Isotopes and trace elements as geographic origin markers for biosecurity pests

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A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy
at
Lincoln University
by
Peter Wilfred Holder

Lincoln University
2012

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Protecting a nation's primary production sector and natural estate is heavily dependent on the ability to rapidly determine the risk presented by incursions of exotic species. Accurate point of origin discrimination in such biosecurity incursions can direct appropriate operational responses in exotic pest eradication and post-border incursion campaigns, as well as identify risk pathways.

Reading natural abundance biogeochemical markers via mass spectrometry methods is a powerful tool for tracing ecological pathways as well as provenance determination of agricultural products and items of forensic interest. However, the application of these methods to trace insects – man's most damaging competitors – has been under utilised to date because our understanding in this field is still in a phase of basic development.

The internationally distributed moth, *Helicoverpa armigera* (Noctuidae), has been used to examine processes that are fundamental to the location-to-plant-to-insect imprinting of light and heavy element isotope ratios and trace element profiles. The validity of using multivariate biogeochemical profiles to differentiate insects of New Zealand natal origin from insects of exotic origin was also assessed.

This study initially required the development of an integrated method for the collection of natural abundance δ^2H, $^{87}Sr/^{86}Sr$, $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios and trace element concentration profiles from single insect specimens. In a comparison of moths from Australia and New Zealand, none of these biogeochemical markers were individually able to separate moths from the different experimental regions (150 to 3000km apart). Conversely, the region of origin was able to be distinguished for approximately 75% of individual *H. armigera* samples using multivariate analysis. Therefore, the determination of whether a biosecurity sample has originated from its collection point, or not, is likely to be successful.

In addition, the primary constraints and limitations of natural abundance biogeochemical science in an entomological context have been quantified sufficiently to enable further application of biogeochemical markers. These limiting factors are: 1) the expression of short term, input signal δ^2H variation in insects, not the average annual rainfall δ^2H; 2) hydrogen turn-over in adult moth wing tissue over the first four days post emergence affects the δ^2H signal; 3) adult diet affects trace elements expression in whole insect preparations; 4) polyphagy introduces biogeochemical signals from more than one host species into the insect population, and affects both light element isotope and trace element biogeochemical signals; and 5) there is a significant degree of intra-population variation in marker expression.

These limiting factors need to be either resolved by subsequent research, or taken into account when using biogeochemical markers in entomology. Given the geographic resolution demonstrated, with further adaptation of the biogeochemical methodology to accommodate additional global regions and insect-host relationships, this approach to geo-location has considerable potential for biosecurity as well as other disciplines including forensics, ecological studies and pest management.

Keywords: hydrogen, Sr, Pb, isotope, trace-element, natal, place-of-origin, geo-location, natural-abundance, biogeochemical-markers, biosecurity, insect, *Helicoverpa armigera*, Lepidoptera.

Acknowledgements

In the spirit of 'Toy Story,' picture me as one of the little green dash-board toys. To the following people, "I am eternally grateful...!"

To my loved ones, Heather and our kids, I owe my biggest and deepest heartfelt thanks. They have supported me and kept their faith in me throughout this epic journey. Although undoubtedly, it's been tougher on Heather than on me!

This project has been a real collaborative effort. Specialists, technical staff and growers have all been most generous with time, knowledge, physical grunt and access to labs and land. My sincerest gratitude goes to my supervisor, Karen Armstrong, who has both needled and supported me throughout – at the same time full of understanding of the struggle and allowing me the freedom to learn and work in my own way. The other members of project team, Tim Clough and Russell Frew (Otago University) have been both fantastically generous and veritable fonts of wisdom.

I owe special thanks – and I seem to recall at least one good hangover – to Joel Baker (Victoria University) who had faith in my crazy plan to get heavy isotopes from insects, contributed to the 'partitioning of the bug' scheme and had sufficient patience to allow me to continue using his fantastic lab – even after several explosions.

I single person whom above all others enabled this project to succeed is Marc-Alban Millet (Victoria University). If you get to read this Marc, I owe you big time for your knowledge and drive, not to mention the good grace to answer my semi-constant calls for help, information and even more lab time.

A smorgasbord to international specialists have all had a shoulder on the wheel at several points. I hope I have recalled them all! Judith Willis (University of Georgia) for helping understand an insects' skin; Len Wassenaar & Keith Hobson (Environment Canada) who, apart from being great guys, helped shape and steer my project, Wolfram Meier-Augenstein (The James Hutton Institute) is always encouraging and speedy to supply answers to curly questions, and Thure E. Cerling (University of Utah) gave me the code that enabled me to get the Reaction Process Variable going, and he emailed it to me while in the field in Africa!

Laura Font (University of Amsterdam) who put me on the path to Sr isotope right-ness, Troy Baisden and Liz Keller (GNS Science) continue to be super generous with δ^2H modelling, the knowledge and help of Scott Hardwick (Landcare Research) and Dave Murray (QDPI) got me started with this love-hate thing I have going with *Helicovepa armigera*, Brett Robinson

(Lincoln Uni) has got me to begin to understand the nature of trace elements, Régis Doucelance (Clermont-Ferrand Magmas et Volcans laboratory) did my TIMS analysis – and brilliantly too!

The stats part of this work has also had many wonderful people involved. These have been David Baird (VSN NZ Ltd), James Ross and Dave Saville (Lincoln), and my PERMANOVA saviours Rebecca McLoed (Otago) and Marti Anderson (Massey University). Plus, Sam Brown (Lincoln) has been super generous in helping me R the graphs.

I also want to say a special thanks my mate Darryl who has given me a home and friendship in my forced Victoria Uni exile, and Andy and Lisa who have done the same for my prolonged Otago Uni sessions. Rebecca Hood-Nowotny, Rob Van Hale and Dave Murray, are also among the kind souls who have hosted this science gypsy.

The real back-bone of this, like almost all other science effort, is the support of the technical staff. Anne Barrington (Plant and Food) has given me huge help for which I AM eternally grateful. Roger Cresswell and Lynn Clucas have been super helpful and efficient in getting my soils program sorted. And last but certainly not least my send a huge thanks to the great folk in the Otago Uni Mass Spec scene, especially Robert Van Hale, Dianne Clarke, Emad Ehtesham, Malcom Reid, Claudine Stirling and Dave Barr.

Table of Contents

List of Tables

List of Figures

Chapter 1
Introduction

1.1 Project rationale

1.1.1 New Zealand's biosecurity

'Biosecurity' is the prevention of exotic pest and disease introductions, as well as the mitigation of such introductions, to protect a nation's economy, environment, and public health. The economy of New Zealand is based largely on export oriented primary production and has an international advantage in being relatively pest free. As a consequence, the economy and environment of New Zealand is "more dependent on successful biosecurity than any other developed country" (Biosecurity-Council, 2003). There is a high risk of exotic pest introduction (Gevrey & Worner, 2006; Goldson et al., 2005), and the direct and indirect financial losses are large. In 2002 the annual cost to the New Zealand economy of total exotic insect pests was estimated to range from NZ$0.8 to $2 billion (Barlow et al., 2002; Bertram & Hackwell, 1999) – of the 2002 GDP of $114 billion (IMF, 2011). The average cost for a Biosecurity New Zealand response[1] to an exotic organism incursion is $540,000 per new unwanted organism (Kriticos et al., 2005).

The increasing biosecurity risks faced by New Zealand, arising from the increased international mobility of people and trade products (ISSG, 2004; Plodkin & Kimball, 1997), are illustrated by the number of new incursions and the cost of incursion management. Between 2001 and 2005/ 2006, the number of shipping containers increased by 140 000 pa. to 0.5 – 0.6 million units pa. (Biosecurity-New-Zealand, 2006), with 14% – 24% of these containers having external biosecurity contamination and 14.9% - 18% with internal contamination. Similar risk profiles exist for used vehicles and machinery imports, international travellers and international mail (Biosecurity-New-Zealand, 2006, 2007). Since the late 1990's, approximately 40 new-to-New Zealand organisms have been reported each year (Biosecurity New Zealand PPIN data 2007, (J. A. Wilson et al., 2004), and this trend is expected to continue.

Effective biosecurity has several integrated aspects, including risk assessment, pathway risk management, border inspection, pest surveillance and diagnostics and incursion responses. Incursion responses can be investigations of possible post-border pest incursion, or

[1] 'Response' in a biosecurity context is an emergency response to a recently detected exotic organism incursion.

eradication responses to established pest populations. Biosecurity data is also used to assure New Zealand's trading partners of her pest-free status for several high impact pests, and hence maintain access to those markets.

1.1.2 A need for place-of-origin determination in biosecurity

A biosecurity requirement for the accurate determination of geographic place of natal origin for insect samples has emerged with the growing impacts and risks from biosecurity incursions; as it is often unclear whether exotic organisms that are intercepted in surveillance networks represent established populations or are newly arrived, not established post-border individuals. Reliable place-of-origin information will allow biosecurity agencies to direct appropriate response actions because the magnitude of response actions – and concomitant expenditure of resources and funds – are vastly different for established pest population eradication responses, which routinely cost $0.5 – 10+ million, compared to post-border specimen investigations, which generally cost less than $50,000 (Kriticos et al., 2005). Cases where natal origin could not be determined have included high-impact biosecurity pests like Asian gypsy moth (*Lymantria dispar*, Lymantriidae) in Hamilton, 2003-2005 (assumed established, response cost $12.4M) (Ross, 2005); and Asian tiger mosquito (*Aedes albopictus*, Culicidae) in Auckland, 2007 (assumed established, response cost approximately $500,000) (Holder et al., 2010). A similar example concerns exotic fruit flies (Tephritidae). In New Zealand it is difficult to known whether the detection of one or two fruit fly in surveillance traps represent established populations or isolated unsuccessful incursions e.g., *Bactrocera tyroni*, Whangarei 1995, *Bactrocera papaya*, Auckland 1996 (Stephenson et al., 2003). In these types of cases, point-of-origin differentiation would direct the response to be either an eradication campaign or an enhanced surveillance program as appropriate (Anon., 2008). Further, if it can be shown that the detected sample does not represent an established population, scientific vigour can be applied to quickly reversing trade embargoes that are placed on horticultural trade so long as the possibly of New Zealand produce being infested remains uncertain.

In addition to guiding response actions, dependable point of origin discrimination will inform risk assessment and refine biosecurity policy and so mitigate biosecurity risks. This includes cases when the risk pathway is uncertain in post-border incursions; examples include the detection of *Thrips palmi* (Thysanoptera: Thripidae) in recycled flower boxes in Auckland (Biosecurity New Zealand Post Border case 2008/542), ants found at container terminals (Ormsby, 2003) and mosquitoes found in aircraft (Hood-Nowotny & Knols, 2007). Another

biosecurity risk aspect that could benefit from point-of-origin differentiation is when the import risk is a natural, unregulated pathway such as the currently unknown point of origin of parasites on migratory birds (Hood-Nowotny & Knols, 2007) – with New Zealand being especially susceptible to flea and tick incursion via this pathway (Mackereth et al., 2006) and bird vectors of avian influenza and avian malaria (including novel strains of the latter) (Derraik et al., 2008; Tompkins & Poulin, 2006).

The specific impetus for this PhD project was the quandary arising from the detection of five painted apple moth (PAM) (*Teia anartoides*, Lymantriidae) and four fall web worm (FWW) (*Hyphantria cunea*, Arctiidae) specimens in Auckland, New Zealand, over early 2005 to May 2006. These detections presented serious political and technical issues, as they occurred *after* the controversial 1999-2004 aerial spraying eradication responses (that cost $62 and $6.7 million for PAM and FWW respectively). It was unknown if these finds represented new independent incursions, indicating border security failure; or residual individuals of the targeted population – that was considered to have been eradicated – indicating control failures. In an attempt to answer this question, stable isotope ratio analyses of H and C was engaged, as this technology has given spatial resolution in monarch butterfly (*Danaus plexippus*, Nymphalidae) migration studies in eastern North America (Hobson, Wassenaar, et al., 1999). However, it was only possible to draw tentative conclusions from analyses of PAM and FWW specimens (Husheer & Frew, 2005), as the assumptions supporting this technology had not been examined in sufficient detail for a biosecurity application.

Unlike the monarch study of (Hobson, Wassenaar, et al., 1999), with a single host plant[2] system in continental isoscapes and robust sample sizes, biosecurity interceptions usually involve specues that are polyphagous[3], have sample sizes of only one or two insects and are accidentally introduced, and so from an unknown and unpredictable place, point in time and host. Further, the spatial distribution of δ^2H in New Zealand may not be reliable, and location to host plant and host to insect fractionation variables are un-quantified and potentially overwhelm any New Zealand signal. The distribution of $\delta^{13}C$ is unknown, and hence the geo-location value of $\delta^{13}C$ is uncertain, especially for polyphagous insects (Section 1.2).

With such significant knowledge gaps the accuracy of this method is uncertain, and the point of origin of biosecurity pests cannot be confidently assigned using H and C isotope ratios. These unquantified, fundamental parameters constitute the primary basis for the research

[2] Host plant is the plant/ plant species that supports the development of the immature life stage of phytophagous insects.
[3] Polyphay is defined as insect speces that have many different species of host plant.

programme presented in this thesis, and are discussed in further detail in Sections 1.3.1 – 1.3.3.

Additional research needs have been recognised here, based on the possibility that superior point-of-origin determination may be achieved through the use of additional natural abundance geo-location markers, such as heavy element isotopes and trace element abundances (Hobson, 2005; S. D. Kelly et al., 2005; Rubenstein & Hobson, 2004). These further research needs are discussed in detail in Sections 1.3.4 – 1.3.5.

1.2 Principles of isotope and trace element markers

The geographical distribution of isotopes in the environment is affected by a variety of physical, geological and biological processes. Similarly, the distribution of trace elements is primarily a function of soil geology, fertilisation and pollution sources. The global distribution of the most common biologically active elements, H, C and O, are broadly understood and are predictable in time and in space (many references (e.g., Bowen, 2007; IAEA, 2002; Korner et al., 1991)).

Trace element and isotope profiling and tracing is based on the principle that plants and animals acquire the isotopic and elemental composition of the local physical components of their environment ('markers'). The trace element and isotope markers of the plants and animals can then be measured, using mass spectrometry, and compared to the known distribution of the elements in the environment. The relative similarities and differences between the environmental and samples' values is then used to infer linkages and movements within the trace element and isotope distribution 'landscapes'.

Isotope and trace element makers have been shown to have the potential to trace the origin of a wide range of both non-biological and biological materials (Oulhote et al., 2011). However, the application of natural abundance biogeochemistry in insect provenance assignment is limited (Sections 1.3.4 and 1.3.5).

The reporting convention for trace element data is to give the concentration of the element. The usual convention for reporting heavy element isotopes (e.g., Sr or Pb) is to use the molar ratios, e.g., $^{87}Sr/^{86}Sr$. In contrast, stable isotope composition from light bio-elements (H, C, N, O, S) is usually reported in a standardized 'delta' (δ) notation, which is calculated:

Equation 1.1

$$\delta(‰) = \left[\frac{R_{\text{sample}}}{R_{\text{std}}} - 1\right] * 1000$$

Where R is the ratio of the heavier isotope over the lighter isotope. This standardizes the very small natural abundance differences of the isotopes, relative to international calibration reference standards. The standards are given in Table 1.1 and all delta values in the text of this thesis are given relative to these standards. An excellent background reference for isotope ratio mass spectrometry (IRMS), providing basics principles and supporting literature is (Brenna et al., 1997).

Table 1.1 Natural abundances of the stable isotopes H, C, O, N and S, and reference materials used for delta scale calibration[3, 4].

