Mn, and Zn concentrations in honey bees and their products vary sufficiently to discriminate between uncontaminated areas adjacent to national parks (Galston, Gordon), Sydney metropolitan areas (Coogee, Randwick, Sydney CBD, Surry Hills, Newtown, Marrickville, Mascot), and the Pb−Zn−Ag mining city of Broken Hill. Even after excluding Broken Hill data, the Pearson correlation between Pb concentrations in Sydney metropolitan worker honey bees and corresponding dust samples remained significant ($r = 0.731$, $p = 0.025$). By contrast, analysis for associations of Pb concentrations between other paired data (i.e., those in beehive products and corresponding environmental samples) was not significant after the removal of the Broken Hill data ($r = -0.022$−0.650, $p = 0.058$−0.955). Although a relationship between Sydney metropolitan Pb concentrations in dust and soil was evident, it was marginally statistical insignificant ($r = 0.650$, $p = 0.058$). Previous studies of environmental contaminants within the Sydney metropolitan region reveals that multielemental concentrations in soil and dust across Sydney metropolitan suburbs are heterogeneous.[28,91−93] Given that honey bees forage an area over 7 km^2,[11,12,75] it likely that concentrations of trace elements found in honey bees and their products reflect a more generalized proxy of contamination at a suburb level. This would have the effect of smoothing variations in anthropogenically sourced contaminants measured at the different Sydney metropolitan suburb sites. Local geogenic factors that could influence the Pb isotopic composition of honey bees and their products is vastly different to low radiogenic Broken Hill type ores that were used predominantly in Australian leaded petrol and have contaminated Sydney soils and dusts.[52,68] The Pb isotopic composition of honey bees, honey and wax from Sydney metropolitan sites ($^{206}Pb/^{207}Pb$ = 1.082−1.106, $^{207}Pb/^{208}Pb$ = 2.352−2.388), match closely the Pb isotopic compositions of aerosols ($^{206}Pb/^{207}Pb$ = 1.067−1.148, $^{207}Pb/^{208}Pb$ = 2.341−2.410) collected from 1978−2004 (Table S5, Figure 4), the period when peak leaded petrol emissions were beginning to decline.[17] Several studies have shown that aerosols collected during the period of leaded petrol consumption correspond closely to the Pb used in petrol at that time,[68,94−96] and it is apparent that its signature remains.

In terms of geogenic sources, bedrock geology across the Sydney basin is composed predominantly of rocks and sediments of Triassic age or younger,[97] is more radiogenic than former lead petrol emissions (Figure 4), and corresponds more closely to samples from background areas. Since the withdrawal of Australian leaded petrol in 2002, there remain only limited atmospheric lead emissions in the city of Sydney. The largest emitter in the city of Sydney was from a used lead acid battery (ULAB) approximately 2 km from the Mascot bee hive site, which reported 400 kg of Pb emissions to the air in 2015−2016.[98] Moreover, the ULAB site is also approximately 2 km from the Australian Nuclear Science and Technology Organisation's air quality station at Mascot and its emissions do not appear to influence seasonal or long-term downward trend of lead in air concentrations since before 2002 (Figure S1).[99−101] Annual Pb in air peaks occur during the cooler Australian months of June to September, with mean concentrations from 2015 at 6.14 ng/m^3 and monthly maximum average concentrations not exceeding 15 ng/m^3; Figure S1). In the warmer months of the sample period (November 2015 to April 2016) when honey bees are foraging intensively, Pb in air was <5 ng/m^3; Figure S1). Despite the reducing contemporary atmospheric Pb sources in Sydney, the study indicates that honey bees, honey and wax have good potential application for their use as environmental indicators. This is particularly the case where there are markedly different sources such as natural background, urban-residential (legacy) and mining, and where Pb is the focus trace element.[31] However, further work applying the same approach to other areas is required to confirm the wider applicability of this approach.

The data show that honey bees and their products are suitable markers of contemporary contamination emanating from mining operations in Broken Hill where total Pb emissions to the atmosphere over 2015−2016 were estimated at 28000 kg.[98] Lead in air concentrations (as total suspended particulates) some 1.6 km from the Broken Hill honey bee sampling site averaged 185 ng/m^3, with a maximum of 527 ng/m^3 between January 2015 and April 2016 (Figure S1).[102] In contrast to Sydney, peak Pb in air concentrations in Broken Hill occur in the Australian summer months when honey bees are most active (Figure S1). Given the significant current Pb and other dust contaminant emissions in Broken Hill,[47,73,74] it is not surprising that trace element concentrations from Broken Hill samples were tens to hundreds of times greater than those from Sydney background sites adjacent to national parks (Table S2). The sensitivity of honey bees and their products as environmental markers is supported by the fact that the Pb

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Environ. Sci. Technol. 2018, 52, 991−1001

isotopic compositions of samples from Broken Hill were within 95−98% of the Broken Hill ore body (Figure 4). Other studies of Broken Hill contamination have shown that ambient dust is similar in its Pb isotopic composition to the Broken Hill ore body and that Pb isotopic composition analysis of ambient dust is an effective analytical tool for discriminating sources of contamination in that city.[47,73,103,104]

Recent studies of Australian soils,[45] sediments,[105,106] wines,[68] peat bogs,[107] seagrass,[108] lichens,[62,109] and wildfire ash[110] have all been shown to contain varying proportions of legacy petrol Pb with a Broken Hill signature, indicating its widespread significance as a contaminant. Moreover, Broken Hill Pb has been found across the globe due its former common use in leaded petrol.[50,51,111,112] Although Pb emissions from petrol have also been eliminated in Australia and its adverse health effects diminished, it remains a persistent contaminant in the environment from its seven-decade period of use between 1932 and 2002.[17,52,113] Therefore, in the absence of other contemporary Pb sources in Sydney, the data in this study indicate that legacy Pb isotopic compositions in contemporary honey bees and their products reflect the dominant source of Pb in the ambient environment. Recent analysis of Sydney aerosols before, during, and after wildfires show that Pb compositions have shifted back toward natural values following the cessation of leaded petrol use in 2002.[52] However, assessment of ash produced during wildfires[110,114] contemporary lichens,[62] wine,[68] and now in honey bees and their products demonstrate how easily legacy Pb is being recycled into food and ecological systems. Such persistence should act as a warning to the use and unregulated release of other chemicals that may be harmful to humans and ecosystems.[115]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04084.

Supplementary tables and figures detailing trace element and statistical analyses, Pb isotopic compositions in honeybees, honey, wax, soil and dust, and current Pb sources in Sydney and Broken Hill, Australia (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mark.taylor@mq.edu.au.

ORCID

Xiaoteng Zhou: 0000-0002-1900-6037

Mark Patrick Taylor: 0000-0001-7598-9982

Author Contributions

X.Z. helped develop the project design and undertook the majority of field sampling, laboratory analysis, data assessment, and write up. M.P.T. devised the original project idea and design, assisted with field sampling, and undertook data analysis, interpretation, and write up. P.D. and S.P. assisted with sample design and data write up.

Notes

The authors declare no competing financial interest.

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Chapter 4: Trace elements in the environment and the Australian native bee (*Tetragonula carbonaria*)

Chapter 4 presents the following paper:

Xiaoteng Zhou (80 %), Mark Patrick Taylor (15 %) & Peter J. Davies (5 %). Tracing natural and industrial contamination and lead isotopic compositions in an Australian native bee species. *Environmental Pollution*, **242**, pp 54-62.

This chapter builds on the findings from the previous chapter that identified the most common bee species, the European honey bee (*Apis mellifera*), as a practical and suitable bio-indicator of current and legacy contamination. Chapter 4 applies a similar approach to that utilised in the previous chapter to examine the Australian native bee, *Tetragonula carbonaria*, and its products for their potential use as a bio-indicator of contamination. In addition, this chapter compares the efficacy of *T. carbonaria* as a bio-indicator to that of the European honey bee, *A. mellifera*. *T. carbonaria* has a shorter foraging distance than *A. mellifera* (0.3–0.7 km vs. 5–9 km), lower annual honey production (1 kg vs. 50 kg) and a longer lifespan (~100 days vs. ~40 days). The study finds that these factors are likely to be important due to the more significant correlations measured between *T. carbonaria* and ambient trace element values compared to those measured in corresponding *A. mellifera* samples.

In summary, the data reveals a stronger relationship between soil and dust trace elements at a suburban level in native bee hive products than those associated with *A. mellifera*. Paralleling the *A. mellifera* study in Chapter 3, Pb isotopic composition analysis of *T. carbonaria* and its products also shows that legacy Pb depositions from former leaded petrol emissions continue to be recycled in contemporary food and ecological systems. This chapter complements the previous study by confirming that the native bee species, *T. carbonaria*, can also be utilised as an effective bio-indicator to trace current and legacy contamination in the environment.

However, the data also indicate that this species may be more responsive to variations in trace element contamination over relatively small areas, suggesting that the efficacy of bees as bio-indicators is related to their biological and behavioural features.

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Tracing natural and industrial contamination and lead isotopic compositions in an Australian native bee species☆

Xiaoteng Zhou [a, *], Mark Patrick Taylor [a, b, **], Peter J. Davies [a]

[a] Department of Environmental Sciences, Faculty of Science and Engineering, Macquarie University, North Ryde, Sydney, NSW 2109, Australia
[b] Energy and Environmental Contaminants Research Centre, Faculty of Science and Engineering, Macquarie University, North Ryde, Sydney, NSW 2109, Australia

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ABSTRACT

This study investigates trace element concentrations (arsenic (As), manganese (Mn), lead (Pb) and zinc (Zn)) and Pb isotopic compositions in an Australian native bee species, *Tetragonula carbonaria,* and its products of honey and wax. Co-located soil and dust samples were simultaneously analysed with the objective of determining if the bees or their products had potential application as a proxy for monitoring environmental contamination. The most significant relationships were found between Pb concentrations in honey ($r = 0.814$, $p = 0.014$) and wax ($r = 0.883$, $p = 0.004$) and those in co-located dust samples. In addition, Zn concentrations in honey and soil were significantly associated ($r = 0.709$, $p = 0.049$). Lead isotopic compositions of native bee products collected from background sites adjacent to Sydney national parks ($^{206}Pb/^{207}Pb = 1.144$, $^{208}Pb/^{207}Pb = 2.437$) corresponded to local geogenic rock and soil values ($^{206}Pb/^{207}Pb = 1.123–1.176$, $^{208}Pb/^{207}Pb = 2.413–2.500$). By contrast, inner Sydney metropolitan samples, including native bees and wax ($^{206}Pb/^{207}Pb = 1.072–1.121$, $^{208}Pb/^{207}Pb = 2.348–2.409$), co-located soil and dust ($^{206}Pb/^{207}Pb = 1.090–1.122$, $^{208}Pb/^{207}Pb = 2.368–2.403$), corresponded most closely to aerosols collected during the period of leaded petrol use ($^{206}Pb/^{207}Pb = 1.067–1.148$, $^{208}Pb/^{207}Pb = 2.341–2.410$). A large range of Pb isotopic compositions in beehive samples suggests that other legacy sources, such as Pb-based paints and industrials, may have also contributed to Pb contamination in beehive samples. Native bee data were compared to corresponding samples from the more common European honey bee (*Apis mellifera*). Although Pb isotopic compositions were similar in both species, significant differences in trace element concentrations were evident across the trace element suite, the bees and their products. The statistical association between *T. carbonaria* and co-located environmental contaminant concentrations were stronger than those in European honey bees, which may be attributable to its smaller foraging distance (0.3–0.7 km versus 5–9 km, respectively). This implies that *T. carbonaria* may be more suitable for assessing small spatial scale variations of trace element concentrations than European honey bees.

1. Introduction

Urban development and industrial activities have caused extensive trace element contamination of surface soil, dust and water across terrestrial environments in major cities around the world (Su et al., 2014). A large number of studies have reported elevated levels of trace elements in urban environments, giving rise to concern about potential harmful effects on human health from exposure to air, water, dust or soil contamination (e.g. Li et al., 2001; Turer et al., 2001; Islam et al., 2015; Rodriguez Martin et al., 2015; Trujillo-Gonzalez et al., 2016).

The rapid urbanisation of Sydney following European occupation in 1788 has resulted in trace element contamination of soils and dusts, including arsenic (As), lead (Pb), manganese (Mn) and zinc (Zn) (e.g. Gulson et al., 2006; Gulson et al., 2008; Birch et al., 2011; Laidlaw and Taylor, 2011; Gulson et al., 2014; Rouillon et al.,

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* Corresponding author.
** Corresponding author. Department of Environmental Sciences, Faculty of Science and Engineering, Macquarie University, North Ryde, Sydney, NSW 2109, Australia.
E-mail addresses: xiaoteng.zhou@students.mq.edu.au (X. Zhou), mark.taylor@mq.edu.au (M.P. Taylor).

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2017; Zhou et al., 2018), all of which have known potential to impair human neurological systems, behavioural and cognitive abilities (Siegel, 2002). Sources of enriched trace elements are associated with emissions from vehicular traffic (Davis and Birch, 2010; Huber et al., 2016), urban and industrial wastes (Zhang et al., 2013), and mining and smelting of industrial activities (Kristensen and Taylor, 2016; Dong and Taylor, 2017). In addition, the former use of Pb-based paints and leaded petrol remain persistent in the environment (Gulson et al., 1995; Kristensen, 2015; Kristensen et al., 2017), and have contaminated urban soils (Birch et al., 2011; Rouillon et al., 2017) and dusts (Davis and Gulson, 2005; Laidlaw and Taylor, 2011; Dong et al., 2015). Across Australian cities, former leaded petrol depositions have also been measured in lichens (Wu et al., 2016b), fungi (Wu et al., 2016a), wildfire ash deposits (Kristensen et al., 2014; Wu et al., 2017) and seagrasses (Serrano et al., 2016).

The common European honey bee (*Apis mellifera*) has been assessed previously for its potential as bio-indicators of trace element contamination in urban (Perugini et al., 2011; Zarić et al., 2016), industrial (Bromenshenk et al., 1991; Matin et al., 2016) and mining areas (Lopes et al., 2010; Satta et al., 2012; Alvarez-Ayuso and Abad-Valle, 2017), as well as for apportioning Pb sources (Zhou et al., 2018). However, to the best knowledge of the authors, there is no equivalent research examining the utility of local native bees for the same purposes. The keeping of *T. carbonaria* has been promoted extensively in recent years in Australia (Halcroft et al., 2015; Heard and Dollin, 2015) as part of government and permaculture programs to connect people to nature. In the tropics, *T. carbonaria* is a commonly used bee species to assist with pollination services (Heard, 2000).

Compared to European honey bees (*A. mellifera*, introduced to Australia in 1822), the Australian native bee (*T. carbonaria*) has a number of important biological and behavioural differences. *T. carbonaria* has a smaller body (4 mm vs. 15 mm) (Smith et al., 2017), smaller foraging distance (0.3–0.7 km vs. 5–9 km) (Beekman and Ratnieks, 2000; Smith et al., 2017), shorter foraging time per day (7 h vs. 10 hrs) (Heard, 1999), produces less honey per year (1 kg vs. 50 kg) (Heard, 2016a), and has a longer lifespan (100 days vs. 40 days) (Heard, 2016b).

Given that previous studies have shown that European honey bees and their products can be used as proxy indicators to trace environmental contamination sources, it was considered that native bees could also be used as a bio-indicator. We hypothesise that their smaller foraging area may confer an advantage over European honey bees in that it may reduce the effect of contaminant heterogeneity that is known to exist in soils and dusts, even over small areas in urban environments (Schwarz et al., 2012; Rouillon et al., 2017). Various studies of European honey bees and their products have shown that they cannot distinguish subtle differences in trace element contamination across heterogeneous urban environments (Jones, 1987; Fakhimzadeh and Lodenius, 2000; Conti and Botre, 2001; Saunier et al., 2013; Alvarez-Ayuso and Abad-Valle, 2017). We also hypothesise that lower honey production in native bees may result in higher trace element concentrations in honey and wax providing a more robust measure of local environmental contamination, where present. Our previous work investigating European honey bee hive products across Sydney suburbs revealed that they were suitable bio-indicators for tracing As, Pb, Mn and Zn contamination (Zhou et al., 2018). This study, builds on these findings by investigating the same trace elements in the Australian native bee, *T. carbonaria*, and its honey and wax in order to: (a) to investigate its utility as a proxy marker for urban contaminants and its sources; and (b) assess its efficacy compared to European honey bees, which are more commonly used as a contaminant bio-indicator.

2. Materials and methods

2.1. Study area

Eighteen Australian native bee hives were sampled across Sydney, New South Wales (NSW) (Fig. 1). Eleven of those hives were located in Roseville, with other suburbs having only one native bee hive available for sampling. For comparative purposes, nine European honey bee hives (i.e. one European honey bee hive per native bee hive per site) close to the sampled native bee hives were also included in the study analysis (Fig. 1). Sample and analytical details for the European honey bee data have been published in Zhou et al. (2018). The locations of all the sampled bee hives range from sites in outer Sydney, coastal Sydney and inner Sydney, close to the central business district (CBD) (Fig. 1). All locations have been exposed to varying degrees of metal contamination, as a consequence of industrial activity (Birch et al., 2011; Rouillon et al., 2017), the use of Pb-based paints and emissions from vehicles previously using leaded petrol (Kristensen, 2015; Rouillon et al., 2017).

2.2. Sample collection

Australian native bees are very temperature dependent (Norgate et al., 2010) with their most intensive foraging period being over the warmer Australian summer months from November to early April. During this peak foraging time, pollen and nectar are collected and enough honey produced to sustain the native bees over the cooler winter months.

Over the sampling period (2015–2016), 30 live native bees were collected at the entrance of each beehive when they were observed entering or exiting the hive. Apiarists (beekeepers), normally split their native bee hives for propagating purposes in the spring (November) of each season. At this time, raw, unprocessed samples of native bee honey ($n = 20$) and wax ($n = 21$) were collected twice—once in November 2015 and then in November 2016. The native bee data were compared with co-located soil, dust and European honey bees samples, collected between November 2015 to April 2016 (Zhou et al., 2018).

Dust deposition samples gauges, were constructed and deployed in accordance with the Australian Standard 3580.10.1–2003 (Standards Australia, Dong and Taylor, 2017; Zhou et al., 2018). These were used to collect atmospheric dust samples monthly from November 2015 to April 2016, incorporating the most intensive foraging period for both the native and European honey bees. Surface (0–2 cm) soil samples ($n = 37$) were collected around each beehive in November 2015, and were dried at 40 °C for 48 h and sieved to < 2 mm for trace element analysis.

2.3. Sample and data analysis

Samples of native bees, honey, wax, soil and dust were analysed at the National Measurement Institute (NMI), using National Association of Testing Authorities accredited in-house reference methods for food, soil and dust. Raw, unprocessed native bees and honey samples were used for analysis. Wax samples were rinsed with Milli–Q™ (Milli–Q) water until all honey residues were removed and then oven dried at 60 °C. One decigram of native bees was digested with 1 ml HNO_3 (15.6 M) and 1 g honey and wax were digested with 3 ml HNO_3 before heating at 100 °C for 2 h.

Soil samples were digested by adding HCl (12.1 M) and HNO_3 (15.6 M) (3 ml and 3 ml, respectivelyl) followed by heating at 100 °C for 1 h. After cooling, 10 ml of Milli–Q water was added and the sample reheated to 100 °C for another hour. Dust deposition samples were collected on glass microfibre filters (Whatman 934-AH™, 47 mm diameter), and after weighing, digested using HCl (12.1 M)

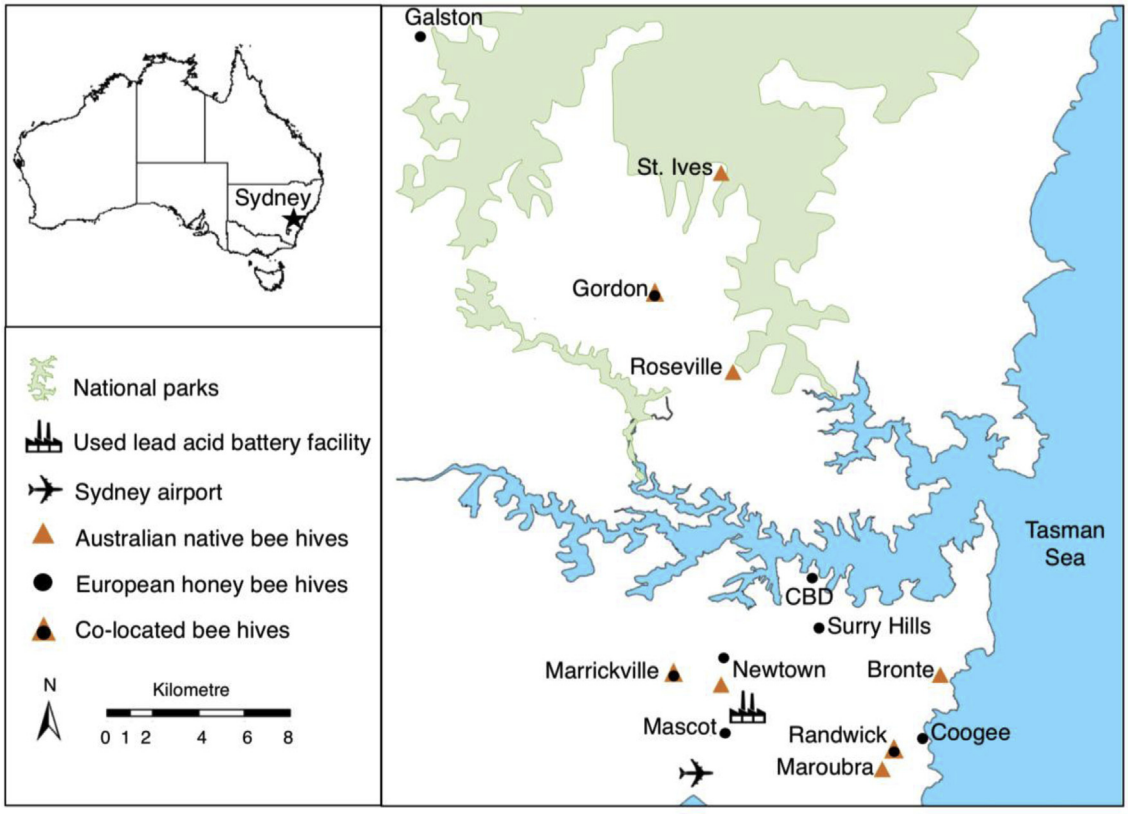

Fig. 1. Beehive locations of the Australian native bee hives (*T. carbonaria*) and European honey bee (*A. mellifera*) hives in Sydney, New South Wales (NSW). Locations of all the beehives range from sites in outer Sydney (Galston, St Ives, Gordon, Roseville), coastal Sydney (Bronte, Coogee, Randwick, Maroubra) and inner Sydney (Sydney Central Business District (CBD), Surry Hill, Newtown, Marrickville and Mascot).

and HNO_3 (15.6 M) (2 ml and 6 ml, respectively) for 2 h. Digested native bees were topped to 10 ml with Milli–Q water with the other samples (honey, wax, soil and dust) topped up to 40 ml. Samples were diluted (*2 for native bees, honey and wax; *5 for dust; *20 for soil) prior to analysis for As, Mn, Pb and Zn concentrations on an Inductively Coupled Plasma Mass Spectrometer (Agilent 7900 equipped with an Integrated Sample Introduction System). Each sample batch (n = 20) contained a laboratory reagent blank and duplicate, blank spike, blank matrix, duplicate sample and matrix spikes.

Procedural blanks were below NMI's Limit of Reporting (LOR) of 10 μg/kg for As, Mn, Pb, and Zn in honey bees, honey and wax. Dust sample blanks were < LOR of 0.1 mg/kg and 0.05 mg/kg for As and Zn, Pb and Mn, respectively. Procedural blanks for the soil samples were < LOR of 0.05 mg/kg for As, and 0.01 mg/kg for Pb, Mn and Zn. The procedural blanks (i.e. the filter material used in the dust analysis process and reagent solutions) were used to correct trace elements concentrations and Pb isotopic compositions.

The NMI's internal reference materials AGAL-10 (Hawkesbury River Sediment, n = 4) and AGAL-12 (bio-soil, n = 4) were processed with soil and dust samples. Elemental relative standard deviations (RSDs) for AGAL-10 and AGAL-12 were < 5.5% and < 4.8%, and mean recovery rates were 101% and 108%, respectively. Sample RSDs for As, Pb, Mn and Zn were < 1.8% for soil, < 4% for dust, < 6% for native bees and <4% for native bee honey and wax. Recovery rates of the AGAL-10 and AGAL-12 reference materials for As, Pb, Mn and Zn were 96–99% for soil, 98–100% for dust, and matrix spike recovery rates were 81–98% for native bees, 97–100% for honey, and 92–99% for wax.

Australian native bee samples (bees n = 5) and wax (n = 19) were subjected to Pb isotopic composition analysis ($^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$), after optimising sample volumes based on their Pb concentrations (where > 1 μg/kg of Pb after digestion). The National Institute of Standards and Technology (NIST) SRM981 (natural Pb isotope composition standard) was used to correct for mass discrimination. Analytical uncertainties for Pb isotopic compositions (expressed for the NIST SRM981) were $^{204}Pb/^{206}Pb = 0.0590 \pm 0.0005$, $^{204}Pb/^{207}Pb = 0.065 \pm 0.0005$, $^{206}Pb/^{207}Pb = 1.093 \pm 0.005$, $^{208}Pb/^{207}Pb = 2.370 \pm 0.01$, respectively. The mean RSDs for NIST981 $^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$ were 0.55%, 0.23%, 0.22%, respectively. The mean RSDs for sample analysis $^{204}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$ were 0.29%, 0.56% and 0.31%.

Data were analysed using GraphPad Prism 7.0 statistics software for arithmetic mean, standard deviation, minimum and maximum values and tests of significance (Mann–Whitney *U* test). Minitab was used for generating linear regressions and tests of significance (Pearson correlations). Concentrations of As, Mn, Pb and Zn in Australian native bees, honey and wax with < LOR (10 μg/kg) were treated as having a concentration of 5 μg/kg for statistical analysis purposes. Trace element concentrations in various beehive products and environmental samples of soil and dust were non-normally distributed and were $\log_{10}$ transformed for correlation analysis. Pearson correlation coefficients and two-tailed tests of significance were applied to assess the relationships between bee and hive products (native bees, honey, wax) and the environment (soil, dust) across the sampling sites. The data pertaining to European honey bees and their products is from our previous work (Zhou et al., 2018). These data were compared to the results of this study using the Mann–Whitney *U* test to compare concentrations

of As, Mn, Pb and Zn in beehive samples between Australian native bees and European honey bees.

3. Results

3.1. Trace element concentrations in native bee samples and corresponding dust and soil

Arsenic and Pb concentrations in native bees, honey, wax, soils and dusts were greatest from the inner city Sydney metropolitan areas of Newtown and Marrickville and lowest at sites distant from Sydney CDB (St. Ives, Gordon and Roseville, Fig. 1). The concentration of As in all native bee honey samples was lower than the LOR of 10 μg/kg. Minimum As concentrations in native bees (21 μg/kg) were measured in Roseville samples, with the lowest concentrations of As in wax (12 μg/kg) and dust (2 mg/kg) being measured in samples from the northern Sydney areas of St. Ives and Gordon, respectively. Arsenic concentrations in wax and dust samples were typically more elevated in the Sydney city metropolitan city, with the notable exception of Roseville which recorded 102 mg/kg of As in dust (Supplementary Table S1).

Native bee honeys contained low levels of Pb with many being < 10 μg/kg, with the maximum concentration being measured in samples from the inner city suburb of Marrickville (34 μg/kg). This area is characterised with elevated soil lead levels (811 mg/kg, this study (Supplementary Table S1); and 689 mg/kg, Rouillon et al. (2017)). The lowest concentrations of Pb in native bees (24 μg/kg), wax (100 μg/kg) and dust (45 mg/kg) were found at Gordon, which were 85 times, 34 times, 16 times lower, respectively, than corresponding samples from the inner Sydney area of Newtown (Supplementary Table S1). The concentrations of Mn and Zn in native bee, honey or wax samples were not spatially consistent across the Sydney sites unlike corresponding concentrations of As and Pb. However, maximum values were recorded for Mn (46,400 μg/kg) and Zn (70,600 μg/kg) in native bees and honey (Mn 2700 μg/kg) from the inner Sydney areas of Newtown or Marrickville (Supplementary Table S1).

Lead concentrations in honey ($r = 0.814$, $p = 0.014$) and wax ($r = 0.883$, $p = 0.004$) were significantly related to its corresponding concentrations in dust, and Pb in honey was associated with its co-located soil ($r = 0.658$, $p = 0.076$) (Fig. 2a–c). The Zn concentrations in honey also significantly correlated to those in soil ($r = 0.709$, $p = 0.049$) (Fig. 2d). By comparison, no statistically significant relationships were evident in the data set for As or Mn concentrations in beehive products with respect to corresponding soil or dust samples ($r = -0.390–0.501$, $p = 0.206–0.984$) (Table S2).

3.2. Comparison of trace element concentrations in different bees and their products

Arsenic, Pb, Mn and Zn concentrations of Australian native bees, honey and wax products are presented in Supplementary Table S1. The concentrations measured in Australian native bee hives are compared below to data from co-located European honey bee hives, published in Zhou et al. (2018).

The data show that Australian native bees had significantly higher concentrations of As (median 72 μg/kg, range 21–140 μg/kg) than European honey bees (median 31 μg/kg, range 11–95 μg/kg) ($p < 0.001$) from all sites collected across Sydney (Fig. 3, Supplementary Table S3). By contrast, Mn and Zn were significantly lower in Australian native bees when compared to European honey

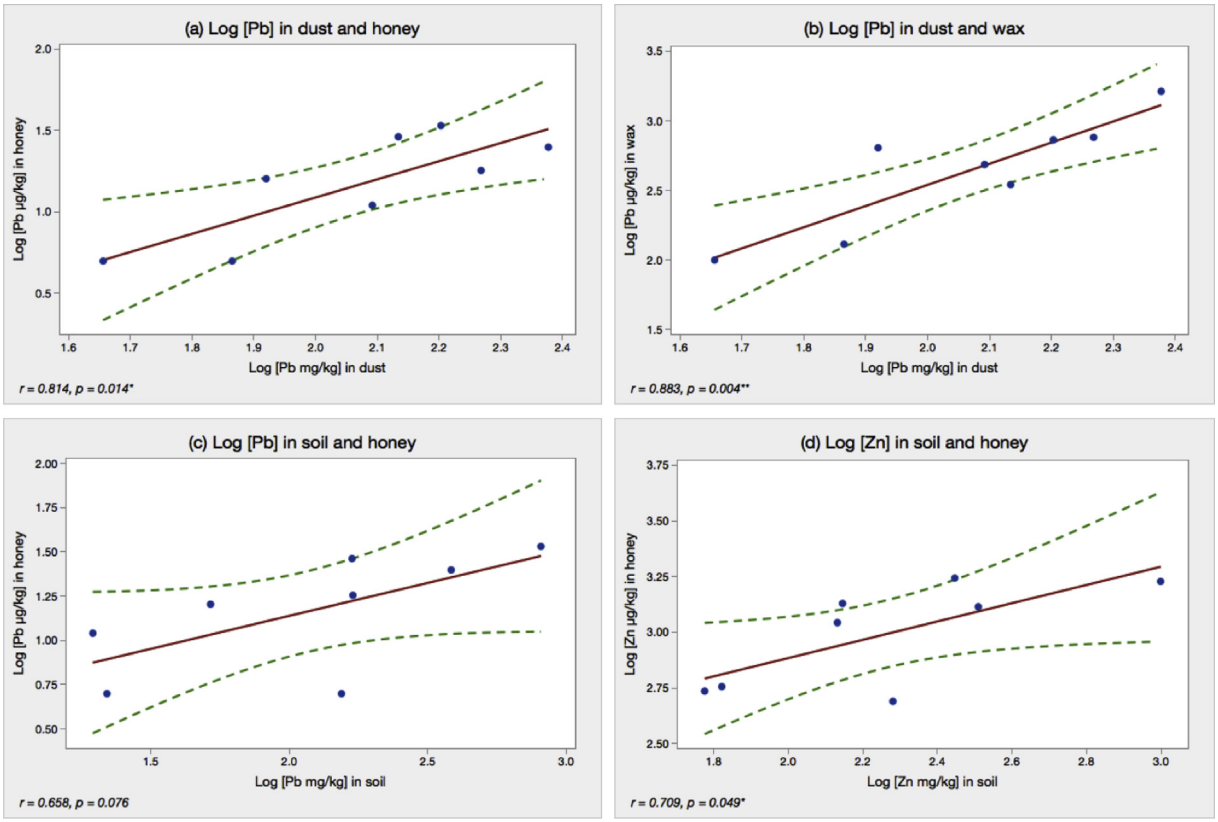

Fig. 2. Linear relationships with Pearson correlation coefficients (r) and *p* values between various Australian native bee products and soil or dust. Pearson correlation significance (*p*) at the following levels: * = 0.05 and ** = 0.01.

