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Title: Pursuing sensitivity limits of biochemical geographic discrimination as generic tool for high risk pest plants

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Contents

1. Executive Summary

There is an urgent need for a method of provenance determination of biosecurity samples, insect incursions in particular. Biogeochemical markers, which are non-inherited but linked to geographic variation, are presently the only viable option for this. However, the existing techniques are not fit-for-purpose, as they are too slow, expensive, and lack the required analytical sensitivity.

A new method of Sr isotope analysis has been developed that allows for $^{88}Sr/^{86}Sr$ ratios to be obtained with 99.8% precision on 2-4 ng of Sr using a Thermo iCap TQ ICP-MS in MS-MS mode This equates to approximately 0.4 mg of insect tissue. In addition, we investigate the Pb concentration in insects and link this to preliminary method development for Pb isotopes that indicate Pb isotope ratios can be obtained on the Thermo iCap TQ ICP-MS on 4 mg of Pb. This equates to minimum of 5 mg of insect tissue. These methods now enable the use of Sr and Pb isotope analysis to help answer biosecurity questions about provenance as it allows for very small amounts of tissue to be analysed. The technique is relatively inexpensive and has a fast turnaround, and is thus suitable for biosecurity operations as well as population-scale studies.

Here we assessed how variation in feeding styles affects the concentration of the provenance sensitive elements Sr and Pb in insects, and therefore if there were limits to the use of single specimens for measurement of their isotopes. Ten insect species were studied to include the functional groups of leaf-chewing moths, sap-sucking bugs, wood boring beetles, root feeders, nectar feeders and carnivores. There was no distinction in Sr or Pb concentrations for the ten different insect species. From this we conclude that functional group does not influence the Sr and Pb concentration of insects and the weight of single specimens suffice for analysis except very small species such as scale insects.

2. Introduction

The ability to discriminate geographic origin of a biosecurity threat is immensely valuable for biosecurity decision-making (Heinrich and Collins, 2017; Holder et al., 2014; Holder et al., 2015; Hoogewerff et al., 2019; Lin et al., 2019). Government agencies responsible for biosecurity need to be able to evaluate as quickly as possible whether or not an intercepted insect pest species is of a local origin or not. A local origin implies an extant breeding colony requiring a decision on quarantine and response, and with possible implications for interrupted trade. If the intercepted pest insect is not local then there is no indication of an established population and the response can be more circumspect. In either case the government agencies need to identify the invasive pathway using any evidence as to geographic source. Therefore biosecurity agencies need to interrogate intercepts with multi-pronged approaches to discriminate geographic origin, such as the use of Sr, Pb, O and H isotopes and genetic analysis with fast turnaround. Unfortunately, when a biosecurity threat is encountered there is generally limited material available for these analyses. A 1 cm long insect may only have a dry mass of 100 mg and a 2-3mm insect as little as 5 mg. Therefore, to know how much material is required for successful analysis of provenance tools such as Sr and Pb isotopes in different species will pre-empt subsampling needs to allow for timely outcomes.

The chemical similarity of Sr with Ca leads-to relatively high concentrations of Sr in many biological tissues (Ashcroft and Stanfield, 1981; Ashcroft and Stanfield, 1982). Thus, given the high spatial variation of Sr isotopesthe ratio $^{87}Sr/^{86}Sr$ is an ideal provenance tracer. The variability of Sr isotope ratios is related to a combination of their inherent variation in different bedrock geologies and from variable environmental and anthropogenic sources (Bentley, 2006; Faure and Mensing, 2005; Hoogewerff et al., 2019; Willmes et al., 2018). Similarly Pb isotope ratios ($^{208}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$ and $^{206}Pb/^{204}Pb$,) have high spatial variation (Faure and Mensing, 2005), but this isotope system has not been used as frequently for biological provenance as Sr isotopes because the natural signals are commonly disrupted by anthropogenic activity; which confound ecological and archaeological applications, but create a largely untapped potential for forensic applications.