Element and stable isotopes of interest	Natural abundance of the heavy isotope (%)[4]	Reference materials used for the delta scales[5]
Hydrogen $^{2}H/^{1}H$	0.015574	Vienna Standard Mean Ocean Water (VSMOW)
Carbon $^{13}C/^{12}C$	1.1056	Vienna Pee Dee Belemnite (VPDB)
Nitrogen $^{15}N/^{14}N$	0.36630	Air (AIR)
Oxygen $^{18}O/^{16}O$	0.20004	VSMOW
Sulphur $^{34}O/^{32}O$	4.19719	Vienna Cañon Diablo Troilite (V-CDT)

The distributions of the isotopes of H, C and O are the most commonly used biogeochemical location markers. Over broad latitudinal ranges, the distribution of the stable isotope ratios of H and O are driven by isotopic fractionation during water evaporation and condensation – with the fractionation being strongest at lower temperatures. Thus, as precipitation migrates further from the ocean to higher, cooler altitudes and/or with increasing latitude, the vapour-condensation fraction increases, leading to progressively 'lighter'[6] $\delta^{2}H$ and $\delta^{18}O$ values ((Craig, 1961). The terrestrial bio-expression of ^{13}C also decreases with latitude due to the carbon isotopic fractionation differences in the plant photosynthetic pathways of C_3 and C_4 plants (Peterson & Fry, 1987) and the higher proportion of C_4 plants in the tropics and a gradient toward dominance of C_3 plants with increased latitude in (in J. F. Kelly, 2000). In addition, there is a progressive equatorial to polar decrease in ^{13}C discrimination by C_3 plants (Korner et al., 1991), probably due to the increasing carboxylation efficiency with decreasing temperature and atmospheric pressure and increasing humidity.

[4] Data from Coplan, et al. (2002); Ding, et al. (2001) and Fry (2006)
[5] (Coplen, 1996; Ding et al., 2001; IAEA, 2009b)
[6] 'lighter' refers to isotopically lighter; i.e., having fewer heavy isotopes, and therefore a lower delta value

Although $\delta^{13}C$ has been reported as an important migration tracing stable isotope marker (Hobson, 1999; Hobson & Wassenaar, 2008; Tieszen et al., 1988), careful interpretation of $\delta^{13}C$ values in geo-location applications is required (J. F. Kelly, 2000) and $\delta^{13}C$ data is frequently not point of origin informative. Within countries, and even continents, the ^{13}C distribution in plants is non-uniform (Hobson, Wassenaar, et al., 1999). Therefore, in order to use $\delta^{13}C$ for geo-location one needs to know the ^{13}C distribution over the host plants' distribution. However, in the biosecurity arena, the host plant is generally unknown (as the adults are usually the intercepted life stage) which compromises the use of $\delta^{13}C$ as a geo-location marker. In addition, the north-south $\delta^{13}C$ relationship may be distorted by ecological complexities (e.g., host specialization), as well as both the inter- and intra- host-to-insect natural variation of +/− 5‰, due largely to diet quality and other ecological variables (e.g., temperature and plant water stress) effecting the $\delta^{13}C$ of the host plant (McCutchan et al., 2003; Post, 2002; Vander Zanden & Rasmussen, 2001). Further, the New Zealand $\delta^{13}C$ C_3/C_4 signal is predominantly C_3, as it will be for other temperate regions from which biosecurity risks will arrive. Therefore, unless a clear C_4 signal is expressed, $\delta^{13}C$ will not be informative regarding the country of origin. In this regard, (Abney et al., 2008) reported that within the range of C_3 host plants of *Heliothis virescens* (Noctuidae), C isotope signatures were not sufficiently unique to enable determination of natal origin for this species. Given the limited biosecurity geo-location potential of ^{13}C, and the relatively complex method required for both C and H isotope ratio analyses from the same tissue sample (described in Hobson, Wassenaar, et al., 1999), ^{13}C was not investigated in this project, so time and resources could be focused on other markers. Furthermore, the expression of $\delta^{13}C$ in insects is relatively well understood and so experimentation in this regard in this study will not significantly contribute to the body of scientific knowledge.

1.3 The research needs

Unlike $\delta^{13}C$, the use of $\delta^{2}H$ as an entomological point-of-origin marker suffers from the fact that it is only partially known how $\delta^{2}H$ is expressed in phytophagous insects. The most significant unquantified fundamental parameters are:

- The effect of environmentally driven variation in $\delta^{2}H$ is not understood, yet may overwhelm any New Zealand signal. Most significantly,
    - The effect of precipitation $\delta^{2}H$ variation (short tern fluctuation) on the expression of insect $\delta^{2}H$ is not quantified, and
    - The effect of physiological H fractionation variation in response to fluctuating environmental parameters is unknown.

- The effect that different host plants' δ^2H signal will have in polyphagous insect populations' δ^2H expression is unknown.
- It is unknown if H turnover in adult insect tissues will cause adult feeding input to be expressed, distorting the natal point of origin δ^2H acquired during larval feeding.

Additional research needs arise from the knowledge that superior point-of-origin determination may be achieved through the use of other natural abundance geo-location markers (Hobson, 2005; S. D. Kelly et al., 2005; Rubenstein & Hobson, 2004). However, there are no analytical methods published to measure, in insects, the suite of additional biogeochemical markers that may be informative with regard to the geographical origin. Further, similar questions as posed above for δ^2H then emerge as to how these additional markers are expressed in phytophagous insects.

Lastly, it is unknown if these additional markers, or δ^2H, have sufficient geographic discrimination to distinguish New Zealand specimens from those of exotic origin.

Current knowledge around each of these aspects is considered below, toward forming a platform for the research presented in this thesis.

1.3.1 Environmentally driven hydrogen isotope variation

Variation in δ^2H at a given location mainly arises from fluctuation in precipitation δ^2H and variation in physiological fractionation in response to irregular environmental parameters (Marshall et al., 2008). To enable an evaluation of δ^2H as an entomological point-of-origin marker, the influence of both of these factors on insects' δ^2H requires examination.

1.3.1.1 Precipitation δ^2H variation and different sources of δ^2H signal

A fundamental tenet in stable isotope science is that the fluctuating precipitation δ^2H input is 'averaged out' in the soil and water table (Clarke & Fitz, 1997; Gat, 1971) and plant δ^2H is closely correlated with that of the local water (Gleixner & Mügler, 2007). This relationship between the local water and the plant is then largely expressed in vertebrates' δ^2H, with about 90% of animal δ^2H signal arising from the H consumed (approximately 60% food and 30% from drinking water) (Fry, 2006). This generally dependable expression facilitates reliable regional isotopic marking. However, this precept has not been validated for insects. Uncertainty arises because 1) immature insects have feeding periods that can be as short as one or two weeks, and it is unknown if the short term, high variation in precipitation input δ^2H during the larval feeding period is expressed in adult insects; and 2) insects may

proportionally use more leaf water – a highly variable mixing pool – than vertebrates. Therefore, the averaging out principle may only partially apply.

In regard to the first issue, New Zealand has a highly variable, mid-latitude, island climate, with both maritime polar and maritime tropical air masses bringing precipitation (NIWA, 2007). This causes large variation in precipitation δ^2H values within monthly and seasonal timeframes, and monthly precipitation δ^2H deviations of 25‰ or more from the long term weighted average are common (Frew et al., 2011). Therefore the short term spatial distribution of ^2H may not be a reliable indicator of latitude in New Zealand. Consequently, it is unknown if the principle of using the broad latitudinal δ^2H ranges across continents as an insect point-of-origin marker, as established by Hobson, Wassenaar, et al. (1999); Wassenaar & Hobson (1998), can be applied for New Zealand caught insects. Specifically, it is unknown how such environmental variation in δ^2H input signal gets expressed in plants and in turn, if that 'input noise' gives a similar degree of variation in insects δ^2H signal. Accordingly, it is unknown if this δ^2H marker variation is greater than between country differences, affecting the confidence in discrimination between insects reared in New Zealand and those of exotic origin.

Indeed, examination of the small amount of intra-region insect δ^2H data available (Husheer & Frew, 2006b; Wassenaar & Hobson, 1998), suggests that insects will express the high short term variation in the growth season rainfall δ^2H (over late-spring and summer in New Zealand), rather than the reliable long-term or even annual average rainfall δ^2H pattern. This variation in an insects' isotopic signal will, in part, be dependent on the proportion of water a particular host plant uses from either ground or surface water sources (see J. W. C. White, 1988), with many plants accessing isotopically variable surface water rather than more constant ground water (Schulze et al., 1996).

In consideration of the second issue, namely whether the intrinsic δ^2H of adult insects is derived from the plant or leaf water H, and not that of the plant solid[7], Husheer & Frew (2006a) observed that insects reared on artificial diet appear to assimilate the water component H more readily than the solid H. Given this, it is theorized here that phytophagous insects will express the δ^2H of the leaf water.

If this is the case, the intra-population variation in insects' δ^2H will more closely match the highly dynamic and generally unquantified leaf water δ^2H values, rather than the longer term averages expressed in cellulose (e.g., Yapp & Epstein, 1982). And, as the leaf water δ^2H can

[7] 'Plant solid' is used to refer to total organic H and C throughout this document.

vary by as much as 40‰, driven by fluctuating temperature, humidity (Yakir, DeNiro, & Gat, 1990) and, for surface feeding plants, by variation in the rainfall δ^2H (e.g., see fig. 10.2 in J. W. C. White, 1988), the effect that these environmental variables have on plant feeding insect δ^2H values may be large and also difficult to predict.

The suggested mechanisms given above, whereby phytophagous insects are accessing isotopically varied water sources (surface and leaf water), is a possible explanation for the within region δ^2H variation of 25‰ or more observed by Husheer & Frew (2006b) in *Epiphyas postvittana* (Lepidoptera: Tortricidae) in Auckland. Similarly, the Wassenaar and Hobson (1998) monarch butterfly data set shows within location variation of approximately +/− 10‰.

This potential for variation in insect δ^2H values is unquantified and could be larger than the differences in average δ^2H values between New Zealand and potential overseas points of origin. Therefore, the confidence of accurately discriminating between insects reared in New Zealand and those of exotic origin, using δ^2H analysis alone, is reduced. For example, over the period the PAM and FWW specimens referred to in Section 1.1.2 would have been developing (2005 to early 2006), the Auckland precipitation δ^2H values were -22 to -38‰ on average. This range overlaps, or is close to the average values of the potential sources of the infestations for the same period, with New South Wales (NSW) and southern Japan having average precipitation δ^2H values of -14 – -38 and -40 – -71‰ respectively.

1.3.1.2 Physiological fractionation variation

The extent of fractionation and fractionation variation within the system of interest need to be quantified or at least estimated, in order to interpret and maximise confidence in the stable isotope results of ecological studies (Gannes et al., 1997; Hobson, 1999; McCutchan et al., 2003). In this regard, the water-to-plant H fractionation is another large and influential variable that needs to be taken into account in a phytophagous insect geo-location system. Photosynthetic δ^2H depletion of -40 to-70‰ or more is the norm (e.g., Ziegler et al., 1976), yet H fractionation is irregular within a plant species. This variation is caused by changeable photosynthetic responses to inconsistent environmental inputs e.g., temperature, humidity, air vapour δ^2H (Ehleringer & Dawson, 1992; Marshall et al., 2008; J. W. C. White, 1988; Yakir, DeNiro, & Ephrath, 1990), water stress (with plant solid δ^2H enriched by 30‰ + (John Roden leaf water model 1999; Roden et al., 2000), and atmospheric pollution (Savard et al., 2005).

Furthermore, the degree of δ^2H 'consumer enrichment' fractionation is unknown or only partially understood for many specific tissues – including moth wings. The magnitude of ^2H

fractionation from plant to adult insect was only 1 – 4‰ in both monarch and *Epiphyas postvittana* studies (Hobson, Wassenaar, et al., 1999; Husheer & Frew, 2006a). However, it is unknown if this value will be the same in other host-insect systems. In addition, there is no knowledge regarding the effect of variable metabolic stresses on consumer enrichment (Wassenaar, 2011).

The unknown extent of host plant fractionation and the unknown degree of fractionation variation were the major factors that restricted the confidence of the PAM stable isotope results (Husheer & Frew, 2005). As the host plant and its history are unknown in many biosecurity specimens, ^{2}H data correction is unreliable without a better understanding of these parameters. It is also not known to what degree this uncertainty affects confidence of interpretation of stable isotope data in insect geo-location studies.

1.3.2 Polyphagy

Successful, invasive insect species are frequently polyphagous (N. Martin & Paynter, 2010), as are most high impact biosecurity insect pests (Stephenson et al., 2003). Individuals of such insects generally feed on a single plant; however, on a population level the varied larval diet will include several different plant species biogeochemical signals. Other than for $\delta^{13}C$ and $\delta^{15}N$ (Ponsard & Arditi, 2000) the effect that polyphagy has on biogeochemical marker expression in insect populations has not been studied. It is reasonable to expect that the range of plant species in an insect populations' diet, will be expressed as a concomitant range of $\delta^{2}H$ and other biogeochemical markers values in the insects' signals. Therefore the effect of polyphagy on entomological biogeochemical markers is potentially very large (Menozzi et al., 2007; Sherlock et al., 1985).

For example, cellulose $\delta^{2}H$ values vary between different plant species under the same environmental conditions from more than 40‰ (e.g., see figure 10.6 in J. W. C. White, 1988) to as high as 160‰ (Sternberg, 1988; Yakir, 1992). This range of variation arises largely from biochemical differences in the C_3, C_4 and CAM photosynthetic pathways (Luo & Sternberg, 1991; Yakir, 1992), as well as within C_3 group differences of 20 – 30‰ (Chikaraishi et al., 2005). Different plant species used by polyphagous insect species will also have broad differences in their concentrations of trace element biogeochemical markers (Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Bonnin, 1983; Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Kuennen, 1983).

Furthermore, in polyphagous species there is evidence of differences in the plant-to-insect ^{2}H fractionation in individual insects reared on different host plant species (Husheer & Frew,

2006b), which will contribute to the variation in fractionation discussed in the previous section.

1.3.3 Biogeochemical marker turnover in adult tissue

Contrary to assumptions made by Wassenaar and Hobson (Hobson, Wassenaar, et al., 1999; Wassenaar & Hobson, 1998), adult insect cuticle[8] is not metabolically fixed at the time of emergence, but undergoes some degree of physical and chemical change (Chapman, 1998; Neville, 1983). Thus, biogeochemical markers in adult insects' exoskeletons may reflect adult feeding input, rather than the natal origin geo-chemical signature, so that a new incursion might be obscured by subsequent feeding in New Zealand. Adult diet has been shown to be expressed in adult moth and beetle wing $\delta^{13}C$ values, with a shift from the larval $\delta^{13}C$ signal of up to 4.8‰ (Gratton & Forbes, 2006; Ponsard et al., 2004). In addition, the rate of metabolic activity of the insect appears to affect the turn-over rate; and as metabolic activity is variable within a population, this may add additional variation to wild insects (Ponsard et al., 2004). However, the effect adult feeding has on ^{2}H expression, has never been evaluated, but must be anticipated given the turnover of C demonstrated by these authors. Similarly, the turnover of other potential biogeochemical markers in adult insect tissues is also unknown.

Along with the potential sources of variation mentioned above, it is necessary to understand the influence of biogeochemical marker turnover in adult insect tissues in order to have confidence in the interpretation of biogeochemical marker data.

1.3.4 Constraints in current methods of isotope and trace element insect tracking

Tracking insect movement is an important and challenging component within many aspects of entomological science, including understanding linkages in ecological and pest management settings, as well as biological control enhancement. A biosecurity requirement for point-of-origin discrimination of insect samples has also emerged, as discussed in Section 1.1.2.