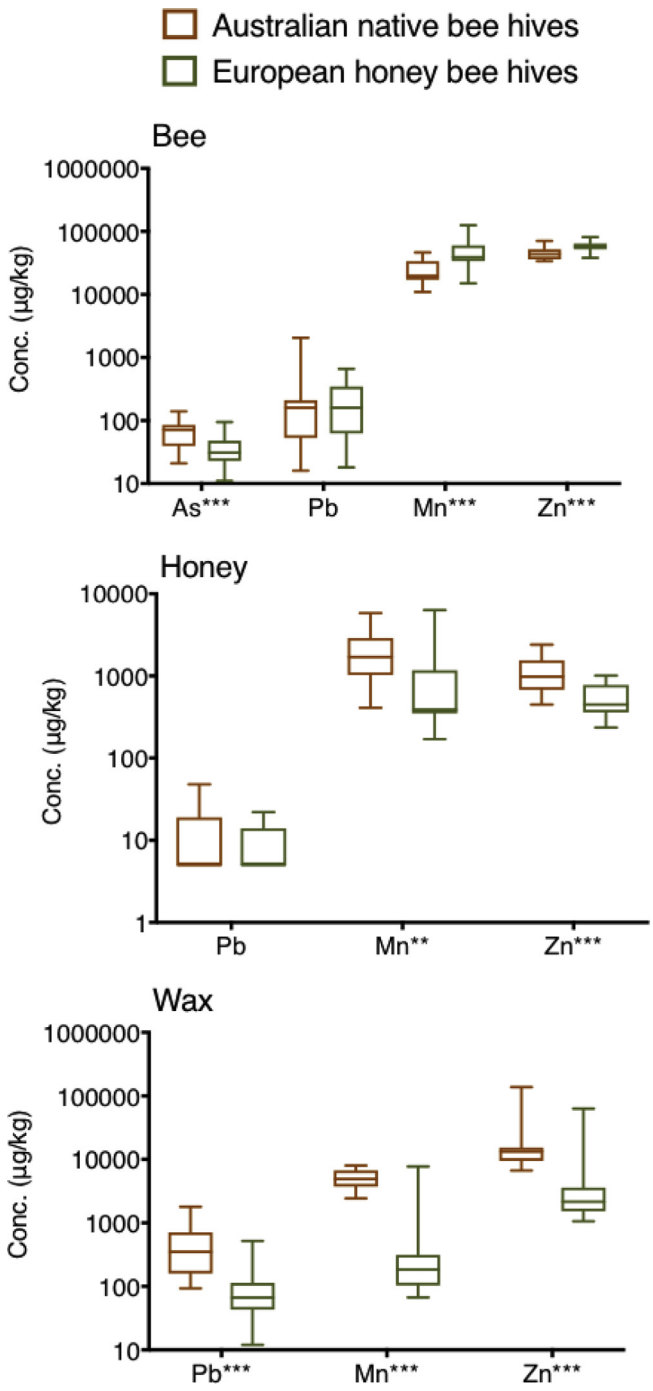

Fig. 3. Box-whisker plots for As, Pb, Mn and Zn concentrations (μg/kg) in native bees (n = 16), their honey (n = 20) and wax (n = 21) compared to European honey bees (n = 37), their honey (n = 15) and wax (n = 14). The *p* value significant at the following levels: *0.05, **0.01 and ***0.001.

bees (Mn $p < 0.001$, Zn $p < 0.001$). Trace element concentrations in the products of Australian native bees (honey and wax) were greater than those from European honey bees (Fig. 3): native bee honey contains more Mn (1700 (range: 410–5800) μg/kg vs. 390 (range: 170–6300) μg/kg, $p = 0.001$) and Zn (980 (range: 450–2400) μg/kg vs. 450 (range: 235–1010) μg/kg, $p < 0.001$) than that in honey produced by European honey bees (Supplementary Table S3). By contrast, Pb concentrations in native and European honey bees had identical median values of 160 μg/kg, with ranges of 16–2050 and 18–660 μg/kg, respectively (Fig. 3, Supplementary Table S3). Wax from native bee hives had significantly greater concentrations of Pb ($p < 0.001$), Mn ($p < 0.001$) and Zn ($p < 0.001$) than that from European honey bee hives.

3.3. Lead isotopic compositions in bees and their products

The mean values of Pb isotopic compositions ($^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$) of Australian native bees and their products are plotted alongside co-located European honey bee data (Fig. 4). The Broken Hill ore body (Gulson, 1984; Kristensen and Taylor, 2016) is included as an ultimate end member, given that it was the primary source of Pb used in leaded petrol in Australia (Kristensen, 2015; Kristensen and Taylor, 2016). Lead isotopic compositions of Sydney basin sub-surface soils and rocks, along with aerosols (1979–2004) are from Wu et al. (2016b). Additional potential contaminant sources of environmental Pb are included in Fig. 4. These Pb isotopic composition data include Australian coal (Díaz-Somoano et al., 2009), industrial contaminants from fly ash and smelter fumes (Chiaradia et al., 1997) and Pb paints (Laidlaw et al., 2014; Rouillon, 2017).

Native bee samples (honey and wax) collected to the north of Sydney CBD close to national parks at St Ives, Gordon and Roseville had Pb isotopic compositions that could be differentiated from Sydney metropolitan samples (Table 1) and plotted either within or close to the envelope for natural Sydney Pb (Fig. 4). The mean Pb isotopic composition of native bee wax from Gordon ($^{206}Pb/^{207}Pb$ 1.120, $^{208}Pb/^{207}Pb$ 2.409) and Roseville ($^{206}Pb/^{207}Pb$ 1.115, $^{208}Pb/^{207}Pb$ 2.396) (Table 1) either overlap or are close to the Sydney surface soil and dust envelope determined in this study. Interestingly, the values also match the Pb isotopic compositions for surface soils ($^{206}Pb/^{207}Pb$ 1.098–1.121, $^{208}Pb/^{207}Pb$ 2.388–2.432) as measured by Wu et al. (2016b), which collectively implies that the natural composition of soils even at distance to the CBD have been adulterated by atmospheric depositions from leaded petrol (cf. Gulson et al., 1981).

The mean Pb isotopic compositions of Australian native bees and their wax ($^{206}Pb/^{207}Pb$ 1.072–1.121, $^{208}Pb/^{207}Pb$ 2.348–2.405) from Sydney metropolitan areas correspond closely to the more restricted range associated with aerosol values (1979–2004) compared to other possible sources of Pb contamination. Lead contamination from petrol consumption was dispersed widely across urban environments resulting in significant soil and dust contamination (e.g. Davis and Birch, 2010; Laidlaw et al., 2017; Rouillon et al., 2017). The atmospheric lead concentrations were collected in Sydney mainly during the period dominated by Pb-petrol use, which ended in 2002 (Gulson et al., 1985; Kristensen, 2015; Kristensen and Taylor, 2016; Wu et al., 2016b). The native bee data analysed here parallels that measured in European honey bees and their wax from hives across Sydney ($^{206}Pb/^{207}Pb$ 1.082–1.106, $^{208}Pb/^{207}Pb$ 2.352–2.388) (Zhou et al., 2018). Although the native bee data matches the envelope characterising the dominant historic source of atmospheric Pb contamination from leaded petrol (Fig. 4), it is notable that there is a variation of Pb isotopic compositions in native bees, wax and dust from each site (Table 1). This indicates that there are a range of Pb sources (e.g. paints (Laidlaw et al., 2014; Rouillon, 2017), industry emissions (Chiaradia et al., 1997) and coal (Díaz-Somoano et al., 2009)) present across the Sydney metropolitan area in addition to the main source of deposition from historic leaded petrol.

4. Discussion and conclusions

The median Pb concentration in *T. carbonaria* was the same as that in European honey bees (160 μg/kg) (Supplementary Table S3).

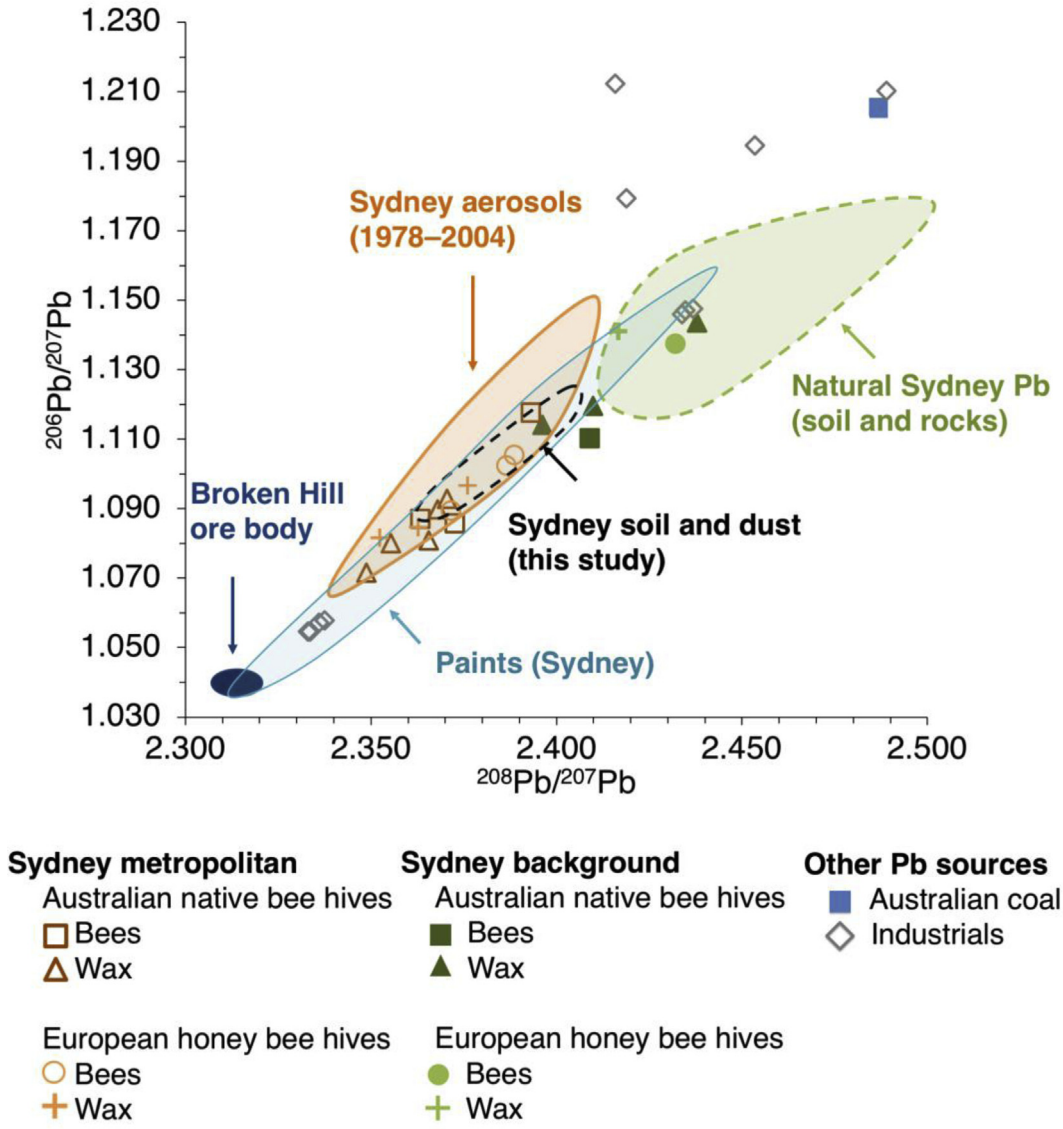

Fig. 4. Mean lead isotope compositions ($^{208}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$) for Australian native bee and European honey bee products across the Sydney region. Data comprises of Australian native bees (n = 5) and wax (n = 19) (Table 1); European honey bees (n = 15) and their wax (n = 3) (Zhou et al., 2018). The shaded areas represent characteristic signatures of: green – Sydney background (Wu et al., 2016b); orange – Sydney aerosols (Wu et al., 2016b); light blue – Sydney Pb-based paints (Laidlaw et al., 2014; Rouillon, 2017); dark blue – Broken Hill ore body (Gulson, 1984; Kristensen and Taylor, 2016). Coal and industrial data were obtained from Díaz-Somoano et al. (2009) and Chiaradia et al. (1997), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

However, *T. carbonaria* showed a greater concentration range over equivalent sampling areas (16–2050 μg/kg vs. 18–660 μg/kg) (Fig. 3, Supplementary Table S3). This large variation in Pb concentrations in *T. carbonaria* may relate to its longer lifespan of *T. carbonaria* compared to that of European honey bees (100 days vs. 40 days) (Heard, 2016b), increasing exposure time and potential for uptake of environmental contaminants. The length of the native bees life cycle may also explain higher concentrations of As in *T. carbonaria* than those in European honey bees ($p < 0.001$) (Supplementary Table S3). However, essential elements of Mn ($p < 0.001$) and Zn ($p < 0.001$) had lower concentrations in *T. carbonaria* than European honey bees, which may be attributed to metabolic processes to support specific nutrient requirements (Ben-Shahar et al., 2004; Zhang et al., 2015) or its body size.

Although As concentrations were measurable in Australian native bees (median 72 μg/kg, range 21–140 μg/kg), concentrations of As in honey were below the laboratory reporting limit of 10 μg/kg (Supplementary Table S1). This finding is consistent with our recent investigation of European honey bees and their honey in Sydney (Zhou et al., 2018) as well as with other studies (Alvarez-Ayuso and Abad-Valle, 2017). Lower trace element concentrations in honey versus bees is thought to be due to metabolic 'filtering' and storage within the body of the bee (Leita et al., 1996; Fakhimzadeh and Lodenius, 2000; Porrini et al., 2002; Bogdanov et al., 2003; Bogdanov 2006; Ruschioni et al., 2013). Trace element contaminants are also excreted in bees faecal mass (Zhelyazkova et al., 2010).

Some trace element concentrations in honey (Mn $p = 0.01$, Zn $p < 0.001$) and wax (Pb $p < 0.001$, Mn $p < 0.001$ and Zn $p < 0.001$) from native bee products were significantly higher than those from European honey bees. Although not statistically significant ($p = 0.744$), median Pb concentrations in native bee honey (23 μg/kg) were greater than those in European honey bees (15 μg/kg) as were Zn concentrations in wax (13,200 μg/kg vs. 2150 μg/kg,

Table 1
Lead isotopic compositions ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$) in native bees, wax, soil and dust across the Sydney metropolitan region, Australia. The range of isotopic composition measurements for different environmental media are provided, where available, in parentheses.

Sites	Sample	$^{206}Pb/^{204}Pb$	$^{206}Pb/^{207}Pb$	$^{208}Pb/^{207}Pb$
St. Ives	Native bees (n = 1)	17.435	1.111	2.408
	Wax (n = 1)	18.022	1.144	2.437
	Soil (n = 3)	17.127 ± 0.459	1.100 ± 0.019	2.378 ± 0.017
		(16.671–17.589)	(1.081–1.118)	(2.362–2.395)
	Dust (n = 4)	17.382 ± 0.357	1.118 ± 0.024	2.4398 ± 0.031
		(16.922–17.673)	(1.091–1.147)	(2.363–2.434)
Gordon	Wax (n = 1)	17.061	1.120	2.409
	Soil (n = 3)[a]	16.864 ± 0.585	1.095 ± 0.026	2.371 ± 0.029
		(16.525–17.540)	(1.077–1.125)	(2.350–2.404)
	Dust (n = 3) [a]	17.419 ± 0.160	1.122 ± 0.019	2.403 ± 0.019
		(17.314–17.602)	(1.111–1.143)	(2.389–2.424)
Roseville	Wax (n = 10)	17.282 ± 0.385	1.115 ± 0.017	2.396 ± 0.024
		(16.752–17.862)	(1.096–1.139)	(2.368–2.444)
	Soil (n = 7)	16.784 ± 0.405	1.087 ± 0.023	2.365 ± 0.023
		(16.280–17.366)	(1.064–1.119)	(2.340–2.401)
	Dust (n = 8)	16.980 ± 0.459	1.090 ± 0.026	2.368 ± 0.025
		(16.339–17.592)	(1.045–1.120)	(2.322–2.391)
Maroubra	Wax (n = 1)	16.549	1.081	2.355
	Soil (n = 2)	16.861	1.090	2.372
		(16.495–17.227)	(1.070–1.111)	(2.349–2.395)
	Dust (n = 5)	17.207 ± 0.105	1.106 ± 0.004	2.385 ± 0.012
		(17.072–17.315)	(1.099–1.110)	(2.363–2.393)
Bronte	Native bees (n = 2)	16.780	1.088	2.363
		(16.639–16.920)	(1.080–1.095)	(2.346–2.380)
	Wax (n = 1)	16.613	1.081	2.365
	Soil (n = 3)	17.359 ± 0.274	1.121 ± 0.007	2.405 ± 0.004
		(17.050–17.570)	(1.113–1.126)	(2.401–2.409)
	Dust (n = 5)	17.150 ± 0.243	1.104 ± 0.010	2.382 ± 0.011
		(16.863–17.505)	(1.090–1.114)	(2.371–2.392)
Randwick	Native bees (n = 1)	16.926	1.086	2.372
	Wax (n = 2)	16.728	1.090	2.367
		(16.715–16.741)	(1.089–1.091)	(2.364–2.371)
	Soil (n = 8) [a]	17.140 ± 0.190	1.102 ± 0.012	2.382 ± 0.015
		(16.887–17.524)	(1.090–1.129)	(2.366–2.412)
	Dust (n = 5) [a]	17.133 ± 0.199	1.109 ± 0.012	2.389 ± 0.010
		(16.851–17.358)	(1.093–1.120)	(2.372–2.398)
Newtown	Native bees (n = 1)	17.315	1.118	2.393
	Wax (n = 2)	16.539	1.072	2.348
		(16.344–16.734)	(1.063–1.081)	(2.340–2.357)
	Soil (n = 8) [a]	17.379 ± 0.373	1.116 ± 0.020	2.398 ± 0.022
		(16.872–17.901)	(1.089–1.142)	(2.369–2.427)
	Dust (n = 5) [a]	17.021 ± 0.218	1.099 ± 0.009	2.381 ± 0.018
		(16.692–17.222)	(1.1092–1.115)	(2.365–2.411)
Marrickville	Wax (n = 1)	16.923	1.093	2.370
	Soil (n = 7) [a]	17.107 ± 0.190	1.103 ± 0.010	2.385 ± 0.014
		(16.867–17.364)	(1.087–1.112)	(2.363–2.400)
	Dust (n = 4) [a]	17.248 ± 0.135	1.115 ± 0.004	2.401 ± 0.004
		(17.055–17.348)	(1.110–1.120)	(2.397–2.404)

Analytical uncertainties for Pb isotopic compositions were as follows: $^{204}Pb/^{206}Pb = 0.0590 \pm 0.0005$, $^{206}Pb/^{207}Pb = 1.093 \pm 0.005$, $^{208}Pb/^{207}Pb = 2.370 \pm 0.01$, respectively.
[a] Lead isotopic composition data from Zhou et al. (2018).

$p = 0.114$). Elevated trace element concentrations in native bee products is likely influenced by the significantly smaller amount of honey produced each year when compared to European honey bees (1 kg vs. 50 kg of honey) (Benecke, 2007; Heard, 2016c) and the smaller volume of wax produced per beehive box (8 L vs. 40 L) per year (Heard, 2016a).

Statistical analysis showed that Pb in honey ($p = 0.014$), wax ($p = 0.004$) and Zn in honey ($p = 0.049$) from native bees were strongly correlated to corresponding dust Pb or soil Pb/Zn concentrations, respectively (Fig. 2). This is not surprising because Pb depositions from petrol emissions blanketed Sydney during its use between 1932 and 2002 and remain persistent in the environment, including in European honey bees (Zhou et al., 2018). There are very limited current sources in the city of Sydney, with the largest single emitter being a used lead acid battery recycling facility (ULAB, Fig. 1) that released 400 kg of Pb to the atmosphere in 2015–2016 (NPI, 2017). By comparison, Sydney European honey bee data (Zhou et al. (2018) were more weakly associated to co-located soil (i.e. $p = 0.404$ vs 0.049 for honey Zn) and dust (i.e. $p = 0.340$ vs 0.014 for honey Pb) values than native bee samples (Fig. 2). The fact that Pb and Zn in Australian native bee samples are more strongly associated with surrounding dust and soil concentrations is possibly due to the markedly different life cycle and foraging behaviour of Australian native bees. Australian native bees live for ~100 days (cf. European honey bees' lifespan of ~40 days) and their foraging distance is much smaller at 0.3–0.7 km vs. 9 km in European honey bees. The strong association between Australian native bees (and their products) to corresponding environmental dust Pb and soil Zn levels indicate that they are more sensitive bio-indicators than European honey bees.

European honey bees and Australian native bees and their wax from sites in inner Sydney corresponded closely to the Pb isotopic compositions in historic aerosols (1978–2004) sampled when the use of leaded petrol began to decline (Kristensen, 2015) (Fig. 4).

Lead isotopic compositions of Australian native bees show that historic leaded petrol depositions remain a persistent contaminant in biological samples, as confirmed in a number of other local (Wu et al., 2016a, 2016b) and international studies (e.g. Farmer et al., 2002; Flegal et al., 2010; Odigie and Flegal, 2011; Hansson et al., 2017). Aside of leaded petrol depositions, other Pb sources including Pb-based paint (Laidlaw et al., 2014; Rouillon et al., 2017), traffic emissions (Birch and Scollen, 2003), coal combustion (Díaz-Somoano et al., 2009; Cohen et al., 2012) and industrial sources (Cohen, 1999; Cohen et al., 2002; Davis and Gulson, 2005; Gulson et al., 2007; Marx et al., 2010; Wu et al., 2016b) may also be present in the environment. Indeed, the variation of Pb isotopic compositions at individual sample sites suggests that a range of Pb sources persist in the Sydney environment (Table 1).

Overall, this study indicates that Australian native bees have clear potential to be used as a bio-indicator species to evaluate environmental trace element concentrations and Pb sources at small scales (up to 0.7 km) over different land use types (cf. Holt and Miller, 2011). For example, data from native bee hives could be used to supplement standard soil sampling investigations as part of rezoning and development approval processes, which often result in the reuse of former industrial sites as residential land or public open space. The data suggest that Australian native bees are at least equivalent to those of European honey bees as a proxy environmental marker and for certain media (e.g. dust and wax) may be even more sensitive. The increased interest in and keeping of all bee types in Australia and elsewhere presents an opportunity to supplement traditional aerosol and dust deposition sampling that typically relies on a limited number of monitors to characterise air quality. Given the persistence of legacy contaminants in the environment (e.g. Pb) and the emergence of new compounds of concern including those listed on the Stockholm Convention (United Nations, 2015), bees and their products provide an obvious choice for measuring the prevalence, distribution and recycling of toxic pollutants in food and ecological systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.06.063.

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Chapter 5: Trace elements in global honeys and their use in the identification of the geographical origin of honey

Chapter 5 presents the following paper:

Xiaoteng Zhou (80 %), Mark Patrick Taylor (10 %), Helen Salouros (5 %), Shiva Prasad (5 %). The authenticity and geographical origin of global honeys determined using carbon isotope ratios and trace element analysis. Submitted to *Scientific Reports*.

Chapters 3 and 4 revealed there were strong, positive correlations between trace elements in the environment and honey produced in co-located hives at a relatively small city-wide scale. This chapter applies a suite of statistical methods to characterise trace element signatures in honey to authenticate its geographic origin, and is comprised of an analysis of five raw honey samples that were collected directly from Sydney beehives and 95 commercial honeys. The commercial honey products were from Australia (n=38) and 18 overseas countries (n=54) covering Africa, Asia, Europe, North America and Oceania. Three samples were blended with no specific geographic origin listed. Genuine pure honey is derived primarily from C-3 plants foraged by bees. It is a common practise for pure honey to be adulterated by adding cheaper cane and/or corn C-4 sugars, but the marketed product is sold as pure honey. This study applied carbon isotopic analysis to honey and its protein to measure the sugar composition (C-3 and C-4) present in commercial honeys to ascertain their authenticity according to multiple Association of Official Analytical Chemists methods (AOAC 991.41, 1995; AOAC 978.17, 1995; AOAC 998.12, 2014) and a recent study by Dong et al. (2016). The five raw honeys collected directly from Sydney beehives were used as genuine pure sample benchmarks to both confirm the laboratory method and that they matched the relevant criteria.

Of the 95 commercial honeys analysed, 69 samples (73 %) met the official criteria for pure honey. These samples were then subject to trace element analysis to ascertain if elemental

concentrations could be used for geographical origin determination. The trace elements K, Mn, P and Sr were found to be the most effective in distinguishing Australian honey from overseas honey, as well as distinguishing different continental honeys.

This chapter embraces a key aspect of this PhD thesis, in that it examines the relationship between trace elements in the environment and corresponding values found in the food web to ascertain if these values have potential to be used as discriminant criteria at a regional/continental scale. This is the first study to use a combination of carbon isotope ratios and trace element analysis to evaluate global honeys to verify both authenticity and geographic origin. Moreover, it is the first study to investigate Australian honey quality and attempt to distinguish it from overseas honey. Findings presented in this chapter verify the use of trace element signatures in honey to characterise and identify its geographic origin, providing a promising and feasible approach to solve the issue of mislabelling in the global honey market. The study presented in this chapter generated significant public interest after it reported by ABC 7:30 and covered in Sydney Morning Herald, The Age, ABC News and The Conversation (Appendix E).

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Authenticity and geographic origin of global honeys determined using carbon isotope ratios and trace elements

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Xiaoteng Zhou[1], Mark Patrick Taylor[1,2], Helen Salouros[3] & Shiva Prasad[4]

Honey is the world's third most adulterated food. The addition of cane sugar or corn syrup and the mislabelling of geographic origin are common fraudulent practices in honey markets. This study examined 100 honey samples from Australia (mainland and Tasmania) along with 18 other countries covering Africa, Asia, Europe, North America and Oceania. Carbon isotopic analyses of honey and protein showed that 27% of commercial honey samples tested were of questionable authenticity. The remaining 69 authentic samples were subject to trace element analysis for geographic determination. One-way ANOVA analysis showed a statistical difference ($p < 0.05$) in trace element concentrations of honey from Australian regions and different continents. Principal component analysis (PCA) and canonical discriminant analysis (CDA) coupled with C5.0 classification modelling of honey carbon isotopes and trace element concentrations showed distinct clusters according to their geographic origin. The C5.0 model revealed trace elements Sr, P, Mn and K can be used to differentiate honey according to its geographic origin. The findings show the common and prevalent issues of honey authenticity and the mislabelling of its geographic origin can be identified using a combination of stable carbon isotopes and trace element concentrations.

Genuine pure honey is classified as a natural product produced entirely by bees. Formally, it is defined as "…the natural sweet substance, produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature"[1,2].

Honey is a natural sweetener containing sugars and small quantities of minerals, vitamins, proteins, fatty acids, amino acids. Its nutritious components make honey suitable for a wide range of applications in the food industry, such as cooking, baking, desserts and beverages. Honey also has medicinal properties due to its antioxidant and antimicrobial activities, resulting in specific types of honey, for example New Zealand manuka honey[3], having significant commercial value.

Manuka honey is New Zealand's most iconic honey and commands a premium price due to its claimed health-related benefits[3]. The annual production of manuka honey in New Zealand is only 1,700 tons[4]. However, it is estimated that as much as 10,000 tons of New Zealand manuka honey is sold globally each year[4]. The global market value of honey was worth an estimated US$6.6 billion in 2015[5].

There was an escalation in the practice of adulterating honey in world markets from the 1970s following the introduction of high fructose corn syrup[6]. Corn syrup and sugar cane, both a cheaper sugar source than honey, are added commonly to honey to increase product volume, which is then traded as a genuine pure honey[7]. Corn syrup and sugar cane are sourced from C-4 plants with the produced sugars reflecting their original carbon

[1]Department of Environmental Sciences, Faculty of Science and Engineering, Macquarie University, North Ryde, Sydney, New South Wales, 2109, Australia. [2]Energy and Environmental Contaminants Research Centre, Macquarie University, North Ryde, Sydney, New South Wales, 2109, Australia. [3]Australian Forensic Drug Laboratory, National Measurement Institute, North Ryde, Sydney, New South Wales, 2113, Australia. [4]Analytical Service Branch, National Measurement Institute, North Ryde, Sydney, New South Wales, 2113, Australia. Correspondence and requests for materials should be addressed to X.Z. (email: xiaoteng.zhou@students.mq.edu.au) or M.P.T. (email: mark.taylor@mq.edu.au)

isotopic composition. By contrast, bees collect nectar and pollen for honey production primarily from the flowers of C-3 plants, and to a lesser extent from the flowers of C-4 plants[7]. Sugar syrups produced by the C-4 metabolic pathway exhibit a $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$) that differs from sugars derived from the C-3 metabolic pathway (−10‰ to −20‰ for C-4 plants, and −22‰ to −33‰ for C-3 plants)[8,9].

The $\delta^{13}C$ value in pure honey is relatively uniform, with $\delta^{13}C$ values >−23.5‰ classified as adulterated with lower $\delta^{13}C$ syrups according to the AOAC (Association of Official Analytical Chemists) Official Method 978.17[10]. Also, honey is considered to be adulterated if it contains C-4 sugars >7%, according to the following equation (1)[11]:

$$C-4\ Sugars,\ \% = \frac{\delta^{13}C_{protein} - \delta^{13}C_{honey}}{\delta^{13}C_{protein} - (-9.7)} \times 100 \quad (1)$$

where $\delta^{13}C_{protein}$ and $\delta^{13}C_{honey}$ are $\delta^{13}C$ values (‰), for protein and honey, respectively, and −9.7 is the average $\delta^{13}C$ value for corn syrup (‰).

In addition, honey with C-4 sugars <−7% can also be classified as adulterated[12]. Although the analysis of $\delta^{13}C$ in honey and calculation of the proportion of C-4 sugars is useful for detecting adulteration by the addition of syrups, false positive results may occur if honey is produced naturally from C-4 plants[13]. High values of the non-peroxide activity (NPA >10+) and methylglyoxal (MGO >250 mg/kg) in New Zealand manuka honey can increase the possibilities of false positive results[14]. This limitation of the carbon isotope method can potentially be addressed via a comparison of the carbon isotope ratios of bulk honey to honey protein[7]. The protein acts as an internal control, given that its $\delta^{13}C$ value is unaffected by adulteration[9]. By contrast, the $\delta^{13}C$ value of honey changes with addition of sugar causing a difference to occur between $\delta^{13}C$ values in honey and protein[15]. A difference >1‰ in $\delta^{13}C$ values ($\delta^{13}C_{honey\text{-}protein}$ expressed as $\delta^{13}C_{h\text{-}p}$) is considered to indicate that the protein and the bulk honey have different origins[16] resulting in such honeys being classified as adulterated[8,15,17–20]. Consequently, stable carbon isotope ratio analysis has been used for decades as an analytical tool to detect honeys that have been adulterated with C-4 sugars[13,17,21].

As well as the addition of sugar to honey, mislabelling of the geographic origin of food is a growing worldwide problem[22–24] including in the honey market[25]. According to the Codex Alimentarius Commission Standards[2] and European Commission[1], the geographic origin of honey should be the same as the area declared on its label. Deliberate mislabelling of honey origin has been reported frequently in the media[26–30]. This not only compromises the confidence of customers with respect to the authenticity of certified regional products, but raises health and safety concerns as blended honey of unknown origin has been known to contain antibiotics, toxins, irradiated pollen or even alkaloids with the potential to cause organ damage[31].

Although honey trace element profiles have previously been related to their geographic source at a regional scale[32–35], no study has investigated if samples can be separated at a broader continental level to authenticate their origin. Further, while trace element analysis has been used to confirm the authenticity of honey labelled with place of origin in Spain[36], Turkey[32], Argentina[33], Slovenia[37], Brazil[34], Italy[35] and Romania[38], no study has evaluated Australian honey with the aim of developing a method to distinguish its honey from international products. Australia is the world's 4th largest exporter of honey[39]. The Australian honey industry, including pollination services was estimated to be worth at least AUD$4–6 billion per annum in 2008[40]. Australian honey is characterised as safe and high quality since it is produced in a "clean and green" country[41] with one of the world's most rigorous apicultural management systems. However, recent scandals in which the "Australian product" logo was used falsely on products purporting to be Australian honeys[26,27] has raised public concern about the stated authenticity of honey origin and quality.

This study uses stable carbon isotope ratio analysis to investigate the authenticity of honey from mainland Australia (n = 29), Tasmania (n = 9) and 54 honeys from 18 other countries across five continents. The non-Australian samples were derived from Africa (n = 1), Asia (n = 21), Europe (n = 21), North America (n = 9) and Oceania (n = 2 from New Zealand), with three samples of an unknown origin. Trace element analyses of Australian and international honeys were undertaken with the aim of ascertaining if there were separate and distinct concentrations according to regions and continents and if this approach could be used to verify geographic origin.

Results

Five raw honey samples were collected from Sydney (New South Wales) and Calliope (Queensland) hives, to: (a) demonstrate that they matched the AOAC criteria for $\delta^{13}C$ in pure honey, its protein and C-4 sugar and (b) benchmark $\delta^{13}C$ and C-4 sugar values measured in 95 commercial honeys.

The 95 commercial honey samples from 19 countries were assessed according to the following criteria for potential adulteration: $\delta^{13}C_{honey}$ > −23.5‰[10]; C-4 sugar >7%[11] and <−7%[12]; and $\delta^{13}C_{h\text{-}p}$ >1‰[8,15,17–20]. Honey samples that passed the C-4 sugar criteria were assumed to be authentic given that C-4 sugar is the only adulterant that has an official detection method[42].

Honey samples that passed the C-4 criteria were subjected to multi trace element analysis. The 16 trace element concentrations coupled with $\delta^{13}C$ values in bulk honey and its protein were used to determine the geographic origin of the honey using PCA and CDA statistical analysis. In addition, C5.0 classification modelling, a commonly used tool in data mining, was used to evaluate $\delta^{13}C$ values and trace element concentrations to ascertain if samples could be separated according to their geographic origin. The full dataset for $\delta^{13}C$ in honey and protein is provided in Supplementary Table S1. Trace element concentrations of Al, Ba, B, Ca, Cu, Fe, Mg, Mn, Ni, P, K, Rb, Na, Sr, Sn and Zn are provided in Supplementary Table S2.

Sample No.	Countries	$\delta^{13}C_{honey}$ (‰) Criterion[10]: $<-23.5^{a}$	$\delta^{13}C_{protein}$ (‰)	$\delta^{13}C_{h\text{-}p}$ (‰) Criterion[8,15,17–20]: $\leq 1^{b}$	C-4 sugar (%) Criteria[11,12]: $\leq 7^{c}$ or $>-7^{d}$
M-AUS-25	Australia	-26.40 ± 0.05	-25.21 ± 0.06	−1.19	***−7.66*** ^{d}
M-AUS-26	Australia	-26.74 ± 0.06	-25.62 ± 0.14	−1.12	***−7.02*** ^{d}
M-AUS-27	Australia	-26.66 ± 0.04	-25.10 ± 0.18	−1.56	***−10.14*** ^{d}
M-AUS-28	Australia	-25.33 ± 0.09	-26.80 ± 0.12	***1.48*** ^{b}	***8.63*** ^{c}
M-AUS-29	Australia	-24.37 ± 0.09	-25.44 ± 0.11	***1.07*** ^{b}	6.80
TAS-8	Australia	-23.68 ± 0.08	-25.17 ± 0.09	***1.49*** ^{b}	***9.64*** ^{c}
TAS-9	Australia	-23.84 ± 0.06	-25.22 ± 0.26	***1.38*** ^{b}	***8.91*** ^{c}
AS-36	China	-25.25 ± 0.06	-23.70 ± 0.09	−1.55	***−11.04*** ^{d}
AS-37	China	***−15.52*** ^{a} ***± 0.04***	-23.71 ± 0.10	***8.18*** ^{b}	***58.43*** ^{c}
AS-38	India	-24.68 ± 0.11	-27.02 ± 0.12	***2.34*** ^{b}	***13.52*** ^{c}
AS-39	Indonesia	***−21.58*** ^{a} ***± 0.06***	-26.05 ± 0.17	***4.47*** ^{b}	***27.35*** ^{c}
AS-40	Indonesia	-24.49 ± 0.06	-27.33 ± 0.06	***2.84*** ^{b}	***16.13*** ^{c}
AS-41	Iran*	***−17.47*** ^{a} ***± 0.22***	-22.30 ± 0.20	***4.83*** ^{b}	***38.30*** ^{c}
AS-42	Iran	***−13.35*** ^{a} ***± 0.07***	-23.07 ± 0.12	***9.71*** ^{b}	***72.66*** ^{c}
AS-43	Iran*	***−17.42*** ^{a} ***± 0.04***	-22.94 ± 0.13	***5.52*** ^{b}	***41.72*** ^{c}
AS-44	Iran	***−19.45*** ^{a} ***± 0.17***	-23.07 ± 0.14	***3.63*** ^{b}	***27.13*** ^{c}
AS-45	South Korea*	***−20.94*** ^{a} ***± 0.16***	-27.53 ± 0.20	***6.58*** ^{b}	***36.93*** ^{c}
AS-46	China	***−15.50*** ^{a} ***± 0.02***	—	—	—
EU-47	Greece*	-24.31 ± 0.10	-25.67 ± 0.14	***1.36*** ^{b}	***8.50*** ^{c}
EU-48	Hungry	-24.77 ± 0.06	-25.97 ± 0.16	***1.19*** ^{b}	***7.32*** ^{c}
EU-49	Macedonia*	***−17.46*** ^{a} ***± 0.08***	-22.49 ± 0.03	***5.03*** ^{b}	***39.30*** ^{c}
EU-50	Macedonia*	***−17.68*** ^{a} ***± 0.10***	-23.13 ± 0.14	***5.44*** ^{b}	***40.55*** ^{c}
EU-51	Romania*	-24.84 ± 0.004	-25.92 ± 0.12	***1.08*** ^{b}	6.64
EU-52	Serbia*	***−17.37*** ^{a} ***± 0.04***	—	—	—
OA-53	New Zealand*	-25.13 ± 0.09	-23.66 ± 0.06	−1.47	***−10.55*** ^{d}
OA-54	New Zealand*	-25.58 ± 0.20	-26.78 ± 0.06	***1.20*** ^{b}	***7.04*** ^{c}

Table 1. Data for $\delta^{13}C_{honey}$ (‰), $\delta^{13}C_{protein}$ (‰), $\delta^{13}C_{h\text{-}p}$ (‰), C-4 sugar (%) and detection criteria in adulterated commercial honey samples from mainland Australia (M-AUS, n = 5), Tasmania (TAS, n = 2), Asia (AS, n = 11), Europe (EU, n = 6) and two Oceanic samples (OA) from New Zealand. Values in bold indicate honey samples that did not meet the specific criteria. Data are expressed as Mean ± 1SD with triplicates, and "—" means no extractable protein. *International honeys obtained from local food markets and commercial supermarkets in Australia. Manuka honey (OA-53) had the MGO (methylglyoxal) value of 30+ (mg/kg) on its label; manuka honey OA-54 had no MGO or NPA (non-peroxide activity) information on its label. [a]Detection criterion for $\delta^{13}C_{honey} < -23.5$‰ according to the AOAC Official Method 978.17[10]. [b]Detection criterion for $\delta^{13}C_{h\text{-}p} \leq 1$‰ according to Padovan *et al.*[8], White and Winters[15], Simsek *et al.*[17], Tosun[18], Guler *et al.*[19], Elflein and Raezke[20]. [c]Detection criterion for C-4 sugar ≤7% according to the AOAC Official Method 998.12[11]. [d]Detection criterion for C-4 sugar where >−7% according to Dong *et al.*[12].