Here we investigate the Sr and Pb concentration in ten insect species with different feeding types, including the functional groups of leaf-chewing moths, sap-sucking bugs, wood boring beetles, root feeders, nectar feeders and carnivores. These include species that are either on the National Priority Plant Pests list (NPPP, 2016) or are proxy for insects on the NPPP and allows us to asses if the different feeding types have an influence on the Sr and Pb concentrations in individual specimens. This will enable biosecurity experts to be informed on the quantity of insect tissue required for Sr and Pb isotope analysis.

We use individual specimens of these insects to assess our newly developed technique for measuring Sr isotopes. This technique uses Mass Spectrometry-Mass Spectrometry Inductively Coupled Plasma Mass Spectrometry (MS-MS ICPMS; Bolea-Fernandez et al., 2016) on a Thermo iCap TQ system (Murphy et al., 2019). The small size of many pest insect species and the requirement for subsampling for other techniques means that the quantity of Sr available for isotope analysis is commonly sub optimal for conventional Sr isotope analysis using Thermal Ionisation Mass Spectrometry (TIMS) and Multi Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICPMS). The MS-MS ICPMS method requires significantly lower quantities of Sr than conventional Sr isotope analysis albeit with lower precision (Bolea-Fernandez et al., 2016; Murphy et al., 2019). Our method also has the advantage of having a much faster turnaround time for analysis because the isobaric interference between ^{87}Rb and ^{87}Sr can be removed during analysis rather than requiring an additional chemical separation step as is the case for conventional Sr isotope analysis. Time for analysis is effectively reduced from several weeks to a few days, making it eminently more suited to biosecurity needs.

3. Aim

To estimate the amount of tissue required for Sr and Pb isotope analysis on insects with different feeding styles, using model species, by:

1. Quantifying the variability of Sr and Pb concentrations in the different insect types and to assess how this may relate to their mode of feeding.

2. Assessing how much insect tissue is required to achieve an accurate and precise Sr isotope analysis using the MS-MS ICPMS method (Bolea-Fernandez et al., 2016; Murphy et al., 2019) and Pb isotope analysis using the single quadrupole ICPMS method of Ulrich et al. (2010).

4. Methods/Process

We have collected samples of ten insect species with different feeding behaviours in terms of diet and from a broad region around Brisbane. Insects are from existing collections or caught from the wild. The insect collections from which insects where sourced include pinned insects from the entomology collections at Queensland University of Technology, The University of Queensland, The Queensland Museum and the Biosecurity Queensland, Queensland Department of Agriculture and Fisheries. Insects from the wild were caught in various locations around Brisbane using standard insect collection techniques. The details of sample preparation and mass spectrometry are provided in Murphy et al. (2019)

5. Achievements, Impacts and Outcomes

5.1 Insect description

Phoracantha semipunctata (Fabricius 1775) (Coleoptera: Cerambycidae). Commonly known as the Australian Eucalyptus longhorn. This species is a woodborer which specialises on Eucalyptus (Hanks et al., 1995; Hanks et al., 2005) and adults are typically 22-28 mm in length. The larvae of these beetles bore into the trunk of the tree feeding on the cambium tissues and can cause serious damage which can result in tree mortality (Hanks et al., 2005; Paine et al., 2010). Adults have also been known to eat pollen and nectar of Eucalyptus (Hanks et al., 1995). This species and its close relative *P. recurva* are considered to be pests of Eucalyptus plantations in several parts of the world including in California (Hanks et al., 1995), and South Africa (Paine et al., 2010).

Rhopaea magnicornis (Blackburn 1888) (Coleoptera: Scarabidae). Is commonly known as the brown cockchafer or cane grab for which adults are typically 21-30 mm long (Miller and Allsopp, 2000; Miller et al., 1999). As larvae, this species feeds on the roots of cane crops and grass, which in turn stunts the plant's growth and reduces yield; plants can unintentionally be pulled out at harvest time. Given Australia's sugar cane industry, this species is considered an agricultural pest due to the damage it can cause (Allsopp and Lambkin, 2006; Robertson et al., 1995). A closey related species, *R. verreauxi*, has been known to feed on plant roots and organic matter (Cairns, 1982).

Tribolium castaneum (Herbst 1797) (Coleoptera: Tenebrionidae). This is known as the red flour beetle. This small beetle grows to 2.2-4.4 mm and is thought to have originated from the Indo-Pacific is now found almost globally; it is the cause for much agricultural concern (Boukouvala et al., 2019). This well-known species is a major pest of cereal and post-harvest grains, and the consequence of infestation is major decrease in yield (Boukouvala et al., 2019; Hill, 2002; Rahimi Namin et al., 2018). Like most beetles, this species uses a chewing mechanism for feeding type.