Most insect tracking studies involve applying a 'mark' to a set of samples, then releasing and recapturing as many samples as possible. Insect tracking by chemical marking was reviewed by Akey (1991) and more recently by Holden (2006) and Hagler & Jackson (2001). The once common practice of insect tracking by radioactive isotope marking (see Service, 1993) has been replaced by trace element and stable isotope artificial enrichment. These artificial

[8] Insect wings are a specialized region of the cuticle.

enrichment methods have been used in a wide array of invertebrate tracking applications, including tracking mass reared, sterile insect technique (SIT) tephritid fruit flies and male mosquitoes with ^{13}C (Hood-Nowotny et al., 2009; Hood-Nowotny et al., 2006), parasitic wasps with ^{44}Ca (Wanner et al., 2006; Wanner et al., 2007), and aphids, stoneflies and mayflies with ^{15}N (Hershey et al., 1993; Macneale et al., 2004, 2005; Nienstedt & Poehling, 1998). ^{15}N enrichment marking of phytophagous insects and their parasites, as well as flower-visiting insects by labelling plant tissue was further developed by Steffan (2001). There have also been a number of studies that labelled phytophagous insects with artificially enriched Rb, Cs and Sr via translocation into the host plant (Armes et al., 1989; Hagler & Jackson, 2001; Stadelbacher, 1991). The inherent limitations of 'marking' insects for geo-location research include: the practical challenges of applying the mark to the insects, the cost of placing and/or reading the marking signal, population density dependent distortions and generally low recapture efficiency (Akey, 1991; Hagler & Jackson, 2001; Holden, 2006; Lavandero et al., 2004). Further, as there is no opportunity for marking in biosecurity, forensic and many ecological studies, threte is a need for additional and more effective migration monitoring tools, which drives us to consider natural abundance, intrinsic markers of insects (Hood-Nowotny & Knols, 2007). Such markers have the distinct advantage of being recorded innately in every member of the population – i.e., every collected wild sample potentially has point of origin information incorporated within its tissues.

In contrast to the common practice of artificial biogeochemical enrichment, there are few natural abundance insect geo-location studies published – in fact, the Wassenaar and Hobson monarch studies (Hobson, Wassenaar, et al., 1999; Wassenaar & Hobson, 1998) are generally the only papers ever cited in this field. In a watershed study, these workers used the intrinsic δ^2H and $\delta^{13}C$ label in migrating adult monarch wings to reveal their natal origins over 100s to 1000s of kilometres within eastern North America by comparison with the δ^2H and $\delta^{13}C$ distribution of non-migrating butterflies. At the time of writing (April 2012), the only other studies found in the literature concerning tracking insect movement using natural abundance biogeochemical markers, are the following: Field populations of a variety of serieous pest species were able to be charaterized using a small series of 'common elements' (e.g., the abundant trace elements P, S, Cl, K, Ca, Fe, Cu, Zn), including the ambosia beetle, *Gnathotrichus sulcatus* (Scolytidae) (McLean & Bennett, 1978); and western spruce budworm, *Choristoneura occidentalis* (Lepidoptera, Tortricidae) (McLean et al., 1979). The more comprehensive studies of Turner & Bowden (1983) reported promising regional separation of widely distant populations of *Nilaparvata lugens* (Homoptera: Delphacidae), and Bowden, et al. (1979) demonstrated the inter-regional heterogenity in *Noctua pronuba*

(Lepidoptera, Noctuidae) that could enable discrimination. However, the confounding effects of polyphagy, adult feeding and gender differences in elemental expression were all intimated as masking the point-of-origin signals (Bowden, et al., 1984; Dempster, et al., 1986), when using the suite of common elements able to be analysed with the X-ray spectrometry techniques available at the time, as these elements are also biologically active (Mertz, 1981). Further, Kowalski, et al. (1989) showed that the effect of insect weight also corrupted X-ray spectrometric profiles. Natural abundance entomological biogeochemistry was not developed further; until the work of Wassenaar and Hobson in the late 1990's. Subsequently, Dockx, et al. (2004) used plant derived steroid markers extracted from adult monarchs (to determine host), along with δ^2H and $\delta^{13}C$ to successfully resolve migration patterns in Cuba. The preliminary study of Menozzi, et al. (2007) was able to estimate the natal host of some wild, trapped *Helicoverpa armigera* (Lepidoptera: Noctuidae) adults using $\delta^{13}C$; however, their sample sizes were too small to trace the migration of *H. armigera* and the hoverfly *Episyrphus balteatus* (Syrphidae) between western Africa and south-west France using δ^2H. Brattström, et al. (2008) measured δ^2H, $\delta^{13}C$, and $\delta^{15}N$ from wild *Inachis io* (Lepidoptera: Nymphalidae) over a 250 km^2 region in Sweden. These authors found an informative north /south relationship in δ^2H, a weak expression in $\delta^{13}C$, and no regional differences in $\delta^{15}N$. However, the within site δ^2H variation (≈ 20‰ at each site) and the inter-annual variation in both δ^2H and $\delta^{13}C$ was too large to assign within region provenance. Prasifka & Heinz (2004) used the C_3/C_4 difference in $\delta^{13}C$ to trace natural enemies, while Dalecky, et al. (2006), Nagoshi, et al. (2007) and Gould, et al. (2002) used the same relationship to trace the movements of the moths *Ostrinia nubilalis* (Crambidae), *Spodoptera frugiperda* and *Helicoverpa zea* (Noctuidae), respectively. Forbes & Gratton (2011) used within insect differences in $\delta^{13}C$ turnover between muscle and exoskeleton to infer inter-crop dispersal of two species of Coccinellidae (Coleoptera). Additional workers have revealed host use dynamics using the same C_3/C_4 photosynthetic pathway discrimination (e.g., Bontemps, et al. (2004); Malausa, et al. (2005); Ostrom, et al. (1997); Ponsard, et al. (2004). While these later studies were not explicitly designed to study insect provenance or movement, the results indicated migration themes. In contrast, Abney, et al. (2008) found that within a range of C_3 hosts of *Heliothis virescens,* the range of $\delta^{13}C$ values overlapped, and there was "no significant correlation between the $\delta^{13}C$ values of moths vs. the $\delta^{13}C$ value of plant tissue on which they were reared", and the natal origin of this species was not able to be determined using $\delta^{13}C$. In a more sophisticated, although preliminary, analysis Tigar & Waldron (2002) also used the C_3/C_4 plant $\delta^{13}C$ differentiation, but in combination with natural abundance $\delta^{15}N$ and trace element signatures (using pooled insects) to monitor host plant and place-of-origin

of the larger grain borer *Prostephanus truncatus* (Bostrichidae). They suggested that $\delta^{13}C$ along with S, Cr and Mn concentrations may have the potential for tracking this beetle; however the trace element data were not given and the geo-location analysis was not completed.

It is considered unlikely that any provenance resolution achieved with the existing systems, reviewed above, will enable New Zealand insects to be distinguished from those of exotic origin. The studies that employed trace elements i.e., Tigar & Waldron (2002) and the 1970's and 80's trace element studies, were inconclusive. And although the isotope studies used the spatial separation across continental $\delta^{2}H$ and $\delta^{13}C$ contours or the C_3/C_4 difference, the scale of resolution from isoscapes of these light elements (Bowen, 2010; IAEA, 2002) can be too coarse for confident geo-location – as shown by Abney, et al. (2008); Brattström, et al. (2008) and Menozzi, et al. (2007). This is due to insufficient differences between study areas and/or within region environmentally driven isotopic variation, as described in the previous sections, resulting in variation in signal that is greater than the between region differences (Sagers & Goggin, 2007; Spence & Rosenheim, 2005). Significantly, the $\delta^{2}H$ values for northern New Zealand show broad overlaps with the $\delta^{2}H$ values of the parts of Australia, southeast Asia and China that are the origin of most of New Zealand's imports, and hence biosecurity threats, for most months of the year (IAEA, 2002). The broad scale of spatial resolution and within region variation are of particular consequence in forensic or biosecurity applications, where the sample sizes are usually only a few insects, and so statistically confident provenance assignment becomes improbable (Lancaster & Waldron, 2001). In addition, the existing natural abundance insect provenance studies are generally specific to a single insect species / single host system, within a pre-defined time and space. In contrast, forensic specimens are generally polyphagous and accidentally introduced, and so from an unknown and unpredictable place, point in time and host, which all confound isoscape to insect corrections.

1.3.5 Toward more reliable point of origin discrimination in entomology: Combining heavy element isotope and trace element markers with light element isotopes

To improve on the provenance capability over the large spatial and/or ecological dimensions of the commonly used 'light' element stable isotopes discussed above (Bowen, Wassenaar, et al., 2005; Fry, 2006; Hobson & Wassenaar, 2008), several recent reviews have all concluded that reliable natural abundance geo-location requires a large number of variables to be assessed, and stressed the geo-location potential of multiple isotope assessment combined with trace elemental analysis (Hobson, 2005; S. D. Kelly et al., 2005; Oulhote et al., 2011;

Rubenstein & Hobson, 2004). The combination of information from the global and continental scale distribution patterns of δ^2H and other light element isotopes, with the finer spatial scale of geological markers, such as Sr and Pb isotopes, can provide closer spatial resolution (Aggarwal et al., 2008; S. D. Kelly et al., 2005). For example, Sellick, et al. (2009) improved allocation to the correct region of origin of tree swallow specimens (*Tachycineta bicolor*) to 79% by combining δ^2H and $^{87}Sr/^{86}Sr$ ratio data, whereas correct region of origin assignment was less than 40% using either variable alone. Similarly, different African elephant (*Loxodonta* sp.) populations have been distinguished using the combination of N, C, Sr and Pb isotopes in the samples' bones and tusks, yet the individual variables overlapped significantly between regions (Vogel et al., 1990).

The provenance determination potential of the isotopic composition of heavy elements, such as Sr and Pb, derives primarily from the geological differences between regions (Aggarwal et al., 2008). These isotopic signatures are transferred from the rocks to their associated soil and water bodies through physical and chemical processes related to mineral weathering, and ultimately to the plants and animals linked to these resources. Strontium has four naturally occurring isotopes, all of which are stable. The quantity of three of the four stable isotopes of Sr (^{84}Sr, ^{86}Sr, ^{88}Sr) remain constant, whereas the amount of ^{87}Sr increases through time, due to the decay of radioactive ^{87}Rb (Capo et al., 1998; Sun & McDonough, 1989). The amount of ^{87}Sr relative to the other Sr isotopes is most commonly expressed as $^{87}Sr/^{86}Sr$. The Pb isotopic composition arises from several separate systematic pathways. Radioactive ^{238}U emits alpha-particles during decay through the Uranium/Radium series to ^{206}Pb; ^{235}U decays through the Actinium series to^{207}Pb; and ^{208}Pb is the stable radiogenic daughter nuclide of the ^{232}Th decay chain (Audi et al., 2003). As a result, rocks of different ages have differing amounts of radiogenic Sr and Pb isotope daughter products relative to the non-radiogenic isotopes. The geological and biological fractionation of heavy elements is reported to be small (Blum et al., 2000; Capo et al., 1998; Chamberlain et al., 1997; Holzl et al., 2004). Consequently, heavy-element isotopic compositions are assumed to be conserved from bedrock to soil to plant to animals (Aberg, 1995; Bentley, 2006), and are thus considered the biogeochemical variables most likely to be robust point of origin markers (Holzl et al., 2004). The heterogeneous distribution of Sr and Pb isotope ratios have been used in provenance determination applications ranging from mammoths (*Mammuthus* spp.) (Hoppe et al., 1999), to cheese (Fortunato et al., 2004) (both Sr isotopes), to humans (*Homo sapien*) (Gulson et al., 1997) (Pb isotopes).

Trace element concentration profiles in ecological provenance determination are less well researched, although, the potential of these markers has been demonstrated in tracking the dispersal of migratory birds (Donovan et al., 2006; Norris et al., 2007; Szep et al., 2003), as location markers in criminal investigations (Ruffell & McKinley, 2005), and in tracing the origin of agricultural products (S. D. Kelly et al., 2005; Thiel et al., 2004). Trace element profiles are very specific to the location or origin (S. D. Kelly et al., 2005) and so show greater near distance geo-location resolution than δ^2H and $\delta^{13}C$ (Poesel et al., 2008). This suggests that trace element profiles will have good potential for biosecurity provenance applications, where the interception site and possible origin of infestation may be of similar latitude (and hence similar δ^2H), and/or the point of origin assignment is compromised by poor resolution from inherent uncertainty in δ^2H and $\delta^{13}C$ values.

Most multivariate biogeochemical (i.e., multi-element and multi-isotope analysis) tracing research has been conducted in the food authentication arena. Several effective methods have been developed, and this is an active and ongoing field of research (see reviews by S. D. Kelly et al., 2005; S. D. Kelly & Lees, 2003; Luykx & van Ruth, 2008; Ogrinc et al., 2003; Oulhote et al., 2011; Rossmann, 2001). Anderson & Smith (2005) Brescia, et al. (2005) S.D. Kelly, et al. (2002) and Pillonel, et al. (2005) are all examples that have demonstrated the highest place-of-origin allocation value was obtained from screening a wide range of variables, including trace elements. Other instances of multivariate biogeochemical tracing across large distances include Crittenden, et al. (2007) and Rossmann, et al. (2000), wherein the potential for the stable isotope ratios of C, N, O, S and Sr to discriminate the point of origin of dairy products were examined. The K. A. Anderson & Smith (2005) study was able to identify 'home' samples for Pistachio nuts (*Pistacia vera*) with 100% accuracy through elemental and stable isotope analysis. Strontium concentration was the highest discriminant, followed by Ca, K, Mg and P. Supporting geo-location was found in $\delta^{13}C$, and by plotting the total C and N ratio against $\delta^{15}N$. Unlike S.D. Kelly, et al. (2002), a subsequent Anderson and Smith study (K. A. Anderson & Smith, 2006) found that the discriminating power of stable isotope values was not negated by seasonal variation for that commodity and the areas examined. Powerful geo-location and temporal classification has similarly been claimed in a tea (*Camellia sinensis*) study using isotope ratio analysis of H, N, C with trace element analysis (Pilgrim, 2007; Pilgrim et al., 2010)[9]. Conversely, ecological examples of multivariate biogeochemical provenance determination are fewer (Rubenstein & Hobson, 2004). Successful examples include Sellick, et al. (2009) (discussed above) and Chang, et al.

[9] All of these multi-element studies employ, by necessity, powerful statistical interpretation and visualisation methodology. Subsequent point-of origin determination is by means of various discriminant analyses.

(2008) who employed $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{2}H$ to determine the provenance of cormorants (*Phalacrocorax carbo sinensis*).

Despite the high provenance determination potential of natural abundance heavy element isotopes and trace element biogeochemical markers, the application of these profiles to successfully trace insect movement has been limited. The earlier attempts at insect provenance determination using trace elements (e.g., Bowden et al., 1984; Dempster et al., 1986) were compromised (see Section 1.3.4). However, the more recent trace element studies in other sciences (e.g., Norris et al., 2007; Szep et al., 2003), that used superior mass spectrometric techniques and considered less common elements, may be free of these confounding limitations. In regard to $^{87}Sr/^{86}Sr$, although no examples of natural strontium abundance use in insect tracing research have been found, the trophic linkage studies of Blum, et al. (2001) and Blum, et al. (2000) demonstrated that achieving $^{87}Sr/^{86}Sr$ signal is possible from insects samples.

The studies reviewed in this section suggest that the need for more reliable entomological point of origin discrimination may be achieved through the multivariate combination of the global scale distribution patterns of $\delta^{2}H$ with the finer spatial scale of Sr and Pb isotopic ratios and trace element concentration geological markers.

1.4 Research aims and scope

The research question is "Can isotope analyses determine the natal geographic origin of potentially invasive insects collected in New Zealand?" The fundamental uncertainties from the review above are i) that variation in light element isotope expression in insects, from environmental fluctuation and additional host signals, may be too high to allow spatial discrimination, and ii) that the isotopic composition of heavy elements and trace element concentrations may provide greater spatial resolution. Given this, the Null Hypotheses is that $\delta^{2}H$ on its own cannot discriminate New Zealand bred insects from overseas reared insects. The Alternative Hypothesis is that reliable provenance assignment will be possible using $\delta^{2}H$ combined with trace element concentration, light and heavy element isotope ratio data.