Carbon isotope analyses. The $\delta^{13}C$ values in raw honey samples (n = 5) were <−23.5‰[10], and their C-4 sugars were <7%[11] (Supplementary Table S1). Also, the difference in $\delta^{13}C$ values between the bulk honey and its protein met the criterion used widely, that being ≤1‰ ($\delta^{13}C_{h\text{-}p}$ ranged from −0.61‰ to −0.08‰) (Supplementary Table S1), corresponding to <7% added C-4 sugars[8,15,17–20]. The most recent AOAC 998.12[11] sets the upper acceptable limit for C-4 plant sugars in honey at ≤7%. The linear relationship for raw honey and its protein was: $\delta^{13}C_{protein} = 0.655 \times \delta^{13}C_{honey} - 8.67$ ($R^2 = 0.932$, $p = 0.008$).

Commercial honey samples (n = 95) had $\delta^{13}C$ values for honey and protein that ranged from −27.91 to −13.35‰ and −27.78 to −22.30‰, respectively. Seventeen of these commercial honey samples (17.9%) were found to be potentially adulterated (Table 1) according to the AOAC 998.12[11], which states that honey samples are considered to be adulterated if they contain C-4 sugars >7%.

According to the AOAC 991.41[16] along with corresponding studies[8,15,17–20], the value of $\delta^{13}C$ of the honey and its protein should differ by no more than 1‰ ($\delta^{13}C_{h\text{-}p} \leq 1$‰), which is equivalent to <7% added corn or cane sugar. In this study, 19 honey samples (20% of total commercial samples analysed) had $\delta^{13}C$ values in their honey and protein that were >1‰ (1.07–9.71‰) (Table 1). Two samples (AS-46 and EU-52) had no extractable protein (Table 1) but their honey carbon isotopic ratios −15.50 and −17.37‰ where >−23.5‰ indicated they had been adulterated with C-4 sugars, such as those from corn or sugar cane[10].

In addition to the above standards to detect authentic honey, a recent study by Dong *et al.*[12] indicated that honey with C-4 sugar content <−7% ought to be also classified as being adulterated. Two Australian samples marginally exceeded this criterion (M-AUS-25, −7.66%; M-AUS-26, −7.02%) along with two overseas samples (AS-36, −11.04%; OA-53, −10.55%), indicating that they had been potentially adulterated (Table 1).

The adulterated honeys (n = 26, 27% of the total (n = 95) commercial honey samples) had $\delta^{13}C$ values ranging from −26.74 to −13.35‰ in honey and −27.53 to −22.30‰ in protein. By contrast, the range of $\delta^{13}C$ values in

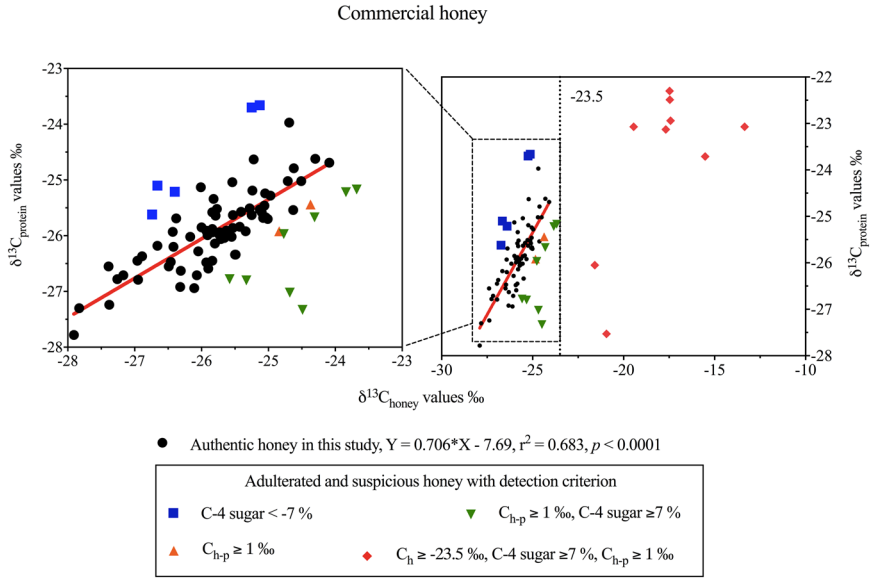

Figure 1. Bivariate plot of $\delta^{13}C_{honey}$ and $\delta^{13}C_{protein}$ values of commercial honey from Australia (mainland and Tasmania) (n = 38), overseas (n = 54) and unknown origin (n = 3). Circles represent authentic honey (Supplementary Table S1); triangles, squares and diamonds represent adulterated honey (Table 1).

pure honeys (n = 69) was much narrower from −27.91 to −24.09‰ in honey and −27.78 to −23.97‰ in protein, resulting in a regression analysis of $\delta^{13}C_{protein} = 0.706 \times \delta^{13}C_{honey} - 7.69$ ($R^2 = 0.683$, $p < 0.0001$) (Fig. 1).

Trace element analysis. The 69 commercial honeys that passed the C-4 sugar criteria were further analysed for their trace element concentrations. The 69 authentic honeys were from mainland Australia (n = 24), Tasmania (n = 7), Asia (n = 10), Europe (n = 15), North America (n = 9) and Africa (n = 1), with three samples having no clear geographic information on their label. One-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison was used to determine the difference between each pair of geographic categories (three samples of an unknown origin and one African honey were excluded) with significance being identified when $p < \alpha = 0.05$ (Fig. 2). The full dataset of trace element concentrations and p values between different geographic groups is provided in Supplementary Tables S2 and S3, respectively.

There were significant differences between honey samples from mainland Australia and Tasmania in the trace elements Ca ($p < 0.001$), Mg ($p = 0.049$) and Sr ($p = 0.014$). Authentic commercial honey samples from mainland Australia and overseas (Asia, Europe and North America) had significant differences ($p = <0.001$–0.042) in the trace elements Ba, Ca, Fe, Mn, P, Na and Sr. However, Tasmania honey had significantly different concentrations in Fe ($p = 0.004$) and P ($p = 0.008$) compared to international honey from Asia and Europe only. Asian and European honey also showed significant differences ($p = 0.003$) in the trace elements Fe and P (Fig. 2).

PCA and CDA analysis. Trace elements Ba, Ca, Fe, Mg, Mn, P, Na and Sr showed statistical differences between honey samples according to their geographic origin (Fig. 2). However, a combination of any two of these trace elements alone could not be used to visually depict geographic origin (Supplementary Fig. S1). Therefore, multivariate methods of principal component analysis (PCA) (Fig. 3a,b) and canonical discriminant analysis (CDA) (Fig. 3c,d) were applied to reduce the number of variables needed to describe the variation between individual samples, resulting in group differences being visible with a low-dimensional view for geographic classification.

PCA generates principal components that are linear combinations of the original variables. The PCA results for all seven geographic groups (mainland Australia, Tasmania, Asia, Europe, North America, Africa and unknown origin) showed that the first six components accounted for 77.2% of the total variability, with no visual clustering according to honey's geographic origin in the first two components (Fig. 3a). Interestingly, PCA results for honeys from mainland Australia and Tasmania showed the first six components accounted for 81.5% of the total variability, with clustering evident in the first two components (Fig. 3b).

In contrast to the PCA, CDA produced clear groupings for Australia and overseas (Fig. 3c). Also, international honeys were further separated according to their respective continental origins of Asia, Europe, North America and Africa (Fig. 3d). The most important parameters to separate Australian honey (mainland and Tasmania) from overseas honey were Ca, Mn, P, K and Sr (Fig. 3c). Classification results from CDA showed that 84.8% of the original grouped cases were correctly classified according to their different geographic origin (Supplementary Table S4). Cross validation analysis returned a 75.8% correction rate, revealing that CDA is a reliable and suitable model for predicting the geographic origin of honey (Supplementary Table S4). Also, the classification results indicated that one of the unknown origin honey samples was from mainland Australia with the other two being grouped with overseas samples (Fig. 3c and Supplementary Table S4).

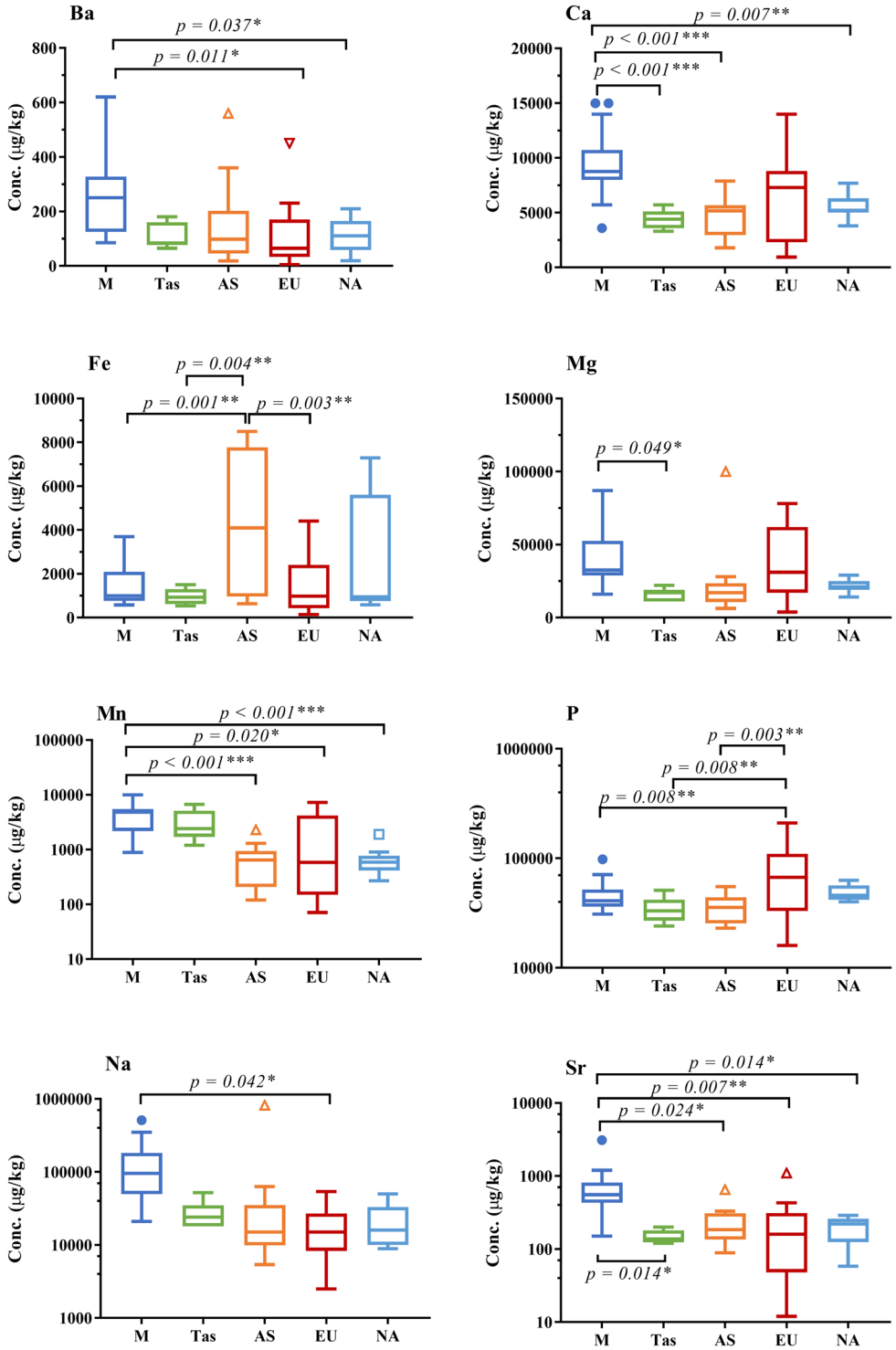

Figure 2. Boxplot with Tukey whiskers showing trace element concentrations (µg/kg) of Ba, Ca, Fe, Mg, Mn, P, Na and Sr for authentic commercial honey samples of known geographic origin (n = 65; with the three samples of an unknown origin and the single African honey sample excluded) collected from mainland Australia (n = 24), Tasmania (n = 7), Asia (n = 10), Europe (n = 15) and North America (n = 9). Significant differences were determined using a One-way ANOVA with Tukey's multiple comparison at ***$p = 0.001$, **$p = 0.01$ and *$p = 0.05$ levels.

Model analysis. The PCA and CDA displayed a clear visual clustering for honeys according to their different geographic origin covering both regions (Fig. 3b,c) and continents (Fig. 3d). In order to identify a potential approach for distinguishing honey from different regions and continents, a C5.0 classification model with 92.3%

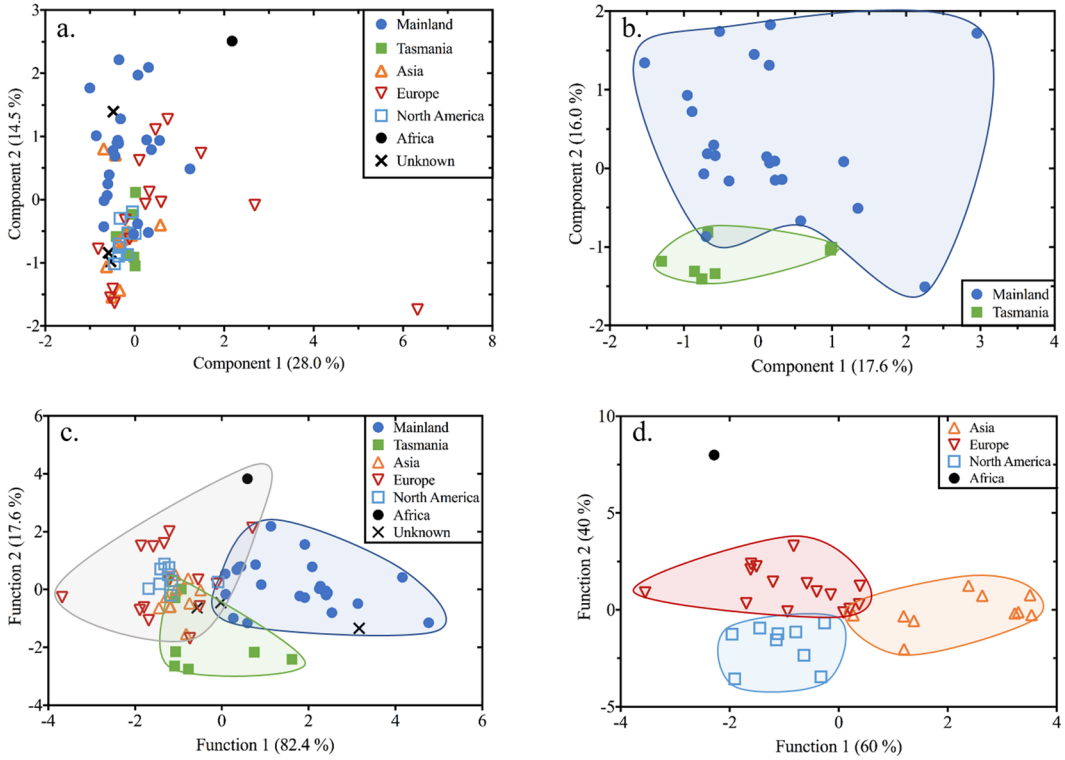

Figure 3. Score plots of PCA (**a**,**b**) and CDA (**c**,**d**) analysis used to distinguish all authentic honey samples (n = 69) from mainland Australia (n = 24), Tasmania (n = 7), Asia (n = 10), Europe (n = 15), North America (n = 9), Africa (n = 1) and unknown geographic origin (n = 3). (**a**) Important parameters are Cu, Ni, P and Rb in Component 1, and Ba, Ca, Mg and Mn in Component 2. (**b**) Important parameters are $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$ and Ba in Component 1, and Ca, Mg and Na in Component 2. (**c**) Honey from mainland Australia (blue shaded area), Tasmania (green shaded area) and overseas (grey shaded area) were used as the input groups to train the CDA model. CDA was then used to predict the geographic origin of the three honeys produced in an unknown geographic location (Supplementary Table S4). The important parameters are Mn and Sr in Function 1, and Ca, P and K in Function 2. (**d**) Honey from Europe (in red shaded area), Asia (yellow shaded area) and North America (light blue shaded area) were used as the input groups. The important parameters are Ba, Ca, Fe, Na and Sn in Function 1, and $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, Al, B, Cu, K, Mg, Mn, Ni, P, Rb, Sr and Zn in Function 2. Loading values for the PCA and CDA results are in Supplementary Table S5 and S6, respectively.

overall accuracy was applied to honey carbon isotopic values and trace element concentrations. This model was used to test its predictive accuracy to correctly group honeys according to its geographic origin. Sixty-five authentic commercial honey samples with clear geographic information were used for model analysis (the single African sample was excluded). Training (90% sample size, n = 60) and testing sets (10% sample size, n = 5) were used to build and evaluate the model. The training set is used to train the candidate algorithms, and the testing set is used to measure the predictive ability of the model. Results from both sets showed 100% classification rates in grouping honey from different geographic origins according to the C5.0 model.

Trace element profiles in honey revealed predictor importance in the C5.0 classification model (Fig. 4 and Supplementary Fig. S2). The C5.0 model demonstrated that trace elements Sr, P, Mn and K can be used to differentiate 95% (19 of 20) samples from the Australian mainland, 71% (5 of 7) samples of Tasmanian, and 71% (10 of 14) samples of European honey (Fig. 4) in four end nodes.

Discussion

Honey is ranked third in the list of globally adulterated products[43]. The common fraudulent practice of adulterating commercial honey is confirmed by this study of global honeys (Supplementary Fig. S3). We found that 52% of Asian honey samples tested were adulterated (11 of 21 samples) (Table 1); of which three were from China (3/7), one from South Korea (1/1), one from India (1/2), two from Indonesia (2/2) and four from Iran (4/4). Six honey samples from Europe, from a total of 21 tested, contained added sugar. These honeys originated from Macedonia (2/3), Romania (1/2), Serbia (1/1), Greece (1/5) and Hungary (1/3). Australia has a lower adulteration rate (18.4% in total), with honey from its mainland having an adulteration rate of 17.2% (5/29) compared to 22.2% of samples from Tasmania (2/9). Both New Zealand manuka honey samples tested (2/2) were adulterated.

Manuka honey is prone to failing the C-4 sugar test carried out using the AOAC 998.12[11], as it can present "false-positive" results for sugar adulteration as a result of insoluble material (such as pollen or dust) being retained in honey or due to the removal of pollen by filtration and centrifuging, affecting its $\delta^{13}C$ protein value[44–47]. Manuka sample OA-53 returned a value of −10.55% for C-4 sugar (<−7% cf. Dong *et al.*[12]) and OA-54 had

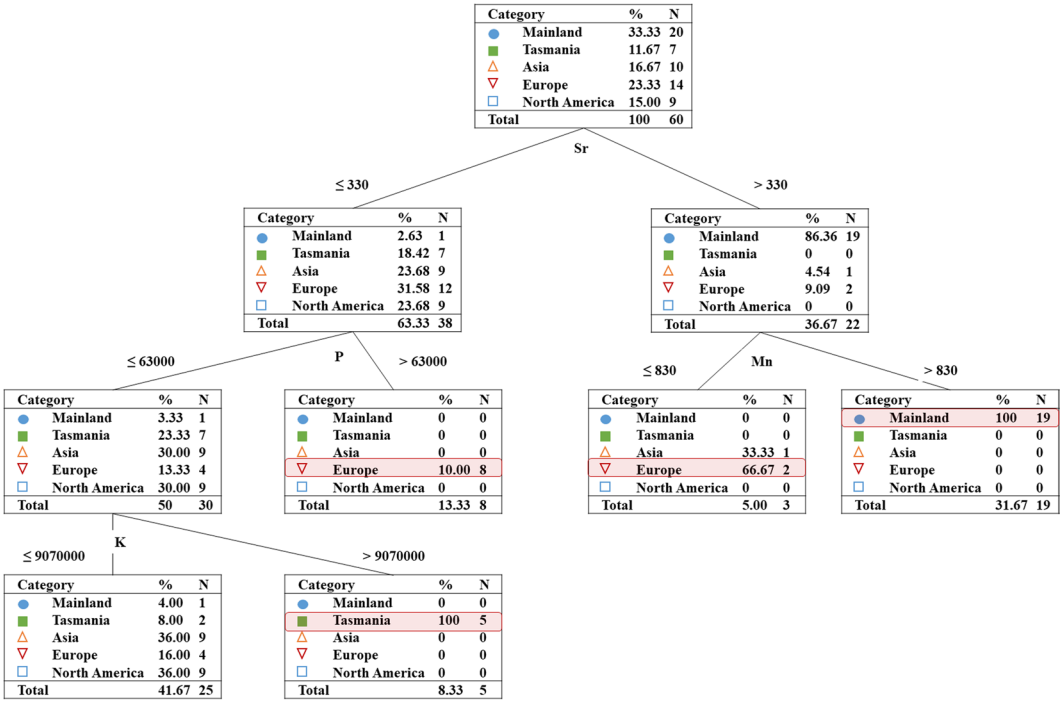

Figure 4. Decision tree outcomes (partial results) with four end nodes to separate honey according to concentrations (μg/kg) of Sr, P, Mn and K. Full results are provided in Supplementary Fig. S2.

a 7.04% C-4 sugar value (>7% cf. AOAC 998.12[11]) and 1.20‰ in $\delta^{13}C_{h\text{-}p}$ (>1‰)[8,15,17–20], classifying them as adulterated. Moreover, the manuka samples had $\delta^{13}C_{honey}$ values of −25.13‰ and −25.58‰ respectively, which is inconsistent with Rogers *et al.*[45] who suggested that $\delta^{13}C$ values in authentic manuka honey should not be less than −24.7‰. Therefore, the multiple criteria used here to assess honey authenticity indicates that both the manuka honey samples analysed were non-authentic products. Rogers *et al.*[45] also analysed manuka honey samples and found that 15% (113 of 757) were adulterated.

Stable carbon isotopic ratio analysis has gained increasing importance in the determination of the geographic origin of honey[21]. The $\delta^{13}C$ values of honey and protein are strongly influenced by climatic conditions[48] and agricultural practices[19]. Nine honey samples were collected from the island state of Tasmania, 240 km south of the Australian continental mainland. The different geographic environment of mainland Australia and Tasmania appears to be reflected in the carbon isotope ratios of their respective honey and honey protein values. The mean values of $\delta^{13}C_{honey}$ in authentic Australian honey samples were Tasmania (−25.57‰) > mainland (−25.85‰) > raw honey (−26.15‰) (Supplementary Table S1). These three groups of Australian honey that ranged from −27.91‰ to −24.71‰, correspond to the range determined by the Australian government[49] who established authentic honeys had $\delta^{13}C$ values from −27.47‰ to −24.28‰. Tasmanian honey (−25.68‰) also has higher mean $\delta^{13}C$ values for protein than Australian mainland honey (−26.08‰) and raw honey (−25.79‰) (Supplementary Table S1). The $\delta^{13}C_{protein}$ (−27.78‰ to −25.02‰) for all authentic Australian honeys (comprising raw, and commercial mainland Australia and Tasmania samples) also corresponds with authentic honeys identified in the Australian government investigation (−27.20‰ to −23.22‰)[49]. Hence, the differences in carbon isotopes are related not only to the source locations but also to whether the honey is raw or has been commercially processed.

Trace element analysis can also be used to discriminate the geographic origin of food[50]. However, two or three individual trace elements are typically ineffective for differentiating foods according to their geographic origin (Supplementary Fig. S1)[51]. Hence, multivariate statistical methods can provide better discrimination between geographic regions[52]. PCA and CDA are both commonly used statistical methods to reduce the dimensions of variability for clustering. CDA is a supervised learning method that can classify unknown samples (Supplementary Table S4). In this study, CDA resulted in more effective clustering than PCA as it was able to separate authentic commercial honey samples according to their geographic origin, similar to Kropf *et al.*[37], Efenberger-Szmechtyk *et al.*[53], and Zakaria *et al.*[54] in their analysis of the geographic classification of honey.

Advanced statistical analyses, such as decision trees, have been used previously to verify the most relevant trace elements for classifying the geographic origin of food[50,51,55]. In this study, we applied the C5.0 classification model to separate honey at regional and continental scales: that is, samples originating from mainland Australia, Tasmania, and also Asia, Europe and North America. The C5.0 classification model is consistent with the CDA statistical analysis in that they both show elemental concentrations of Mn and Sr in honey can be used to distinguish honeys from mainland Australia from those sourced from Tasmania and overseas (95% of samples and 82.4% of the total variation, respectively) (Fig. 3c and 4). The trace elements Mn and Sr together with K and P can be used to separate 71% of Tasmanian honey (5 in 7) from the other samples with a specific geographic identity.

This outcome was also reflected in the CDA analysis where the same four elements were included in Function 1 (Mn and Sr) and Function 2 (K and P) (Fig. 3c). Further, the same four elements were also included in the second function for classifying different continental honey (Fig. 3d). This is consistent with the C5.0 classification model that showed 71% (10 in 14) of European honeys were differentiated from samples from Asia, North America, Tasmania and mainland Australia (Fig. 4). Other studies have also shown that the trace elements K[36,56], Mn[56,57], P[58] and Sr[36,59] were critical for the classification of honey according to their geographic origin.

This study investigated the authenticity of global commercial honeys, covering 19 countries from five continents. The issue of authentication of honey cannot be ignored in the international honey market, not only in Asian countries but also European[60] and Oceanic countries. Analyses of $\delta^{13}C$ values in honey and its protein can be an effective tool for the authentication of honey where it has been subject to adulteration with C-4 sugars. Further, analysis of trace elements in honey for fingerprinting its geographic origin shows significant potential to assist in product authentication. Thus, this study shows that a combination of $\delta^{13}C$ and statistical analysis of trace element concentrations can be used to expose the common fraudulent practice of adding C-4 sugars along with the mislabelling of a honey's geographic origin.

Limitations

This study contains a number of potential limitations. Firstly, the study is comprised of a relative small sample size (n = 100). The study was not intended to be a systematic survey of global honeys, but rather a snapshot of randomly sampled honeys to ascertain the prevalence of adulteration. Indeed, the results presented here are consistent with other assessments of honey adulteration rates[60]. While the analysis of honey's trace element concentrations showed clear potential to discriminate between samples from different geographic locations, a larger sample size would be required to further confirm this study's preliminary findings. However, to fully characterise the variance of trace elements across a region may require prohibitively expensive large data sets. An alternative lower cost approach could involve establishing if trace element concentrations of an unknown product are consistent with genuine reference products and are different to those sourced from potential risk locations.

While the study showed that some 73% of commercial honeys analysed were classified as pure according to AOAC criteria[10,11,16] and previous studies[8,12,13,15,17–20] it is entirely possible this overestimated the actual number of pure honeys. This is because honey is sometimes adulterated using C-3 sugars, such as those from sugar beet[7]. It is unfortunate that the methods used here cannot detect this form of adulteration[61]. Consequently, detection of adulteration of this nature remains a challenge[7].

In addition to C-3 sugars, other adulterants in honey are difficult to detect due to the development of new and more sophisticated practices and lack of officially accepted analytical techniques. The use of EA-IRMS (Elemental Analysis - Isotope Ratio Mass Spectrometry) is the only official detection method for addition of C-4 sugar in honey[42]. This study also relied on a number of AOAC criteria[10,11,16] to determine if honey samples were pure. As shown in Table 1, 65% (17 of 26) of samples classified as adulterated did not meet the $\delta^{13}C_{h\text{-}p}$ and the C-4 sugar criteria; 9 samples also failed to meet the relevant criterion for $\delta^{13}C_{honey} < -23.5‰$[10]. While the C-4 sugar criterion is considered robust and has broad application, the AOAC 998.12[11] method does identify that a small percentage of genuine pure honeys fall outside of the accepted criterion for non-adulterated honey ($\leq$7%).

Method

Sample collection. Raw and unprocessed honey samples (n = 5) were collected for this study from beehives across Sydney, New South Wales (*Apis mellifera*, n = 3) and Calliope, Queensland (*Tetragonula hockingsi*, n = 2), Australia. Commercial honeys were obtained from local food markets and commercial supermarkets. Of the twenty-nine Australian mainland commercial honeys (M) labelling information indicated two were from NSW, six from Queensland, seven from Victoria, six from Western Australia, four from South Australia, with four of the Australian honeys not providing state of origin information on their labels. A further nine Australian Tasmanian honey samples (TAS) were collected for analysis.

Fifty-four commercial honey samples were acquired overseas from five continents; Africa (n = 1 from Kenya), Asia (AS, n = 21), Europe (EU, n = 21), North America (NA, n = 9) and Oceania (OA, n = 2 from New Zealand). Asian countries investigated in this study include China (n = 7), India (n = 2), Indonesia (n = 2), Iran (n = 4), Japan (n = 3), Saudi Arabia (n = 2) and South Korea (n = 1). European commercial honeys were collected from England (n = 3), Greece (n = 5), Hungary (n = 3), Italy (n = 4), Macedonia (n = 3), Romania (n = 2) and Serbia (n = 1). Honey from USA (n = 7) and Canada (n = 2) were included as North American samples. Nineteen of the overseas honey samples were purchased in Australian supermarkets (marked with an asterisk in Supplementary Table S1 and Table 1) with the remainder being acquired from overseas. Three commercial honey samples with an unclear origin of production (U) were purchased in Australia. Two had their origin listed as "made in Australia from local and imported ingredients", with the other having no geographic identification. All samples (raw n = 5, and commercial honey samples n = 95) were analysed at the National Measurement Institute (NMI), North Ryde, Sydney.

Sample preparation and analysis. *Trace element analysis.* One gram of honey was digested with 3 mL HNO_3 before heating at 100 °C for 2 h. Each digested sample was topped up to 40 mL with Milli-Q (18.2 MΩ.cm) deionised water. Samples were diluted two times prior to analysis for their trace element concentrations on an Inductively Coupled Plasma Mass Spectrometer (Agilent 7900 equipped with an Integratated Sample Introduction System). Each sample batch (n = 20) contained a laboratory reagent blank and duplicate, blank spike, blank matrix, duplicate sample and matrix spikes. Sixty-four trace elements were measured in the samples: Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Cs, Cr, Co, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hg, Hf, Ho, Rb, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, Os, P, Pb, Pd, Pt, Pr, Re, Ru, Se, Sb, Sr, Sm, Sn, Ta, Tb, Te, Th, Tl, Tm, Ti, U, V, W, Y, Yb, Zn and Zr. Forty eight of these trace elements were below the NMI's Limit of Reporting (LOR) of 10 µg/

kg in 70–100% of samples. As a result, the concentrations of 15 trace elements (Ba, B, Ca, Cu, Fe, Mg, Mn, Ni, P, K, Rb, Na, Sr, Sn and Zn) that were above the LOR in 71–100% of samples were used in the statistical analyses. Although only 46% of samples had Al at concentrations >LOR, its exclusion had potential to cause erroneous statistical outcomes because of its median concentration (760 µg/kg) and large range (23–21000 µg/kg). In all cases, sample procedural blanks were below the NMI's LOR. The combined mean RSD (relative standard deviation) for the individual trace elements in honey was 2.4%. Matrix spike recovery rates for all trace elements in honey was 75–110%. Analytical uncertainties for all trace elements was 14–24%.

Carbon isotopic ratio analysis. Proteins from honey were isolated according to the AOAC Official Method 998.12[11]. Honey samples (10–12 g) were weighed into a centrifuge tube (50 mL) and mixed with 4 mL Milli-Q water, followed by the addition of 2 mL of 0.335 mol/L H_2SO_4 and 2 mL of Na_2WO_4 10% (w/v). This mixture was homogenized and heated to 80 °C until flocculation of proteins were visible and a clear supernatant observed. In cases where flocculation did not occur, H_2SO_4 (2 mL, 0.335 M) was added to the solution. Sample tubes were filled to 50 mL with Milli-Q water, mixed thoroughly and centrifuged at 1500 × g for 5 min. The supernatant was subsequently discarded. This procedure was repeated for a minimum of five times, until the supernatant was clear. The precipitated protein was dried in an oven (75 °C) for a minimum of 3 h until dry.