Theseus modestus (Stal 1865) the gum tree shield-bug and *Austromalaya reticulata* (Westwood 1837), the brown long-headed shield-bug are both shield-bugs (Hemiptera: Pentatomindae). Insects from this family typically feed on xylem and are noted as stem-sucking insects (Miller et al., 1999). *Theseus medestus* is about 14-16 mm long and is found Australia-wide mostly in eucalyptus forests (Department of Agriculture and Water Resources 2017), while *A. reticulata* is 13-18 mm long and is also found Australia-wide (Department of Agriculture and Water Resources 2017). Neither species

has been the subject of much research and seem to have little impact to agriculture, but they both serve as a proxy for another well-known pest pentatomidae, *Halyomorpha halys* or the Brown Marmorated stink bug which is of global pest concern (Lee et al., 2013, Department of Agriculture and Water Resources 2017).

Icerya seychellarum (Westwood 1855) (Hemiptera: Monophlebidae). This species is a scale insect sometimes referred to as the Seychelles scale insect, which is found throughout Australasia, South America and Indomalaya (Unruh and Gullan, 2008). These species grow to 10 mm in length and is polyphagous, feeding on the phloem of trees (Hill and McC., 1980). While the impact and pest status of *I. seychellarum* is thought to be low, its close relative *I. purchasei* is of much more concern. Due to close relationship and same feeding mechanisms *I. seychellarum* is used as a proxy for *I. puchasei*.

Amegilla cingulata (Fabricius 1775) (Hymenoptera: Apidae), the blue banded bee. This species grows to 14 mm long (Leijs et al., 2017). Bees of the genus *Amegilla* are known to forage pollen and nectar on a wide range of plants and to be pollinators of many Australian plants (Leijs et al., 2017) and could produce adequate pollination for commercial greenhouse tomatoes (Hogendoorn et al., 2007). This Australian native species currently presents no pest status but may be of commercial value.

Apis mellifera (L 1758) (Hymenoptera: Apidae). This species in commonly named the European honey bee which grows up to 17 mm in body length. This insect is one of the most recognised and studied insects due to its ability to provide honey and pollination services for many crops. (vanEngelsdorp and Meixner, 2010). Because of this, the species is now found globally. This species is of great commercial value and is not considered a plant pest.

Abispa ephippium (Fabricius 1775) (Hymenoptera: Vespidae). Commonly known as the Australian hornet. Adults grow up to 30 mm long and is found Australia-wide. Adult *Abispa* species build nests for their offspring to mature in. Adults will hunt caterpillars and bring them back to their nests to allow larvae to feed on them during early instars (Matthews and Matthews, 2004). This is the only species used in this study that represented a predatory feeding mechanism.

Lophyrotoma (Ashmead 1998) (Hymenoptera: Pergidae). The insects are known as sawflies as adults and spit-fire grubs as larvae. They are found along the east and west coasts of Australia. Larvae of some *Lophyrotoma* species grow to 32 mm. *Lophyrotoma zonalis* larvae are gregarious leaf feeders who often “skeletonize” leaves and can defoliate trees (Burrows and Balciunas, 1997) which has led to it being used as a biocontrol for *Melaleuca* (Myrtaceae) in Florida (Buckingham, 2001). Adults of *L. zonalis* don’t feed. None of the species within *Lophyrotoma*. are currently considered pests, even given their plant defoliation potential in large numbers, but other sawflies such as The European wheat stem sawfly (*Cephus pygmeus*) are of concern.

Euploea core-corinna (Cramer 1780) (Lepidoptera: Nymphalidae), is known as the common crow. Adults have a wingspan of up to 95 mm.This butterfly species has a large distribution range across the top end of Australia and down its eastern coast (Scheermeyer, 1985). This polyphagous insect is known to feed on at least three different plant families (Scheermeyer et al., 1989). Larvae feed on plant leaves while adults feed on nectar. While *E.core* is not considered a pest species it may be informative for understanding other lepidopterans.