To test these hypotheses, the objectives of this project were to:

- Examine various factors that could influence the imprinting of the ^{2}H signature in insects as an indicator of New Zealand versus exotic natal origin. The primary non-quantified variables considered were a) the environmental water ^{2}H variation, including the concomitant leaf water versus plant solid H partition question; b) the fractionation relationship within a model plant/insect system, including the potential range in

fractionation variation; and c) the effect of polyphagy and adult feeding on ^{2}H expression in the insect.

- Assess the feasibility and benefits of integrating heavy element stable isotope and trace element analysis with light element isotopes for point of origin discrimination of insect pests of biosecurity importance to New Zealand. This requires an evaluation of the representation of heavy element isotope and trace element signatures in plant and insect tissues, as well as the development of an integrated series of methods to enable these variables to be measured from the limited mass of insect samples.
- Verify whether multivariate signals from New Zealand specimens are distinguishable from overseas specimens.

1.5 Thesis structure

The objectives of this project have been addressed by conducting complementary field and laboratory controlled-environment experiments using the model soil-plant-insect system.

In Chapter 2 the methodological development required to reliably read light and heavy isotope and trace element profiles from single insect samples is given; along with general materials and methods used in the following experimental program.

Chapter 3 constitutes a series of experiments that are designed to quantify the major uncertainties in biochemical geo-location marker expression in insects. The first two experiments consider how the variation in precipitation $\delta^{2}H$ signal gets expressed in plants, and in turn to what extent that 'input noise' is expressed as variation in adult insect $\delta^{2}H$ values. The influence of larval polyphagy and adult feeding on trace element and $\delta^{2}H$ marker expression are examined in separate experiments in the same chapter.

Chapter 4 is a test of the potential for multivariate signals to distinguish New Zealand specimens from those of overseas origin.

Chapter 2
Materials, methods and methodology development

2.1 Model insect and host plant system

Helicoverpa armigera (Hübner 1805) [Lepidoptera: Noctuidae: Heliothinae] (tomato fruit worm) was selected as the model insect for this research because the following characteristics make it an appropriate model for invasive species and facilitated the execution of the project: *Helicoverpa armigera* is polyphagous, enabling comparisons with samples from several hosts. It is able to be reared on artificial diet and, under laboratory conditions, on host plants; is commercially available from a disease free colony year round; and both insect and its hosts are easily and quickly grown. *H. armigera* has moderate to abundant wild populations, is readily field collectable. It is also distributed widely internationally, including in the countries that present New Zealand's main biosecurity risk pathways, facilitating an international collection effort in the most appropriate locations, as well as a range of regions expected to have biogeochemical signature differences ranging from large to small. *H. armigera* also has relatively large mass – for an insect, which improved the chance of isolating measurable quantities of the minor elements, and thus Sr and Pb isotopic profiles. Further, as *H. armigera* is a serious pest, many scientists are actively involved in research related to this species. Therefore, the fundamental biological parameters of this species, such as phenology, host preferences and distribution patterns are well understood, and assistance with international collections was possible.

Zea mays (corn) has been identified as the most suitable model plant for the inter-regional comparison as well as most of the controlled environment experiments. It is a productive *H. armigera* host, on which this insect has comparatively low levels of parasitism. Further, *Zea mays* does not support the morphologically similar species *Helicoverpa punctigera* (Wallengren), facilitating the field collection of the correct species. *Zea mays* is also grown extensively in all the areas that the field reseach was carried out.

2.2 Collection

2.2.1 Insect

Helicoverpa armigera adults are highly dispersive (Scott et al., 2006), and so reliable natal origin cannot be assumed through trapping the adults. Therefore, non-mobile late instar larvae and pupae were collected to ensure samples could be attributed an accurate point of

origin. Field collected larvae were subsequently reared individually (*Helicoverpa* larvae are cannibalistic) on maize or corn cobs from the same location, until they emerged; whereas the field collected pupae were simply held under secure conditions until emergence. The reared insects were held until emergence at 25°C at a 16:8 dark: light cycle, at ≈ 65% RH, to prevent the pupae from entering diapause (Hardwick, 2008, pers. comm.). Following eclosion, the moths were not given access to food or water (unless need for experimentation, Chapter 3), to ensure the adult specimens had only the signals of their natal origin.

2.2.2 Plant material

Only the part of the host plant that the larvae consumed was used for analysis. With corn, the larvae of *H armigera* feed primarily in the tips of the corn cobs and almost exclusively on the kernels, therefore only apical corn kernels were collected. Plant samples were collected directly in 50 mm polycarbonate tubes. Sample plants were at least 10 m from the nearest road.

2.2.3 Soil

The TRACE Project soil sampling procedures (TRACE, 2005) were used as a guide for this project. At the same time as the insects and the host plant samples were collected, a single top-soil sample was collected from the root-ball area, below the leaf litter zone, of the same plant that vegetation was sampled from. A stainless steel trowel was used to collect 50 ml of soil into glass tubes. Living vegetation, along with visible litter and roots were removed from the soil samples.

2.3 Development of a multivariate biogeochemical analytical method

In the previous chapter, it was proposed that confident provenance assignment of insects in forensic applications, such as in biosecurity, requires the analysis of multi-element and multi-isotope natural abundance variables, as well as the quantification of fractionation and the effect of environmental variation in insect biogeochemical signals. Accordingly, this study has aspired to reach a level of natal geographical origin resolution sufficient for biosecurity purposes using combined H, Pb and Sr isotope and trace element profiles, from single insect samples. However, the low natural abundance of many elements and light mass of a single insect, along with the complex chemical structure of the tissues, makes achieving these biogeochemical variables technically challenging. The majority of other geochemical applications have available greater size and weight, elemental abundance and/or simpler

chemical structure (Beauchemin, 2010; Oulhote et al., 2011). A review of the methods for reading biogeochemical markers from insects (Section 1.3.4, Table 2.1), shows that there were no existing methods appropriate to meet the objectives of this study. No previous entomological studies have considered the intended broad suite of variables, with nearly all entomological biogeochemical studies being restricted to light isotope methods (Hood-Nowotny & Knols, 2007). The methods used by the studies that have considered trace element concentration profiles from insects either lack the required degree of sensitivity, and so could only provide data for common (and biologically active) elements (Bowden et al., 1984; Dempster et al., 1986; Sherlock et al., 1985, X-ray spectrometry), generally required pooling of several whole insects (Barker et al., 1998), and/or were unable to achieve detectable or accurate trace element values (Tigar & Waldron, 2002, ICP-AES), or required more mass (100 mg per analysis) than is available in almost all individual insects (Gongalsky, 2006, Neutron activation analysis).

Table 2.1 Existing analytical methods for reading natural abundance biogeochemical markers from insects. A selected review of the literature most closely aligned to the objectives of this project.

Isotope/ variable	Specimen preparation	Analytical methods	Reference
Tracking insect movement			
δ^2H	Wing tissue only; chloroform: methanol wash; break-seal tubes	Dumas combustion/ H_2 cryogenic transfer; dual inlet IRMS	(Dockx et al., 2004; Hobson, Wassenaar, et al., 1999; Wassenaar & Hobson, 1998)
$\delta^{13}C$	As above	cryogenic separation of CO_2; dual inlet IRMS	(Dockx et al., 2004; Hobson, Wassenaar, et al., 1999; Wassenaar & Hobson, 1998)
δ^2H and $\delta^{13}C$	Wing tissue only, freeze dried	EA/IRMS	(Menozzi et al., 2007)
$\delta^{13}C$	Whole beetles; RO H_2O wash; dried; tin capsules	CG/C/IRMS	(J. Prasifka & Heinz, 2004; J. R. Prasifka et al., 2004)
$\delta^{13}C$	Wing tissue only 95% ethanol storage; tin capsule	EA/IRMS	(Nagoshi et al., 2007)
$\delta^{13}C$	Wing tissue only 95% ethanol storage; tin capsule	cryogenic separation of CO_2; IRMS	(Gould et al., 2002)
$\delta^{13}C$	Whole beetles; UHP H_2O wash; dried; tin capsules	EA/IRMS	(Tigar & Waldron, 2002)
$\delta^{15}N$	As above	Simultaneous	(Tigar & Waldron, 2002)
Trace element (not specified) conc.	Whole beetles; UHP H_2O wash; dried; pooled,15M HNO_3+H_2O_2 microwave digestion	ICP-AES	(Tigar & Waldron, 2002)

Isotope/ variable	Specimen preparation	Analytical methods	Reference
Na, Mg, P, S, Cl, K, Ca, Al, Mn, Fe, Cu, Zn conc.	Whole insect, ashed, "pettlised" + sucrose	X-ray fluorescence spectrometer	(Bowden et al., 1984; Sherlock et al., 1985)
Trophic level linkages			
$^{87}Sr/^{86}Sr$	Whole caterpillars, ashed, HNO_3+HCl digestion	TIMS	(Blum et al., 2000)
Ca, Sr, Ba conc.	As above, aliquot	ICP-MS	(Blum et al., 2000)
Turn over			
$\delta^{13}C$	Wing and leg tissue; tin capsules	(EA)/ CF-IRMS	(Ponsard et al., 2004)
$\delta^{13}C$	Various tissues; dried; tin capsules	EA/CF-IRMS	(Gratton & Forbes, 2006)
Monitoring pollutants (reviewed by (Cortet et al., 1999)			
Trace element conc.	Several taxa, whole, pooled (4 -6 insects). HCl digestion	Atomic absorption spectrophotometer	(Barker et al., 1998)
Trace element conc.	Whole Orthoptera and beetles; one beetle sp. pooled (needs 100mg samples),	Neutron activation analyser	(Gongalsky, 2006)
Hg, Cd, Pb conc.	Several taxa, whole, dried, ashed, Hg extraction: H_2SO_4–HNO_3–V_2O_5 Cd, Pb $HClO_4$–HNO_3	Cold Vapor Atomic Fluorescence Spectrophotometer (Hg), ICP-MS (Cd, Pb)	(Z. Zhang et al., 2009).

On-the-other-hand, Blum, et al. (2000) and Font, et al. (2007) have demonstrated, respectively, that accurate measurement of natural abundance Sr isotope markers can be achieved from single insect and very light mass samples. These studies inspired further development of existing, traditionally geomorphological methods for the suite of desired markers in this study.

2.3.1 Sample description and analytical requirements

Pilot study ICP-MS analyses were carried out on test samples of the model insect and plant species and representative soils, to assess the biological representation of trace elements in plants and insect tissue, and preliminarily investigate trace element differences between Australian and New Zealand samples (Table 2.2). The test moth (wings and bodies separately) and corn samples underwent quartz distilled 15M HNO_3 digestions in Savillex Teflon beakers for 2 h at 90°C. The soil samples were subject to 1M quartz distilled HNO_3 hot extraction, then 2.5 ml of the leachate pippetted off. Analysis was carried out using an Agilent 7500CS Quadrupole ICP-MS, with He collision cell engaged. Calibration was achieved by internal standards. This trial confirmed that many potential geo-location

discrimatory elements are expressed and detectable in insects and that both trace element and multiple-element isotope analyses are possible from single insects. However, the restricted mass of insects presents a limited quantity of material for analysis. Many of the rare earth elements were below detectable limits, and the potentially geo-location informative elements La, Nd and Pb were less that 10 ppb and were therefore unreliable without further optimization. It was also ascertained that the moth wings do not have sufficient trace element and heavy element mass for the analytical methods currently available, and that the whole insect body is required.

Consequently a method was developed that efficiently used the limited sample mass and minimises contamination. In this method, individual adult moths were cleaned and partitioned. A small section of a wing was used for TCEA IRMS non-exchangeable δ^2H analysis (TC/EA-IRMS methods having replaced the more laborious methods used by Hobson, et al. (1999) (e.g., Bowen, Chesson, et al., 2005). The rest of the moth was subject to ultra-clean solution based methods. The moths were cleaned in N_2 gas, and then digested with HNO_3 and H_2O_2. An aliquot was used for trace element concentration determination by ICP-MS. Sr and Pb were extracted from the remaining solution by element specific resins in micro-columns. MC-ICP-MS was used for Pb isotope ratio analysis and TIMS for Sr isotope ratio analysis[10]. The specifications for these analyses, together with a summary of the methodological development process and contextual discussion, are given in the following sections.

[10] A single leg was also removed for DNA analysis, and the molecular markers were enquired for population characterisation; however, this aspect will be reported elsewhere (Holder & Armstrong, in prep) and is not included in this thesis.

Table 2.2 Pilot study ICP-MS analyses values (ppb) for moths (wild and lab reared), corn and soil. Dark grey cells are New Zealand samples; yellow Australian; lab moth values are white; blanks light grey. Moth average dried weight = 68 mg; corn samples were approx. 100 mg, both in 2.5 ml solution; soil samples diluted to 0.8 mg /ml. Data expressed as zero < the limit of detection