Bulk honey (0.55–0.60 mg) and extracted protein (0.48–0.57 mg) were weighed into tin capsules (3.3 mm × 5 mm; IVA Analysentechnik, Meerbusch, Germany) and introduced into a Flash Elemental Analyzer coupled to an Isotope Ratio Mass Spectrometer (EA-IRMS, Thermo Finnigan Delta V Plus) via a ConFlo IV interface. Data was acquired using ISODAT 3.0 (Version 2.84) (ThermoScientific, Bremen, Germany). High purity oxygen (>99.5%), ultra-high purity helium (>99.99%), high purity carbon dioxide (>99.5%) were obtained from BOC gases (Sydney, Australia) and zero enrichments were performed for CO_2 reference gas prior to sequence acquisitions. The δ-values of nine 20 s gas pulses were measured, and the standard deviation determined to be less than 0.2‰.

A typical sample sequence for $\delta^{13}C$ analysis was initiated with the thermal combustion of two blank tin cups to ensure the system was void of contamination. Each honey and protein sample were analysed in triplicate alongside standards USGS 24 graphite ($\delta^{13}C$ −16.05‰) and NBS 22 oil ($\delta^{13}C$ −30.03‰), which returned mean RSDs of 0.36% and 0.33%, respectively. Three reference materials were employed as quality control samples in this study: CCQM-K140 ($\delta^{13}C$ −24.09‰; analysed 6 times) returning a RSD of 0.27% and measurement uncertainties ranging between 0.08% and 0.28%[62]; USGS 40 L-glutamic acid ($\delta^{13}C$ −26.39‰) and IAEA-CH-3 cellulose ($\delta^{13}C$ −24.72‰) were analysed in quadruplicate and had a mean RSD of 0.27% and 0.35%, respectively.

Data processing. The isotope ratios of $^{13}C/^{12}C$ in honey and protein are expressed as $\delta^{13}C$ in units of ‰. Corrected values were obtained according to equation (2):

$$\delta^{13}C(‰) = \left(\frac{R_{means\ (sample)} - R_{means\ (Std\ 1)}}{R_{means\ (Std\ 2)} - R_{means\ (Std\ 1)}}\right) \times (R_{true\ (Std\ 2)} - R_{true\ (Std\ 1)}) + R_{true\ (Std\ 1)} \quad (2)$$

where $R = {}^{13}C/{}^{12}C$, $R_{means\ (sample)}$ is the measured $^{13}C/^{12}C$ ratios of the honey or protein samples, $R_{means\ (Std\ 1)}$ and $R_{means\ (Std\ 2)}$ are the measured $^{13}C/^{12}C$ ratios of the standards USGS 24 graphite and NBS 22 oil, respectively; $R_{true\ (Std\ 1)}$ and $R_{true\ (Std\ 2)}$ are the true $^{13}C/^{12}C$ ratios for USGS 24 graphite and NBS 22 oil, which are −16.05‰ and −30.03‰, respectively.

Data were analysed using GraphPad Prism 7 for boxplot presentation with Tukey whiskers and a one-way ANOVA test. Multivariate approaches were used to investigate the feasibility of characterising the geographic origin of authentic commercial honey samples based on the measured variables of $\delta^{13}C_{honey}$ and $\delta^{13}C_{protein}$ together with trace elements of Al, Ba, B, Ca, Cu, Fe, Mg, Mn, Ni, P, K, Rb, Na, Sr, Sn and Zn. Principal component analysis and canonical discriminant analysis were carried out using IBM SPSS v22.0, and classification analyses using C5.0 model were implemented via IBM SPSS Modeler v18.0. Concentrations of trace elements with <LOR (10 µg/kg) were considered as 5 µg/kg for statistical analyses.

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Author Contributions

X.Z. developed the project and undertook the majority of laboratory analysis, data assessment and write up. M.P.T. provided critical feedback and helped shape the research design, analyses and manuscript write up. H.S. assisted with carbon isotopic analysis and data write up. S.P. assisted with commercial honey collection, trace element analysis and write up.

Additional Information

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Chapter 6: Discussion

6.1 Summary of thesis

The principal aim of this thesis was to investigate the relationship between trace elements in the environment and in beehive products. In order to achieve this research goal, this thesis identified and quantified trace element contamination in soil and dust in the environment, and then used trace elements and Pb isotopic compositions in beehive products to trace contaminant sources across various regions. Although there have been previous studies examining the quality of honey from around the globe and the authenticity of product labelling, there has been very little holistic research into evaluating the full cycle of the relationship between trace elements in specific environments and those measured in honey end products. More specifically, there is a paucity of research examining whether trace element concentrations in honey can generate unique signatures nor if they can then be used to identify geographic source of origin and whether, along with other beehive products (bees and wax), such data can be used to measure and source environmental contamination.

Bees as bio-markers of environmental contamination

European honey bees (*Apis mellifera*) are the most common bee species in the world. Given their strong interaction with the environment, the use of European honey bees as bio-indicators for trace element contamination was first proposed by Svoboda (1962) who tested Sr in bees to indicate atmospheric contamination. In the following decades, numerous studies demonstrated the potential value of this monitoring method by revealing that higher trace element concentrations were present in European honey bees and their products when sampled from polluted areas compared to those sampled from unpolluted areas [4] (see references in Supplementary Table S1-1, Appendix A). However, the practicality and efficacy of using European honey bees or their products as reliable bio-indicators has had mixed success, as trace

[4] Contamination is the presence of a substance that should not be present naturally, while pollution is considered to have occurred when the levels of contamination is such that it causes harm to organisms or ecosystem (Sciortino J. A. and Ravikumar R. 1999).

element concentrations in beehive products sampled from polluted areas are not always higher than those from background areas (see references in Supplementary Table S1-2, Appendix A). Also, given that European honey bees are exposed directly to atmospheric contamination, it is surprising that direct evidence of the link between trace elements in dust and honey bees, or their products, has not been established. Further, prior to this PhD study, there had been no research examining the use of European honey bees for tracing contamination sources via lead (Pb) isotopic composition analysis.

The European honey bee (*Apis mellifera*) is the most studied bee species in terms of its use as a bio-indicator for trace element contamination. In Australia, and specifically the main study area of Sydney, the native bee *Tetragonula carbonaria* is also present in urban areas (Figure 1-3, Chapter 1). Given that *T. carbonaria* also has a strong interaction with the environment but has different biological behaviours (i.e. foraging distances, honey production rates and lifespans) to European honey bees (Table 1-2, Chapter 1), this thesis proposed and verified the hypotheses that native bees and their products can also be used as bio-indicators.

Honey – a global food source

Honey, the most popular product produced by bees, is one of humankind's oldest food products (Zábrodská and Vorlová 2015). It is appreciated not only for its taste and nutritional value, but also for its health benefits (Arawwawala and Hewageegana 2017, Miguel et al. 2017). However, researchers have reported a plethora of adulteration issues (Johnson 2014, Ayza and Belete 2015, Lohumi et al. 2015, Siddiqui et al. 2017, Vetrova et al. 2017), which have attracted significant media attention (Laskawy 2011, Schneider 2011, Radio France International 2013, Robb 2014, Clemons 2016) such that honey has been recognised as the world's third most adulterated food product (Garcia and Phipps 2017). Adulteration of honey by the addition of C-4 sugars and mislabelling of place of geographic origin are, unfortunately, common occurrences in the global honey market. In order to try and detect and combat this issue, official

analytical methods (AOAC 978.17, 1995; AOAC 991.41, 1995; AOAC 998.12, 2014) have been established by governments and international agencies to detect C-4 sugars in honey. In contrast, the determination of geographic authenticity still remains a challenge for the global honey market (Figure 6-1).

In Australia, about 30,000 tonnes of honey is produced annually (worth AUD$101 million), (Dijk et al. 2016) and of this, 25–30 % is exported (O'Brien 2007). Australian honey is highly sought after by both local and overseas customers as it is considered to be safe and of high quality, and is produced according to one of the most rigorous apicultural management systems in the world (Centre for International Economics 2005). Unfortunately, the highly respected reputation of Australian honey has led to deliberate mislabelling by some industrial suppliers who have used other sources of honey but purport that their product is premium Australian honey (Robb 2014, Clemons 2016). To resolve this issue, as part of this thesis, honey from Tasmania (n = 9) and mainland Australia (n = 29), together with international samples (n = 54) from 18 different countries covering Africa, Asia, Europe, North America and Oceania in addition to three samples having unknown origin (no geographic origin was stated on their label), were collected and measured to determine if trace elements could be used to distinguish their geographic origin.

Thesis research framework and aims

The studies undertaken as part of this thesis contribute to an integrated picture of trace elements in the environment and beehive products. Initially, trace elements in soil and dust deposits across various land uses were investigated, along with the potential application of the most common bee species (European honey bees) as a bio-indicator for contamination (Chapter 3). The approach was then extended to an Australian native bee species (*T. carbonaria*) (Chapter 4). Positive correlations were found between trace elements in the environment and beehive products — specifically Pb and Zn, and Pb isotopic compositions were able to be used to

apportion sources of historical and ongoing emissions. The resulting evidence verified the use of European honey bees (Aim 1 in Figure 6-1) and Australian native bees (Aim 2 in Figure 6-1) as suitable bio-indicators for trace element contamination.

In addition to raw beehive samples, global commercial honeys were collected and subject to carbon isotopic analysis for authentication investigation (Aim 3 in Figure 6-1) and to trace element analysis for geographic identification (Aim 4 in Figure 6-1). The analyses showed that 27 % of commercial honeys (26 out of 95 samples) contained significant artificial sugars, supporting the prevailing view that adulteration is prevalent in the global honey markets. Statistical analysis of trace elements in honey showed visual clustering according to Australian regions and different continents, indicating that trace element signatures in honey can be used to distinguish its declared geographic origin. The overall goal of this thesis research was to use evidence-based data to propose a robust approach for environmental monitoring using bees and their products and an effective method to establish the authenticity of honey. The development and establishment of robust approaches from this thesis research could be used more broadly to provide the public with greater surety in regard to food quality and its relationship to the environment.

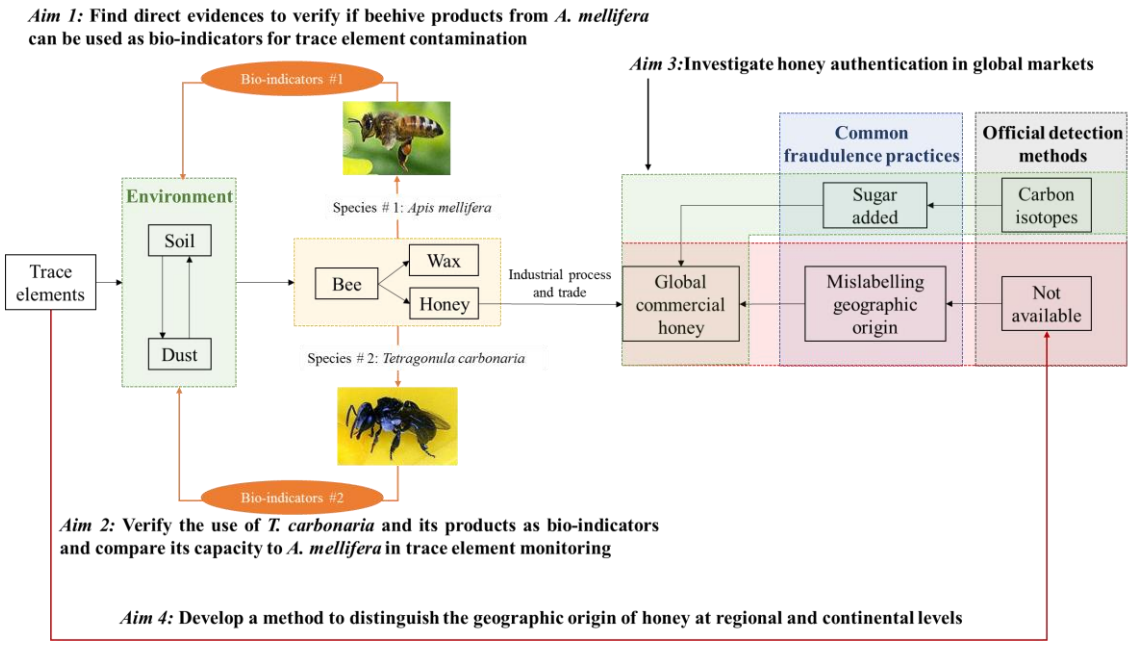

Figure 6-1. Thesis research framework and aims.

6.2 Verifying beehive products as bio-indicators for trace element contamination

Previous researchers have identified the use of beehive products as potential bio-indicators due to existing indirect evidence that bees and their products sampled from polluted areas contain more trace elements than those from unpolluted areas (see references in Supplementary Table S1-1). However, despite this significant body of research, findings were not sufficiently conclusive to support the use of honey bees and their products as bio-indicators. Direct evidence of European honey bees carrying thousands of airborne particles from neighbouring areas was presented in recent investigations by Negri et al. (2015) and Pellecchia and Negri (2018). The researchers found that contaminated particles attached to each single European honey bee's head, wings and legs were derived from the co-located soil at the industrial and post-mining areas near to where the bees foraged (Negri et al. 2015, Pellecchia and Negri 2018). The study presented in Chapter 3 of this thesis demonstrates that trace element concentrations in individual European honey bees varies within the same sampling area (Figure 6-2), suggesting that European honey bees are sensitive to environmental contaminants as they age (Zhou et al. 2018b).

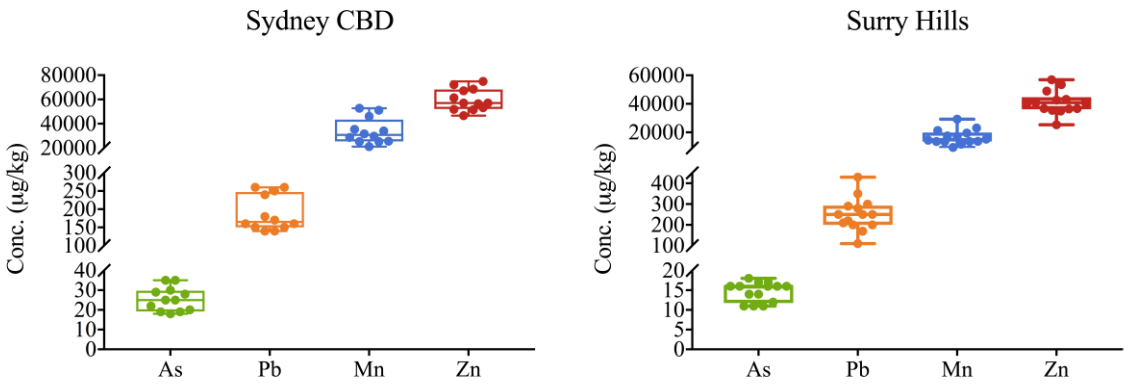

Figure 6-2. Concentration of trace elements As, Pb, Mn and Zn (µg/kg) in individual European honey bees collected from the Sydney CBD (n = 12) and Surry Hills (n = 14) areas. The boxplots depict the median values and 25th and 75th percentiles of the data with whiskers representing minimum and maximum values. The relative standard deviations (RSDs) for As, Pb, Mn and Zn were 24 %, 26 %, 31 % and 15 % in the Sydney CBD. The RSDs for As, Pb, Mn and Zn were 17 %, 31 %, 31 % and 20 % in Surry Hills. Data were obtained from Zhou et al. (2018b).

A study by Cozmuta et al. (2012), which measured trace element concentrations in drones and worker honey bees, also found direct evidence of honey bees carrying airborne particles. Drones are relatively inactive and usually have three or less flights per day, totalling about 30 min around the beehive (Tofilski 2012). By contrast, worker honey bees have about 10 flights per day, totalling up to 90 min foraging for nectar and pollen (Van der Steen 2015). Cozmuta et al. (2012) found that worker bees were more contaminated with trace element Pb than drones. This finding is consistent with our results reported in Chapter 3 (Figure 6-3a) that found the mean values of trace element concentrations in drones were lower than those in worker honey bees. This suggests that worker bees are more likely to be contaminated by atmospheric and substrate particles while foraging beyond the hive (Zhou et al. 2018b).

Based on this finding, Chapter 3 investigated trace elements (As, Pb, Mn and Zn) in both live and dead worker honey bees that were collected on the same sampling day. The trace element results demonstrated that dead individuals contained higher concentrations (p = 0.005–0.572) of these elements than live ones. This further confirms the finding that worker bees are exposed to contamination from the surrounding environment and implies that they are incrementally contaminated as they age (Figure 6-3b) (Zhou et al. 2018b). Thus, the evidence shows that worker bees can be considered a sentinel species for trace element monitoring across their local environment. Even though there is emerging evidence that bees can be contaminated from the environment during their foraging, the sources (or toxicity effects) of contaminated particles attached to their hairy bodies, from current or legacy emissions, have not been investigated through Pb isotopic composition analysis. As such, this thesis also sought to fill this research gap (Chapters 3 and 4).

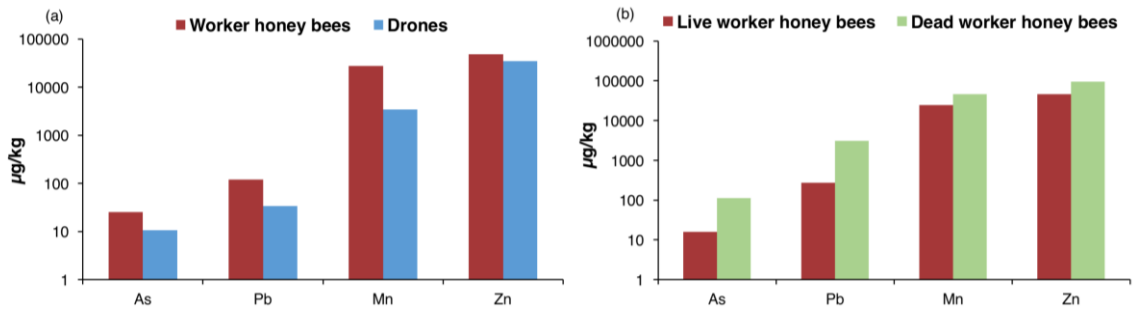

Figure 6-3. Trace element concentrations (µg/kg) of As, Pb, Mn and Zn in live and dead worker honey bees a well as drones (source: Zhou et al. 2018b).

Overall, the paucity of robust and consistent evidence regarding a positive correlation between environmental sources of contaminants and those recovered in beehive products, along with apportionment Pb sources, has undermined the legitimacy of using bees as sentinel species for monitoring trace element contamination in the environment. In summary, the studies presented in this thesis (Chapter 3 and 4) attempt to address this gap via application of trace element

analysis and Pb isotopic composition analysis, to provide evidence to evaluate and support the suitability of beehive products as bio-indicators.

6.2.1 Investigating the association between trace elements in beehive products and the environment

The absence of direct positive and statistically significant correlations ($p < 0.05$) between trace elements in the environment and beehive samples during the last five decades is unlikely to be a random occurrence. Deductive reasoning would indicate it is likely related to specific factors. In order to better understand this conundrum, all relevant literature was reviewed to evaluate the relevant factors that might limit the use of bees as environmental monitors of contamination.

A hidden clue relating to the spatial aspects of sampling when using European honey bees as bio-indicators was indicated in an American study conducted by Bromenshenk et al. (1985), which used European honey bees to monitor trace element contamination from an arsenic and copper smelter across Puget Sound, an ocean inlet located along the north-western coast of the USA. The study drew the following conclusion:

> '*Our results show that beekeepers can effectively use colonies of bees as a self-sustained system for environmental monitoring over large geographical areas*.' (Bromenshenk et al. 1985)

However, 'large geographical areas' were not defined in the study and there was no further explanation in subsequent studies as to why this approach should be applied and under what circumstances. Two studies by Van der Steen et al. (2012, 2015) investigated trace elements in European honey bees and air quality across three locations (distances between the sites were 2.0, 6.6 and 14.6 km) in the Netherlands. The researchers found that there were no significant differences of Cd and Pb concentrations in European honey bees between locations, and that

trace elements in European honey bees had no significant positive correlation (p = 0.92, r = 0.00 for Pb; p = 0.008, r = -0.23 for Cd) with atmospheric concentrations (ng/m^3) of the same elements. While Van der Steen et al. (2012, 2015) contended that the relatively small-scale sampling was the reason why their beehive products failed as bio-indicators, no further information was provided in regard to defining the appropriate geographic areas when using European honey bees as bio-indicators.

Chapter 3 reports on the use of beehive products as an environmental monitoring method across the city of Sydney and also at the regional town of Broken Hill, NSW (1100 km west of Sydney). The study samples one European honey bee hive located adjacent to the Pb-Zn-Ag mining operations in Broken Hill and nine beehives located on land of diverse use around the city of Sydney, including national parks, coastal residential suburbs, high density inner city areas, residential-industrial areas, a major international airport and light industrial, commercial, and residential areas. Beehive samples of European honey bees and their honey and wax, along with environmental samples of soil and dust were collected from each location. All the samples were subject to trace element analysis by ICP-MS at the National Measurement Institute, North Ryde, Sydney, NSW.

In our study of NSW beehive products, the data shows that European honey bees accumulate more Pb than other trace elements (As, Mn and Zn) during foraging (Zhou et al. 2018b), indicating that honey bees are sensitive to atmospheric Pb contamination (Lambert et al. 2012, Ruschioni et al. 2013). This argument is further substantiated by the absence of statistical significance after the removal of Broken Hill data. Specifically, following the removal of the Broken Hill data (which are extreme values at the upper end of the contamination spectrum) (Figure 6-4), trace elements As, Mn and Zn in beehive products and corresponding environmental samples are not significant (r = -0.022−0.650, p = 0.058−0.955) (Zhou et al.

2018b). However, Pb in European honey bees remains significantly correlated with Pb in dust across the Sydney metropolitan area (r = 0.731, p = 0.025) (Figure 6-5c).

Although the Pb in European honey bees (p = 0.0008) (Satta et al. 2012) and honey (p = 0.004) (Czipa et al. 2017) was found to be linked with co-located soil, Chapter 3 (Zhou et al. 2018b) was the first study to observe the positive and statistical correlations of Pb between various beehive products and co-located dust. This directly supports the argument for the use of European honey bees and their products as bio-indicators for atmospheric contamination (Gutiérrez et al. 2015). Our study also demonstrates that dust Pb is derived from soil Pb (p = 0.005) (Zhou et al. 2018b). While the correlation between Pb in dust and soil has been verified by local (Laidlaw and Taylor 2011) and international studies (Mielke et al. 2011, Zahran et al. 2013), Chapters 3 is the first study to demonstrate this relationship using European honey bees as bio-indicators.

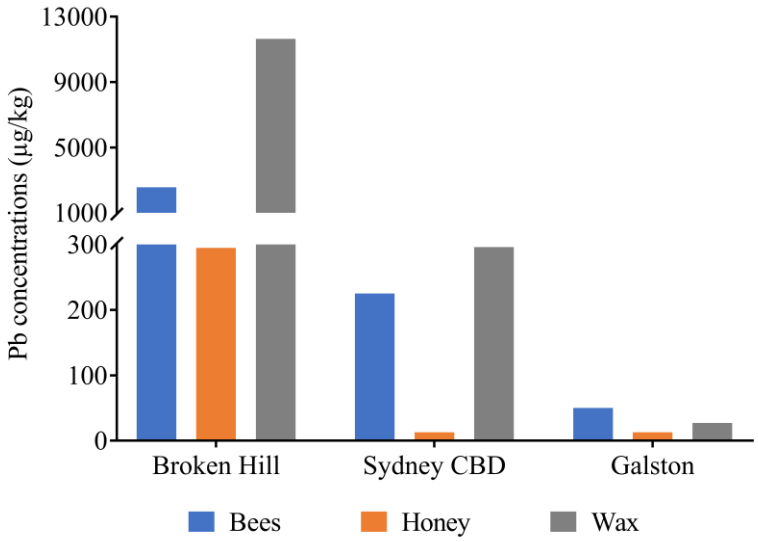

Figure 6-4. The Pb concentrations (µg/kg) in European honey bees and their products of honey and wax adjacent to the Berowra National Park at Galston, Sydney CBD and the Pb-Zn-Ag mining town of Broken Hill (source: Zhou et al. 2018b).

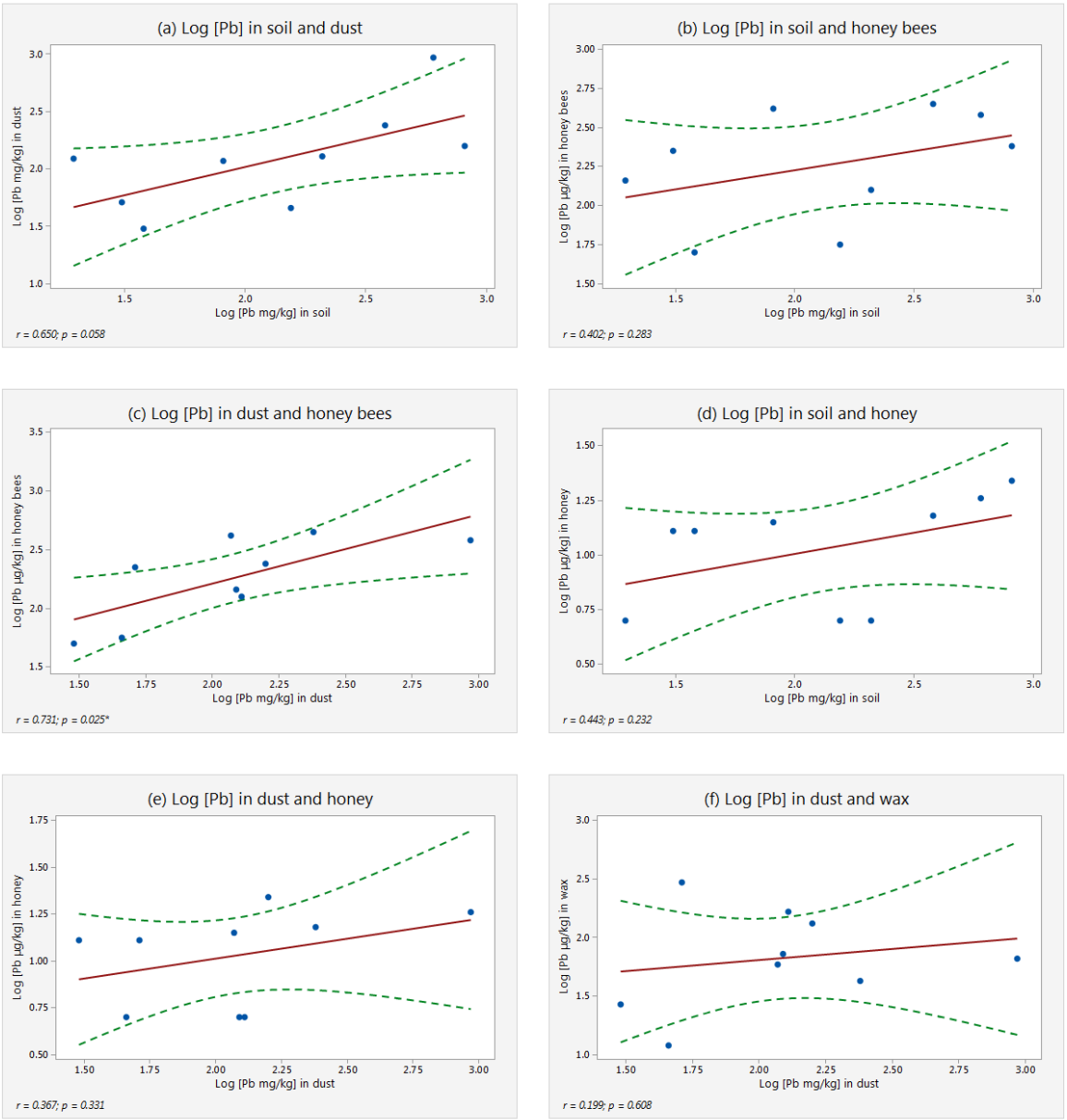

Figure 6-5. Relationships (with 95 % confidence intervals) in $\log_{10}$ transformed mean Pb concentrations between honey bees (µg/kg, n = 38), honey (µg/kg, n = 15), wax (µg/kg, n = 14) and co-located soil (mg/kg, n = 41) and dust (mg/kg, n = 40), as well as the correlation between co-located soil Pb and dust Pb samples across Sydney metropolitan areas. Pearson correlation significance is at the *0.05 level (source: Zhou et al. 2018b).

Aside from European honey bees (*Apis mellifera*), other bee species have been employed in studies aiming to determine trace element contamination in the environment. Szentgyörgyi et al. (2011) measured trace elements in bumblebees (*Bombidae*) and found that Cd and Pb in bumblebees were significantly related to the soil contamination levels (p = 0.001–0.009). Local

native bee species were also used as bio-indicators of trace element contamination in a study by Nascimento et al. (2018). In this case, the researchers examined trace elements in pollen stored by a Brazilian stingless bee species (*Tetragonisca angustula*). Pollen collected from post-mining areas exhibited higher levels of Ba, Cr, Cu, Ni and Fe than samples from natural protected areas. Also, a linear correlation in trace element concentrations between pollen and suspended particles was determined (Nascimento et al. 2018).

However, to date, there have been no studies comparing the capacities of different bees as bio-indicators in the assessment of trace element contamination. As there are a number of biological and behavioural differences among bee species (Michener 2007), the hypothesis in Chapter 4 proposes that there is a difference between bee species and their propensity to be contaminated by particles from the environment. Statistical analysis verifies that trace element concentrations in Australian native bees (*T. carbonaria*) and their products are significantly ($p < 0.05$) associated with those in co-located soil and dust across an equivalent area to that of the European honey bee hives (Figure 6-6) (Zhou et al. 2018a, Zhou et al. 2018b). This strong association between Australian native bee hive products and dust and soil within the immediate environment suggest that they are more sensitive as bio-indicators than European honey bees (Figure 6-5 vs. Figure 6-6).

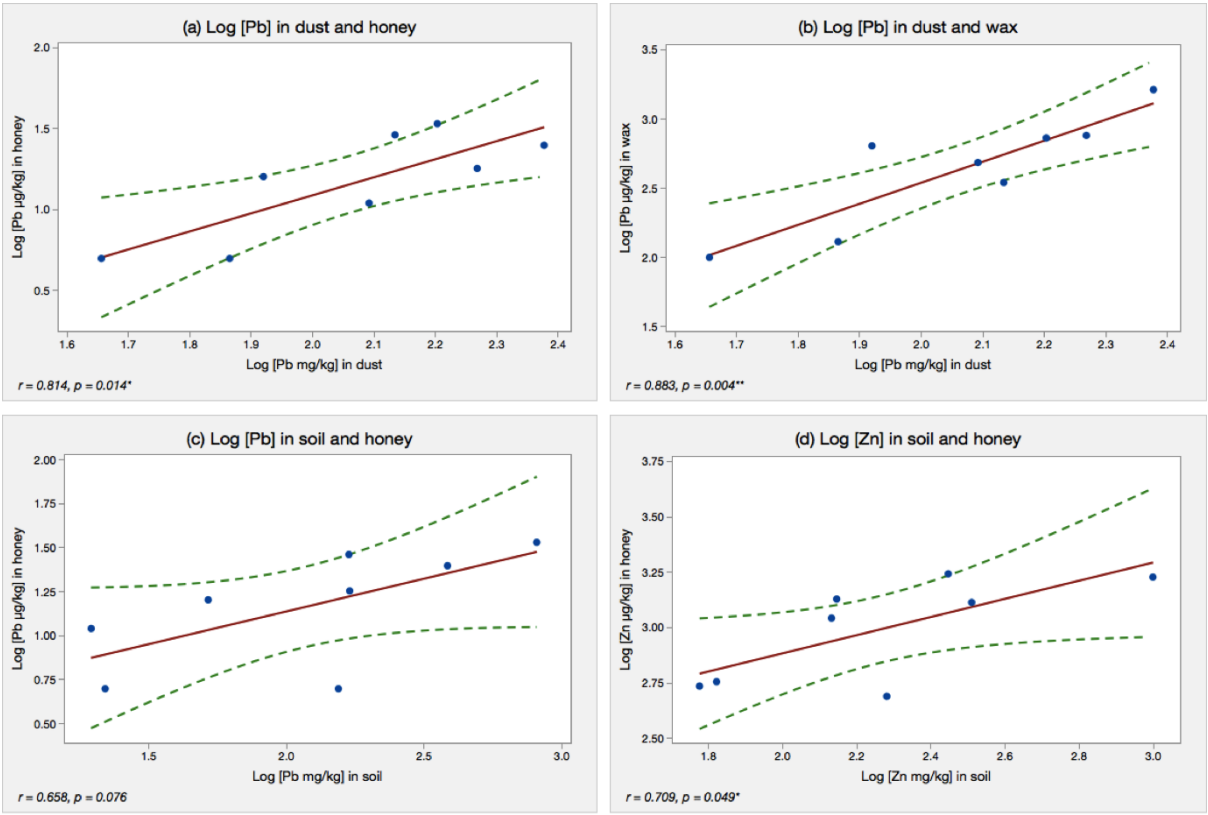

Figure 6-6. Linear relationships with Pearson correlation coefficients (r) and *p* values between various Australian native bee products and soil or dust. Pearson correlation significance (*p*) is indicated at the following levels: * = 0.05 and ** = 0.01 (source: Zhou et al. 2018a).

Overall, the findings presented in Chapter 3 and 4 demonstrate that the use of bees as bio-indicators for trace element contamination should be undertaken over a large geographic area where land use types are markedly different (Zhou et al. 2018b). Also, the distance between beehives should be larger than bees' foraging distances (5–9 km for *A. mellifera*, 0.3–0.7 km for *T. carbonaria*) to avoid 'smoothing' concentrations variations of environmental contaminants across the sample area.

6.2.2 Identifying sources of Pb contamination in beehive products using the Pb isotopic composition

Statistical analysis shows that bees, honey and wax from different bee species have demonstrable potential for their use in the assessment of Pb contamination in the environment (Figure 6-2 and 6-3) (Zhou et al. 2018a, Zhou et al. 2018b). However, trace element analysis alone cannot provide information on the source(s) of Pb contamination carried by bees and stored in their products. Such sources can include mining areas, smelting activities, leaded petrol and other industrial emissions (De Leon 2012).

Mining

European honey bees were identified as 'active samplers' of airborne dust across mining areas after Negri et al. (2015) and Pellecchia and Negri (2018) showed that thousands of contaminated particles were mainly derived from Pb-Zn ore deposits associated with mining areas. Furthermore, the fact that concentrations of Pb and Cd in European honey bees collected from the same and other mining areas in Italy were higher than those from control areas further supports this argument (Massidda et al. 2007, Satta et al. 2012), which is consistent with our own Australian study (Zhou et al. 2018b).

However, trace elements in honey and wax collected from mining areas are not always higher than those from background areas. Specifically, the trace elements As, Cd and Zn in honey from mining areas were found to be below the LOR of 0.05 mg/kg (Alvarez-Ayuso and Abad-Valle 2017) or within the concentration range of honey collected from control areas (Tong et al. 1975, Iskander 1996). The low concentrations of Cd, Pb, Tl and Zn were also found in wax samples collected from mining areas (Losfeld et al. 2014). This is consistent with Zhou et al. (2018b) who showed that As, Mn and Zn in honey and wax collected from the Pb-Zn-Ag mining town of Broken Hill were similar to Sydney data. Hence, trace element analysis alone is not adequate

to support the capacity of beehive products in assessment of contamination caused by intensive mining activities.