For the remainder of the document the common name for each insect species will be used.

5.2 Sr and Pb concentrations in insects

All insects were analysed for 43 elements including Sr and Pb (Murphy et al., 2019). The Sr concentration of the insects ranged from 0.2 to 9 ppm, with the vast majority of insects (76 %) having Sr concentrations between 1 and 3.5 ppm (Figure 1a). The different insect species do not demonstrate any significant difference in Sr concentration, although the common crow and the blue banded bee do show a greater range of Sr concentrations than the other insect species. The quantity of Sr in nanograms (ng) in an insect relates the Sr concentration to the insect mass (Figure 1b). While there is no significant variation in Sr concentration between insect species investigated, the different species have considerable differences in mass (Murphy et al., 2019). Therefore, there is substantial variation in the quantity of Sr among the different species that is controlled by insect mass (Figure 1b).

The Pb concentration of the insects is far more variable than Sr (Figure 1c) with a range from 0.003 to 23 ppm, although the majority of samples (51 %) have Pb concentrations between 0.01 and 0.35 ppm. In addition, six samples had Pb concentrations below the detection limit. All insect species demonstrate at least an order of magnitude variation in Pb concentration (Figure 1c). Similar to Sr, the different insect species do not demonstrate any significant difference in Pb concentration. The wide range of Pb concentrations also results in a high degree of variability in the quantity of Pb in the different insects.

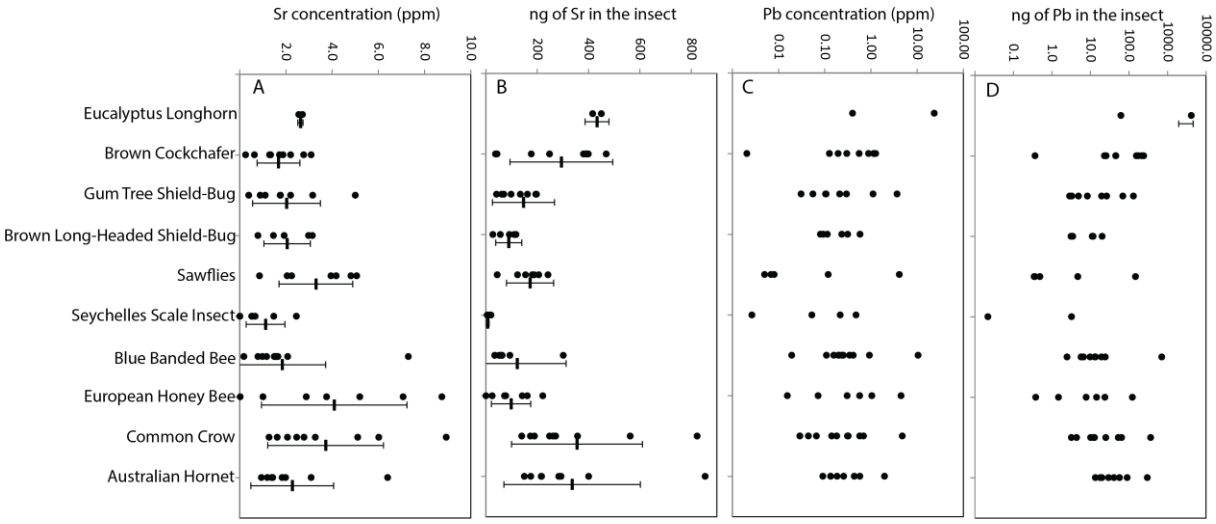

Figure 1: Plots of variation in Sr concentration (A), the quantity of Sr (B), the Pb concentration (C) and the quantity of Pb (D) in individual specimens of the ten insect species investigated (modified after; Murphy et al., 2019). Note that the scale for A and B is linear while the scale for C and D is logarithmic. Only for A and B is the mean and standard deviation included.