Sample:	^{7}Li	^{11}B	^{23}Na	^{24}Mg	^{27}Al	^{29}Si	^{39}K	^{43}Ca	^{51}V	^{53}Cr	^{55}Mn	^{57}Fe	^{59}Co	^{60}Ni	^{63}Cu	^{66}Zn	^{69}Ga	^{75}As	^{78}Se	^{82}Se	^{85}Rb	^{88}Sr	^{89}Y	^{90}Zr	^{93}Nb	^{107}Ag	^{111}Cd	^{118}Sn	^{121}Sb	^{133}Cs	^{138}Ba	^{139}La	^{140}Ce	^{141}Pr	^{146}Nd	^{147}Sm	^{153}Eu	^{157}Gd	^{163}Dy	^{165}Ho	^{166}Er	^{169}Tm	^{172}Yb	^{175}Lu	^{178}Hf	^{181}Ta	^{182}W	^{208}Pb	^{232}Th	^{238}U
Blank1	0.0	1	6	3	6	33	2	43	0	6	0	37	0.0	2	18	10	2	0.0	-0.1	-0.3	0.0	0.1	0	0	0	0	0.0	1	0	0.0	7	0	0	0	0.00	0	0	0	0	0	0	0	0	0	0	0	0.6	0.4	0	0.0
WHBod61	-0.1	3	2246	12670	41	93	47214	7215	1	203	185	1980	0.5	70	112	1211	3	0.4	1.9	2.2	6.0	16.7	1	0	0	0	0.3	1	1	0.0	14	1	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	5.5	1.4	0	0.0
WGBod	0.0	12	4621	16510	32	120	62880	4405	1	103	527	1968	0.7	42	207	3084	17	0.9	0.3	0.1	2.5	30.3	0	0	0	0	1.9	3	1	0.2	69	2	0	0	0.06	0	0	0	0	0	0	0	0	0	0	0	3.3	1.8	0	0.0
Lab moth 3, body	0.8	31	5078	18860	23	320	75640	9765	1	454	877	3101	2.0	179	259	5832	7	2.1	5.3	5.5	28.4	20.1	0	0	0	0	1.6	2	1	0.3	28	2	0	0	0.00	0	0	0	0	0	0	0	0	0	0	0	9.7	1.3	0	0.0
Lab moth 2, body	0.1	14	3881	13410	20	220	56970	7202	2	546	1597	3667	2.2	197	245	5015	9	1.9	4.5	5.3	6.4	20.3	0	0	0	0	1.7	2	1	0.2	35	3	0	0	0.03	0	0	0	0	0	0	0	0	0	0	0	12.0	1.3	0	0.0
Lab moth 1, body	0.0	15	3518	16980	21	120	37730	11880	0	9	598	559	0.6	7	149	5856	3	1.3	1.8	1.8	27.2	10.9	0	0	0	0	1.5	1	0	0.3	10	2	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	3.7	1.3	0	0.0
Blank2	0.0	1	26	13	7	32	36	40	0	27	4	118	0.2	22	17	70	0	0.1	-0.2	-0.4	0.0	0.1	0	0	0	0	0.0	1	0	0.0	2	0	0	0	0.00	0	0	0	0	0	0	0	0	0	0	0	0.3	0.2	0	0.0
WHWing61	0.0	3	74	181	13	38	645	157	1	201	14	877	0.7	80	14	37	1	0.1	0.0	-0.5	0.1	0.2	0	0	0	0	0.1	1	1	0.1	5	1	0	0	0.00	0	0	0	0	0	0	0	0	0	0	0	2.4	0.9	0	0.0
WGWing	0.0	7	268	420	25	48	1111	370	11	3440	228	14460	12.1	1389	92	220	16	1.4	-0.3	-0.5	0.1	1.5	0	0	1	0	0.2	8	2	0.1	64	2	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	37.0	1.7	0	0.0
Lab moth 2, wing	-0.1	7	50	90	17	43	256	149	0	7	1	65	0.1	3	38	32	0	0.1	-0.1	-0.2	0.1	0.2	0	0	0	0	0.1	0	1	0.1	1	2	0	0	0.07	0	0	0	0	0	0	0	0	0	0	0	0.6	1.0	0	0.0
Lab moth 2, wing	0.0	22	134	198	11	56	869	155	0	15	7	93	0.2	18	20	38	0	0.1	-0.1	-0.5	0.1	0.3	0	0	0	0	0.0	1	0	0.3	1	2	0	0	0.00	0	0	0	0	0	0	0	0	0	0	0	0.6	0.7	0	0.0
Lab moth 1, wing	-0.1	13	85	110	15	39	443	454	0	26	4	145	0.1	14	36	55	0	0.1	-0.2	-0.2	0.3	0.3	0	0	0	0	0.0	1	0	0.0	2	6	1	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0.8	1.2	0	0.0
Blank3	-0.1	1	69	15	14	30	57	199	0	26	2	119	0.1	12	20	62	1	0.0	-0.1	-0.5	0.0	0.3	0	0	0	0	0.2	2	0	0.0	4	0	0	0	0.00	0	0	0	0	0	0	0	0	0	0	0	0.4	0.6	0	0.0
Corn, site 10, MC	0.1	23	81730	35620	50	390	194200	10120	0	16	274	632	0.4	99	132	817	3	0.7	0.0	-0.1	133.4	56.2	0	0	0	0	0.4	3	0	0.2	11	4	0	0	0.04	0	0	0	0	0	0	0	0	0	0	0	0.5	3.2	0	0.0
Corn, site 36, BP	0.0	30	35720	20910	30	410	86030	4103	1	153	210	1252	0.8	75	96	745	7	0.4	0.4	-0.2	233.8	24.3	0	0	0	0	0.3	4	1	2.7	26	2	0	0	0.04	0	0	0	0	0	0	0	0	0	0	0	2.5	1.6	0	0.0
Corn, site 45, QLD	0.0	30	36140	31450	28	370	70870	4657	0	8	135	516	1.1	66	86	435	2	0.2	5.6	4.9	62.0	43.3	0	0	0	1	0.1	5	1	0.1	8	1	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0.3	2.9	0	0.0
Corn, site 57, NSW	0.1	30	39830	41750	39	420	76800	5589	0	15	348	563	0.6	23	118	553	3	0.3	4.3	4.5	87.4	47.8	0	1	0	2	0.2	7	1	0.1	11	3	0	0	0.04	0	0	0	0	0	0	0	0	0	0	0	0.4	2.1	0	0.0
Soil, site 10, MC	11.6	1	117	1905	6067	520	786	1944	8	7	163	9173	2.8	7	4	49	10	3.9	0.6	1.1	7.1	12.8	10	7	2	0	0.1	1	0	0.7	29	24	47	3	11.0	1	0	1	1	0	0	0	0	0	0	0	0.4	8.4	9	0.5
Soil, site 17, AK	1.4	1	50	269	16740	300	420	6480	30	12	2074	27160	6.3	5	26	68	31	5.7	1.5	2.5	6.3	18.6	10	120	2	0	0.3	1	0	2.0	84	17	180	3	9.6	1	0	1	1	0	1	0	1	0	2	0	0.4	28.0	26	2.0
Soil, site 36, BP	0.5	0	69	251	10510	340	173	2998	8	3	215	12720	1.5	3	8	39	58	2.5	1.6	4.8	2.5	15.0	32	130	2	0	0.2	1	0	1.1	187	36	79	6	19.0	3	0	2	2	0	2	0	2	0	2	0	0.4	9.5	10	1.1
Soil, site 45, QLD	0.4	0	268	3356	8400	48	905	9398	4	13	406	13820	12.6	30	10	63	41	1.0	3.0	7.9	4.0	114.5	44	8	0	0	0.1	1	0	0.1	118	50	110	9	36.0	6	1	4	3	1	2	0	2	0	0	0	0.1	4.2	6	0.2
Soil, site 57, NSW	0.9	1	533	2105	6619	68	1337	3570	4	14	185	11750	4.0	15	8	38	29	1.5	2.0	6.4	13.9	39.6	32	3	0	0	0.1	1	0	0.3	91	46	96	8	28.0	4	1	3	2	0	2	0	2	0	0	0	0.1	9.3	19	0.2

2.3.1.1 Insect

Insect cuticle is a complex structure of chitin fibres embedded in a matrix composed mainly of various proteins, plus water, catechol, lipids and wax (Chapman, 1998; Gunderson & Schiavone, 1989; Rebers & Willis, 2001; Willis et al., 2005). As reported for other organic samples (Font et al., 2007; Sellick et al., 2009; Spalla et al., 2009), the complex molecular structure of insect tissues along with the constraints of the low abundance of many potentially informative elements in a single insect (Table 2.2) and the restricted sensitivity of the analytical methods and instruments available, directed the adaption and evolution of the clean sample preparation and efficient extraction of geochemical methods to achieve accurate biogeochemical signals. The method needed to achieve:

1. Effective yet non-depleting cleaning methods (discussed in Section 2.5);
2. Sample preparation and analytical methods optimised to cope with low quantities of target element relative to contamination errors. The pilot study ICP-MS analyses indicated that *H. armi*gera, with an average dried weight of 68mg and an average Sr and Pb concentrations of 24 and 3 ppb respectively, yields 1.63 ng Sr and 0.20 ng Pb per wild adult moth. (Subsequently improved cleaning and digestion methods significantly increased recovery, and yielded averages of 66.95 ng Sr and 2.72 ng Pb per adult wild moth). Despite conventional 'clean lab' methods being used for the exploratory digestions, the blanks revealed contamination of up to 12% of the moth signal for some elements. As the heavy elements are easily and commonly contaminated (e.g., Tsuji et al., 2009), especially Pb, which corrupts isotopic analysis (e.g., Marx et al., 2010), all subsequent digestions were switched to the ultra-clean laboratories at Victoria University, New Zealand (protocols given in Section 2.6.2.1), where column processing and all vessel cleaning was also conducted. Ultra clean Sr blank errors given in the literature range from <40 pg to 76 pg (Blum et al., 2000; Chamberlain et al., 1997); and Pb reported blanks range widely from 5 pg (Inoue & Tanimizu, 2008) to 60 pg Pb (Bollhofer & Rosman, 2000). Thus reported ultra clean lab 'blank errors' represent < 1% of Sr and < 3% Pb recoverable from the model insect, representing a significantly improved contamination error from the pilot study analyses.
3. Thorough digestion for ICP and TI MS analysis, as un-decomposed organic compounds interfere with the preparatory elemental separation and mass spectrometric analysis (Section 2.6.2);
4. Optimized recovery of elements (Sections 2.6.2.2 – 2.6.2.6); and
5. Sensitive and high precision analyses (Sections 2.6.2.7 – 2.6.2.11).

Similar methodological development for samples of small size and hence restricted availability of the element(s) of interest, was described by Charlier , et al. (2006) and Font, et al. (2007) for single crystals in magmatic rocks and sedge warbler (*Acrocephalus schoenobaenus*) feathers respectively. The large difference in the elemental abundance found in the pilot study analyses (Table 2.2) and the abundance values reported in Table 2.5 (repeated Moth Standard) reveal the improvements in analytical sensitivity achieved by the refined methods.

2.3.1.2 Plant material

The results from pilot study ICP-MS analyses of Australian and New Zealand corn (Table 2.2) confirmed, as anticipated (Koeppe, 1977; Ming & Bing, 1998), that the Sr and Pb abundances in the corn were also limited. The abundance of these and other potentially informative heavy elements was even less than for the insect samples – with approximately 40 ppb Sr and 3 ppb Pb observed , or ≈ 4 ng Sr and ≈ 0.3 ng Pb per 100 mg sample. Hence the plant samples have similar analytical requirements to the moth, namely; thorough digestion and efficient methods of element extraction, along with under ultra-clean lab conditions to minimise contamination and mass spectrometry matrix-effects[11] interference (Ming & Bing, 1998; Spalla et al., 2009; Zarcinas et al., 1987), along with sensitive analyses.

To increase elemental yield, in particular for the uncommon but potentially informative elements, 300 mg plant samples and more complete microwave digestion methods (Section 2.6.2.3) were subsequently used. These methods increased elemental yield to an average of ≈ 300 ng Sr and 27 ng Pb per sample.

2.3.1.3 Soil

The pilot study ICP-MS analyses of Australian and New Zealand soils resulted in average Sr and Pb concentrations of 40 ppb and 12 ppb per 100 fold diluted 200 mg soil extract (Table 2.2). These results indicated that average abundances of 50 and 3.75 mg/kg for Sr and Pb respectively from the proposed research sites could be expected, and that there were no special analytical requirements for the soil samples.

Subsequent, total-recoverable digests of the soils from all of the research sites resulted in average Sr and Pb concentrations of 53.45 mg/kg and 17 mg/kg respectively. Soil extraction and digestion methods are further discussed in Section 2.6.2.4.

[11] In the context of chemical analyses, 'matrix' refers to the composition of the sample

2.4 Sample preservation and partitioning

The moths were reared and stored individually. The adult moths were kept alive for three to four days after emergence and then euthanized by freezing. The specimens were then confirmed as the target species by morphological identification (Matthews, 2008). All samples were stored dry in sealed glass vials at -20°C. Although no literature specific to the optimal preservation of δ^2H during the storage of solid samples has been found, the research of M.S. Edwards, et al. (2002) Saraknios, et al (2002) and Hobson, et al. (1997), demonstrated that dried frozen storage is acceptable for the maintenance of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ signals in biological samples. Storage in alcohol is probably also acceptable for δ^2H signal in solid samples (Wassenaar, 2008, pers. comm.), but as it diminishes Sr and other trace elements in samples (see moth cleaning trial, Section 2.5.1.1), was deemed not appropriate for this study.

Before sample cleaning and preparation, the moths were partitioned, with a set of wings prepared for H analysis and the rest of the body used for all the other element analyses. The left hand side fore- and hind-wings were manually broken off at the bases, and transferred to a 1.5 ml clip top C-tube for chloroform/ methanol washing prior to δ^2H analysis. A foreleg was broken off and stored in 0.5 ml clip top C-tube of 95% ethanol for molecular analysis; and the rest of the body was maintained in dry storage in the original glass vials at -20°C before preparation for solution based analyses (all the other elements).

Plant material was stored frozen in sealed polycarbonate tubes, so as to preserve both the plant water and solid δ^2H signatures. Subsequently the material was partitioned, with approximately 10 grams of the apical corn kernels retained frozen in the original sealed tubes for cryogenic extraction of the plant water component; and the other portion of the corn samples were transferred to glass vials for cleaning and drying for solid δ^2H, trace element concentration and heavy element isotope analyses.

Soil samples were stored in sealed glass vials and kept in the dark at room temperature until processing.

2.5 Sample preparation and cleaning

2.5.1 Insect

For light element isotopic analysis of solid substrates lipids are generally removed (J. F. Kelly, 2000; Ricca et al., 2007) as they are depleted in ^{13}C and ^{2}H (e.g., DeNiro & Epstein, 1977). Accordingly, the moth wing samples used for δ^2H analysis were washed three times with a solution of 2:1 chloroform: methanol (AR grade) in 1.5 ml C-tubes to remove oils

(Dobush et al., 1985; Wassenaar & Hobson, 1998); then air dried for 12 h. Six, ≈ 200 µg sections (three replicate pairs) were dissected from the distal costal region of the dried wing, and weighed into 3 X 5 mm elemental analyzer silver cups (Elemental Microanalysis); these were loosely crimped to allow H equilibration before δ^2H analysis. The remainder of the wing samples were archived.

The other proportion of the moth samples (for trace element and heavy element analysis) were 'gentle dried' at 60°C for 24 – 48 h, before a separate cleaning process.

2.5.1.1 Moth cleaning trial

For high precision trace element and heavy element isotope analyses of insect samples it was necessary to develop a pre-digestion washing method that minimised external contamination, yet did not leach cations from the sample (Section 2.3.1.1). Four methods were evaluated.

1. The same triple 2:1 chloroform: methanol solution as used for the ^2H sample preparation was trialled. However, this gave low Sr recovery (< 30 ng/g Sr), probably from loss of Sr when removing the lipids. This led to exploration of washing methods that maintained the maximum Sr signal.
2. Washing moths using three Milli-Q (MQ, 18.2 MΩ) water rinses minimised Sr loss, but as discussed by Font, et al. (2007), the disadvantages of MQ water washes is that the depleted ion status of the water leaches some other ions from the samples. Further, washing with water only partially removed external contamination, see Figure 2.1.
3. Various numbers of post weighing 1M Seastar HNO_3 washes were also trialled. However, the 1X and 3X wash treatments had the same Pb isotope ratio and precision as the 2:1 chloroform: methanol wash (number 1 above), indicating no improvement in cleaning. In this regard Weyers, et al. (1988) also found that solution based cleaning of biological samples did not completely remove external dust particle contamination. In addition, even at this mild HNO_3 concentration, partial digestion of the insect samples was observed. Therefore, although HNO_3 washing treatment is undoubtedly an effective Pb contamination removal for geological substrates (Baker, 2009, pers. comm.), this method is considered not appropriate for insect biogeochemical samples.
4. The most appropriate washing method for insect samples evaluated was to pass high pressure N_2 over the samples (Figure 2.1). Individual moths were 'washed' in a custom built filtered chamber by passing two 30 second 250 kPa+ streams of high purity N_2 through the chamber. Further advantages of gas cleaning are the low potential for additional contamination and it does not prematurely digest nor leach cations from the

samples as may occur during solution based cleaning. Sr recovery using this N_2 cleaning method was ≈ 980 ng/g. This method was used for all subsequent moth cleaning.