In light of this, Chapter 3 presents Pb isotopic compositions in European honey bees, honey and wax from the mining town of Broken Hill. The Pb isotopic compositions of the Broken Hill beehive samples were compared to the local ore body data (Gulson 1984, Kristensen and Taylor 2016). Results showed that the data from the beehives was nearly identical (95–98 %) to the composition of the local ore body, indicating that the current mining operation is a key factor in driving trace element contamination in the local food and ecological systems, although there were no elevated concentrations of trace elements (As, Mn and Zn) in honey and wax from Broken Hill (Zhou et al. 2018b).

Isotopes of trace elements other than Pb, such as ^{137}Cs (Porrini et al. 2003) and ^{226}Ra (Horn et al. 1996, Barišic et al. 2002), have been used in European honey bees, honey and wax to assess the activity of radionuclides in mining areas. Surprisingly, Pb isotopic composition analysis of European honey bees and their products has received limited interest from researchers previously, Zhou et al. (2018b) being the first to publish the use of Pb isotopes for source apportionment in bees. This study along with other international studies (Horn et al. 1996, Barišic et al. 2002, Porrini et al. 2003) have confirmed the potential for using isotopic analysis in European honey bees and their products to identify contamination derived from mining operations.

Leaded petrol

Petrol mixed with tetraethyl lead was first used in the 1920s and phased out and banned for use in automobiles across most of the globe by the early 2000s (Filella and Bonet 2017). Australia used leaded petrol for seven decades between 1932 to 2002 (Kristensen 2015). As a consequence, *inter alia*, Australian soils (Laidlaw et al. 2014), aerosols (Gulson et al. 1983),

dust (Laidlaw et al. 2014), wines (Kristensen et al. 2016) and lichens (Wu et al. 2016a, Wu et al. 2016b) have been shown to be contaminated with varying proportions of petrol Pb across Australian urban areas. These studies examined the effect of leaded petrol on various Australian environmental media and correspond to other global studies due to leaded petrol's pervasive use and persistence as an environmental contaminant (e.g. Véron et al. 1999, Vallelonga et al. 2002, Schwikowski et al. 2004, Ayrault et al. 2014).

Although previous Pb emissions from leaded petrol have left a legacy of elevated concentrations in the environment, this source of environmental Pb has declined throughout the years with consequent changes in Pb isotopic composition ratios. Indeed, a recent review by Kristensen (2015) showed that the annual Pb emissions from leaded petrol combustion in Australia dropped from 7,000 tonnes to 100 tonnes between the period 1970s to 2000s. These downwards shifts in atmospheric Pb emissions were reflected in reduced blood Pb concentrations in children, falling from 20 µg/dL in the 1970s to < 5 µg/dL in the 2000s. Lead isotopic compositions in the air sheds of major cities shifted from Broken Hill type Pb isotopic composition signatures (predominant source of leaded petrol) toward ratios that more closely matched local uncontaminated soil and rocks (Kristensen et al. 2017, Wu et al. 2017b). The changes of Pb concentrations and isotopic compositions were also reflected in European honey bees. For example, given that the use of leaded petrol ceased in 2011 in Serbia, a decline in Pb concentrations was expected in European honey bees. Indeed, Zaric et al. (2018) found a clear reduction in Pb concentration in European honey bees from 4.0 mg/kg in 2013 to 0.3 mg/kg in 2015. Furthermore, the Pb isotopic compositions in European honey bees shifted from $^{206}Pb/^{207}Pb$ 1.179 and $^{208}Pb/^{207}Pb$ 2.67 in 2013 to $^{206}Pb/^{207}Pb$ 1.163 and $^{208}Pb/^{207}Pb$ 2.923 in 2015, reflecting contemporary local environmental Pb sources (i.e. wood areas, agricultural land, highways and coal fired thermal power plants) in the neighbouring area (Zaric et al. 2018).

Other sources

Apart from mining and leaded petrol deposition, variations in Pb isotopic compositions in beehive products from both native and European bee species sampled at the individual sites suggest that a range of Pb sources persist in the Sydney environment (Figure 6-7) (Zhou et al. 2018a, Zhou et al. 2018b). According to previous Australian studies, other Pb sources in beehive products could include those from former Pb-based paints (Laidlaw et al. 2014, Rouillon 2017), traffic exhausts (Birch and Scollen 2003), coal combustion (Díaz-Somoano et al. 2009, Cohen et al. 2012) and industrial emissions (Chiaradia et al. 1997, Cohen 1999, Cohen et al. 2002, Davis and Gulson 2005, Gulson et al. 2007, Marx et al. 2010, Wu et al. 2016b).

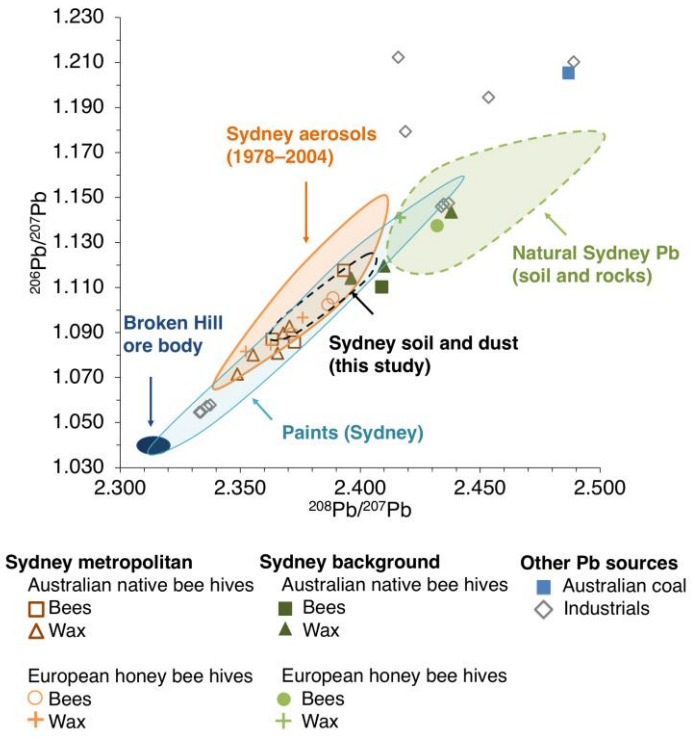

Figure 6-7. Mean lead isotope compositions ($^{208}Pb/^{207}Pb$, $^{206}Pb/^{207}Pb$) for native and European beehive samples across the Sydney region. The shaded areas represent characteristic signatures of: green – Sydney background (Wu et al. 2016b); orange – Sydney aerosols (Wu et al. 2016b); light blue – Sydney Pb-based paints (Laidlaw et al. 2014, Rouillon 2017); dark blue – Broken Hill ore body (Gulson 1984, Kristensen and Taylor 2016). Coal and industrial data were

obtained from Díaz-Somoano et al. (2009) and Chiaradia et al. (1997), respectively (source: Zhou et al. 2018a).

Chapters 3 and 4 show that the Pb isotopic compositions in beehive products collected from different locations indicate the corresponding sources of environmental contamination. This is consistent with unpublished study conducted by Weis (2016) (Figure 6-8), which demonstrated that Pb isotopic compositions in honey collected from Vancouver metropolitan areas were derived from park trees and also anthropogenic sources.

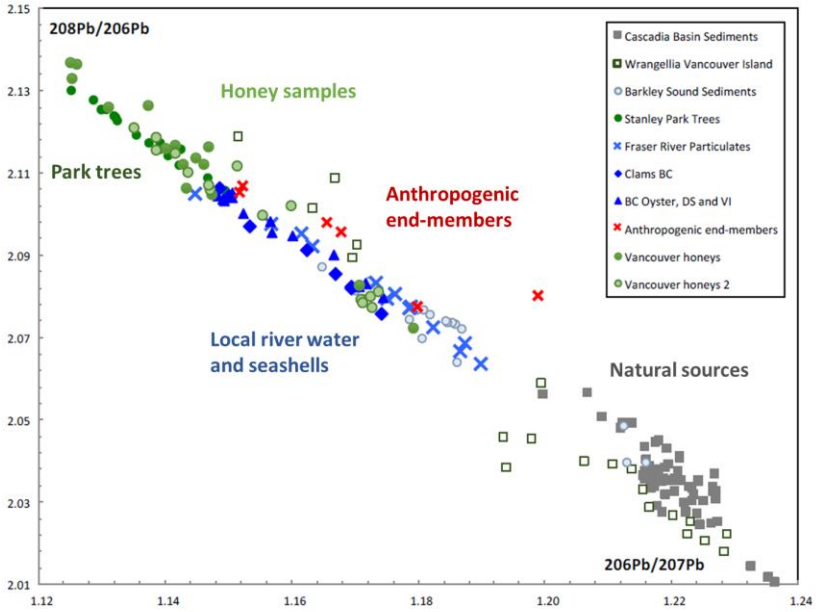

Figure 6-8. Lead isotopic compositions of $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ in Vancouver honey samples and surrounding environmental samples, i.e. park trees, water, seashells and sediments (source: Weis 2016). The author notes that this figure is subject to revision following additional research which is subject to an article by D. Weis that is currently under review (D. Weis, pers. comm).

The combination of positive correlations between beehive products and the environment as well as the apportionment of trace element sources provides substantial support for the practical application of beehive products as bio-indicators, not only for evaluating contamination levels

but also for tracing current and legacy emissions. This is significant for contemporary monitoring methods that detect changes in environmental contamination. The traditional approach for assessing trace element contamination in the environment (i.e. via hi-volume air sampling or through the collected of dust in passive deposition gauges) is labour intensive and requires an evenly distributed set of sample collection sites to adequately characterise contamination across a specific environment (Holt and Miller 2011). Measurement of trace element contamination across isolated and inaccessible areas can also be extremely difficult in broad scale geographic studies (Harry et al. 2011). As such, the alternative approach of using beehive products as bio-indicators is relatively low cost and far less labour intensive. The data presented in this thesis and other studies suggesting this method has clear potential as an efficient and sensitive approach for indicating environment stress caused by trace element contamination, and could provide an early warning system to signal potential risks.

6.3 Investigating honey quality and developing a method to identify its geographic origin

Chapter 3 and Chapter 4 use isotopic analysis of Pb to trace contamination sources, but isotope techniques have other applications, including applications in food authentication (Dennis 1998, Reid et al. 2006). Carbon isotopic ratios have been recognised as an official method (AOAC 978.17, 1995; AOAC 991.41, 1995; AOAC 998.12, 2014) to detect sources of sugar in honey, including industrial processed sugars and sugars derived from plants foraged by bees.

Chapter 5 of this thesis uses multiple AOAC methods in addition to other analytical approaches (Dong et al. 2016) to investigate honey purity, i.e. whether C-4 sugars have been added to commercial honey samples (n = 95) collected from 19 countries covering Africa, Asia, Europe, North America and Oceania. The results show that 27 % of the global commercial honeys tested (26 out of 95 samples) were of questionable authenticity due to adulteration with C-4 sugars (Figure 6-9). Most of the honeys that were identified as being adulterated (52 %) were from

Asian countries (Figure 6-9). In Australia, 15 % (5 out of 33) of honeys from mainland regions and 22 % (2 out of 9) from Tasmania were also identified as likely to have been subject to adulteration with C-4 sugars. The adulteration rate of honey reported in Chapter 5 is possibly underestimated, given that new adulteration practices, including the addition of C-3 sugars to honey, which are unfortunately difficult to detect using the current AOAC methods (Soares et al. 2017, Wu et al. 2017a).

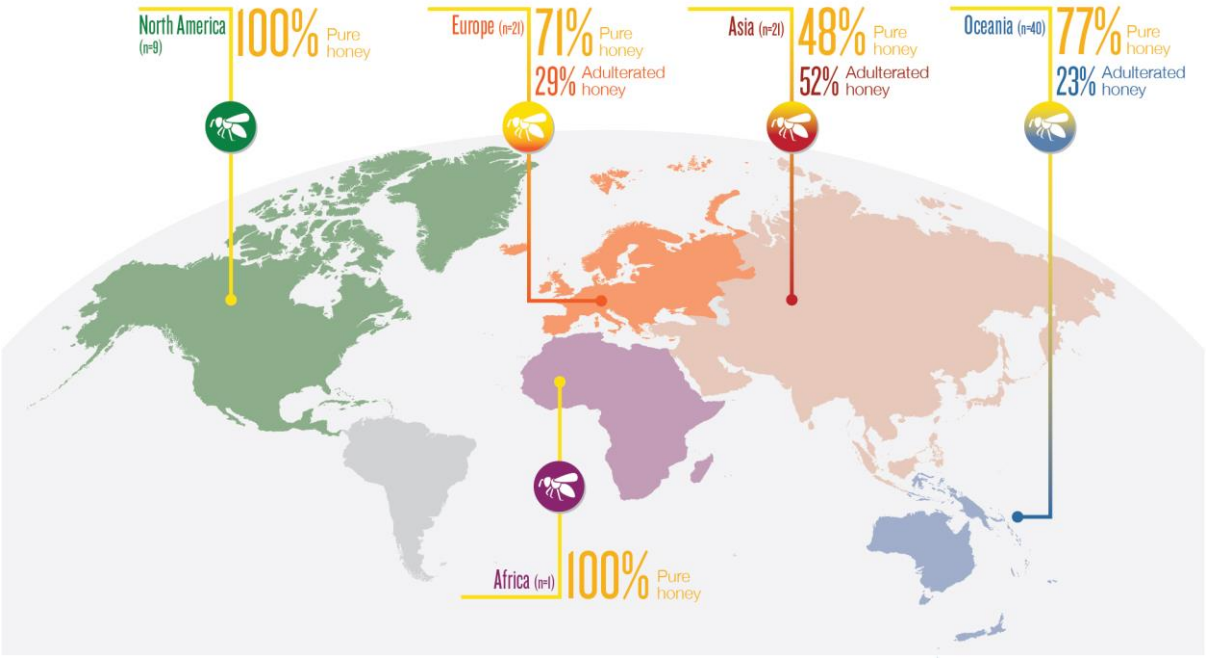

Figure 6-9. Investigation of purity of global honey with respect to adulteration with C-4 sugars (source: Zhou et al. 2018c).

In addition to the adulteration of honey through the inclusion of C-4 sugars, deliberate mislabelling of the place of geographic origin is also one of the most common fraudulent practices in the global honey market (Figure 6-10) (Soares et al. 2017). However, the detection of mislabelling remains a challenge and has yet to be resolved (Wang and Li 2011).

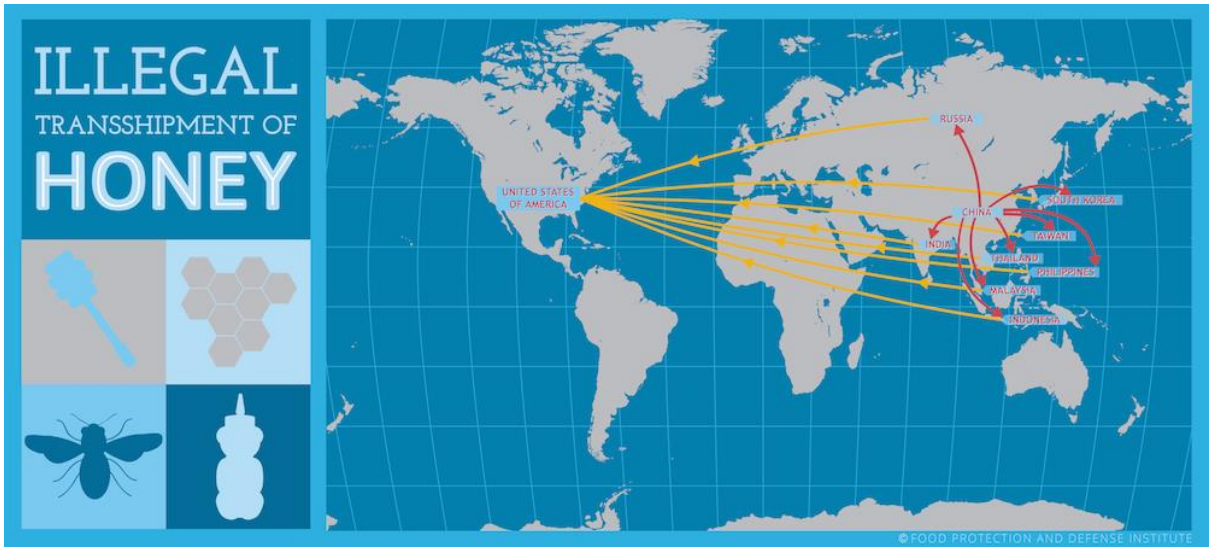

Figure 6-10. An example of illegal honey trans-shipment (honey laundering) from China to the United States of America. Honey from China attracts a high tariff when imported directly into the USA, and thus is shipped to various other countries, including Russia, South Korea, India, Thailand, Taiwan (China), Malaysia, Indonesia and the Philippines where it is re-labelled with a country of origin label indicating it is from the intermediate country. The honey is then shipped to the USA, thereby avoiding the higher tariff (source: Everstine 2013).

Although trace elements have been used previously as signatures for geographic authentication of honey at a regional scale (Tuzen et al. 2007, Baroni et al. 2009b, Batista et al. 2012, Pellerano et al. 2012, de Andrade et al. 2014, Di Bella et al. 2015), no study has investigated whether honey can be separated at the broader continental level to authenticate its origin. Chapter 5 measured multiple trace elements (Al, Ba, B, Ca, Cu, Fe, Mg, Mn, Ni, P, K, Rb, Na, Sr, Sn and Zn) in authentic honey samples (n = 69) which passed the criteria of C-4 sugar testing. Analysis using PCA and CDA showed distinct clusters in honey from mainland Australia and Tasmania as well as honey from different continents (Figure 6-11). The modelling results further demonstrate that K, Mn, P and Sr are the key trace elements that differentiate 95 % (19 of 20 samples) of honey samples from mainland Australia, 71 % (5 of 7 samples) of honey samples from Tasmania and 71 % (10 of 14 samples) of European honey samples in four end nodes (Zhou et al. 2018c).

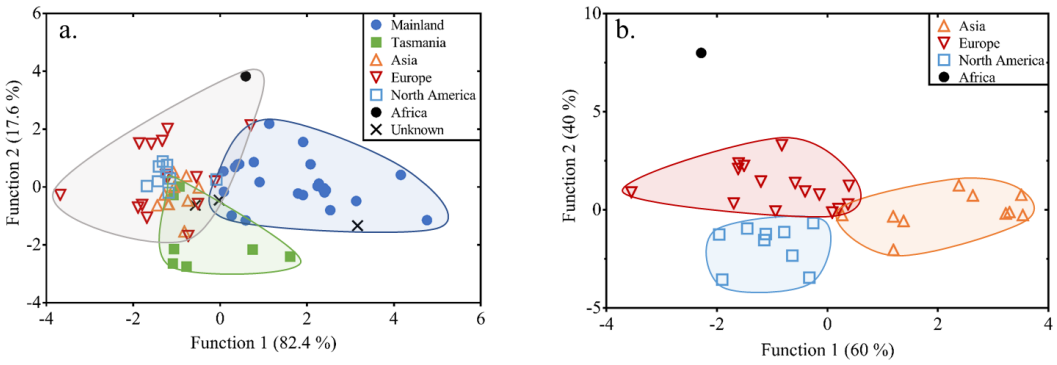

Figure 6-11. Score plots for two functions to distinguish all authentic honey samples (n = 69) from mainland Australia (n = 24), Tasmania (n = 7), Asia (n = 10), Europe (n = 15), North America (n = 9), Africa (n = 1), unknown geographic origin (n = 3) (source: Zhou et al. 2018c).

The findings presented in Chapter 5 are of particular significance to the Australian honey industry, whose honey is characterised internationally as safe and of high quality, but has been revealed in some cases to be adulterated by impure honey and deliberately mislabelled (Robb 2014, Clemons 2016). Moreover, it was recently reported that a leading Australian supermarket chain (Coles) removed imported honey (Figure 6-12) from their shelves from July 2018, in order to '*meet the needs of customers*' (Claughton 2018). This reflects a rise in consumer awareness and importance of a food's origin that may be linked to safety and sustainability. For honey, this suggests that the labelling of its origin, and the ability to authenticate this, is of increasing importance to consumers. Hence, developing a method to identify the geographic origin of honey is a practical research outcome. Sniderman et al. (2018) used melissopalynology, the pollen analysis of honey, to identify Australian honey through the number of pollen morphotypes per sample of Australian Eucalyptus honey (mean = 4.6), which was found to be different from that in honey produced in Mediterranean regions and South America (≤ 2). This study together with the approach detailed in Chapter 5 have clear potential to provide effective methods to detect the authenticity and origin of Australian honey, as well as to differentiate it from overseas samples.

Figure 6-12. The honey brand Allowrie was removed from store shelves in the Australian supermarket chain Coles (July 2018) due to the fact that the product's labelling concealed its country of origin from consumers. Country of origin honey labelling (OZBargain 2018) must follow the standards set by the Australian Competition and Consumer Commission (ACCC 2017).

6.4 Future directions

6.4.1 Potential future work on bio-indicators

Although there are around 25,000 bee species on earth (Gould 2015), only a limited number of these species (such as *A. mellifera, Bombae, T. carbonaria* and *T. angustula*) have been investigated for their capacity as bio-indicators in the assessment of trace element contamination. Chapters 3 and 4 verified that the two species *A. mellifera* and *T. carbonaria* are robust and reliable bio-indicators of trace element contamination. Given the wide distribution of both species (Figure S6-1 and S6-2, Appendix F) these species also have clear potential to be applied as bio-indicators in other countries, especially tropical areas which have both *A. mellifera* and *T. carbonaria.*

Although total trace element concentrations in *A. mellifera* and *T. carbonaria* are detailed in this thesis, no information on trace element bioaccessibility was undertaken. Data on trace element bioaccessibility reflects the fraction of total trace elements that can enter the systemic circulation of an organism, potentially resulting in adverse health outcomes at certain

concentrations (Intawongse and Dean 2006, Turner and Ka-Hei 2007). In order to address this research gap on trace element bioaccessibility, it is recommended that future studies assess the effect of trace elements on bees and their influence on foraging behaviour.

6.4.2 Potential future work on source apportionment

The Pb isotopic composition analysis ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$, $2^{08}Pb/^{207}Pb$) is an established technique for determining possible sources of Pb (i.e. natural or anthropogenic) that are present in the environment (Patterson and Settle 1987, Bollhöfer and Rosman 2000, Bollhöfer and Rosman 2001, Komarek et al. 2008). In Chapters 3 and 4, Pb isotopic composition analysis was applied to identify distinguish geogenic Pb from legacy Pb depositions associated with the use of leaded petrol in raw beehive samples. The results also demonstrated that there were other sources that contributed Pb contamination to beehive products (Chapter 4). Other potential Pb contamination sources includ the former use of Pb-based paints, coal combustion and other industrial sources (Chapter 4). However, it was not possible to identify these other specific Pb contaminant sources because of a lack of Pb isotope data.

Trace element concentration ratios can be utilized as a complementary and additional method to Pb isotopic composition analysis to identify the relative contributions of natural and anthropogenic sources in environmental samples (Ayuso and Foley 2008, Desaules 2012, Dewan et al. 2015, Ledoux et al. 2017). For example, the ratios of Cr/Mn and As/Cd were used to distinguish geogenic contributions from hydrothermal activity versus those sourced from industrial activities found in local soils, slags, scums and landfill materials (Tarzia et al. 2002). Lin et al. (2015) used trace elemental ratios based on Cu (i.e. Fe/Cu, Ba/Cu, Sb/Cu, Sn/Cu and Ga/Cu), which is considered to be a tracer of traffic-related brake wear abrasion. The ratio of Pt and Pd concentrations were used to identify traffic contamination in residential soils that was derived from three-way catalytic converters (Cicchella et al. 2003). However, with respect to the analysis of contaminants in beehives there has been no published use of trace element

concentration ratios to identify contamination sources in beehive samples. Given the successful application of trace element ratios in source apportionment across soil and air and other biological materials (e.g. Varrica et al. 2000, Tarzia et al. 2002, Dolgopolova et al. 2006, Dewan et al. 2015, Ledoux et al. 2017), its application in future beehive contamination research is warranted.

6.4.3 Potential future work on honey authentication

Chapter 5 investigates the quality of Australian honey and develops a promising approach to distinguish Australian honey from overseas honey. Recommended future investigations could shift the focus to identifying honey sourced from different Australian states. This would be of particular significance to honey produced from Western Australia, Jarrah and Marri, which have been verified as having high antimicrobial properties, comparable to the prized New Zealand Manuka honey (Irish et al. 2011, Fitzgerald 2017, Roshan et al. 2017, Murphy 2018, Pancia 2018). Compiling a database of trace element signatures for Jarrah and Marri honey *inter alia* would be beneficial in the detection of potential adulteration in the growing honey market, protecting the reputation of this specialist regional Australian honey.

Unadulterated honey should be genuinely pure, i.e. produced by bees from the nectar of plants, and should comply with prescribed product information as labelled. Improving techniques to detect adulterated honey still remains a challenge, particularly in the face of continuous development of new and more sophisticated adulteration practices (i.e. adding C-3 rice sugars in honey and indirect practice of feeding bees with C-3 sugars) coupled to that lack of official accepted analytical methods to detect these new fraudulent practices (Zábrodská and Vorlová 2015). Existing official methods (AOAC 978.17, 1995; AOAC 991.41, 1995; AOAC 998.12, 2014) are currently only used to detect C-4 sugars in honey, and are not adequate for distinguishing other adulteration practices, such as the intentional mislabelling of honey. The study detailed in Chapter 5, together with other international studies (Spain – Hernández et al.

2005, Turkey – Tuzen et al. 2007, Argentina – Baroni et al. 2009a, Slovenia – Kropf et al. 2010, Brazil – Batista et al. 2012, de Andrade et al. 2014, Italy – Di Bella et al. 2015, and Romania – Oroian et al. 2015), demonstrates the potential utility of using honey trace element signatures identifying geographic origin. However, it is evident that stricter regulation is required in the global honey market, coupled with a need to develop better methods of validation and standardisation in order to overcome persistent and costly fraudulent practices and guarantee food safety and quality.

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Conclusion

This thesis applies evidence-based data to evaluate the relationships between trace elements in the environment and those in beehive products, inclusive of raw samples through to commercially processed honey. The thesis, to the best of the author's knowledge, is the first study to:

(a) find a statistical positive relationship between trace element concentrations in soil and dust and those in beehive products—even though bees have been examined for their potential as bio-indicators since the early 1900s.

(b) use Pb isotopic analysis in beehive products to trace current and legacy contamination in the environment.

(c) compare different bee species, and their respective products, as suitable bio-indicators for determination of trace element contamination.

(d) use a combination of carbon isotopic ratios and trace element analysis to evaluate global honey samples for their authenticity and geographic origin.

The research presented in this thesis shows that European honey bees have strong interactions with the environment and can be used as effective 'samplers' for the presence and source of environmental contaminants. Given the worldwide distribution of *A. mellifera*, this study indicates that European honey bees and their products have the potential to also be used as bio-indicators of contamination globally.

In addition to European honey bees, the Australian native bee *T. carbonaria* also has demonstrable capacity to be used to assess levels of environmental contamination. *A. mellifera* forage over distances of ~ 5-9 km whereas *T. carbonaria* only forage over distances of up to 0.7 km, meaning that the native bee species has more utility for detailing contamination over smaller spatial areas. The finding that *T. carbonaria* and *A. mellifera* have demonstrable utility

as bio-indicators suggests that future research should assess the usefulness of other endemic bee species.

The results from this thesis add to the body of work examining bees and their products over the last 56 years for use bio-indicators for trace element contamination. Environmental monitoring via bees and their products can replace or supplement the higher cost and more intensive sampling processes required for traditional air and dust sampling methods. Such an approach enables more detailed spatial and temporal information to be collected, providing greater opportunity for the early warning of potential environmental health risks.

The research in this thesis also examines global honey authenticity and reveals that the practice of adding sugar to honey is an ongoing and pervasive problem. This issue is of wider concern to *bona fide* producers as well as the general public, who purchase and consume honey in the expectation that the product they are buying is just that—pure honey made from the nectar and pollen of flowering plants. The evidence in this thesis highlights the need for further and more intensive remedial action by appropriate authorities to curb this fraudulent practice. The additional finding that trace elements in honey can be used to fingerprint a product's geographic origin, at both regional Australian level and continental scale, is of particular use and significance in protecting the Australian honey industry. The research also has implications for the potential use of trace elements to solve the issue of geographic source of origin mislabelling that is commonplace in the global honey market. Future work on the identification of the geographic origin of honey should shift the focus to specific honeys that are sourced from unique locations, such as Jarrah and Marri honey from Western Australia, which have desirable antimicrobial properties. Such research will be valuable in helping to protect the inherent advantages and market benefits of regional-specific beekeepers, as well as maintaining the reputation of locally produced honey.

Appendices

Appendix A: Supplementary Information for CHPATER ONE

Supplementary Table S1

Supplementary Table S2

Appendix B: Supplementary Information for CHPATER TWO

Supplementary Table S1

Supplementary Table S2

Appendix C: Supplementary Information for CHPATER THREE

Supplementary Table S1

Supplementary Table S2

Supplementary Table S3

Supplementary Table S4

Supplementary Table S5

Supplementary Figure S1

Supplementary media coverage S1

Supplementary media coverage S2

Supplementary media coverage S3

Supplementary media coverage S4

Appendix D: Supplementary Information for CHPATER FOUR

Supplementary Table S1

Supplementary Table S2

Supplementary Table S3

Appendix E: Supplementary Information for CHPATER FIVE

Supplementary Table S1

Supplementary Table S2

Supplementary Table S3

Supplementary Table S4

Supplementary Table S5

Supplementary Table S6

Supplementary Figure S1

Supplementary Figure S2

Supplementary Figure S6

Supplementary media coverage S1

Supplementary media coverage S2

Supplementary media coverage S3

Supplementary media coverage S4

Supplementary media coverage S5

Supplementary media coverage S6

Supplementary media coverage S7

Supplementary media coverage S8

Appendix F: Supplementary Information for CHPATER SIX

Supplementary Figure S1

Supplementary Figure S2

Appendix A

Supplementary Table S1. Monitoring studies using European honey bees and their relative products to examine trace element contamination across polluted and unpolluted areas.

Country	Relative products	Trace elements	References
Ardabil	Honey	As, Cd, Cr,Cu, Ni,Pb, Zn	Aghamirlou et al. (2015)
Argentina	Honey	As, Br, Cu, Cr, Mn, Pb, Rb, Se, Sr, Ti, Zn,	Enrich et al. (2007)
Belgium	Honeybees	Al, As, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, V, Zn.	Van Der Steen et al. (2012)
Bulgaria	Honeybees, fecal mass, honey, pollen, wax	Cu, Zn, Pb, Cd, Ni, Co, Mn, Fe	Zhelyazkova (2010)
Brazil	Honey	Br, Ca, Cu, Cr, Fe, Ni, K, Se, Sr, Ti, Mn, Zn,	Ribeiro et al. (2015)
Bulgaria	Honeybees, excrements	Cu, Zn, Pb, Cd, Ni, Co, Mn, Fe	Zhelyazkova (2012)
Bulgaria	Honey	As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Zn	Atanassova et al. (2016)
Croatia	Honey	Ca, Cr, Cu, ^{137}Cs, Fe, ^{40}K, Mn, Ni, Pb, Rb, Sr, Zn	Barišić et al. (1999)
Croatia	Honey	As, Cd, Cu, Hg, Pb	Bilandžić et al. (2011)
Czechoslovakia	Honeybees, drones, larvae, honey, propolis, wax	Pb, Cd, Cu, Zn	Velemínský et al. (1990)
Egypt	Honey	Co, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn	Rashed et al. (2009)
Egypt	Honeybees, honey, pollen	Cd, Cu, Fe, Pb, Zn	Naggar et al. (2013)
Egypt	Honeybees, honey, pollen	Cd, Cu, Fe, Pb, Zn	Naggar et al. (2013)
France	Honeybees, honey, pollen	Pb	Lambert et al. (2012)
Germany	Honey	Pb	Voget (1989)
Germany	Honey	Al, B, Ba, Bi, Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Si, Ti, V, Zn	Raeymaekers (2006)
Hungary	Honey	As, Ba, Cu, Ni, Pb, Sr Mn, Zn	Czipa et al. (2017)
Italy	Honeybees, dead Honeybees, honey	Cd, Pb, Zn	Leita et al. (1996)
Italy	Honey	Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, Pb, Sb, Se, Sr, Th, Tl, U, Zn	Pisani et al. (2008)
Italy	Honeybees	Cr, Cd, Hg, Pb	Perugini et al. (2011)
Italy	Honeybees	Cd, Cr, Pb	Satta et al. (2012)
Italy	live and dead honeybees, honey	Cd, Cr, Ni, Pb	Ruschioni et al. (2013)
Italy	Honeybees	As, Bi, Cd Cu, Co, Cr, Ni, Pb, Sr, V, Zn	Giglio et al. (2017)

Country	Relative products	Trace elements	References
Italy	Honey	Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Ge, Hg, Mn, Mo, Pb, Sb, Se, Sn, Sr, Ti, Tl, U, V, Zn	Quinto et al. (2016)
Iran	Honeybees	Hg, Ba, Ca, Fe, Mn, Li, As, Na, K	Sadeghi et al. (2012)
Macedonia	Honey	Ca, Cd, Cu, Fe, Mg, Mn, Na, K, Zn	Stankovska et al. (2008)
Netherlands	Pollen	Pb	Ernst and Bast-Cramer (1980)
Netherlands	Honeybees	Al, As, Ba, Cd, Co, Cr, Cu, Li, Mn, Mo, Ni, Sb, Se, Sn, Sr, Ti, V, Zn	Van Der Steen et al. (2016)
Nigeria	Honey	Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn	Iwegbue et al. (2015)
Poland	Honey, Pollen	Cd, Cu, Pb,	Jablonski et al. (1995)
Poland	Honey	Cd, Pb, Zn	Przyby-lowski and Wilczyńska (2001)
Poland	Honeybees, honey	Cu, Se, Pb, Cd	Roman (2010)
Poland	Honey, propolis, wax, pollen	Cd, Ni, Pb, Fe, Mg, Zn	Formicki et al. (2013)
Romania	Honey	Pb	Codreanu et al. (2009)
Romania	Honeybees, honey, pollen	Cd, Cu, Fe, Mn, Ni, Pb, Zn	Bordean et al. (2007)
Romania	Pollen	Fe, Mn, Zn	Dima et al. (2012)
Romania	Honeybees, drones, propolis, wax, bee larvae, honey, royal jelly	Pb	Mihaly Cozmuta et al. (2012)
Serbia	Honeybees	Al, Ba, Cd, Co, Cr, Cu, Fe, Li, Mn, Na, Ni, Pb, Sr, Zn	Zarić et al. (2016)
Slovak	Honey	As, Cd, Cr, Hg, Pb	Lazor et al. (2012)
Spain	Honey	Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, Zn	Rodríguez García et al. (2006)
Spain	Honeybees	Cd, ,Cr, Pb, Ni	Gutiérrez et al. (2015)
Turkey	Honey	Cu, Cd, Fe, Mg, Mn, Ni	Erbilir and Erdoğrul (2005)
Turkey	Honey	Al, Cd, Cu, Cr, Fe, Mn, Ni, Pb, Se, Zn	Tuzen et al. (2007)
Turkey	Honey	Al, Co, Cr, Cu, Fe, Mn, Ni, Zn	Yarsan et al. (2007)
Turkey	Honeybees, honey	Al, Cu, Cd, Cr, Fe, Pb, Ni, Mn, Zn	Silici et al. (2016)
U.K.	Pollen	Cu, Pb, Mn, Zn	Free et al. (1983)
U.K.	Honey	Ag, Cd, Cu, Pb	Jones (1987)
Ukraine	Honeybees, bee tissues, honey, pollen , wax	Cr, Zn. Cu. Fe. Cd, Pb	Fedoruk et al. (2015)
U.S.	Honey	47 trace elements	Tong et al. (1975)
U.S.	Honeybees, pollen	As, Cd, Cu, Pb, Zn	Bromenshenk et al. (1985)
U.S.	Propolis	Al, Ca, Cd, Cr, Cu, K, Mg, Mn, Na, Pb, Zn	Finger et al. (2014)

Supplementary Table S2. The studies showed that the use of European honey bees and their relative products were not suitable bio-indicators to examine trace element contamination.