5.3 Mass of insect tissue required for Sr and Pb isotope analysis

Murphy et al. (2019) analysed Sr isotopes on the Thermo iCap TQ in 0.8 ml of solution diluted to achieve a Sr concentration of between 0.25 to 0.5 ppb, which corresponds to 0.2 to 0.4 ng of total Sr in the solution. This compares to the much greater quantities required previously by Armstrong et al. (2017) who analysed Sr isotopes on the Agilent 8800 in 2 ml of solution diluted to achieve a Sr concentration of 5 ppb, which corresponds to 10 ng of Sr. All but the Seychelles scale insects have Sr quantities in individual specimens greater than 10 ng (Figure 1b) and so those insects could be analysed for Sr isotopes using either the Thermo iCap TQ or the Agilent 8800 methods.

Note, the Agilent 8800 is the first generation triple quadrupole ICPMS while the Thermo iCap TQ along with the Agilent 8900 are the newest generation triple quadrupole ICPMS and so are expected to have similar sensitivities.

Given that the majority of insects (83 %) have Sr greater than 1 ppm, an insect tissue sample of 0.4 mg and 10mg for the Thermo iCap TQ or the Agilent 8800, respectively is required for a successful Sr isotope analysis.

Preliminary Pb isotope experiments on the Thermo iCap TQ indicate that precision similar to that achieved by Ulrich et al. (2010) can be accomplished with 0.8 ml of solution diluted to achieve a Pb concentration of 5 ppb, which corresponds to 4 ng of Pb. This is a lower quantity of Pb than the study of Ulrich et al. (2010) because the Thermo iCap TQ is a newer generation ICPMS than they used. All but the Seychelles scale insects have greater than 4 ng of Pb (Figure 1d) with 70% of the individual insect collected that could be analysed for Pb isotopes using either ICPMS instrument type.

Given that the majority of insects (76 %) have Pb greater than 0.1 ppm, an insect tissue sample of 5 mg is required for a successful Pb isotope analysis on the Thermo iCap TQ.

Of note, because both the Sr and Pb concentrations of the analysed insects show considerable variation (Figure 1), it is advised that the Sr and Pb concentration of a sample be analysed before attempts at measuring the isotope ratios so that samples can be diluted for optimal isotope analysis.

6. Discussion and Conclusion

The relatively limited variation in Sr concentration (107% RSD) observed in the analysed insects was proposed by Murphy et al. (2019) to relate to the chemical similarity of Sr to Ca (e.g. Ashcroft and Stanfield, 1981; Ashcroft and Stanfield, 1982). Insects modulate their Ca content and Murphy et al. (2019) proposed that Sr would be modulated similarly.

The comparatively high degree of variation in Pb concentration (1405% RSD) likely relates to the high degree of variation of Pb in the environment (Holder et al., 2014).

There is no obvious indication that different feeding mechanisms, or diets per se, have an impact on either Sr or Pb concentrations in the different insect species investigated. Therefore insect mass is the critical control on the quantity of these elements available for analysis.

For most of the insects analysed in this project there is sufficient Sr and Pb in a single insect for precise and accurate Sr or Pb isotope ratio determination.

The digestion method and Sr isotope method developed as part of this project is presented in Murphy et al. (2019). This new method will allow for rapid and relatively inexpensive Sr isotope analysis that befits biosecurity requirements for timely, evidence based decisions.

This project was specifically designed to test if there is variation in Sr concentration between insect functional feeding groups. Nevertheless, because the insects analysed came from a wide geographic area around Brisbane, Murphy et al. (2019) the study was able to use the Sr isotope data to investigate the spatial variability of Sr isotopes around Brisbane. This supported the power of Sr isotopes as a spatially sensitive provenance tool.

7. Recommendations

Further research specifically designed to address geographic variation in Sr and Pb isotopes, and demonstrate their reliability as provenance tools, is required. For example, isotope ratios in insects with a restricted spatial diet and in insects with a foraging diet over a large spatial area, or to confirm that different insects feeding on different plants in the same geographic spot have the same isotope ratios.

Strategic development of geographic reference data for bioavailable Sr and Pb isotope ratios is essential for these markers to be useful with biosecurity detections. Using the information from this current study about element quantities, together with this new mass spectrometry method enabling population-scale analysis, generation of on- and off-shore regional isoscapes relevant to particular industries would be a very useful next step.

Additional investigation of urban contaminants or agricultural practices that could influence Sr and Pb isotope ratios is also recommended as a means to potentially improve the spatial resolution possible with otherwise only natural abundance geological sources.

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