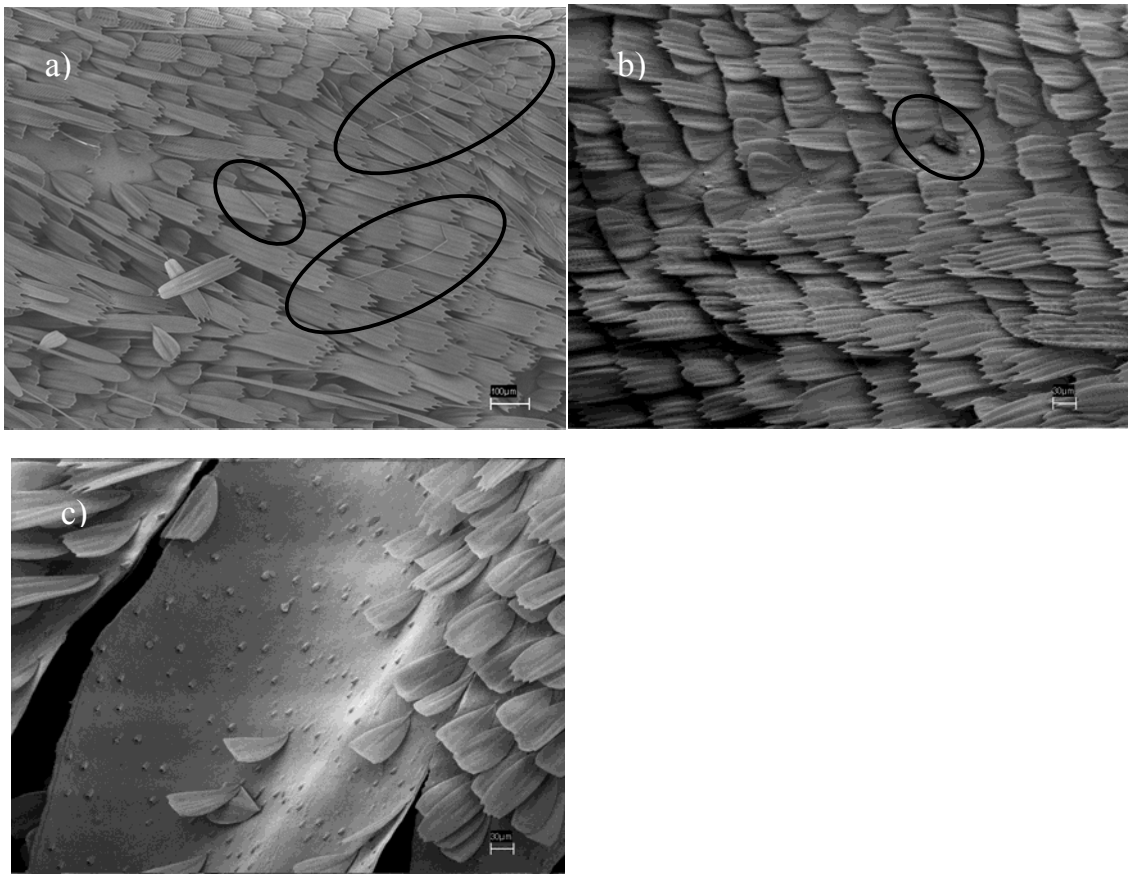


Figure 2.1 SEM images of moth cleaning trial: (a) Un-cleaned moth wing showing several foreign fragments; (b) Moth wing cleaned using MQ water, still with some dust particles on the surface; (c) High pressure N_2 cleaned moth wing – showing all contaminating particles and most of the scales are removed from the wing surface. Image (a) is a larger scale than images (b) and (c).

2.5.2 Plant material

Plant material was examined under a dissecting microscope and cleaned of epibiota, if any present, with gentle scraping. The plant samples for solid δ^2H, trace element and Sr and Pb isotope ratio analysis, were dried at 80°C for 24 hours minimum in labelled glass vials. The dried samples were ground with an acid-washed-quartz cleaned ceramic mortar and pestle. The mortar and pestle were cleaned with AR-grade methanol between samples, and allowed to air dry. The ground samples were stored in labelled glass vials.

2.5.3 Soil

All visible root and plant debris was removed from the soils using fine forceps. The soil was then dried at 80°C in labelled glass vials for at least 48 hours and then gently crushed using a

clean mortar and pestle until even, medium sized aggregates were achieved. Approximately 25 grams of the fine material was stored in numbered sealed glass vials for subsequent digestion and archiving.

2.6 Analytical methods

2.6.1 Hydrogen isotope analysis

2.6.1.1 Plant water extraction

Plant water was extracted from the frozen samples by cryogenic extraction under vacuum using equipment described in Ehleringer & Roden, et al. (2000) and A.G. West, et al. (2006). To circumvent the effects of fractionation from freezing-thawing-freezing, all the water was extracted from the samples, which usually took 60 – 90 min. The extracted water was transferred to 5 ml septum topped tubes, for later analysis.

2.6.1.2 Exchangeable H equilibration in solid samples

Hydrogen covalently bound to non-aromatic C is generally non-exchangeable, and so retains the source $^{2}H/^{1}H$ isotopic and biosynthetic fractionation information incorporated during tissue synthesis. On-the-other-hand, H bound to O, N, or S, which represents 10 to 20% of H in most biological tissues, freely exchanges with H atoms in the isotopically variable atmospheric water vapour, and so partially reflects the $\delta^{2}H$ of vapour the tissue has been recently exposed to, recent H chemical bonding conditions, and steric accessibility (Schimmelmann et al., 1999). Thus, the exchangeable proportion of H needs to be separated from the $^{2}H/^{1}H$ ratio analysis to obtain the 'non-exchangeable' $\delta^{2}H$ from the sample, and hence achieve meaningful information. This can be achieved by chemical replacement of the exchangeable H by nitration for a limited range of biological substrates (e.g., cellulose) (Schimmelmann, 1991; Wassenaar & Hobson, 2000a), however, nitration treatment is not suitable for the majority of complex organic tissues.

Wassenaar & Hobson (2000a) described a comparative equilibration method, where the exchangeable H of reference standards is replaced with H from a series of waters of known, widely dissimilar isotopic composition in an off-line static H equilibration steam manifold. The difference in total $\delta^{2}H$ values of the standards exposed to the isotopically distinct water vapours enables the proportion of the exchangeable (and non-exchangeable) H of the standards to be calculated, as well as the $\delta^{2}H$ of the non-exchangeable fraction. The reference standards are subsequently used to calibrate the non-exchangeable $\delta^{2}H$ values of the sample by achieving equilibration of the sample and standards under identical conditions prior to

analysis Wassenaar & Hobson (2003), ideally to water of known δ^2H (Bowen, Chesson, et al., 2005). More recently, Sauer, et al. (2009) published a paired equilibration steam method in which the exchangeable H on aliquot pairs of the actual sample are simultaneously replaced with H from two reference waters of different δ^2H values, by pumping the waters through corresponding sample equilibration chambers at 115°C. As there were no δ^2H standards material available for the various plant types used in this project, and it was desirable to have a speedier analysis through-put than the conventional four day equilibration time of the comparative equilibration method, the Sauer, et al. (2009) method was trialled here. However, our attempts with this method failed to achieve acceptable precision using internal plant and insect standards (with average difference of repeated samples = 14.49‰, $n = 7$), possibly due to difficult to control and variable vapour pressure and/or fractionation within the chamber, or a failure in this system to completely remove equilibration waters in some samples. A static paired equilibration chamber method developed by Len Wassenaar (2009, pers. comm.) was then tested, and achieved the precision shown in Table 2.3. This became the preparation method used for all subsequent δ^2H analyses.

A pair of sealable aluminium equilibration boxes were built to hold a Costech Zero Blank autosampler carousel. Both have a septum inlet for the injection of equilibration water and a vacuum outlet. The sample pairs (in loosely crimped silver capsules) were loaded into paired autosampler carousels. The loaded carousels were then sealed in the equilibration boxes and placed under a 0.001 atm. vacuum at 110°C for 20 – 30 min, before 0.12 ml of the two waters of known δ^2H_{VSMOW} values, δ-263.88 and δ+539.83, were simultaneously injected into the two equilibration boxes through the septum inlet valves. The septum valves were then closed and the equilibration boxes kept at 110°C for 60 – 90 min. The resulting volume of steam, in the 204 ml airspace inside the chambers, achieved a >50 fold excess H relative to the exchangeable H on the 49 samples, assuming 20% exchangeable H and 200 µg samples. The chambers were then evacuated to approximately 0.001 atm., *via* a liquid nitrogen trap to prevent water entering the vacuum pump, and maintained at 110°C for 30 min, before being cooled under vacuum on the bench for at least another 30 min. The vacuum in the chambers was then replaced by dry N_2, and the carousels immediately transferred to the autosampler on the mass spectrometer. The autosampler chamber was evacuated and replaced with He gas within 1 min.

Table 2.3 Repeated values (vs VSMOW) from static paired equilibration chamber trial. HK = Hoof Keratin, internal lab std.; KHS = Kadou Hoof Standard, inter-lab std., armig ICE = *Helicoverpa armigera* moth wing, internal lab std., PH Corn = maize, internal lab standard, x_e. = the proportion of exchangeable H.

Batch no.	Date	Identifier	^{2}H /H high	^{2}H /H low	x_e	δ^2H‰	Av‰	1SD
128-1	10 June 2010	HK	21.3	-102.2	0.18	-123.5		
		HK	32.2	-103.0	0.20	-127.0		
		HK	32.1	-102.8	0.20	-126.7	-125.7	1.96
128-2	10 June 2010	HK	24.9	-101.2	0.19	-122.8		
		HK	32.2	-102.9	0.20	-126.9		
		HK	32.1	-103.0	0.20	-127.0	-125.5	2.38
128-1	10 June 2010	KHS	70.2	-47.4	0.18	-55.8		
		KHS	66.8	-41.1	0.16	-47.5		
		KHS	71.4	-50.6	0.18	-60.1	-58.0	3.05
128-2	10 June 2010	KHS	63.4	-46.6	0.16	-54.24		
		KHS	71.3	-45.4	0.17	-53.35		
		KHS	70.8	-47.1	0.18	-55.48		
		KHS	72.3	-46.4	0.18	-54.66	-54.4	0.89
128-1	10 June 2010	armig ICE	-44.8	-108.1	0.09	-118.5		
		armig ICE	-38.6	-130.7	0.14	-150.3		
		armig ICE	-44.8	-136.7	0.14	-157.2		
		armig ICE	-39.2	-129.2	0.13	-148.0		
		armig ICE	-43.4	-135.8	0.14	-156.2	-152.9	4.48
408-1	14 Oct 2010	PH Corn	90.2	-76.1	0.19	-56.6		
		PH Corn	87.0	-70.1	0.18	-51.4		
		PH Corn	98.0	-71.0	0.19	-49.7	-52.6	3.60
408-3	16 Oct 2010	PH Corn	95.9	-77.6	0.20	-48.1		
		PH Corn	69.1	-81.6	0.17	-53.4		
		PH Corn	80.5	-78.7	0.18	-53.3		
		PH Corn	109.3	-81.3	0.22	-56.4	-52.8	3.4
408-7	11 Nov 2010	PH Corn	111.2	-77.6	0.22	-53.5		
		PH Corn	106.6	-81.6	0.22	-50.2		
		PH Corn	94.1	-78.7	0.20	-53.7		
		PH Corn	92.3	-81.3	0.20	-53.0	-52.6	1.62

2.6.1.3 Calculation of non-exchangeable δ^2H in solid samples

The proportion of exchangeable H (x_e) was calculated using the following equation from Wassenaar & Hobson (2000a) (equ. 3) and Schimmelmann, et al. (1999) (equ. 4):

Equation 2.1

$$x_e = \frac{\delta D_{SA} - \delta D_{SB}}{(\delta D_{WA} - \delta D_{WB}) * \left(1 + \frac{\varepsilon}{1000}\right)}$$

Where the subscript S refers to sample; W to water; and A and B refer to the isotopically distinct reference equilibration waters; thus δD_{WA} is the δ^2H value of the reference water A,

and δD_{SA} is the δ^2H of the sample that has been was equilibrated with reference water A; ε is the value for the isotopic fractionation (in ‰) between exchangeable H in the sample and the equilibration vapour (Wassenaar & Hobson, 2000a).

Equation 2.2

$$\varepsilon\ (‰) = (\alpha - 1) * 1000$$

Where

Equation 2.3

$$\alpha = R_e/R_w$$

And R_e = the ratio $^2H/^1H$ of the exchangeable H in the sample and R_w = $^2H/^1H$ of the vapour. ε = 80‰ was used for both plant and insect x_e calculations, as Schimmelmann (1991) and Wassenaar & Hobson (2000a) have experimentally determined that a ε of 80‰ is appropriate for plant derived solids as well as more complex organic matrices, including monarch butterfly wing. Landwehr, et al. (2011) have recently proposed that the isotopic fractionation constant, α, presently assumed to = or ≈ 1 (Bowen, Chesson, et al., 2005) ought to be replaced with compound specific constants. However, we have followed current convention to use ε = 80‰, as applied to a broad a range of organic samples (Chesson et al., 2009; Sauer et al., 2009; Schimmelmann et al., 1999; Wassenaar & Hobson, 2006).

The non-exchangeable δ^2H value of the sample (δD_n) was calculated using the equation given in Schimmelmann, et al. (1999) (equ. 3):

Equation 2.4

$$\delta D_n = \frac{\varepsilon * (\delta D_{SA} - \delta D_{SB}) + (\delta D_{SA} * \delta D_{WB} - \delta D_{SB} * \delta D_{WA}) * (1 + \frac{\varepsilon}{1000})}{\delta D_{SA} - \delta D_{SB} + (\delta D_{WB} - \delta D_{WA}) * (1 + \frac{\varepsilon}{1000})}$$

The equations given for x_e and δD_n above are derived from the equation that defines the total measured δ^2H (δD_T), Equation 2.5 (i.e., the sum of non-exchangeable (δD_n) and the exchangeable H (δD_{ex}) pools after equilibration) (Schimmelmann et al., 1999, equ. 1), (Wassenaar & Hobson, 2000a, equ. 1), by solving the equation for x_e and δD_n respectively and using a series of algebraic substitutions (Schimmelmann et al., 1999; Sessions & Hayes, 2005).

Equation 2.5

$$\delta D_T = x_e * \delta D_x + (1 - x_e) * \delta D_n$$

2.6.1.4 δ^2H analysis: TC/EA IRMS

Hydrogen isotope ratios were measured by pyrolysis to H_2 gas in a Thermal Conversion Elemental Analyzer (TC/EA, Thermo, Bremen) followed by isotope analysis of the gas in a Thermo Delta V Isotope Ratio Mass Spectrometer (IRMS) in continuous flow mode. The reactor tube was packed with glassy carbon granulate (MultiLab Ltd, UK). The reactor temperature was 1400°C, and the gas chromatography (GC) column was held at 80^0C. The ≈ 2 m GC column was packed with 5 Å molecular sieve. Helium (99.995% purity, BOC Gases New Zealand Limited) was used as the carrier gas, at 95 – 100 ml/min flow rate. Raw delta values were calculated as sample versus H monitoring gas (BOC Ltd). Data were processed using Thermo Scientific's Isodat NT (version 2.0) proprietary software. The H_3^+ factor was determined daily using a series of five increasing pairs of reference H_2 gas pulses; the signal size of each pulse pair being increased by 2 volts.

For solid samples, two pulses of the monitoring gas were injected before and after each sample combustion.

For the extracted water samples, eight repeat 50 nl samples were injected into the reactor, bracketed by twin injections of monitoring gas. The raw δ^2H results from the H peaks were filtered by removal of values more than 1 standard deviation from the average.

2.6.1.5 δ^2H Standards

Each batch of solid samples was bracketed by nine IAEA-CH-7 reference standards (δ^2H_{VSMOW} -100.3‰). The raw δ^2H values were corrected by the average offset to the accepted IAEA-CH-7 value. Each batch also included four replicates of internal quality assurance standards and/or four replicates of inter-laboratory quality assurance standards. The Principle of Identical Treatment (PIT) requires that the standards are treated identically to the analyte (Werner & Brand, 2001), and where possible, the validation standard be of a similar matrix as the analyte (and cover the range of percent atomic composition of samples), thus presenting the same H exchange characteristics and analytical error for both substrates (Chesson et al., 2009; Jardine & Cunjak, 2005). Accordingly: 1) The CH-7 calibration standards were weighted out to give the same H percent and MS signal strength as the 200 µg (insect) or 600 µg (plant) analyte samples, and 2) KHS (Kadou Hoof Standard -54.1 ± 0.6‰ (Wassenaar & Hobson, 2010)) was used as the quality assurance standard for the insect batches (average precision of measurement over the three months the analyses were carried out = ± 0.8 ‰); and 3) an in-house maize standard used to compare with the plant analyses (calculated δ^2H = -52.7‰ ± 2.6 (1SD), average precision = ± 1.4‰ (1SD)).