Country	Relative products	Trace elements	Conclusions	References
Netherlands	Honeybees	Cd, Pb	No significant correlations were found between honeybees and air quality.	Van Der Steen et al. (2015)
France	Honey, wax	As, Cd, Tl, Pb	Bee products were relatively free of trace element contamination in the mining area.	Losfeld et al. (2014)
Spain	Honey, pollen	As, Cd, Mo, Sb, Zn	Trace elements in honey sampled from mining areas were below the analytical limit of quantification, and honey proved not to be useful as a bio-indicator.	Alvarez-Ayuso and Abad-Valle (2017)
Italy	Honeybees, honey, pollen	Cd, Cr, Pb, Ni	No positive correlations were found between beehive products and the air quality.	Balestra et al. (1992)
Italy	Honeybees, honey, pollen, propolis, wax	Cd, Cr, Pb	Very low concentrations of trace elements were found in honey at polluted areas, and there was no statistical difference in trace elements between honey collected from polluted and unpolluted areas.	Conti and Botre (2001)
Switzerland	Honey	Cd, Pb, Cr, Mn, Fe, Ni, Cu, Zn	Trace element contents in honey were primarily due to botanical origin rather than geographical or environmental exposition.	Bogdanov et al. (2015)
Finland	Honeybees, honey, pollen	Cd, Pb, Cu, Fe, Mn, Zn	The trace element concentrations in honey were very low and there were no significant differences among sampling areas.	Fakhimzadeh and Lodenius (2000)

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Appendix B

Table S1. The sampling information of raw beehive samples coupled with soil and dust collected across Sydney and Broken Hill.

a. The sampling information of European honey bees and their products		
Location	**Sample**	**Date**
Galston	Honeybees, honey and wax	21/11/2015
	Honeybees	19/12/2015
	Honeybees	Feb-16
	Honeybees and honey	9/03/2016
	Honeybees	7/04/2016
Gordon	Honeybees and honey	20/01/2016
	Honeybees	8/03/2016
	Honeybees, honey and wax	9/04/2016
Coogee	Honeybees, honey and wax	22/11/2015
	Honeybees	18/12/2015
	Honeybees	21/01/2016
	Honeybees	9/03/2016
	Honeybees, honey and wax	6/04/2016
Randwick	Honeybees, honey and wax	6/11/2015
	Honeybees	9/12/2015
	Honeybees	20/01/2016
	Honeybees	9/03/2016
	Honeybees	6/04/2016
Sydney CBD	Honeybees, honey and wax	9/12/2015
	Honeybees	21/01/2016
	Honeybees	8/03/2016
	Honeybees, honey and wax	7/04/2016
Surry Hills	Honeybees, honey and wax	20/01/2016
	Honeybees	8/03/2016
	Honeybees and wax	6/04/2016
Newtown	Honeybees, honey and wax	18/12/2015
	Honeybees	20/01/2016
	Honeybees	8/03/2016
	Honeybees	6/04/2016
Marrickcille	Honeybees, honey and wax	18/12/2015
	Honeybees	20/01/2016
	Honeybees	8/03/2016
	Honeybees, honey and wax	6/04/2016
Mascot:	Honeybees, honey and wax	9/12/2015
	Honeybees	21/01/2016
	Honeybees	9/03/2016
	Honeybees, honey and wax	6/04/2016
Broken Hill	Honeybees, honey and wax	2015 Nov-Dec

b. The sampling information of Australian native bees and their products		
Location	**Sample**	**Date**
St. Ives	Native bees	8/03/2016
	Native bees	20/01/2016
	Honey and wax	1/10/2016
Gordon	Native bees	1/11/2015
	Native bees	8/03/2016
	Honey and wax	1/10/2016
Roseville	Native bees, honey and wax	13/11/2015
Maroubra	Native bees and honey	9/10/2015
	Native bees	30/11/2015
	Native bees	18/12/2015
	Honey and wax	1/10/2016
Bronte	Native bees, honey and wax	9/10/2015
	Native bees	9/12/2015
	Native bees	21/01/2016
	Honey and wax	1/10/2016
Randwick	Native bees, honey and wax	9/10/2015
	Native bees	6/11/2015
	Native bees	21/01/2016
	Honey and wax	1/10/2016
Newtown	Native bees and wax	9/10/2015
	Honey and wax	1/10/2016
Marrickville	Native bees and honey	25/10/2015
	Honey and wax	1/10/2016
c. The sampling information of soil and dust across Sydney and Broken Hill		
Location	**Sample**	**Date**
Galston	Dust and soil	25/10/15-21/11/15
	Dust	21/11/15-19/12/15
	Dust	19/01/16-13/02/16
	Dust	13/02/16-09/03/16
	Dust	09/03/16-09/04/16
St. Ives	Dust and soil	13/11/15-08/12/15
	Dust	08/12/15-20/01/16
	Dust	20/01/16-08/03/16
	Dust	08/03/16-07/04/16
Roseville#1	Dust and soil	13/11/15-08/12/15
	Dust	08/12/15-20/01/16
	Dust	21/01/16-08/03/16
	Dust	08/03/16-07/04/16
Roseville#2	Dust and soil	13/11/15-08/12/15
	Dust	08/12/15-07/01/16
	Dust	07/01/16-08/03/16
	Dust	08/03/16-07/04/16
Gordon	Dust and soil	19/12/15-20/01/16
	Dust	20/01/16-08/03/16
	Dust	08/03/16-09/04/16
Coogee	Dust and soil	25/10/15-22/11/15
	Dust	22/11/15-18/12/15

Location	Sample	Date
Coogee	Dust	18/12/15-21/01/16
	Dust	21/01/16-09/03/16
	Dust	09/03/16-06/04/16
Maroubra	Dust and soil	09/10/15-08/11/15
	Dust	08/11/15-18/12/15
	Dust	18/12/15-30/01/16
	Dust	30/01/16-09/03/16
	Dust	09/03/16-07/04/16
Bronte	Dust and soil	09/10/15-06/11/15
	Dust	06/11/15-09/12/15
	Dust	09/12/15-21/01/16
	Dust	21/01/16-08/03/16
	Dust	09/03/16-07/04/16
Randwick	Dust and soil	09/10/15-06/11/15
	Dust	06/11/15-09/12/15
	Dust	09/12/15-21/01/16
	Dust	21/01/16-09/03/16
	Dust	09/03/16-06/04/16
Sydney CBD	Dust and soil	06/11/15-09/12/15
	Dust	09/12/15-21/01/16
	Dust	21/01/16-08/03/16
	Dust	08/03/16-07/04/16
Surry Hills	Dust and soil	06/11/15-20/01/15
	Dust	20/01/16-08/03/16
	Dust	08/03/16-06/04/16
Newtown	Dust and soil	06/11/15-09/12/15
	Dust	18/12/15-20/01/16
	Dust	20/01/16-08/03/16
	Dust	09/10/15-08/11/15
	Dust	08/03/16-07/04/16
Marrickcille	Dust and soil	27/11/15-18/12/15
	Dust	18/12/15-20/01/16
	Dust	20/01/16-08/03/16
	Dust	08/03/16-06/04/16
Mascot:	Dust and soil	06/11/15-09/12/15
	Dust	09/12/15-21/01/16
	Dust	21/01/16-09/03/16
	Dust	09/03/16-06/04/16
Broken Hill	Dust and soil	Nov. 2015

Notation:

1) Beehive types: Nine European honey bee hives were Langstroth hives and used wax sheets. All the native bee hives (n = 18) were boxes without frames, and one of the European honey bee hive was a top bar hive which does not require frames with wax sheets.
2) Wax: The newly produced combs (white to light yellow) were collected.
3) Honey: Honey collected in this thesis was nectar honey due to the blossoms throughout the year in Sydney and Broken Hill.

Table S2. The sample information of 95 commercial honeys purchased from 19 countries on five continents.

Sample No.	Area	Honey types	Donators
N17/021848	Australia (Brisbane, QLD)	Macadamia honey	--
N17/021850	Australia (Ocean View, QLD)	Yapunyah honey	--
N16/028469	Australia (Kuranda, QLD)	Wildflower	--
N17/021840	Australia (Kuranda, QLD)	Kuranda flower	Barry Wong
N17/021841	Australia (Kuranda, QLD)	Tropic Fusion	Barry Wong
N17/021846	Australia (Kuranda, QLD)	Fiddlewood	Barry Wong
N16/025499	Australia (Byron Bay, NSW)	NA	NMI
N16/025501	Australia (Grenfell, NSW)	NA	NMI
N17/021835	Australia (Herne Hill, WA)	Parrot Bush	NMI
N17/021836	Australia (Herne Hill, WA)	Karri	NMI
N17/021837	Australia (Herne Hill, WA)	York Gum	NMI
N17/021838	Australia (Herne Hill, WA)	Mallee	NMI
N17/021839	Australia (Herne Hill, WA)	Bush honey	NMI
N16/025502	Australia (Perth, WA)	Jarrah honey	NMI
N17/021830	Australia (Moorooduc, VIC)	Clover	NMI
N17/021831	Australia (Moorooduc, VIC)	Loval flora	NMI
N17/021832	Australia (Moorooduc, VIC)	Red Gum	NMI
N17/021833	Australia (Moorooduc, VIC)	Organic	NMI
N17/021834	Australia (Moorooduc, VIC)	Banksia	NMI
N17/021847	Australia (Moorooduc, VIC)	Eucalyptus	NMI
N17/021849	Australia (Northcote, VIC)	Eucalyptus	--
N17/021854	Australia (Moonah, TAS)	Leatherwood	Damian Gore
N17/021852	Australia (Perth, TAS)	Leatherwood	--
N17/021853	Australia (Sandy Bay, TAS)	Wildflower	--
N17/021856	Australia (Sandy Bay, TAS)	Wildflower	Damian Gore
N17/021857	Australia (Sandy Bay, TAS)	Leatherwood	Damian Gore
N17/021858	Australia (Sandy Bay, TAS)	Leatherwood	Damian Gore
N17/021855	Australia (TAS) (Meader Valley)	NA	Damian Gore
N17/021851	Australia (TAS)	Leatherwood	--
N16/025500	Australia (TAS)	Letherwood honey	NMI
N17/021845	Australia (Kangaroo Island, SA)	Organic honey	Alison Downing
N17/021842	Australia (Totness, SA)	Organge Blossom	--
N17/021843	Australia (Totness, SA)	Meadow honey	--
N17/021844	Australia (Totness, SA)	Bush Mallee	--
N17/021859	Australia (Supermarket, Capilano)	NA	--
N17/021860	Australia (Supermarket, Capilano)	NA	--
N17/021861	Australia (Supermarket, Capilano)	NA	--
N17/021862	Australia (Supermarket, Capilano)	NA	--
N16/025498	New Zealand	Manuka blend	NMI
N17/021826	New Zealand	Manuka	--
N17/021863	Supermarket, Allowrie	Mixed blossom	--
N17/021864	Supermarket, Macro	organic pure honey	--
N17/021865	Supermarket, Wild flower	NA	--
N17/021819	Canada (Carlisle, Ontario)	Summer blossom	Phil
N17/021820	China (Henan)	Motherwort Honey	Lisa Liu
N17/021821	China (Henan)	Raw honey	Lisa Liu

Sample No.	Area	Honey types	Donators
N17/021822	China(Shanghai)	Linden honey	Angela
N17/021823	China(Shanghai)	Acacia honey	Angela
N17/021799	Greece	Wild flower	--
N17/021800	Greece	Conifers	--
N17/021801	Greece	Thyme	--
N17/021802	Greece	Thyme	--
N17/021803	Hungary	Akacmez	NMI
N17/021804	Hungary	Kosher akacmez	NMI
N17/021805	Hungary	Viragmez	NMI
N17/021824	India	NA	Shaina
N17/021809	Iran	NA	--
N17/021810	Iran	Milk Vetch	Neda Yousefi
N17/021806	Italy	Truffle honey	--
N17/021812	Macedonia	Acacia honey	--
N17/021813	Macedonia	Libereh Mea	--
N17/021814	Macedonia	LIIymcknmea	--
N17/021797	Romania	Multiflower	--
N17/021798	Romania	Acacia honey	--
N17/021807	Saudi Arabia	NA	--
N17/021808	Saudi Arabia	Acacia honey	--
N17/021825	South Korea	NA	--
N17/021811	Serbia	Acacia honey	--
N17/021816	USA (Greeley, CO)	Clover	Minna Hsu
N17/021817	USA (Landover, MD)	NA	Phil
N17/021815	USA (New York)	Natural raw honey	Doug Purdie
N17/021818	USA (Oxnard, CA)	pure honey	Phil
N17/021867	Iran		--
N17/021868	India	Pure honey	Kanchan Joshi
N17/021869	USA (Winchester, VA)	Pure honey	Kanchan Joshi
N17/021870	USA (Hillsboro, KS)	Thrifty bee honey	Kanchan Joshi
N17/021871	China(Shanghai)	Acacia honey	Liqin Wu
N17/021872	China(Guangzhou)	Longyan	Liqin Wu
N17/021873	China (Fujian)	Chrysanthemum	Liqin Wu
N17/021875	Indonesia	NA	Steven Murphy
N17/021876	Indonesia	NA	Steven Murphy
N17/021877	England	NA	Steven Murphy
N17/021878	Iran	NA	Steven Murphy
N17/021879	Europe	NA	Steven Murphy
N17/021880	Europe	NA	Steven Murphy
N17/021882	Japan	Cherry blossom	Steven Murphy
N17/021883	Japan	Linden honey	Steven Murphy
N17/021884	Japan	NA	Vera Sistenich
N17/021886	Italy	Acacia honey	Vera Sistenich
N17/021887	Italy	Sunflower	Vera Sistenich
N17/021888	Greece	Thyme	Vera Sistenich
N17/021889	Italy	Chestnut	Vera Sistenich
NA	Kenya	NA	Mark Taylor
NA	USA	NA	Louise Kristensen
NA	USA-Canada	NA	Louise Kristensen

--honey were purchased by myself from local food markets and commercial supermarkets.

NA: not available.

Appendix C

Supplementary Table S1. Concentrations of As, Mn, Pb and Zn in worker honeybees, drones, living worker honeybees and dead worker honeybees (µg/kg).

	As	Pb	Mn	Zn
Worker honeybees (n = 2)	25 (24 – 26)	120 (79 – 160)	27500 (15000 – 40000)	48500 (39000 – 58000)
Drones (n = 2)	11 (<10 – 21)	34 (26 – 41)	3450 (2100 – 4800)	34500 (31000 – 38000)
Live worker honey bees (n = 2)	16 (14 – 18)	270 (250 – 290)	24500 (22000 – 27000)	46500 (39000 – 54000)
Dead worker honey bees (n = 2)	113 (66 – 160)	3100 (3100 – 3100)	46000 (19000 – 73000)	95500 (41000 – 150000)

Supplementary Table S2. Concentrations of As, Mn, Pb and Zn in honeybees (µg/kg), honey (µg/kg), wax (µg/kg), soil (mg/kg) and dust (mg/kg) across the Sydney metropolitan region and the lead-zinc mining city of Broken Hill, Australia. Trace element concentrations are displayed to 3 significant figures.

Sites	Samples		As	Pb	Mn	Zn
Galston	Honey bees (µg/kg) [n = 5]	Mean ± SD Range	14 ± 3 11 – 20	50 ± 37 18 – 110	71100 ± 35100 33700 – 125000	45400 ± 6660 38000 – 55000
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	13 < 10 – 13	4580 2860 – 6300	465 420 – 510
	Wax (µg/kg) [n =1]	Mean ± SD Range	< 10 –	27 –	930 –	1860 –
	Soil (mg/kg) [n = 3]	Mean ± SD Range	3 ± 1 2 – 4	38 ± 17 18 – 49	267 ± 202 140 – 500	73 ± 7 67 – 80
	Dust (mg/kg) [n = 5]	Mean ± SD Range	38 ± 38 3 – 76	30 ± 14 7 – 42	366 ± 459 63 – 1170	435 ± 294 85 – 840
Gordon	Honey bees (µg/kg) [n = 3]	Mean ± SD Range	21 ± 4 17 – 24	56 ± 21 39 – 80	66000 ± 13100 52000 – 78000	55700 ± 3510 52000 – 59000
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	< 10 –	1320 1170 – 1460	803 780 – 825
	Wax (µg/kg) [n = 1]	Mean ± SD Range	< 10 –	12 –	200 –	2050 –
	Soil (mg/kg) [n = 3]	Mean ± SD Range	7 ± 3 4 – 10	155 ± 61 84 – 190	267 ± 50 220 – 320	323 ± 280 110 – 640
	Dust (mg/kg) [n = 3]	Mean ± SD Range	2 < 0.1 – 2	45 ± 18 34 – 66	121 ± 49 72 – 170	307 ± 127 160 – 380
Coogee	Honey bees (µg/kg) [n = 5]	Mean ± SD Range	36 ± 15 24 – 62	125 ± 108 47 – 300	34000 ± 15200 15000 – 49000	64200 ± 17100 39000 – 81000
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	< 10 –	385 350 – 420	658 525 – 790

Sites	Samples		As	Pb	Mn	Zn
Coogee	Wax (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	167 53 – 280	184 67 – 300	2260 1290 – 3220
	Soil (mg/kg) [n = 6]	Mean ± SD Range	3 ± 2 1 – 6	208 ± 247 15 – 640	61 ± 33 27 – 110	306 ± 350 16 – 810
	Dust (mg/kg) [n = 5]	Mean ± SD Range	75 ± 78 20 – 130	127 ± 62 50 – 200	184 ± 129 70 – 400	354 ± 195 170 – 570
Randwick	Honey bees (µg/kg) [n = 5]	Mean ± SD Range	57 ± 26 26 – 95	146 ± 123 35 – 320	52600 ± 13100 37000 – 65000	62400 ± 8910 56000 – 78000
	Honey (µg/kg) [n = 1]	Mean ± SD Range	< 10 –	< 10 –	435 –	235 –
	Wax (µg/kg) [n = 1]	Mean ± SD Range	< 10 –	73 –	170 –	1550 –
	Soil (mg/kg) [n = 6]	Mean ± SD Range	2 ± 1 1 – 3	20 ± 8 7 – 30	73 ± 38 16 – 120	60 ± 27 16 – 92
	Dust (mg/kg) [n = 5]	Mean ± SD Range	77 ± 69 12 – 170	124 ± 30 88 – 170	186 ± 119 91 – 380	592 ± 394 270 – 1240
Sydney CBD	Honey bees (µg/kg) [n = 4]	Mean ± SD Range	37 ± 18 25 – 63	225 ± 190 130 – 510	53000 ± 12800 37000 – 66000	57500 ± 7590 50000 – 65000
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	13 < 10 – 13	380 370 – 390	385 320 – 450
	Wax (µg/kg) [n =2]	Mean ± SD Range	14 < 10 – 14	296 72 – 520	3930 130 – 7730	32900 2430 – 6330
	Soil (mg/kg) [n = 3]	Mean ± SD Range	2 ± 1 1 – 3	31 ± 5 26 – 35	427 ± 57 380 – 490	183 ± 64 110 – 230
	Dust (mg/kg) [n = 4]	Mean ± SD Range	18 ± 22 2 – 34	52 ± 19 35 – 79	104 ± 20 87 – 130	215 ± 89 130 – 310
Surry Hills	Honey bees (µg/kg) [n = 3]	Mean ± SD Range	30 ± 14 18 – 46	377 ± 148 250 – 540	33000 ± 5570 27000 – 38000	52000 ± 3460 48000 – 54000
	Honey (µg/kg) [n= 1]	Mean ± SD Range	< 10 –	18 –	360 –	590 –

Sites	Samples		As	Pb	Mn	Zn
Surry Hills	Wax (µg/kg) [n =2]	Mean ± SD Range	< 10 –	66 46 – 85	151 81 – 220	1260 1060 – 1460
	Soil (mg/kg) [n = 3]	Mean ± SD Range	11 ± 4 8 –16	603 ± 194 390 – 770	153 ± 35 120 – 190	857 ± 327 480 – 1060
	Dust (mg/kg) [n = 3]	Mean ± SD Range	229 ± 228 68 – 390	940 ± 650 550 – 1690	417 ± 25 390 – 440	540 ± 26 510 – 560
Newtown	Honey bees (µg/kg) [n = 4]	Mean ± SD Range	36 ± 15 20 – 55	443 ± 159 290 – 660	35800 ± 12500 23000 – 53000	60800 ± 7140 51000 – 67000
	Honey (µg/kg) [n =1]	Mean ± SD Range	< 10 –	15 –	290 –	360 –
	Wax (µg/kg) [n = 1]	Mean ± SD Range	< 10 –	43 –	360 –	2150 –
	Soil (mg/kg) [n = 7]	Mean ± SD Range	7 ± 5 2 – 16	384 ± 421 110 – 1320	172 ± 61 63 – 250	191 ± 86 87 – 370
	Dust (mg/kg) [n = 5]	Mean ± SD Range	3 < 0.1 – 3	238 ± 48 180 – 300	286 ± 59 210 – 350	316 ± 144 120 – 490
Marrickville	Honey bees (µg/kg) [n = 4]	Mean ± SD Range	40 ± 10 27 – 50	238 ± 83 150 – 350	35300 ± 2060 33000 – 37000	57800 ± 2630 54000 – 60000
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	22 < 10 – 22	275 170 – 380	360 320 – 400
	Wax (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	131 61 – 200	123 86– 160	3840 2920 – 4750
	Soil (mg/kg) [n = 7]	Mean ± SD Range	23 ± 17 5 – 42	811 ± 585 150 – 1560	239 ± 76 140 – 390	999 ± 747 160 – 1980
	Dust (mg/kg) [n = 4]	Mean ± SD Range	24 ± 11 16 – 31	160 ± 124 66 – 340	116 ± 20 93 – 140	408 ± 441 90 – 1060
Mascot	Honey bees (µg/kg) [n = 4]	Mean ± SD Range	54 ± 15 41 – 75	418 ± 187 200 – 650	34500 ± 5750 30000 – 42000	62500 ± 4660 58000 – 67000
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	14 < 10 – 14	480 220 – 740	695 380 – 1010
	Wax (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	59 43 – 74	180 110 – 250	6280 2150 – 10400
	Soil (mg/kg) [n = 3]	Mean ± SD Range	3 ± 1 3 – 4	81 ± 44 43 – 130	223 ± 67 150 – 280	125 ± 52 66 – 160

Sites	Samples		As	Pb	Mn	Zn
Mascot	Dust (mg/kg) [n = 4]	Mean ± SD Range	2 ± 1 1 – 3	118 ± 10 110 – 130	700 ± 76 590 – 760	510 ± 92 410 – 630
Broken Hill	Honey bees (µg/kg) [n = 1]	Mean ± SD Range	160 –	2570 –	64700 –	36100 –
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	295 240 – 350	6280 5360 – 7190	2180 2150 – 2210
	Wax (µg/kg) [n = 2]	Mean ± SD Range	77 43 – 110	11650 7990 – 15300	3910 2640 – 5180	36000 18100 – 53900
	Soil (mg/kg) [n = 3]	Mean ± SD Range	23 ± 5 19 – 28	3290 ± 223 3120 – 3540	1450 ± 537 1090 – 2070	3890 ± 2000 2650 – 6200
	Dust (mg/kg)[1] [n = 1]	Mean ± SD Range	124 –	4360 –	3100 –	7570 –

– not available.

Supplementary Table S3. Summary Pearson r correlations and P values for trace elements and their relationship to beehive products of honey and wax.

(a) Pb correlations

	Pb bee	Pb honey	Pb wax	Pb soil
Pb honey	0.837			
	*0.003***			
Pb wax	0.789	0.819		
	0.007	*0.004***		
Pb soil	0.674	0.716	0.539	
	0.033	*0.020**	*0.108*	
Pb dust	0.882	0.786	0.730	0.806
	*0.001****	*0.007***	*0.017**	*0.005***

(b) Zn correlations

	Zn bee	Zn honey	Zn wax	Zn soil
Zn honey	-0.813			
	*0.004***			
Zn wax	-0.250	0.469		
	0.486	*0.171*		
Zn soil	-0.587	0.725	0.440	
	0.074	*0.018**	*0.203*	
Zn dust	-0.731	0.725	0.467	0.634
	*0.016**	*0.018**	*0.174*	*0.049**

(c) Mn correlations

	Mn bee	Mn honey	Mn wax	Mn soil
Mn Honey	0.807			
	*0.005***			
Mn wax	0.584	0.564		
	0.076	*0.090*		
Mn soil	0.511	0.636	0.754	
	0.131	*0.048**	*0.012**	
Mn dust	0.137	0.602	0.376	0.547
	0.705	*0.066*	*0.285*	*0.102*

(d) As correlations

	As bee	As wax	As soil
As wax	–		
	–		
As soil	0.310	–	
	0.384	–	
As dust	0.279	–	0.138
	0.435	–	*0.704*

Contents: Pearson correlation (r)

p value significant at the following levels: *0.05, **0.01 and ***0.001.**Supplementary**

Table S4. Lead isotopic compositions ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$)[a] in honeybees, honey, wax, soil and dust across the Sydney metropolitan region and the lead-zinc mining city of Broken Hill, Australia.

Sites	Sample	$^{206}Pb/^{204}Pb$	$^{206}Pb/^{207}Pb$	$^{208}Pb/^{207}Pb$
National park areas (Galston, Gordon)	Honey bees (n = 1)	17.818	1.138	2.432
	Honey (n =2)[b]	18.277 (18.093 – 18.461)	1.159 (1.128 – 1.191)	2.435 (2.416 – 2.453)
	Wax (n = 2)[b]	17.759 (17.438 – 18.081)	1.141 (1.134 – 1.149)	2.417 (2.412 – 2.421)
	Soil (n = 1)	17.527	1.129	2.406
	Dust (n = 4) Range	17.789 (17.698 – 17.863)	1.135 (1.112 – 1.147)	2.415 (2.397 – 2.435)
Residential areas (Coogee, Randwick)	Honey bees (n = 6) Range	16.899 (16.603 – 17.197)	1.090 (1.081 – 1.103)	2.371 (2.353 – 2.383)
	Wax (n = 1)	16.930	1.082	2.352
	Soil (n = 14) Range	17.035 (16.734 – 17.524)	1.095 (1.076 – 1.129)	2.374 (2.349 – 2.412)
	Dust (n = 10) Range	17.051 (16.851 – 17.358)	1.101 (1.087 – 1.120)	2.381 (2.368 – 2.398)
City areas (Sydney CBD, Surry Hill, Newtown)	Honey bees (n = 10) Range	17.109 (16.574 – 17.688)	1.106 (1.093 – 1.126)	2.388 (2.375 – 2.408)
	Wax (n = 1)	17.006	1.097	2.376
	Soil (n = 14) Range	17.427 (16.872 – 17.901)	1.119 (1.089 – 1.142)	2.400 (2.369 – 2.427)
	Dust (n = 12) Range	17.025 (16.338 – 17.506)	1.097 (1.062 – 1.124)	2.377 (2.340 – 2.411)
Industrial and airport area (Marrickville, Mascot)	Honey bees (n = 8) Range	17.121 (16.954 – 17.424)	1.103 (1.096 – 1.117)	2.386 (2.376 – 2.404)
	Wax (n = 1)	16.966	1.085	2.363
	Soil (n = 10) Range	17.204 (16.867 – 17.931)	1.107 (1.087 – 1.137)	2.389 (2.363 – 2.419)
	Dust (n = 8) Range	17.244 (17.043 – 17.425)	1.114 (1.110 – 1.120)	2.399 (2.391 – 2.404)
Sydney	Historical honey (n = 3) Range	17.326 (17.038 – 17.544)	1.106 (1.104 – 1.106)	2.383 (2.380 – 2.385)
Mining area (Broken Hill)[c]	Honey bees (n = 1)	16.008	1.046	2.319
	Honey (n = 3) Range	16.115 (16.036 – 16.253)	1.047 (1.045 – 1.048)	2.327 (2.322 – 2.336)
	Wax (n = 3) Range	15.939 (15.849 – 16.015)	1.041 (1.040 – 1.043)	2.322 (2.317 – 2.326)
	Soil (n = 3) Range	16.121 (16.005 – 16.244)	1.041 (1.1039 – 1.042)	2.325 (2.323 – 2.329)
	Dust (n = 1)	15.898	1.043	2.324

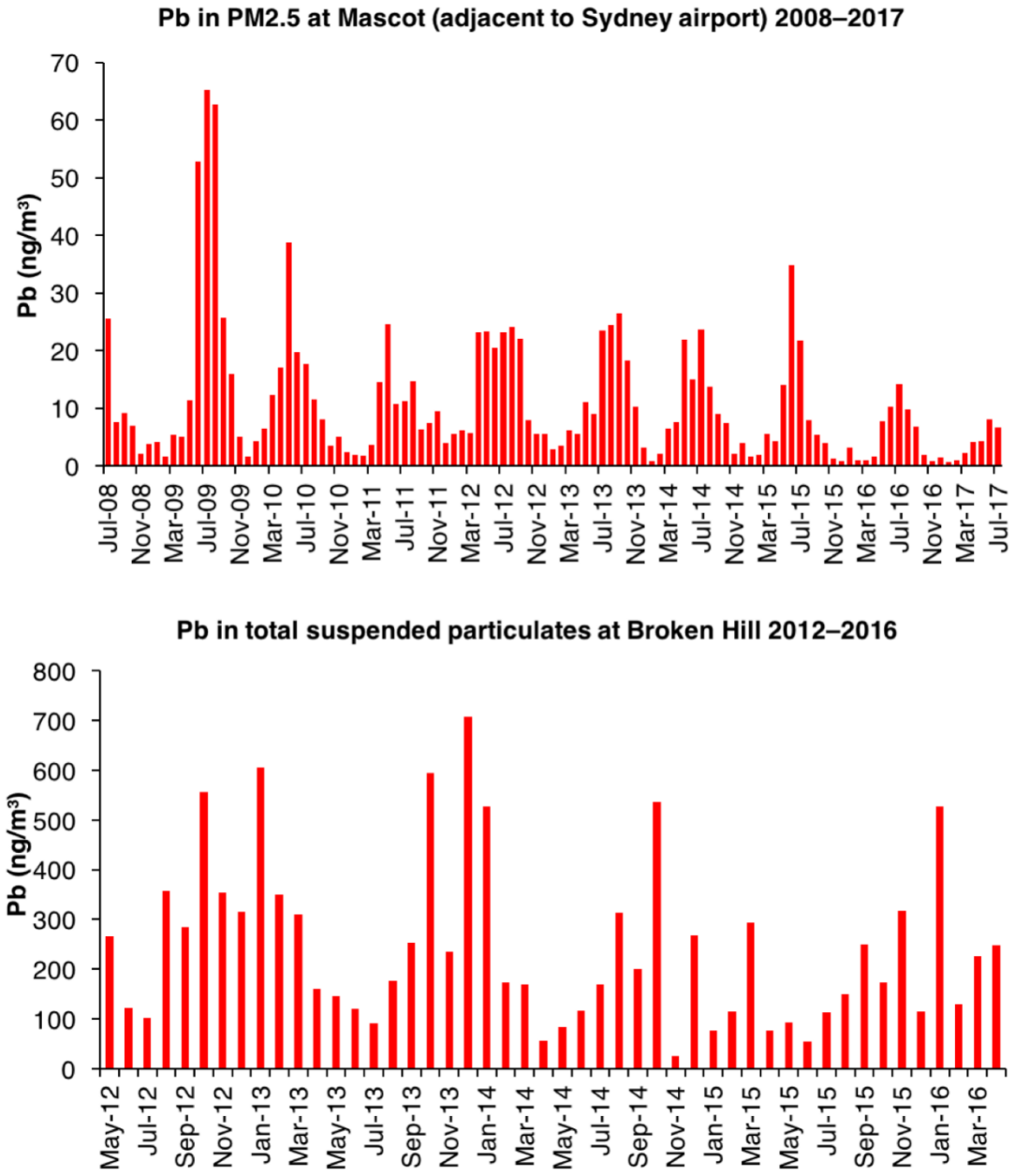
[a] Certified values of $^{204}Pb/^{206}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$ for National Institute of Standards and Technology (NIST) SRM981 (natural Pb isotope composition standard) are 0.059 ± 0.00037, 1.0933 ± 0.00039 and 2.3704 ± 0.0012, respectively.

[b] Honey and wax samples from Galston and Gordon required dry ashing at 550 °C in a muffle furnace to extract adequate Pb concentrations (minimum 1 µg/kg) for Pb isotopic composition analysis.

[c] Broken Hill sampling comprised of a single monthly collection of honey bees and a three-month sample phase for wax and honey. Multiple monthly sampling periods (November 2015 – March 2016) were undertaken across the Sydney metropolitan sites. However, in several cases only one set of samples from a site had sufficient Pb (minimum 1 µg/kg) to permit its isotopic compositions to be measured. In the case of honey only the Sydney metropolitan samples from 2012 had sufficient Pb concentrations for isotopic composition analysis.

Supplementary Table S5. Lead isotopic compositions ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{207}Pb$) of sub-surface soil and rocks, Sydney, Australia.[2]

Sample no.	**Location**	**$^{206}Pb/^{204}Pb$**	**$^{206}Pb/^{207}Pb$**	**$^{208}Pb/^{207}Pb$**
SYD_Rock_1	Sydney Harbour National Park	18.04	1.159	2.478
SYD_Rock_2	Sydney Harbour National Park	17.96	1.160	2.434
SYD_Rock_3	Sydney Harbour National Park	17.84	1.142	2.447
SYD_Rock_4	Sydney Harbour National Park	17.42	1.124	2.413
SYD_Rock_5	Sydney Harbour National Park	18.02	1.158	2.464
SYD_Rock_6	Lane Cove National Park	17.72	1.127	2.434
SYD_Rock_7	Lane Cove National Park	18.13	1.165	2.476
SYD_Rock_8	Lane Cove National Park	18.44	1.176	2.500
SYD_Rock_9	Lane Cove National Park	18.04	1.156	2.467
SYD_Rock_10	Duffys Forest	17.73	1.134	2.426
SYD_Soil_1_30-40	Sydney Harbour National Park	17.86	1.144	2.459
SYD_Soil_2_30-40	Sydney Harbour National Park	17.77	1.146	2.445
SYD_Soil_3_30-40	Lane Cove National Park	17.63	1.123	2.441
SYD_Soil_4_30-40	Duffys Forest	17.71	1.135	2.430



Supplementary Figure S1. Recent monthly average lead in air Pb concentrations at Sydney (Mascot)[3] and Broken Hill.[4] (Data for PM2.5 graph provided by Dr Armand J Atanacio, Australian Nuclear Science and Technology Organisation—more limited data is available on line).[3]

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(1) Dong, C.; Taylor, M. P., Applying geochemical signatures of atmospheric dust to distinguish current mine emissions from legacy sources. *Atmos. Environ.* 2017, *161*, 82-89.

(2) Wu, L.; Taylor, M. P.; Handley, H. K.; Wu, M., Australian atmospheric lead deposition reconstructed using lead concentrations and isotopic compositions of archival lichen and fungi. *Environ. Pollut.* 2016, *208*, 678-687.

(3) Australian Nuclear Science and Technology Organisation (ANSTO), Fine particle pollution-coastal NSW. http://www.ansto.gov.au/Resources/Localenvironment/Atmosphericmonitoring/Fineparticlepollution/index.htm (accessed November 3, 2017).

(4) Perilya Limited, Environmental Reports. http://www.perilya.com.au/health--safety--environment/environment/reports (accessed November 3, 2017).

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Cockburn, P. (2018, January 13) *Lead detected in Sydney and Broken Hill bees due to environmental pollution*. ABC News. https://www.abc.net.au/news/2018-01-13/lead-found-in-sydney-and-broken-hill-bees/9325712

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Murphy, M. (2018, January 17) Lead found in NSW bees and honey. Food Processing. https://www.foodprocessing.com.au/content/ingredients/news/lead-found-in-nsw-bees-and-honey-908529867

Daily Mail AUSTRALIA

Lead found in Sydney and Broken Hill bees

By AUSTRALIAN ASSOCIATED PRESS
PUBLISHED: 21:13 AEST, 15 January 2018 | **UPDATED:** 21:13 AEST, 15 January 2018

Sydney and Broken Hill residents eating local honey are more likely to consume honey contaminated with lead, new research shows.

The Macquarie University research traced contaminating metals in bees, their honey and wax in Sydney and Broken Hill and found the bees were contaminated by ongoing mining emissions and petrol sources.

"The large difference in honey lead concentration demonstrates that local active sources, such as the ongoing lead mining in Broken Hill, can have a substantial impact on the level of metals measured in local food products and ecological systems," Macquarie University Professor Mark Taylor said in a statement on Monday.

Appendix D

Supplementary Table S1. Concentrations of As, Mn, Pb and Zn in native bees (µg/kg) and their honey (µg/kg) and wax (µg/kg), along with co-located samples of soil (mg/kg) and dust (mg/kg) across the Sydney region, Australia (See Figure 1 for sampling details). Trace element concentrations are displayed to 3 significant figures.

Sites	Samples		As	Pb	Mn	Zn
St. Ives	Native bees (µg/kg) [n = 2]	Mean ± SD Range	58 ± 35 33 – 83	588 ± 809 16 – 1160	41000 ± 2970 38900 – 43100	46600 ± 13100 37300 – 55800
	Honey (µg/kg) [n = 1]	Mean ± SD Range	< 10 –	< 10 –	5800 –	570 –
	Wax (µg/kg) [n =1]	Mean ± SD Range	12 –	130 –	8000 –	19000 –
	Soil (mg/kg) [n = 3]	Mean ± SD Range	3 ± 2 2 – 5	22 ± 13 11 – 36	213 ± 184 68 – 420	66 ± 30 44 – 100
	Dust (mg/kg) [n = 4]	Mean ± SD Range	18 < 0.1 – 18	73 ± 18 53 – 95	265 ± 104 110 – 330	273 ± 77 190 – 360
Gordon	Native bees (µg/kg) [n = 2]	Mean ± SD Range	39 ± 1 38 – 39	24 ± 1 23 – 25	29700 ± 2970 27600 – 31800	38900 ± 5730 34800 – 42900
	Honey (µg/kg) [n = 1]	Mean ± SD Range	< 10 –	< 10 –	2900 –	1300 –
	Wax (µg/kg) [n = 1]	Mean ± SD Range	14 –	100 –	5500 –	9500 –
	Soil (mg/kg) [n = 3]	Mean ± SD Range	7 ± 3 4 – 10	155 ± 61 84 – 190	267 ± 50 220 – 320	323 ± 280 110 – 640
	Dust (mg/kg) [n = 3]	Mean ± SD Range	2 < 0.1 – 2	45 ± 18 34 – 66	121 ± 49 72 – 170	307 ± 127 160 – 380
Roseville	Native bees (µg/kg) [n = 1]	Mean ± SD Range	21 –	43 –	17200 –	35000 –
	Honey (µg/kg) [n = 9]	Mean ± SD Range	< 10 –	29 < 10 – 29	2370 ± 862 1100 – 3700	1100 ± 409 580 – 2000
	Wax (µg/kg) [n = 11]	Mean ± SD Range	18 ± 5 11 – 30	348 ± 436 93 – 1620	4930 ± 1660 2430 – 7830	25200 ± 37700 6660 – 137000

Sites	Samples		As	Pb	Mn	Zn
Roseville	Soil (mg/kg) [n = 6]	Mean ± SD Range	5 ± 3 2 – 10	169 ± 228 33 – 610	239 ± 145 52 – 420	136 ± 92 47 – 280
	Dust (mg/kg) [n = 8]	Mean ± SD Range	102 ± 159 < 0.1 – 340	136 ± 69 54 – 270	210 ± 112 100 – 430	253 ± 90 130 – 380
Bronte	Native bees (µg/kg) [n = 3]	Mean ± SD Range	87 ± 13 75 – 100	283 ± 164 160 – 470	16000 ± 4300 11000 – 18600	46000 ± 9150 35700 – 53100
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	18 ± 11 10 – 26	1100 ± 0 1100 – 1100	1750 ± 212 1600 – 1900
	Wax (µg/kg) [n = 2]	Mean ± SD Range	33 ± 1 32 – 34	760 ± 99 690 – 830	3740 ± 92 3670 – 3800	10800 ± 1980 9400 – 12200
	Soil (mg/kg) [n = 3]	Mean ± SD Range	2 ± 1 2 – 4	170 ± 46 130 – 220	64 ± 12 54 – 78	280 ± 78 230 – 370
	Dust (mg/kg) [n = 5]	Mean ± SD Range	68 ± 102 < 0.1 – 220	185 ± 137 73 – 400	189 ± 131 89 – 410	358 ± 250 160 – 730
Randwick	Native bees (µg/kg) [n = 3]	Mean ± SD Range	48 ± 8 41 – 57	131 ± 49 83 – 180	24000 ± 9300 16500 – 34400	44300± 3450 40900 – 47800
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	11 < 10 – 11	935 ± 92 870 – 1000	545 ± 134 450 – 640
	Wax (µg/kg) [n =2]	Mean ± SD Range	30 ± 5 26 – 33	485 ± 163 370 – 600	6910 ± 863 6300 – 7520	14100 ± 1490 13000 – 15100
	Soil (mg/kg) [n = 6]	Mean ± SD Range	2 ± 1 1 – 3	20 ± 8 7 – 30	73 ± 38 16 – 120	60 ± 27 16 – 92
	Dust (mg/kg) [n = 5]	Mean ± SD Range	77 ± 69 12 – 170	124 ± 30 88 – 170	186 ± 119 91 – 380	592 ± 394 270 – 1240
Maroubra	Native bees (µg/kg) [n = 3]	Mean ± SD Range	84 ± 22 70 – 110	167 ± 15 150 – 180	17200 ± 3150 14100 – 20400	49800 ± 10100 43300 – 61400
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	16 < 10 – 16	805 ± 276 610 – 1000	1350 ± 643 890 – 1800
	Wax (µg/kg) [n =1]	Mean ± SD Range	22 –	640 –	4900 –	11000 –
	Soil (mg/kg) [n = 2]	Mean ± SD Range	4 ± 2 2 – 5	52 ± 42 22 – 82	170 ± 99 100 – 240	140 ± 99 70 – 210
	Dust (mg/kg) [n = 5]	Mean ± SD Range	4 < 0.1 – 4	83 ± 42 35 – 150	257 ± 154 86 – 440	276 ± 30 240 – 320

Sites	Samples		As	Pb	Mn	Zn
Newtown	Native bees (µg/kg) [n = 1]	Mean ± SD Range	140 –	2050 –	46400 –	70600 –
	Honey (µg/kg) [n =1]	Mean ± SD Range	< 10 –	25 –	410 –	490 –
	Wax (µg/kg) [n = 2]	Mean ± SD Range	32 ± 11 24 – 40	1630 ± 240 1460 – 1800	5250 ± 2480 3490 – 7000	14900 ± 1270 14000 – 15800
	Soil (mg/kg) [n = 7]	Mean ± SD Range	7 ± 5 2 – 16	384 ± 421 110 – 1320	172 ± 61 63 – 250	191 ± 86 87 – 370
	Dust (mg/kg) [n = 5]	Mean ± SD Range	3 < 0.1 – 3	238 ± 48 180 – 300	286 ± 59 210 – 350	316 ± 144 120 – 490
Marrickville	Native bees (µg/kg) [n = 1]	Mean ± SD Range	83 –	160 –	19300 –	33700 –
	Honey (µg/kg) [n = 2]	Mean ± SD Range	< 10 –	34 ± 20 20 – 48	2700 ± 2120 1200 – 4200	1690 ± 100 980 – 2400
	Wax (µg/kg) [n = 1]	Mean ± SD Range	25 –	730 –	3000 –	73000 –
	Soil (mg/kg) [n = 7]	Mean ± SD Range	23 ± 17 5 – 42	811 ± 585 150 – 1560	239 ± 76 140 – 390	999 ± 747 160 – 1980
	Dust (mg/kg) [n = 4]	Mean ± SD Range	24 ± 11 < 0.1 – 31	160 ± 124 66 – 340	116 ± 20 93 – 140	408 ± 441 90 – 1060

– not available.

*Australian native bee products were collected during each splitting period, i.e. November 2015 and November 2016. However, not every sample site has two samples. Native bees are temperature sensitive and cooler weather at the time of sampling meant that bees were not active and could not be sampled. Each native bee sample comprised 30 individual bees.

Supplementary Table S2. Summary Pearson r correlations and *p* values (in parentheses) for trace elements and their relationship to beehive products and the environment of soil and dust.

(a) As correlations

	As native bees	As wax	As soil
As wax	0.544 (0.164)		
As soil	0.141 (0.738)	-0.066 (0.877)	
As dust	-0.381 (0.352)	0.260 (0.534)	-0.318 (0.443)

(b) Pb correlations

	Pb native bees	Pb honey	Pb wax	Pb soil
Pb honey	0.177 (0.675)			
Pb wax	0.565 (0.145)	0.813 (0.014*)		
Pb soil	0.031 (0.941)	0.658 (0.076)	0.439 (0.276)	
Pb dust	0.572 (0.139)	0.814 (0.014*)	0.883 (0.004**)	0.472 (0.238)

(c) Mn correlations

	Mn native bees	Mn honey	Mn wax	Mn soil
Mn honey	0.023 (0.956)			
Mn wax	0.601 (0.115)	0.146 (0.729)		
Mn soil	0.278 (0.506)	0.501 (0.206)	-0.009 (0.984)	
Mn dust	0.326 (0.430)	-0.390 (0.339)	0.495 (0.212)	-0.171 (0.685)

(d) Zn correlations

	Zn native bees	Zn honey	Zn wax	Zn soil
Zn honey	-0.585 (0.128)			
Zn wax	0.151 (0.721)	-0.567 (0.142)		
Zn soil	-0.343 (0.406)	0.709 (0.049*)	-0.733 (0.039*)	
Zn dust	-0.072 (0.865)	-0.153 (0.718)	-0.384 (0.348)	0.010 (0.981)

Contents: Pearson correlation (r) *p* value significant at the following levels: * = 0.05 and ** = 0.01.

Supplementary Table S3. Trace element concentrations and the significance differences in bees, honey, wax from 18 Australian native bee hives and nine European honey bee hives in the Sydney metropolitan area. Trace element concentrations are presented to 3 significant figures.

Trace element	Beehive product	Concentrations (µg/kg)	Australian native bee hives	European honey bee hives
As	Bee ($p < 0.001$***)	Median Range	72 21 – 140	31 11 – 95
	Honey[a]	Median Range	< 10 --	< 10 --
	Wax[a]	Median Range	20 11 – 40	< 10 < 10 – 14
Pb	Bee ($p = 0.684$)	Median Range	160 16 – 2,050	160 18 – 660
	Honey ($p = 0.744$)	Median Range	23 10 – 48	15 13 – 22
	Wax ($p < 0.001$***)	Median Range	350 93 – 1,800	67 12 – 520
Mn	Bee ($p < 0.001$***)	Median Range	19,900 11,000 – 46,400	39,000 15,000 – 125,000
	Honey ($p = 0.001$**)	Median Range	1,700 410 – 5,800	390 170 – 6,300
	Wax ($p < 0.001$***)	Median Range	4,900 2,430 – 8,000	185 67 – 7,730
Zn	Bee ($p < 0.001$***)	Median Range	43,700 33,700 – 70,600	58,000 38,000 – 81,000
	Honey ($p < 0.001$***)	Median Range	980 450 – 2,400	450 235 – 1,010
	Wax ($p < 0.001$***)	Median Range	13,200 666 – 137,000	2,150 1,060 – 63,300

-- Not available

[a] limited sample numbers for statistical analysis

*Significant at 0.05, **Significant at 0.01, ***Significant at 0.001 between the two types of beehive samples using Mann–Whitney U test.

Appendix E

Supplementary Table S1. Data for $\delta^{13}C_{honey}$ (‰), $\delta^{13}C_{protein}$ (‰), $\delta^{13}C_{h\text{-}p}$ (‰) and C-4 sugar (%) in raw honey samples (RAW, n = 5) and authentic commercial honey (n = 69) from mainland Australia (M-AUS, n = 24), Tasmania (TAS, n = 7), Africa (AF, n = 1), Asia (AS, n = 10), Europe (n = 15), North America (NA, n = 9) and honey of unknown origin (U, n = 3).

Sample No.	Country	$\delta^{13}C_{honey}$ (‰) Criterion: < −23.5 [a]	$\delta^{13}C_{protein}$ (‰)	$\delta^{13}C_{h\text{-}p}$ (‰) Criterion: ≤ 1 [b]	C-4 sugar (%) Criterion: ≤ 7 [c] or > −7 [d]
RAW-1	Australia	−25.65 ± 0.08	−25.57 ± 0.10	−0.08	−0.50
RAW-2	Australia	−25.79 ± 0.04	−25.57 ± 0.19	−0.22	−1.41
RAW-3	Australia	−26.90 ± 0.04	−26.29 ± 0.14	−0.61	−3.71
RAW-4	Australia	−26.49 ± 0.03	−26.06 ± 0.11	−0.43	−2.62
RAW-5	Australia	−25.90 ± 0.05	−25.48 ± 0.02	−0.42	−2.69
M-AUS-1	Australia	−25.80 ± 0.05	−26.14 ± 0.07	0.34	2.09
M-AUS-2	Australia	−25.66 ± 0.07	−26.04 ± 0.12	0.38	2.35
M-AUS-3	Australia	−25.91 ± 0.07	−25.99 ± 0.02	0.09	0.54
M-AUS-4	Australia	−25.74 ± 0.05	−25.97 ± 0.003	0.22	1.38
M-AUS-5	Australia	−25.84 ± 0.01	−26.45 ± 0.13	0.61	3.64
M-AUS-6	Australia	−25.41 ± 0.07	−25.57 ± 0.03	0.16	0.98
M-AUS-7	Australia	−27.91 ± 0.05	−27.78 ± 0.09	−0.13	−0.74
M-AUS-8	Australia	−26.07 ± 0.05	−26.71 ± 0.18	0.64	3.76
M-AUS-9	Australia	−25.10 ± 0.02	−25.58 ± 0.10	0.48	3.04
M-AUS-10	Australia	−25.90 ± 0.11	−26.59 ± 0.17	0.70	4.12
M-AUS-11	Australia	−26.17 ± 0.09	−26.02 ± 0.06	−0.15	−0.93
M-AUS-12	Australia	−25.71 ± 0.15	−26.06 ± 0.13	0.35	2.16
M-AUS-13	Australia	−26.38 ± 0.10	−25.69 ± 0.17	−0.69	−4.32
M-AUS-14	Australia	−27.83 ± 0.03	−27.30 ± 0.14	−0.53	−3.03
M-AUS-15	Australia	−25.28 ± 0.07	−25.51 ± 0.10	0.23	1.48
M-AUS-16	Australia	−26.49 ± 0.09	−26.55 ± 0.09	0.06	0.34
M-AUS-17	Australia	−25.13 ± 0.10	−25.53 ± 0.26	0.40	2.53
M-AUS-18	Australia	−25.05 ± 0.09	−25.24 ± 0.06	0.19	1.20
M-AUS-19	Australia	−25.43 ± 0.07	−25.84 ± 0.04	0.41	2.53
M-AUS-20	Australia	−25.06 ± 0.05	−25.46 ± 0.07	0.40	2.56
M-AUS-21	Australia	−26.31 ± 0.06	−26.63 ± 0.12	0.31	1.85
M-AUS-22	Australia	−25.84 ± 0.06	−25.58 ± 0.02	−0.26	−1.61
M-AUS-23	Australia	−25.08 ± 0.08	−25.68 ± 0.08	0.60	3.76
M-AUS-24	Australia	−25.34 ± 0.15	−25.92 ± 0.08	0.58	3.55
TAS-1	Australia	−25.53 ± 0.01	−25.63 ± 0.09	0.10	0.61
TAS-2	Australia	−25.82 ± 0.09	−25.34 ± 0.14	−0.48	−3.09
TAS-3	Australia	−26.66 ± 0.06	−26.18 ± 0.06	−0.48	−2.89
TAS-4	Australia	−25.77 ± 0.05	−25.52 ± 0.06	−0.25	−1.59
TAS-5	Australia	−24.71 ± 0.12	−25.02 ± 0.07	0.31	2.03
TAS-6	Australia	−25.49 ± 0.07	−26.34 ± 0.22	0.85	5.11
TAS-7	Australia	−25.01 ± 0.04	−25.70 ± 0.08	0.69	4.34

Sample No.	Country	$\delta^{13}C_{honey}$ (‰) Criterion: < −23.5 [a]	$\delta^{13}C_{protein}$ (‰)	$\delta^{13}C_{h-p}$ (‰) Criterion: ≤ 1 [b]	C-4 sugar (%) Criterion: ≤ 7 [c] or > −7 [d]
AF-1	Kenya	−24.69 ± 0.04	−23.97 ± 0.04	−0.72	−5.05
AS-1	China	−25.24 ± 0.04	−25.19 ± 0.21	−0.05	−0.32
AS-2	China	−25.04 ± 0.16	−25.67 ± 0.17	0.63	3.95
AS-3	China	−24.30 ± 0.03	−24.62 ± 0.05	0.31	2.11
AS-4	China	−24.62 ± 0.18	−24.79 ± 0.09	0.17	1.12
AS-5	India	−26.89 ± 0.10	−26.37 ± 0.10	−0.53	−3.17
AS-6	Japan	−27.38 ± 0.11	−27.24 ± 0.04	−0.14	−0.81
AS-7	Japan	−27.26 ± 0.16	−26.78 ± 0.14	−0.48	−2.81
AS-8	Japan	−25.54 ± 0.05	−25.04 ± 0.07	−0.49	−3.21
AS-9	Saudi Arabia*	−25.79 ± 0.07	−25.64 ± 0.23	−0.15	−0.96
AS-10	Saudi Arabia*	−24.63 ± 0.12	−25.54 ± 0.05	0.92	5.78
EU-1	England	−26.96 ± 0.05	−26.45 ± 0.06	−0.51	−3.04
EU-2	Europe	−26.43 ± 0.04	−25.93 ± 0.07	−0.50	−3.10
EU-3	Europe	−26.32 ± 0.10	−26.92 ± 0.04	0.60	3.48
EU-4	Greece*	−25.93 ± 0.06	−26.48 ± 0.04	0.55	3.28
EU-5	Greece*	−25.84 ± 0.10	−25.88 ± 0.05	0.04	0.25
EU-6	Greece*	−25.73 ± 0.01	−25.94 ± 0.15	0.21	1.29
EU-7	Greece*	−25.93 ± 0.09	−25.91 ± 0.07	−0.02	−0.13
EU-8	Hungary	−24.09 ± 0.10	−24.69 ± 0.10	0.59	3.97
EU-9	Hungary	−26.01 ± 0.10	−25.13 ± 0.04	−0.88	−5.70
EU-10	Italy*	−25.55 ± 0.08	−26.02 ± 0.05	0.46	2.85
EU-11	Italy	−25.22 ± 0.03	−24.63 ± 0.12	−0.60	−3.99
EU-12	Italy	−26.00 ± 0.03	−25.85 ± 0.04	−0.15	−0.92
EU-13	Italy	−25.63 ± 0.08	−25.91 ± 0.11	0.28	1.72
EU-14	Macedonia*	−24.51 ± 0.05	−25.02 ± 0.16	0.51	3.34
EU-15	Romania*	−24.97 ± 0.003	−25.28 ± 0.05	0.30	1.96
NA-1	Canada[e]	−26.46 ± 0.14	−26.47 ± 0.14	0.00	0.02
NA-2	USA	−25.84 ± 0.03	−25.94 ± 0.10	0.10	0.61
NA-3	USA	−26.95 ± 0.06	−26.79 ± 0.04	−0.16	−0.93
NA-4	USA	−26.42 ± 0.02	−26.20 ± 0.03	−0.22	−1.31
NA-5	USA	−25.5 ± 0.03	−26.34 ± 0.11	0.84	5.08
NA-6	USA	−26.11 ± 0.06	−26.94 ± 0.02	0.82	4.78
NA-7	USA	−27.17 ± 0.09	−26.71 ± 0.06	−0.46	−2.73
NA-8	USA	−26.05 ± 0.08	−26.28 ± 0.08	0.23	1.40
NA-9	Canada	−25.70 ± 0.17	−26.01 ± 0.08	0.32	1.95
U-1	Unknown*	−25.53 ± 0.09	−25.86 ± 0.14	0.33	2.03
U-2	Unknown*	−25.25 ± 0.05	−25.63 ± 0.01	0.37	2.34
U-3	Unknown*	−27.39 ± 0.04	−26.55 ± 0.07	−0.84	−5.00

All Australian honey (mainland and Tasmania) was obtained from local food markets and commercial supermarkets.

*Nine authentic overseas honeys and three authentic honeys of an unknown origin were purchased in Australia.

[a] Detection criterion for $\delta^{13}C_{honey}$ <−23.5‰ according to the AOAC Official Method 978.17[1];

[b] Detection criterion for $\delta^{13}C_{h-p}$ ≤1‰ according to Padovan et al.[2], White and Winters[3], Simsek et al.[4], Tosun[5], Guler et al.[6], Elflein and Raezke[7];

[c] Detection criterion for C-4 sugar ≤7% according to the AOAC Official Method 998.12[8];

[d] Detection criterion for C-4 sugar where >−7% according to Dong et al.[9];
[e] Sample NA-1 was labelled as a product of USA-Canada but was purchased in Canada.

Supplementary Table S2. Concentrations of trace elements in authentic commercial honey samples (n = 69) from mainland Australia (n = 24), Tasmania (n = 7), Africa (n = 1), Asia (n = 10), Europe (n = 15), North America (n = 9) and honey with an unknown geographic origin (n = 3). Trace element concentrations are displayed to 3 significant figures. Arithmetic mean, error (1 standard deviation) and range are shown.

Trace elements		Mainland Australia	Tasmania	Africa	Asia	Europe	North America	Unknown origin
Al (µg/kg)	Mean ± 1SD	2120 ± 5280	1960 ± 1790	1400	89 ± 139	1860 ± 4720	227 ± 665	< 10
	Range	< 10 – 21000	< 10 – 4800	--	5 – 440	5 – 18000	5 – 2000	< 10
Ba (µg/kg)	Mean ± 1SD	257 ± 145	106 ± 45	700	157 ± 174	114 ± 118	110 ± 63.2	121 ± 42
	Range	85 – 620	65 – 180	--	18 – 560	5 – 450	19 – 210	94 – 170
B (mg/kg)	Mean ± 1SD	5.20 ± 1.75	4.84 ± 2.21	9.7	5.88 ± 3.15	4.97 ± 2.56	7.14 ± 0.87	4.13 ± 0.61
	Range	2.50 – 10.0	3.30 – 9.20	--	3 – 12	1.3 – 10	5.6 – 8.3	3.60 – 4.80
Ca (mg/kg)	Mean ± 1SD	9.38 ± 2.77	4.41 ± 0.89	12	4.77 ± 1.81	6.9 ± 4.1	5.58 ± 1.12	6.90 ± 4.46
	Range	3.60 – 15.0	3.30 – 5.70	--	1.8 – 7.9	0.95 – 14	3.8 – 7.7	3.70 – 12.0
Cu (µg/kg)	Mean ± 1SD	203 ± 168	126 ± 19	1.1	141 ± 144	314 ± 317	149 ± 47.9	73 ± 17
	Range	65 – 770	100 – 160	--	41 – 520	32 – 1300	88 – 230	54 – 86
Fe (mg/kg)	Mean ± 1SD	1.44 ± 0.94	0.93 ± 0.36	2.6	4.14 ± 3.09	1.4 ± 1.18	2.74 ± 2.78	2.63 ± 1.63
	Range	0.58 – 3.70	0.54 – 1.50	--	0.64 – 8.5	0.14 – 4.4	0.59 – 7.3	1.20 – 4.40
Mg (mg/kg)	Mean ± 1SD	40.1 ± 18.32	16.1 ± 4.10	75	24.7 ± 27.3	37 ± 25.1	22 ± 4.47	24.0 ± 16.5
	Range	16.0 – 87.0	11.0 – 22.0	--	6.3 – 100	3.8 – 78	14 – 29	14.0 – 43.0
Mn (mg/kg)	Mean ± 1SD	4.23 ± 2.29	3.14 ± 2.00	3.5	0.75 ± 0.65	2.13 ± 2.6	0.7 ± 0.49	3.23 ± 2.94
	Range	0.89 – 10.0	1.20 – 6.70	--	120 – 2300	0.07 – 7.3	0.27 – 1.9	1.20 – 6.60
Ni (µg/kg)	Mean ± 1SD	33 ± 42	25 ± 15	62	40.5 ± 54.1	132 ± 230	19.1 ± 8.85	10 ± 5
	Range	< 10 – 170	< 10 – 53	--	5 – 160	5 – 860	5 – 28	< 10 – 15
P (mg/kg)	Mean ± 1SD	46.4 ± 15.7	34.3 ± 9.38	130	36 ± 10.7	76.8 ± 49.6	48.9 ± 8.51	25.0 ± 1.00
	Range	31.0 – 98.0	24.0 – 51.0	--	23 – 55	16 – 210	40 – 63	24.0 – 26.0
K (mg/kg)	Mean ± 1SD	8370 ± 3560	9620 ± 3518	7410	5020 ± 4660	10200 ± 8370	4340 ± 1750	6390 ± 6510
	Range	2500 – 15800	4540 – 13000	--	1.02 – 16.9	1620 – 26900	2100 – 7480	2370 – 13900
Rb (mg/kg)	Mean ± 1SD	1.84 ± 1.39	2.18 ± 0.78	4.9	0.89 ± 0.54	1.98 ± 2.41	1 ± 0.81	1.48 ± 1.49
	Range	0.18 – 5.10	0.95 – 3.00	--	0.14 – 1.8	0.16 – 7.4	0.22 – 3	0.56 – 3.20

Trace elements		Mainland Australia	Tasmania	Africa	Asia	Europe	North America	Unknown origin
Na (mg/kg)	Mean ± 1SD	137 ± 122	28.0 ± 12.4	5.8	99.7 ± 254	18.7 ± 14.3	21.3 ± 14.7	90.7 ± 60.2
	Range	21.0 – 510	18.0 – 52.0	--	5.4 – 820	2.5 – 54	8.9 – 50	52.0 – 160
Sr (µg/kg)	Mean ± 1SD	683 ± 564	147 ± 30	890	242 ± 166	240 ± 268	195 ± 78.6	357 ± 272
	Range	150 – 3100	120 – 200	--	89 – 650	12 – 1100	58 – 290	180 – 670
Sn (µg/kg)	Mean ± 1SD	21 ± 14	25 ± 16	5	110 ± 313	17.5 ± 9.19	72.8 ± 128	< 10
	Range	< 10 – 55	< 10 – 58	--	5 – 1000	5 – 35	4 – 380	< 10
Zn (mg/kg)	Mean ± 1SD	1.46 ± 3.75	0.53 ± 0.31	1.1	1.25 ± 1.39	0.95 ± 0.62	1.52 ± 1.34	3.37 ± 5.14
	Range	0.33 – 19.0	0.29 – 1.20	--	0.37 – 4.9	0.18 – 2.4	0.32 – 4.3	0.23 – 9.30

Supplementary Table S3. *p* values of carbon isotopic ratios (‰) and 16 trace element concentrations in authentic commercial honey samples according to their different geographic origin (n = 65, with the three samples of unknown origin and the single African sample excluded). Samples were collected from mainland Australia (n = 24), Tasmania (n = 7), Asia (n = 10), Europe (n = 15) and North America (n = 9). Statistically significant differences (*p* = 0.001***, *p* = 0.01** and *p* = 0.05*) were determined using a One-way ANOVA with Tukey's multiple comparison using the variables of carbon isotopic ratios (‰) of honey and its protein along with 16 trace element concentrations Al, Ba, B, Ca, Cu, Fe, Mg, Mn, Ni, P, K, Rb, Na, Sr, Sn and Zn (µg/kg).

1) $\delta^{13}C_{honey}$

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	0.920			
Asia	0.972	0.999		
Europe	0.960	0.998	>0.999	
North America	0.707	0.443	0.510	0.433

2) $\delta^{13}C_{protein}$

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	0.576			
Asia	0.477	>0.999		
Europe	0.475	1.000	1.000	
North America	0.658	0.154	0.105	0.095

3) Al

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	>0.999			
Asia	0.667	0.880		
Europe	1.000	>0.999	0.818	
North America	0.749	0.913	>0.999	0.871

4) Ba

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	0.062			
Asia	0.248	0.930		
Europe	**0.011***	>0.999	0.924	
North America	**0.037***	>0.999	0.930	>0.999

5) B

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	0.995			
Asia	0.923	0.871		
Europe	0.998	>0.999	0.848	
North America	0.170	0.239	0.718	0.143

6) Ca

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	**< 0.001*** **			
Asia	**< 0.001*** **	0.999		
Europe	0.062	0.292	0.331	
North America	**0.007****	0.918	0.968	0.784

7) Cu

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	0.884			
Asia	0.912	1.000		
Europe	0.425	0.227	0.201	
North America	0.951	0.999	>0.999	0.270

8) Fe

	Mainland	**Tasmania**	**Asia**	**Europe**
Tasmania	0.962			
Asia	**0.001****	**0.004****		
Europe	>0.999	0.978	**0.003****	
North America	0.343	0.268	0.426	0.389

9) Mg

	Mainland	Tasmania	Asia	Europe
Tasmania	**0.049***			
Asia	0.249	0.904		
Europe	0.989	0.160	0.556	
North America	0.147	0.976	0.998	0.387

10) Mn

	Mainland	Tasmania	Asia	Europe
Tasmania	0.718			
Asia	**< 0.001*** **	0.129		
Europe	**0.020***	0.805	0.462	
North America	**< 0.001*** **	0.128	>0.999	0.454

11) Ni

	Mainland	Tasmania	Asia	Europe
Tasmania	1.000			
Asia	1.000	0.999		
Europe	0.086	0.270	0.311	
North America	0.998	>0.999	0.994	0.156

12) P

	Mainland	Tasmania	Asia	Europe
Tasmania	0.825			
Asia	0.836	>0.999		
Europe	**0.008****	**0.008****	**0.003****	
North America	0.999	0.809	0.827	0.105

13) K

	Mainland	Tasmania	Asia	Europe
Tasmania	0.972			
Asia	0.447	0.368		
Europe	0.786	0.999	0.110	
North America	0.295	0.256	0.999	0.064

14) Rb

	Mainland	Tasmania	Asia	Europe
Tasmania	0.986			
Asia	0.464	0.430		
Europe	0.999	0.999	0.410	
North America	0.618	0.541	1.000	0.548

15) Na

	Mainland	Tasmania	Asia	Europe
Tasmania	0.263			
Asia	0.932	0.769		
Europe	**0.042***	1.000	0.506	
North America	0.137	>0.999	0.649	>0.999

16) Sr

	Mainland	Tasmania	Asia	Europe
Tasmania	**0.014***			
Asia	**0.024***	0.986		
Europe	**0.007****	0.984	>0.999	
North America	**0.014***	0.999	0.999	0.999

17) Sn

	Mainland	Tasmania	Asia	Europe
Tasmania	>0.999			
Asia	0.368	0.669		
Europe	>0.999	>0.999	0.413	
North America	0.846	0.948	0.970	0.851

18) Zn

	Mainland	Tasmania	Asia	Europe
Tasmania	0.900			
Asia	0.999	0.975		
Europe	0.968	0.996	0.998	
North America	>0.999	0.928	0.999	0.981

Trace element concentrations with significant differences are plotted in Figure 2 of the main study.

Supplementary Table S4. Classification results from canonical discriminant analysis (CDA) of authentic commercial honey samples (n = 69) comprising mainland Australia (n = 24), Tasmania (n = 7) and overseas honey (n = 35) from Africa (n = 1), Asia (n = 10), Europe (n = 15) and North America (n = 9) in addition to honey samples of unknown origin (n = 3).

Geographic origins			Predicated group membership			Total
			Mainland	Overseas	Tasmania	
Original	**Count**	Mainland	20	2	2	24
		Overseas	2	31	2	35
		Tasmania	0	2	5	7
		Unknown origin	1	2	0	3
	%	Mainland	**83.3**	8.3	8.3	100
		Overseas	5.7	**88.6**	5.7	100
		Tasmania	0	28.6	**71.4**	100
		Unknown origin	33.3	66.7	0	100
Cross-validated [a]	**Count**	Mainland	17	5	2	24
		Overseas	2	29	4	35
		Tasmania	1	2	4	7
	%	Mainland	**70.8**	20.8	8.3	100
		Overseas	5.7	**82.9**	11.4	100
		Tasmania	14.3	28.6	**57.1**	100

CDA analysis resulted in 84.8% of original grouped cases correctly classified and 75.8% of cross-validated grouped cases correctly classified.