For the water samples, the average δ^2H raw results were corrected to international isotope scales using a three-point calibration provided by three laboratory standards analysed before and after every batch of 90 samples. In addition, a control sample was measured after every 12 samples. The three laboratory standards used have a six-year record of calibration against IAEA primary standard material, VSMOW2 (IAEA, 2009b), and have also been measured in a 5-member inter-laboratory comparison exercise. The calibration standards and their consensus values are as follows: ICE (δ^2H = -262.7 ± 0.9‰), TAP (δ^2H = -81.0 ± 2.0‰), SEA (δ^2H = -1.5 ± 0.9‰). The precision of analysis was typically 0.9‰ (1SD).

2.6.2 Trace element profile and Sr and Pb isotope analysis

2.6.2.1 Clean lab protocol

For all the other variables, an ultra-clean lab protocol was applied from the specimen cleaning stage (Section 2.5), at the Geochemistry Laboratory at Victoria University. The lab is positively pressured with the air continuously filtered to Class 100, to minimise contaminants. All samples were handled using tweezers or spatulas to avoid contamination. All surfaces and tools that are linked to the samples were acid washed. Teflon containers were used for solution storage and handling, as well as specimen digestions. Digestion vessels under-went a sequence of 6 M SeaStar HCl and HNO_3 acid washes, each followed by triple MQ water rinse and air drying. Tools were cleaned with MQ water or wiped with Kimwipes® between specimens. All reagent transfer was via acid washed pipette or dripper bottles. Samples were then digested and subsequently diluted in reagents of purity grades appropriate to the analysis, with Seastar chemicals predominantly used. All specimen handling, chemistry and drying was conducted in PicoTrace Class 10 laminar flow workstations.

2.6.2.2 Moth digestion

As introduced in Section 2.3.1, complex organic structures need to be broken down for solution based analysis, as organic molecules interfere both with the columns used for elemental separation and mass spectrometric analysis. This is achieved by a series of digestive steps.

As the literature contained no entomological biogeochemical solution based method for the broad range of variables that were to be considered in this study, it was necessary to develop a method before the research could proceed. An iterative development process was followed, assessing the accuracy and precision of various moth digestion techniques, using the in-house moth standard, PH_arming (i.e., with the same matrix as the insect samples), as well as the external NBS_SRM 1575 standard, with reference to both trace elements and the isotopic

ratios of Sr and Pb. The recovery of Sr and concomitant analysis of $^{87}Sr/^{86}Sr$ proved to be the most problematic variable to achieve. Sr data for individual moths obtained using a series of only HNO_3digestions yielded low Sr recovery and possible matrix effects, with concomitant unacceptably high average $^{87}Sr/^{86}Sr$ 2SD precision values of 0.034 (via ICP MS, data not shown). Following reports by Font, et al. (2007) and Selleck, et al. (2009) where successful Sr isotope results were obtained from feather samples of similar mass to the model insect, using $HNO_3 + H_2O_2$ digestions, evaluation of this digestion method on moth samples yielded average $^{87}Sr/^{86}Sr$ 2SD precision values of 0.003.

The digestion method used was: 2 ml of ultra-pure Seastar 15 M HNO_3 was added to the weighed moth samples in Savillex Teflon digestion beakers (as many elements are volatile, closed vessel digestion and extraction procedures were used (Taggart, 2002) (cf. Zarcinas et al., 1987) via washed pipette. The lids were screwed down tight, and the beakers placed on the hot plate at 120°C for 2 h. If the specimen was not completely dissolved, it was returned to the hot plate until it was completely in solution. Samples were cooled for 20 min and lids slowly removed. Care was taken to ensure that the entire sample was recovered, with the contents of the lids washed back into the beakers following each digestion dry-down. Then 20 µl of Aristar 30% H_2O_2 was added to each cooled sample solution via washed pipette. Samples were then kept at room temperature with the lids on loose for 1 h, then evaporated down at 105°C overnight. The beakers were cooled for 20 min before a 2nd digestion with 2 ml Seastar 15 M HNO_3, followed by 60 µl Aristar 30% H_2O_2 and drying down treatments. A third digestion with 1ml Seastar 15 M HNO_3 was then applied, followed by 60 µl Aristar 30% H_2O_2 and drying down. The final digestion with 10 drops of Seastar 15 M HNO_3 was applied to the dried down samples via dropper bottle. The samples were returned to solution for 1 h, before being evaporated down at 120°C.

The digested moth samples were then cooled before being diluted up in 5 ml 1 M Seastar HNO_3. This and all subsequent dilutions were weighed to maximize accurate trace-element abundance calculation. The capped samples were returned to 120°C for 3 h to ensure they went back into solution.

2.6.2.3 Corn digestion

Three digestion protocols were assessed for the dissolution of corn samples. 1) A HNO_3 digest series, but this resulted in visible residues in the final solutions. 2) A repeated HNO_3 and HF acid digestion series generally gave clear solutions, but some corn samples had precipitates fall out of solution, which impeded column chemistry. In contrast, 3) closed

vessel acid microwave digestion (CEM, 2009) yielded consistently clear sample preparations and reliable reproducibly of results (as also shown by Spalla et al., 2009). This method had the added advantage of avoiding the use of HF.

The acid microwave digestion protocol used was adapted from Blum, et al. (2000) and Spalla, et al. (2009). Five ml of Seastar 15 M HNO_3 and 2 ml Seastar 11 M HCl were added, *via* washed pipette, to the plant samples (≈ 0.3 g) in 55 ml microwave digestion vessels. The lids were screwed down tight and the samples allowed to pre-digest for 24 – 48 h. The vessels were then subject to 20 min ramp to 175°C, then held at 175°C for 15 min. Then 0.4 ml Aristar (30 – 32%) H_2O_2 was added to the cooled solutions and reacted at room temperature for 2 h. The solutions were then transferred to cleaned Savillex beakers and dried down at 120°C, before being twice re-digested in 1 ml 15 M HNO_3 with drying down and cooling after each digestion. The dried samples were taken up in 5 ml 1 M Seastar HNO_3, with all dilutions weighed to maximize accurate trace-element abundance calculation. The samples were returned to 120°C for 3 h.

2.6.2.4 Soil extraction and digestion

There are many methods for extracting trace element and heavy metal isotope ratio profiles from soils. This includes a large body of literature regarding the relationship between the labile phases removed by various extraction procedures and the portion of available elements taken up by plants (biological availability). However, soil digestions and extractions can only provide an estimate of potential trace element and heavy metal availability for plant uptake (Rao et al., 2008). As a result, there is limited consensus and no one 'most appropriate method' for biogeochemical studies (V. H. Kennedy et al., 1997). Extraction methodologies are specific to the element of interest, plant and soil type, as well as project objective (C. A. Wilson et al., 2006). Several researchers have demonstrated that the Pb isotope ratios are more or less equal between ammonium salt extraction, dilute acid leaching and strong oxidative digestion methods (e.g., Prohaska et al., 2005; Sutherland, 2002). In contrast, Sr available to plants, associated with the acid or water exchangeable fraction, can be significantly different from the elemental pool liberated by total-recoverable (pseudo-total, aqua-regia) or total-total (HF + acid) digestions (see Rao et al., 2008 for explanation of terms). Thus, soil Sr isotope ratios may differ depending on the extraction or digestion methods used (Horn et al., 1994; Prohaska et al., 2005), and the $^{87}Sr/^{86}Sr$ ratio in plants will potentially not match whole soil isotope ratios. Blum, et al. (2000) is the only reference found where the Sr isotope ratio transfer from the bio-available proportion of the soil to plant (as well as to consumers) has been validated, and they used 1 M ammonium acetate

($NH_4C_2H_3O_2$) soil leaching. On-the-other-hand, the inappropriateness of total-total digest for soil to plant Sr isotope studies was catalogued by Sillen, et al. (1998). The suitability, or not, of dilute acid leaching or total-recoverable digestion for soil to plant Sr isotope studies appears not to have been evaluated. In a review focused on pollution associated radionuclide and heavy metals, V. H. Kennedy, et al. (1997) recommended 1 M, pH 7 ammonium acetate and 1 M ammonium nitrate (NH_4NO) as the single extraction methods most suitable (i.e., providing acceptable plant available estimations) for a wide range of soil types and elements. The disadvantages to these mild extractions are: 1) the metal recovery ranges from < 2 – 10% of total-recoverable (Li et al., 1998; Rao et al., 2008) and is potentially below the analytical limit of detection; and 2) it is more difficult to achieve salt extractions free of Sr and Pb contamination, than total-recoverable or total-total acid digestions. Similarly, for trace element and heavy metal concentrations values from soils, weak acid (e.g., 0.01 M HNO_3) and salt solution (e.g., 0.5 M $Ca(NO_3)_2$, 1 M NH_4NO_3) soil extractions have been shown to be more suitable for predicting multi-element concentrations in plants than stronger soil extractant methods (e.g., 1 M HNO_3, 0.1 M HNO_3 and 0.05 M EDTA) (Takeda et al., 2006). Although, it needs to be stressed that single extraction trace element values from soils generally do not directly correlate to plant concentration values, regardless of the extraction or digestion method used (Grønflaten & Steinnes, 2005; Li et al., 1998). Therefore, it is prudent to use more than one extraction or digestion method in biogeochemical studies.

Consequently, for the first year of the inter-regional comparison (Chapter 4), a 1 M Seastar HNO_3 leaching of soil samples was used. Two ml of Seastar 1 M HNO_3 was applied to the weighed soil samples, which were then held at 125°C for 2 h in sealed Savillex beakers. The extractant was separated by centrifugation, before being evaporated down and digested again in 15 M Seastar HNO_3, re-evaporated, then diluted up in 1 ml 1 M Seastar HNO_3. However, the trace element data from this method gave poor soil-to-moth correlation and the soil $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios were distinctly different from the plant and insect Pb isotope ratios (Section 4.3.4). Therefore, for the subsequent year's soils, $HNO_3 + H_2O_2$ total-recoverable digest were used, to complement the first year's data. In this method, 5 ml 15 M Seastar HNO_3 and 1 ml Aristar (30%) H_2O_2 were added to weighed (≈ 0.5 g) soil in 55 ml microwave digestion vessels. The lids were screwed down loosely and the samples allowed to pre-digest for 24 – 48 h. The vessels were then subject to a 10 min ramp to 175^{o}C, then held at 175°C for 20 min, and then allowed to cool for 2 h. The solutions were transferred to cleaned 50 ml c-tubes, and diluted to ≈ 20 ml with MQ water. The tubes were then spun at 3300 rpm for 10 min. An aliquot was taken off each of the samples for trace element

analysis. The remainder of the solution was centrifuged again at 3300 rpm for 10 min, then approximately 9 ml was gently pipetted to clean 23 ml Savillex beakers for column chemistry.

Comparing data from the different methods enabled conclusions to be drawn as to the source of the Pb recovered from the insects, although the results are inconclusive regarding most appropriate soil extraction method (Section 4.3.4). Ideally, a 0.05 M $Ca(NO_3)_2$ or similar leaching method could have been used to complement the soil extraction/ digestion methods carried out. However the regents and filters associated with these dilute salt methods could not be made clean enough for Pb isotope analysis.

2.6.2.5 Trace element aliquot

Twenty percent of the insect and plant analyte solutions from the above digestions (i.e., 1 ml) were pipetted to cleaned 10 ml centrifuge tubes for trace element analysis *via* ICP MS. These aliquots were then diluted to ≈ 1% Seastar HNO_3 by the addition of 5 ml MQ water. An aliquot of approximately 50 percent of the soil solutions were pipetted to cleaned 10 ml centrifuge tubes for trace element analysis *via* ICP- OES. The tubes and all dilutions were weighed to maximize accuracy of the trace-element concentration calculations.

The remaining portion of the solutions were dried down then taken up in 2 ml 0.8 M Seastar HBr for Pb and Sr column chemistry. These were returned to 120°C for 2 h, then cooled.

2.6.2.6 Sr and Pb separation: Column chemistry

Sr and Pb isotope analysis require these elements to be separated from the other elements in the sample. Most critically, Sr needs to be separated from Rb and Ca in the samples, as Rb produces a mass interference on ^{87}Sr and Ca interferes with TIMS. The Sr and Pb separation procedures used here followed the column chemistry protocol described by Pinn & Bassin (1992) and Baker, et al. (2004) respectively. Columns were made up from disposable 1 ml pipette tips fitted with pre-cleaned, polypropylene frit (30 μm pore-size) fitted into the tapered section, with the end of the tip below the frit cut off. The columns were cleaned by soaking in 6 M Seastar HCl for two weeks prior to use. The columns were placed in column racks and rinsed three times with MQ water and Seastar 6 M HCl. Pb separation was carried out first. A bed of pre-cleaned Pb resin (Eichrom Technologies, Illinois, USA), approximately 5 mm deep was pipetted onto each column, and then the resin further cleaned with three column volume washes of alternating MQ water and Seastar 6 M HCl. The resin was then equilibrated with one volume of 0.8 M Seastar HBr. The samples, in 2 ml 0.8 M HBr solution, were then loaded carefully onto the Pb resins. The resins were eluted out with one and two half volumes of 0.8 M Seastar HBr, with Sr and other elements collected into clean 7

ml beakers. The original beakers were washed with 6 M Seastar HCl and placed under the columns before the Pb was eluted from the resins in one whole volume and two half volumes of 6 M Seastar HCl. Six drops of 15 M SeaStar HNO_3 were added to both Pb and Sr samples before being dried down. The Pb samples were taken up in 1.5 ml 0.5 wt% Seastar HNO_3 for storage prior to Pb MC ICPMS analysis; and the Sr samples taken up in 2 ml 3 M quartz distilled sub-boiled HNO_3 for Sr columns. The Sr columns were set and washed in the same fashion before a bed of 5 – 7 mm of pre-cleaned Sr-spec resin (Eichrom) was pipetted onto each column. The Sr resin was washed with three column volumes of alternating MQ water and sub-boiled 3 M HNO_3, before being equilibrated with two further volumes of sub-boiled 3 M HNO_3. The samples were then loaded onto the resins, and the elements other than Sr removed in three volumes of sub-boiled 3 M HNO_3. The beakers were then cleaned with 6 M HCl, and the Sr collected in three volumes of MQ water. Six drops of 15 M SeaStar HNO_3 were added to the Sr samples before being dried. Sr samples were then subject to a repeated Sr column chemistry pass, followed by a reverse Aqua Regia digestion (3:1 HNO_3:HCl) and stored dry for either TIMS analysis (moth and plant samples) or MC ICP-MS analysis (soil samples).

2.6.2.7 Insect and plant trace element concentration analysis: ICPMS

The insect and plant trace element aliquots were analysed on an Agilent 7500CS Quadrupole Inductively Coupled Plasma Mass Spectrometer (ICPMS) at Victoria UNiversity. The samples, in 1 wt% Seastar HNO_3, were introduced into the ICP-MS via a Cetac ASX-520 Autosampler, using a 100 µl/min Microflow nebuliser spray chamber.

A calibration standard solution was made up from mono-elemental standard solutions (1000 ppm, Aristar grade, BDH Laboratory Supplies, England). The concentrations in the standard were adjusted to support the suite of elements chosen, ranging from ≈ 1 ppb for Li and the rare earth elements to 508 ppb for Ti and 5270 ppb for Ca; the latter two being more highly concentrated as these elements were measured on their isotopes with low natural abundances. The calibration standard was used to tune the instrument in order to achieve maximum instrument sensitivity whilst maintaining the CeO/Ce ratio below 1.5%. The general instrument parameters and isotopes of the elements of interest are given in Table 2.4.

Element concentrations were calibrated using the multi-element calibration standard solution, as above, and calculated using count-per-second data, the sample's mass, aliquot and dilution weights. Each set of five samples in a run were bracketed with calibration standards that were run in identically treated tubes as the samples. Background counts per second rates were

measured for 90 seconds on an analytical blank before and following each run. The mean background count was subtracted directly from the sample count rates measured during analysis.