[a] Cross validation was completed only for those cases in the analysis. In cross validation, each case was classified by the functions derived from all cases other than that case.

Supplementary Table S5. Loading values of the principal component analysis (PCA) presented in Figures 3a and 3b, respectively. Variables with loading values > 0.600 displayed in the tables are considered as the most important parameters for each component.

Rotated Component Matrix for Figure 3a

Variables	Components					
	1	2	3	4	5	6
δ^{13}_{honey}				0.895		
$\delta^{13}_{protein}$				0.903		
Al					0.700	
Ba		0.716				
B						-0.746
Ca		0.849				
Cu	0.864					
Fe			0.729			
Mg		0.629				
Mn		0.628				
Ni	0.887					
P	0.895					
K						
Rb	0.719					
Na			0.655			
Sr					0.841	
Sn			0.836			
Zn						

Rotated Component Matrix for Figure 3b

Variables	Components					
	1	2	3	4	5	6
δ^{13}_{honey}	-0.901					
$\delta^{13}_{protein}$	-0.716					
Al				0.641		
Ba					0.792	
B	0.914					
Ca		0.911				
Cu						0.848
Fe			0.692			
Mg		0.857				
Mn					0.716	
Ni			0.838			
P					0.765	
K				0.894		
Rb				0.823		
Na		0.789				
Sr						
Sn						
Zn			0.814			

Supplementary Table S6. Loading values of the CDA for Figures 3c and 3d, respectively.

Structure Matrix for Figure 3c

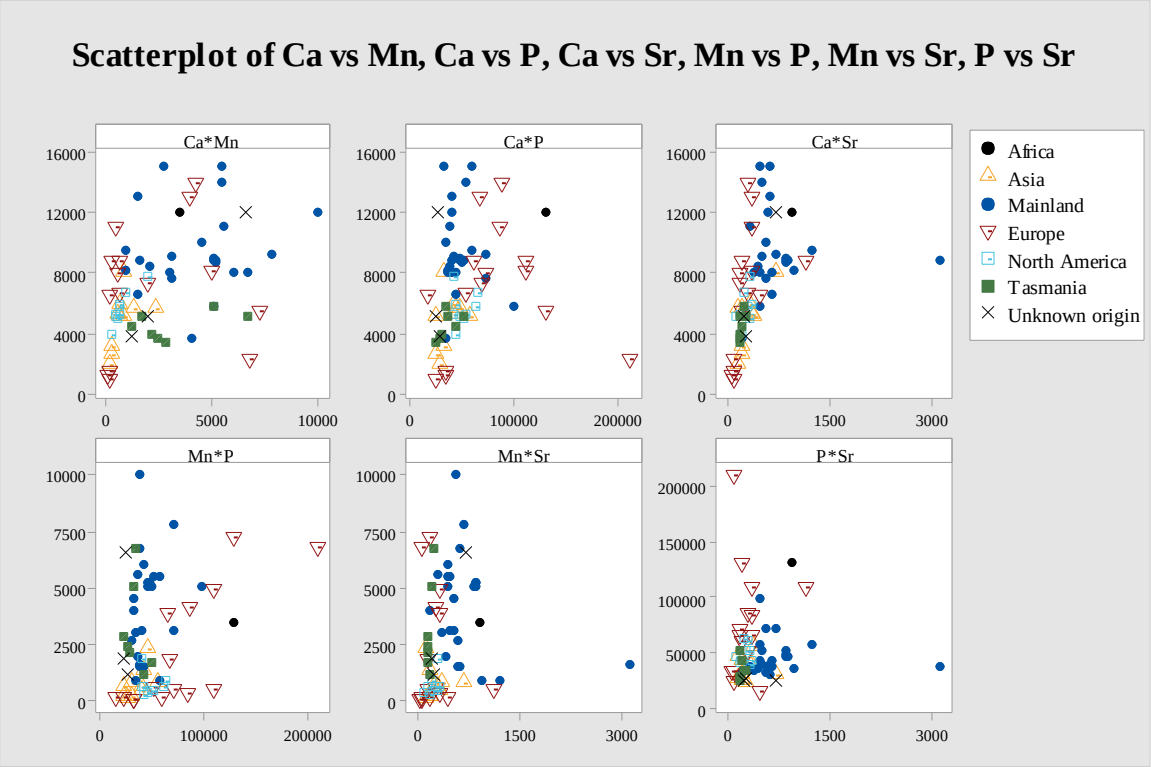
Variables	Functions	
	1	2
Mn	0.475*	-0.243
Sr	0.417*	0.284
Ba[b]	0.399*	0.099
Ni[b]	-0.232*	-0.065
Fe[b]	0.159*	0.026
Zn[b]	-0.130*	0.108
Na[b]	0.121	0.017
Al[b]	0.073*	-0.065
Ca	0.421	0.444*
B[b]	0.004	0.366*
P	-0.136	0.359*
Mg[b]	0.215	0.299*
Cu[b]	-0.072	0.256*
K	0.066	-0.204*
$\delta^{13}C_{honey}$[b]	0.046	-0.101*
$\delta^{13}C_{protein}$[b]	0.010	0.099*
Rb[b]	0.068	-0.082*
Sn[b]	0.036	-0.076*

Structure Matrix for Figure 3d

Variables	Functions	
	1	2
Fe	0.291*	-0.216
Na	0.181*	-0.019
Ca	-0.164*	0.163
Sn	0.120*	-0.112
Ba	0.110*	0.004
$\delta^{13}C_{protein}$	0.155	0.335*
K	-0.135	0.329*
B	-0.002	-0.302*
P	-0.265	0.297*
Mn	-0.133	0.280*
Cu	-0.141	0.257*
Ni	-0.087	0.251*
Mg	-0.082	0.233*
$\delta^{13}C_{honey}$	0.092	0.227*
Rb	-0.123	0.204*
Al	-0.105	0.182*
Zn	0.016	-0.180*
Sr	0.032	0.072*

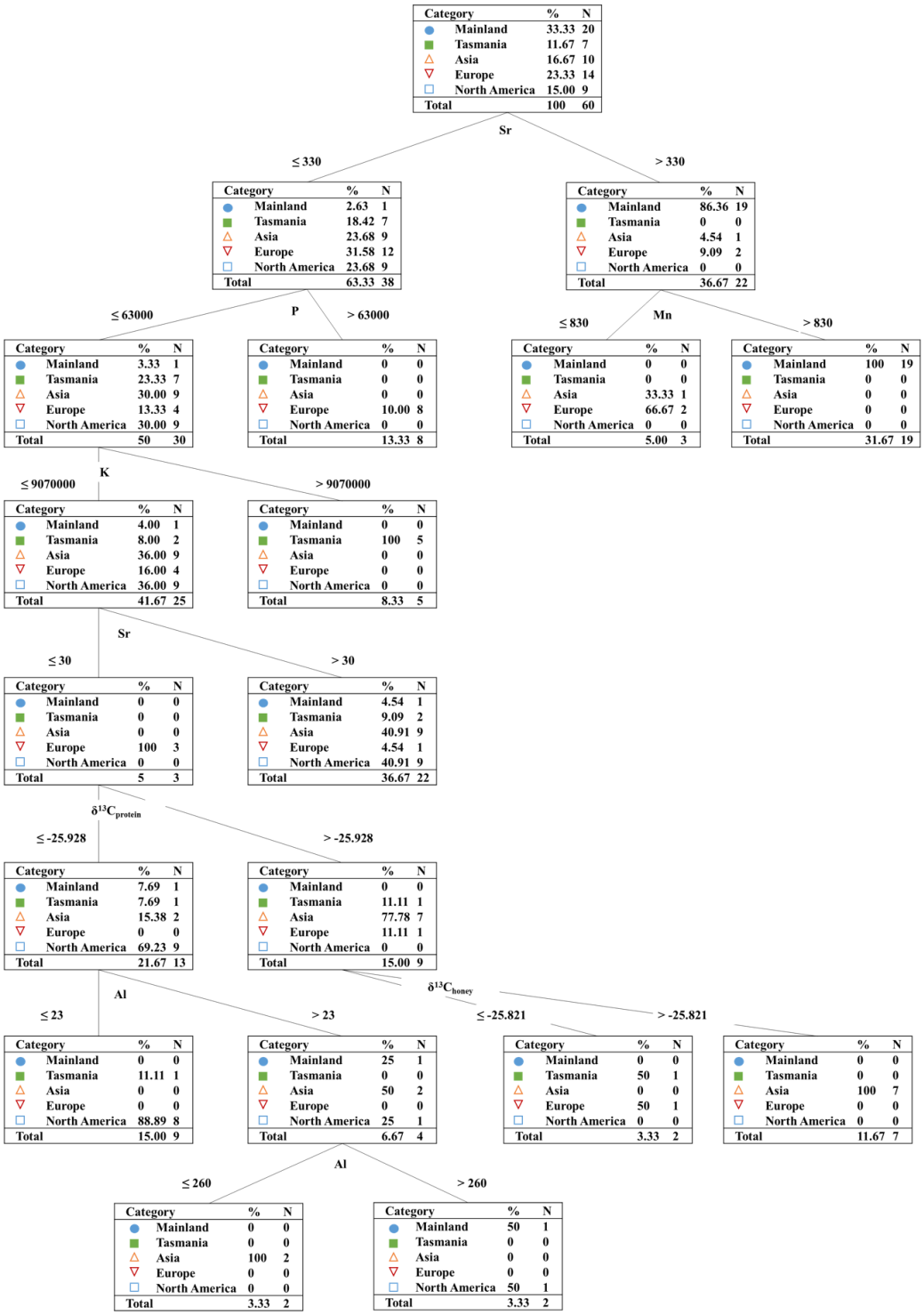
Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions.
Variables ordered by absolute size of the correlation within each function.
* Largest absolute correlation between each variable and any discriminant function
[b] Variable not used in the analysis.

Supplementary Figure S1. Concentrations (µg/kg) of trace elements Ca, Mn, Sr and P in authentic honey samples (n = 69) from mainland Australia (n = 24), Tasmania (n = 7), Africa (n = 1), Asia (n = 10), Europe (n = 15), North America (n = 9), and three unknown samples.

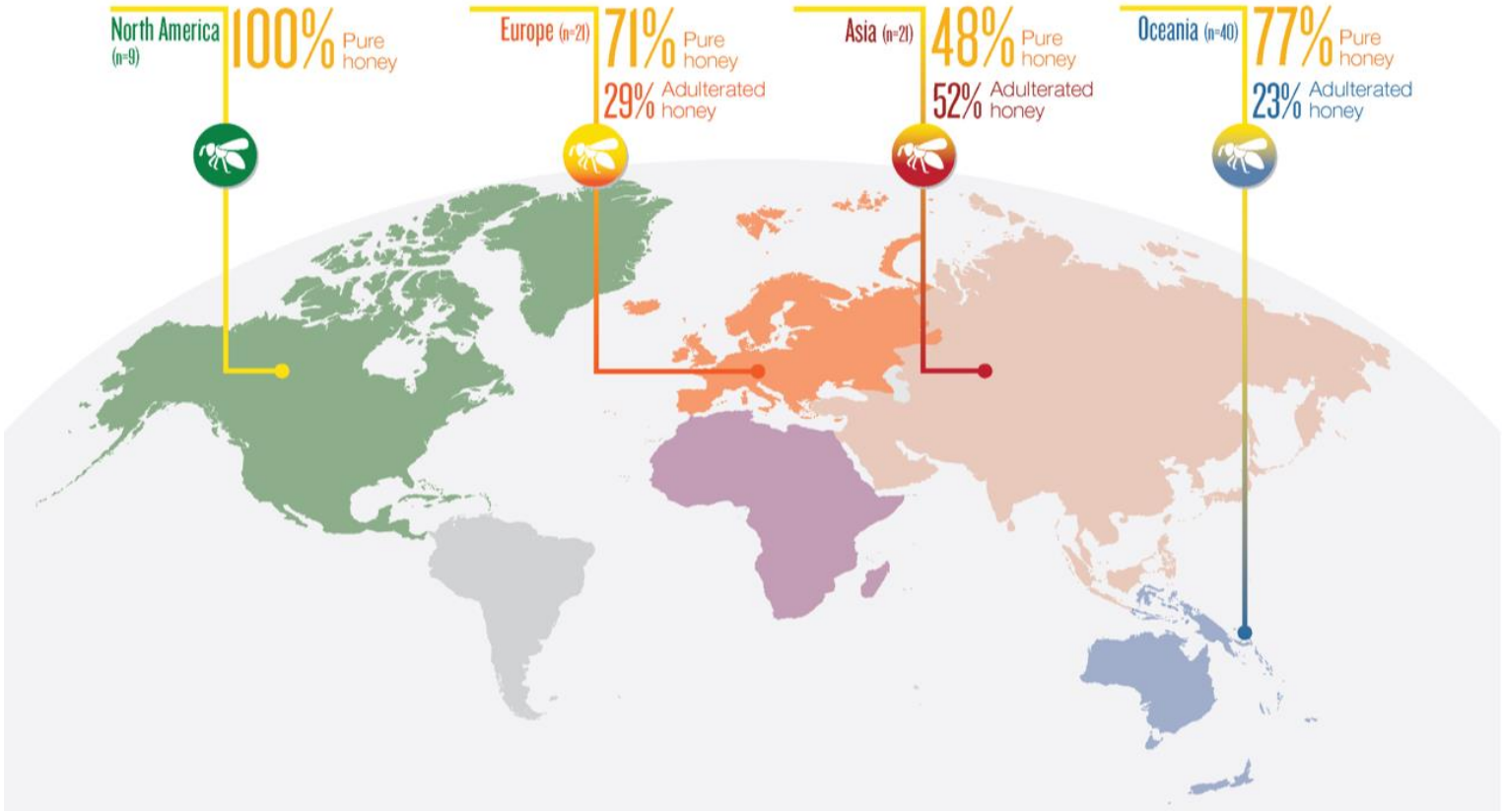


Trace elements Ca, Mn, Sr and P that had highly significant differences (p = <0.001–0.024; 5% significance) in Australian and international honeys (Supplementary Table S3) were selected as examples for scatterplots to show that there was no clear separate clustering according to the different regional and continental origin of honeys. Other paired trace elements (e.g. Ba, Fe, Mg, and Na) also had no clear geographic clustering. Combining any two of these elements did not separate honey according to its geographic origin. Therefore, PCA and CDA multivariate analysis was applied to all of the available variables (carbon isotopic ratios of honey and its protein and 16 trace elements). This approach resulted in clear groupings of honey derived from different geographic locations.

Supplementary Figure S2. Summary results of the C5.0 model for classification of the training set of authentic honey samples of known origin from mainland Australia, Tasmania, Asia, Europe and North America. The honey samples from Africa and those of unknown origin were excluded from the analysis.



Supplementary Figure S3. Summary results of this study's global investigation of honey authentication based upon analysis of honey $\delta^{13}C$, its protein and C-4 sugar content. Although the single African honey (n = 1) sample is excluded from the map it passed the C-4 criteria and is classified as an authentic honey.



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Calling for overseas honey samples

A PhD student working on ways to protect the Australian honey market from fraudulent mislabelling is asking ABA members for help

Australian honey has a well-deserved reputation globally as being 'clean and green' but each time a product is discovered on shelves that has been knowingly or mistakenly contaminated with overseas ingredients, that reputation risks serious damage.

Xiaoteng Zhou, a PhD student at Macquarie University is working to develop a method that will distinguish Australian honey from other sources.

Working with the National Measurement Institute in North Ryde, she is sending honey samples to the lab for carbon and nitrogen stable isotopic analysis to provide information on geographic origins.

This type of analysis has not been used for honey before in Australia but has been used to check the origins of dairy products.

Xiaoteng has been collecting a wide range of Australian honey samples for analysis, but now needs additional overseas samples for comparison. She wonders if some committed honey fans out there have sourced international honeys on their travels – and be prepared to share a small sample for science.

She requires some proof of origin and about 20 ml of honey for testing.

If you can help in this important work – or know of someone else or an organisation who has access to overseas honey – please contact **xiaoteng.zhou@students.mq.edu.au**

ABA Council Meeting: Nowra, August 6

Our next meeting is hosted by the Shoalhaven Beekeepers Association. The meeting will kick off at 9.30 am with forum where members can ask questions of the executive. This will be great opportunity to meet the new ABA president and treasurer and find out what the ABA has been doing to support beekeepers across the state. The regular council meeting will follow the forum. Issues on the agenda include:

- change in ABA President
- new Biosecurity Act and its regulations
- beekeeping registration and ABA recommendations
- 2018 ABA conference
- possible changes to ABA insurance
- standards for amateur extraction and honey packing facilities

VENUE: The West Street Community Centre, 28 West St (Cnr West/Worrigee St), Nowra

MEETING OPEN TO ALL MEMBERS FROM ALL CLUBS

Shoalhaven Beekeepers will be providing an excellent BBQ lunch – all welcome!

Pages 188-209 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Williams P. (2018, October 4) *If honey dissolves in water, is it fake? Scientists cast doubt on DIY 'purity' test*. ABC News. https://www.abc.net.au/news/2018-10-04/fake-honey-test-not-reliable-scientists/10332922

Ferguson, A. & Gillett, C. (2018, October 3) *Almost 20 per cent of Australian honey samples found to not be pure*. ABC News. https://www.abc.net.au/news/2018-10-03/almost-20-per-cent-australian-honey-found-not-pure/10327988

Hatch, P. (2018, October 4) *Honey probe could get extra sting after local samples deemed 'fake'*. The Sydney Morning Herald. https://www.smh.com.au/business/consumer-affairs/honey-probe-could-get-extra-sting-after-local-samples-deemed-fake-20181003-p507k7.html

Ferguson, A. & Gillett, C. (2018, October 3) *Fake honey scandal widens to Australian-sourced brands*, The Sydney Morning Herald. https://www.smh.com.au/business/consumer-affairs/fake-honey-scandal-widens-to-australian-sourced-brands-20181002-p507ar.html

ABC NEWS

Almost 20 per cent of samples of Australian honey not pure

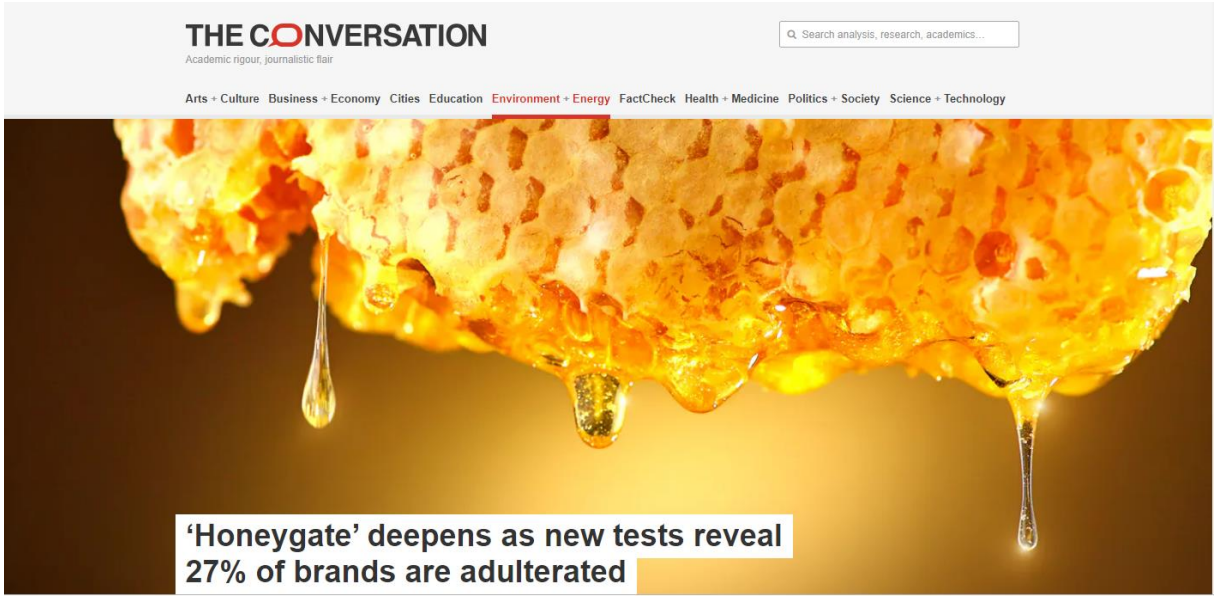
Updated 4 Oct 2018, 6:16am

Australian consumers who've been paying top dollar for pure local honey may have been duped, as the fake honey scandal widens to include premium Australian brands. Scientists have found one in five of the Australian honeys they tested was not 100 percent pure.

Read the statement from the Australian Honey Bee Industry Council here.

Adele Ferguson and Chris Gillett

Supplementary media coverage S7



More than a quarter of commercial honey brands have potentially been watered down with sugar cane, corn syrup or other products, according to our new analysis of 95 products from local food markets and supermarket shelves.

Our discovery is set to deepen the concern over the authenticity of honey for sale in Australia, in the wake of last month's "fake honey" scandal, which revealed the widespread adulteration of honey with cheaper substances.

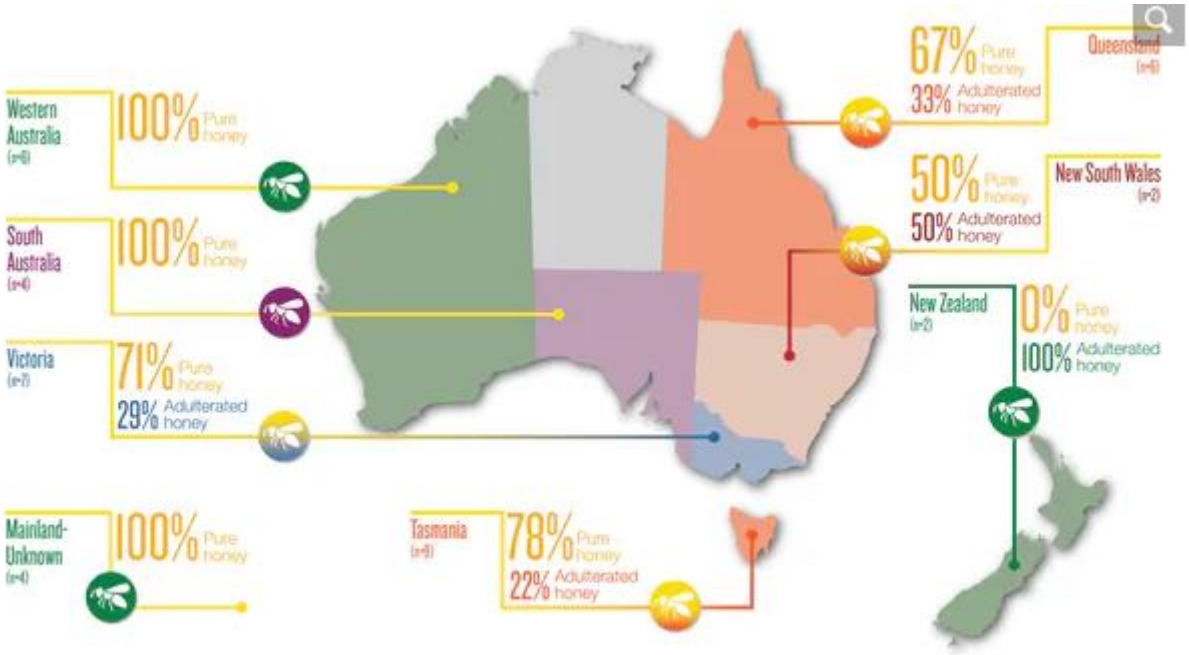
Australia is the world's fourth-largest honey exporter, and the revelations pose a threat to its reputation as a leading producer and supplier of honey.

Read more:** **What is fake honey and why didn't the official tests pick it up?

Our study, published in Nature's Scientific Reports, analysed 100 honeys from 19 countries, including Australia. The study included five raw honey samples (that is, honey direct from the hive) and 95 commercial samples, 38 of them from Australian-based producers.

Analysis of the 95 commercial honeys showed that 27% of them were of "questionable authenticity", meaning they had potentially been adulterated with cane and/or corn syrups. This means they should not be classified as genuine pure honey.

Of the Australian-sourced commercial honeys we analysed, 18% were identified as likely to have been adulterated in a similar way.



Results of pure and adulterated Australasian honeys analysed in this study. Monique Chilton/Copperplate Design

Our study used the only internationally accepted method for determining honey adulteration. This method detects the presence of sugars from a type of plants known as C4 plants – the group that includes corn and sugar cane – as opposed to pure honey, which is made from the nectar of flowers from a different group, called C3 plants.

C4 and C3 plants each have unique isotopic signatures, which allows us to ascertain whether a honey sample is pure (containing only compounds from C3 plants) or whether it has been adulterated with sugars from C4 plants.

An old problem

Honey adulteration is nothing new. It has been on the rise since the 1970s, when cheap high-fructose corn syrup became widely available. Because both corn syrup and sugar cane are cheaper than honey, they are an easy way to increase honey volume and boost profits.

Some operators adulterate honey with rice sugars that enable them to circumvent the C4 test. Some rice syrup producers openly advertise the fact that their products will not cause adulterated honeys to fail the C4 test.

Honey can be adulterated either during or after production. Inadvertent adulteration might happen through overfeeding of sucrose to bees during periods when food sources are limited, or at harvest time. This practice, if done occasionally, can protect colonies at times of low food availability. But if used injudiciously it can also filter through into the finished product.

Of course, our study also comes hot on the heels of recent revelationsthat 12 of 28 Australian honeys were adulterated with rice and other syrups. That discovery was made using a new proprietary method that can reportedly detect adulteration with a wider range of compounds and also identify the geographic origins of the honey.

However, this method does not currently meet the provisions of the Codex Alimentarius Commission, the international body that sets food standards.

Our research group has previously shown that honey can indeed be traced back to its point of origin, by comparing trace chemicals in bees and their honey with those in the dusts and soils where it was produced.

In our latest research, we therefore also investigated whether the commercial honey samples can indeed be tracked back to where they supposedly came from. We found that honey from different continents and regions do indeed have different chemical signatures, which paves the way for detecting mislabelled or geographically fraudulent honey.

Read more:** **How better tests and legal deterrence could clean up the sticky mess left behind by fake honey row

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There is no evidence that adulterated honeys cause any significant health risk (beyond those posed by eating sugary foods anyway). However, in many cases consumers are not getting the supposedly genuine pure honey they have paid for.

But our research, along with previous studies, reveals the scale of the problem.

For Australian honey to retain its premium position in the global market, there needs to be a better framework around the chain of custody and certification of honey. Only then will customers have a guarantee that their “pure” honey is exactly what it says on the label.

The Sydney Morning Herald

Wednesday, October 3, 2018 $3.20 (inc GST)
First published 1831 No. 56,466

INDEPENDENT. ALWAYS.

CBD
Glencore sues tax office boss over Paradise Papers
PAGE 2

ROSS GITTINS
Why do we put profit before people?
COMMENT PAGE 18

WALL STREET CRASH
How GI went from peak to trough
ANDREW WEBSTER SPORT

Genetic testing for new parents

EXCLUSIVE

Kate Aubusson
Health editor

Ten thousand would-be parents will get free testing to detect about 500 genes linked to severe disorders they risk passing on to unborn children in an unprecedented trial that could radically transform family planning in Australia.

Researchers are finalising the extraordinary list of severe and deadly genetic conditions to be included in a large-scale preconception carrier screening trial. It could start recruiting couples in 2019.

The $20 million pilot trial is the cornerstone of the $500 million Australian Genomics Health Futures Mission – the largest investment by the federal government's Medical Research Future Fund.

The Australian Reproductive Carrier Screening Project (ARCSP) will include spinal muscular atrophy (SMA), cystic fibrosis and fragile X syndrome. Eventually, affected couples would be offered subsidised IVF treatment.

It has been dubbed "Mackenzie's Mission" by Health Minister Greg Hunt in honour of baby Mackenzie Casella, who was diagnosed with SMA1. Mackenzie died in October 2017 at seven-months old.

Her parents Rachael and Jonathan Casella successfully campaigned for routine carrier testing. Before Mackenzie's diagnosis, the couple

Continued Page 7

Trust your local honey? One in five Australian samples are fake

Professor Mark Taylor and student Xiaoteng Zhou test honey samples at Macquarie University Photo: Wolter Peeters

About 20 per cent of honey sold in Australia's east – including boutique brands – has been adulterated, research suggests. NEWS PAGE 6

ABC should be so lucky: Milne backed hiring Kylie

EXCLUSIVE

Michael Koziol

Former ABC chairman Justin Milne wanted to hire pop icon Kylie Minogue to sing about the public broadcaster in a multimillion-dollar advertising campaign.

Multiple sources have told the *Herald* the idea was shot down by senior ABC management, including former managing director Michelle Guthrie, because the singer's $750,000 price tag was deemed too expensive.

Ms Guthrie is understood to

Continued Page 4

ABC board scandal bites in Wentworth byelection
NEWS Page 4

Keating savages Turnbull over limp republic effort

Paul Keating has accused Malcolm Turnbull of capitulating to conservatives in the fight for a republic, denouncing the toppled leader and declaring Australians would need a "microscope" to find his true beliefs.

Mr Keating attacked the former prime minister, saying he did too litle during his three years in power and had "failed dismally" to lead the Liberal Party back to the centre of Australian politics.

The rebuke came after Mr Turnbull dismissed Kevin Rudd and Tony Abbott as "miserable ghosts" and said he would keep up his advocacy for a republic.

FULL STORY Page 5

Appendix F

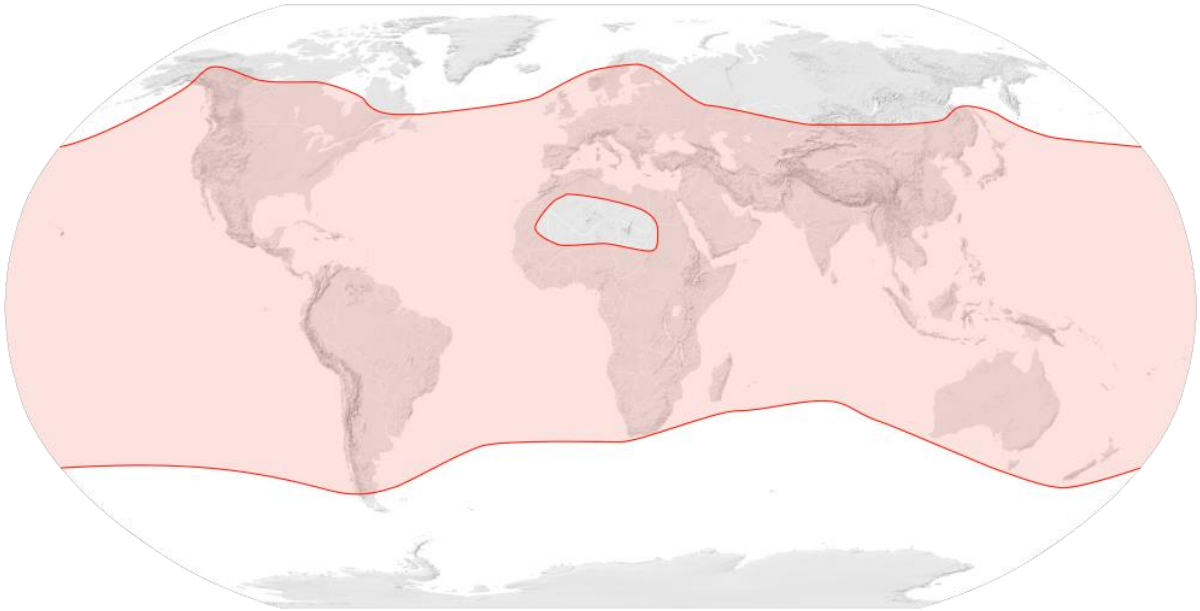

Figure S6-1. Distribution of the European honey bees (*Apis mellifera*) (source: Wood 2014).

(a)

(b)

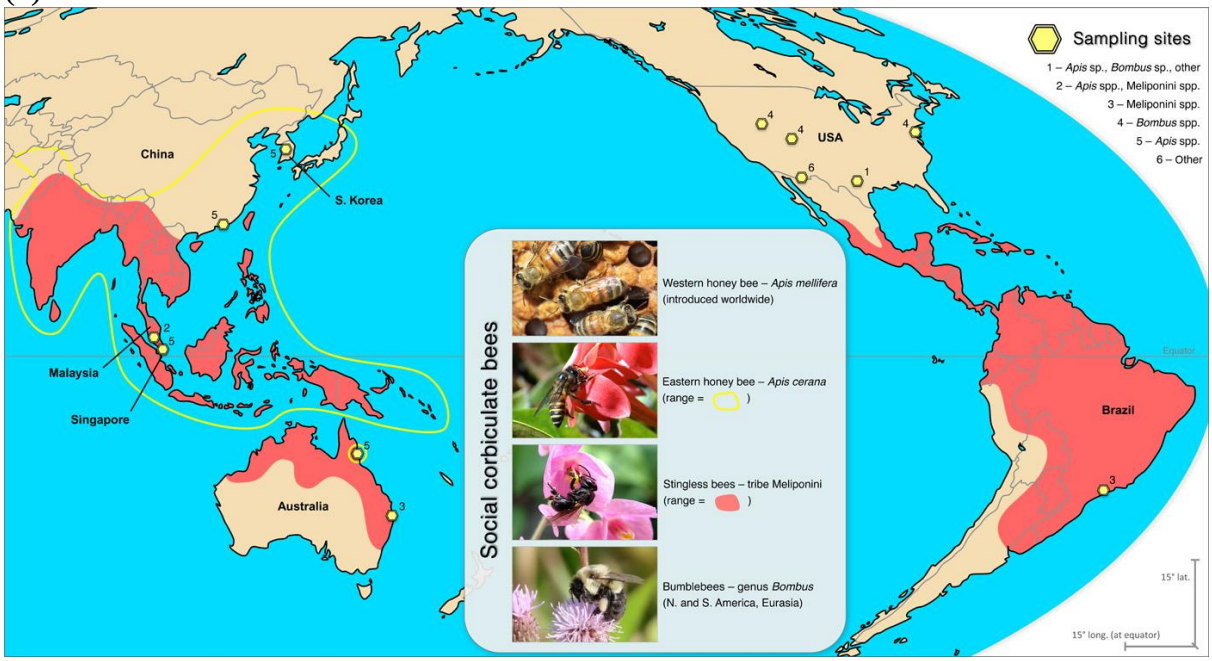

Figure S6-2. Distribution (blue in (a) and red in (b)) of stingless bees (Apidae, Meliponini). The stingless bee studied in this thesis is *Tetragonula carbonaria*, which belongs to the family Apidae, sub-family Apinae and tribe Meliponini. (a) The global distribution map of Apidae Meliponini was sourced from Sakagami (1982). Since 1982 stingless bees have become a more popular bee to be kept domestically across the globe (Hrncir et al. 2016). A recent snapshot of Apidae Meliponini distributution is provided in figure (b).

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