Table 2.4 ICP-MS instrument settings, conditions and method used for trace element analysis of insect and plant samples.

ICP-MS system	Agilent 7500CS octopole
Acquisition mode	Peak hopping
RF power	1400 – 1450 W
RF matching	1.75 – 1.79 V
Carrier gas (Ar) flow rate	0.70 – 0.75 L/min.
Reaction Cell	Not activated
Nebuliser pump	0.14rps
Background acquisition	90 s
Sample/standard acquisition	180 s
Washout time	210 s
Isotopes measured and integration times	10 ms: ^{7}Li, ^{9}Be, ^{27}Al, ^{43}Ca, ^{45}Sc, ^{47}Ti, ^{49}Ti, ^{53}Cr, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{65}Cu, ^{67}Zn, ^{75}As, ^{77}Se, ^{88}Sr 20 ms: ^{85}Rb, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{139}La, ^{140}Ce, ^{182}W, ^{205}Tl 50 ms: ^{206}Pb, ^{208}Pb

To verify the accuracy of the results each run included duplicate dilute (10%) calibration standards, matrix specific in-house standards (moth tissue, PH_armig)[12] and external standards (NBS 1575 Pine needle). These repeated measurements and the average recovery of elements for NBS 1575 versus published values are given in Table 2.5.

The repeated measures of Be, Al, Sc, Se and Tl indicate these may not be reliable variables, as these have high coefficient of variations (%CVs). Be and Tl concentrations in the moth standard and samples were below the limit of reliable detection with the methods available and were not used in the following experiments. Al concentration data was informative in the inter-regional comparison, and so used, albeit with low emphasis. Sc is difficult to measure in biological tissues (Horovitz, 2000) – as reflected in the high %CV observed in the data presented. However, the average Sc values obtained were close to published NBS 1575 values (Freitas et al., 2008) and therefore Sc also used with low emphasis. The variable Se data from the dilute synthetic standard and NBS-1575 was due to Argon dimmers interference on the same mass, and so was not used in subsequent experiments. However, the provenance determination potential of Se concentration (e.g., Thomson & Robinson, 1980) and the moderate %CV in the moth repeated samples suggest future consideration of Se analytical methods is warranted. The values for Ti show probable interference in the biological

[12] PH_armig in-house standard was prepared by rearing 500 adult moths, (≈50 g) on artificial diet; the moths were cleaned (Section 2.5.1), dried, and ground in an acid washed mortar and pestle.

standards – with $^{47}Ti > {}^{49}Ti$ in the NBS 1575 analyses, and $^{47}Ti >> {}^{49}Ti$ in the PH_armig in-house standard. As the concentration of these two isotopes should be the same, it is concluded that ^{47}Ti is interfered, possibly by $^{31}P^{16}0$, a polyatomic ion that forms in the IC plasma. In contrast the ^{47}Ti and ^{49}Ti values in the dilute synthetic standard are approximately equal, which shows that the ICP-MS is reading Ti acceptably. Cr, Co, Ni, Cd, Cs, La, Ce, and W values all have repeatable values that have moderate %CV, although the reliability of these variables appear to be affected by the matrix being analysed. Therefore, these variables may be considered for provenance determination for matrices with low %CV, so long as the uncertainties of the measurements are taken into account (Thiel et al., 2004). The similarity of ^{182}W and ^{183}W counts shows low inference on these masses. The %CV for Cu and the similar counts for ^{63}Cu and ^{65}Cu indicate that the Cu data in this study will be reliable. The Zn and As data also have low %CVs for the biological standards. The element concentrations shown to have high provenance determination potential later in this study (Chapter 4), Rb, Sr and Ba, have low %CV, similar to that reported by Blum, et al. (2000) for the same elements. Pb concentration is also an informative variable, although the high %CV from the Pb values in the repeated in-house moth standard analyses necessitates a cautious use of the Pb concentration data for moth provenance determination. On-the-other hand, the similar counts for ^{206}Pb and ^{208}Pb, and relatively close agreement with the NBS-1575 average Pb recovered concentrations and the certified value demonstrates acceptable precision and accuracy. In this regard, the higher %CV values across almost all of the elements measured in the repeated in-house moth standard analyses than the NBS-1575 may be due to heterogeneity in the in-house standard.

In addition, to assess linearity of measurement a series of four dilutions, each increasing by 50%, were applied to a PH_armig standard preparation. These were analyzed as above. The values also given in Table 2.5 and a selection of indicator elements are compared to the non-diluted PH_armig averages in Figure 2.2. The average distortion on the analytical values, comparing the non-diluted moth standard averages to the most heavily diluted (1:4) was 3.5%, i.e., the dilution series results indicate that there were minimal matrix effects suffered in the ICP-MS analysis.

Table 2.5 Repeated ICP-MS measurements for in-house and external standards. All concentrations are ng/g calculated using sample and dilution weights. %CV = coefficient of variation. Cells with pink shading are considered to be errors. The references for the NBS-SRM 1575 published values are: A = Certificate of Analysis, (Reed, 1993); B = (Freitas et al., 2008); C = (Saitoh et al., 2002); D = (Asfaw & Wibetoe, 2006); E = (Taylor et al., 2007).

Dilute Synthetic Std																																
Date	Batch label	Comment	sample label	^{7}Li	^{9}Be	^{27}Al	^{43}Ca	^{45}Sc	^{47}Ti	^{49}Ti	^{53}Cr	^{59}Co	^{60}Ni	^{63}Cu	^{65}Cu	^{67}Zn	^{75}As	^{77}Se	^{85}Rb	^{88}Sr	^{111}Cd	^{133}Cs	^{137}Ba	^{139}La	^{140}Ce	^{182}W	^{183}W	^{205}Tl	^{206}Pb	^{208}Pb
Aug 28 2009	Batch 1	Contaminated																														
Mar 16 2010	Batch 13A	missing Prim Std values	Secondary Std A	0.9	10.9	101.2	5196.1	9.3	521.0		11.1	11.3	10.9	11.7			1.1	1.6	12.0	11.8	10.8	1.1	10.8	1.1	1.1	10.3		9.6		10.7
Mar 16 2010	Batch 13A	missing Prim Std values	secondary std A	0.9	11.1	109.5	5155.3	9.5	509.6		11.1	11.0	10.9	11.0			1.1	1.0	11.2	11.0	10.2	1.1	10.5	1.1	1.1	10.4		9.7		10.5
Mar 24 2010	Batch 13B		dil. synthetic std	0.9	10.9	111.7	5123.2	9.1	520.0	519.0	11.1	11.1	10.6	10.9	11.0	12.0	1.0	0.9	11.0	11.0	10.4	1.1	10.6	1.1	1.1	10.2	10.3	10.9		11.0
Mar 25 2010	Batch 13B		dil. synthetic std	0.9	10.9	108.6	5070.5	9.2	517.4	514.1	11.0	10.9	10.5	10.6	10.8	11.6	1.1	0.7	11.0	11.1	10.4	1.1	10.6	1.1	1.1	10.3	10.4	10.9		11.0
Apr 9 2010	Batch 13C	all Stds lost																														
Apr 9 2010	Batch 13C2		dil. synthetic std	0.9	10.9	258.6	5160.9	7.3	513.1	516.6	10.9	11.0	10.7	10.9	11.1	10.3	1.0	1.1	11.0	11.1	10.6	1.1	10.8	1.1	1.1	10.4	10.5	11.1		11.3
Jun 22 2010	Batch 13C3		Dil Synth Std	1.0	10.7	183.1	5267.7	10.7	521.1	522.4	11.1	11.2	10.5	10.6	10.6	9.9	1.0	0.7	11.2	11.3	10.3	1.1	10.8	1.1	1.1	10.6	10.6	11.2	11.3	11.3
June 21 2010	Batch 14		Dilute Synthetic Std	1.0	10.9	114.0	5609.6	11.2	544.0	539.0	11.3	11.6	10.9	11.5	11.3	15.4	1.0	1.6	11.5	11.7	10.5	1.1	11.0	1.1	1.1	10.9	11.0	11.6	11.6	11.6
Jun 22 2010	Batch 14		Dilute Synthetic Std2	1.0	10.8	108.1	5294.0	10.4	529.1	530.5	11.0	11.5	10.8	11.2	11.1	10.6	1.0	1.5	11.5	11.5	10.5	1.1	10.9	1.1	1.1	10.7	10.6	11.2	11.4	11.3
Aug 12 2010	Batch 15		Dilute Sythetic Std A1	0.9	10.7	107.9	5337.2	11.0	525.9	531.5	11.0	11.5	10.8	11.2	11.2	11.4	1.1	1.1	11.4	11.4	10.6	1.1	10.9	1.1	1.1	10.5	10.5	11.2	11.3	11.5
Aug 12 2010	Batch 15		Dilute Sythetic Std A2	0.9	10.6	105.7	5167.0	10.8	515.1	520.7	10.9	11.2	10.4	10.9	10.9	8.4	1.0	1.3	11.2	11.3	10.5	1.1	10.6	1.1	1.1	10.4	10.4	11.2	11.2	11.5
Feb 4 2011	Batch 16		Dil Synth Std	0.9	10.6	106.4	5261.7	10.8	531.9	534.4	11.5	11.0	10.8	10.7	11.1	11.6	1.1	1.7	11.1	11.1	10.5	1.1	10.8	1.1	1.1	10.7	10.7	10.6	11.2	10.7
Mean				1	11	108	5240	10	523	525	11	11	11	11	11	11	1	1	11	11	10	1	11	1	1	10	11	11	11	11
SD				0	0	4	146	1	10	9	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
%CV				3	2	3	3	12	2	2	2	2	2	3	2	17	3	29	3	2	2	2	1	2	2	2	2	6	1	3

NBS-SRM 1575 Pine Needle

Date	Batch label	comment	^{7}Li	^{9}Be	^{27}Al	^{43}Ca	^{45}Sc	^{47}Ti	^{49}Ti	^{53}Cr	^{59}Co	^{60}Ni	^{63}Cu	^{65}Cu	^{67}Zn	^{75}As	^{77}Se	^{85}Rb	^{88}Sr	^{111}Cd	^{133}Cs	^{137}Ba	^{139}La	^{140}Ce	^{182}W	^{183}W	^{205}Tl	^{206}Pb	^{208}Pb
Aug 28 2009	Batch 1	contaminated																										
Nov 18 2009	Batch 8	samples lost.																										
Mar 24 2010	Batch 13B		99	6		4069000	45	4427	3355	1882	88	2123	2732	2747	53856	193	47	10924	4192	167	111	6364	86	204	42	44	46		10789
Mar 24 2010	Batch 13B		98	6		4028837	38	3607	3320	1908	89	2114	2729	2743	53993	190	38	10881	4178	170	110	6303	85	197	40	40	46		10632
Mar 24 2010	Batch 13B		103	6		4153131	34	5078	3040	2041	92	2180	2806	2818	55491	196	50	11210	4264	173	114	6498	91	204	42	43	47		11007
Mar 24 2010	Batch 13B		99	6		4038709	30	3586	2704	1880	88	2101	2711	2724	54326	198	30	10995	4182	170	111	6373	87	202	41	41	45		10746
Apr 9 2010	Batch 13C	all Stds lost																										
Apr 9 2010	Batch 13C2		85	6		3858735	80	3883	3143	1565	83	1953	2547	2555	47341	177	50	10391	3930	157	103	5310	73	176	37	37	42		9615
Apr 9 2010	Batch 13C2	sucked dry. Not used	6	0		302357	-53	242	142	101	5	141	170	170	3316	11	-66	811	289	11	8	390	5	12	-5	-6	3		729
Jun 22 2010	Batch 13C3		129	5		3827526	62	5469	4509	2411	96	2137	2648	2670	52964	191	13	10402	4024	161	107	5711	89	214	47	46	41	10735	10514
Jun 23 2010	Batch 13C3		118	5		3726729	50	4654	4050	2325	93	2090	2572	2570	51010	190	10	10204	3932	158	103	5526	86	201	44	44	38	10226	10062
June 21 2010	Batch 14		126	9		3958451	79	6321	5501	2422	103	2188	2808	2722	56133	199	128	10571	4090	169	109	5828	92	209	49	50	43	10801	10300
June 21 2010	Batch 14		124	8		4048866	75	6461	5308	2485	104	2235	2842	2787	56974	203	95	10855	4169	165	111	5893	92	214	49	48	46	10888	10320
June 21 2010	Batch 14		118	9		3846700	62	5695	4228	2423	101	2176	2767	2693	47413	198	105	10539	4032	165	108	5691	90	206	49	47	44	10526	10034
June 21 2010	Batch 14	sucked dry?	100	7		3222192	-19	4362	3516	2030	84	1815	2329	2298	43307	166	5	9208	3538	146	91	4884	76	193	39	44	36	9038	8946
Aug 11 2010	Batch 15		103	7		3740516	113	5320	4204	1718	92	1988	2565	2526	54617	184	62	10045	3849	163	103	5725	72	175	32	32	42	9988	9687
Aug 12 2010	Batch 15		95	7		3602160	52	4484	3156	1462	86	1922	2476	2449	52759	177	59	9661	3698	157	99	5515	69	163	31	29	40	9639	9322
Feb 4 2011	Batch 16		110	9		3618498	43	7878	6469	2163	105	2034	2408	2508	55091	211	122	9913	3874	162	102	5677	101	230	49	49	37	9396	8754
Feb 4 2011	Batch 16		111	10		3383807	40	7515	6146	2028	99	1904	2244	2364	51627	198	100	9195	3597	153	98	5414	97	222	48	48	38	9315	8731
Mean			108	7		3808257	57	5249	4177	2050	93	2064	2612	2612	52460	191	65	10333	3970	162	105	5781	86	201	43	43	42	10055	10055
SD			13	2		265503	23	1328	1187	322	7	123	184	159	3789	12	39	628	225	7	6	449	9	18	6	6	680	680	680
%CV			12	24		7	40	25	28	16	8	6	7	6	7	6	60	6	6	5	6	8	11	9	14	14	7	7	7
Recovery relative to published values (%)						93[A]	96[B]			79[A]	82[B]	95[C]	79[A]		82[D]	91[A]		88[A]	83[A]	89[D]	37[E]	114[C]					84[A]		92[A]

Moth (PH_armig) in-house Std

Date	Batch label	comment	sample label	^{7}Li	^{9}Be	^{27}Al	^{43}Ca	^{45}Sc	^{47}Ti	^{49}Ti	^{53}Cr	^{59}Co	^{60}Ni	^{63}Cu	^{65}Cu	^{67}Zn	^{75}As	^{77}Se	^{85}Rb	^{88}Sr	^{111}Cd	^{115}In	^{133}Cs	^{137}Ba	^{139}La	^{140}Ce	^{182}W	^{183}W	^{205}Tl	^{206}Pb	^{208}Pb
Nov 9 200	Batch 9	intn Std error	moth1	96	10332	197501	1005307	76	18505	11922	1042	147	569	22138	22235	401795	262	168	5252	14214	189	11957	57	11410	71	174	438	439	12583		129
Nov 9 200	Batch 9	intn Std error	moth2	94	10961	165448	991860	56	14631	7805	810	90	391	21490	21595	387754	273	184	4422	13562	166	11540	39	10607	60	146	188	190	12239		132
Nov 9 200	Batch 9	intn Std error	moth3	93	10587	169449	936411	57	13887	7566	682	82	329	20722	20735	349021	238	173	4255	12216	167	10531	37	9765	55	138	178	181	11353		116
Nov 9 200	Batch 9	intn Std error	moth4	99	10949	179993	980124	55	15225	8579	920	92	450	20375	20466	345534	249	196	4480	12898	186	11156	41	10047	60	149	212	212	11888		122
Nov 9 200	Batch 9	intn Std error	moth5	100	11482	185273	1017447	54	14915	8207	950	94	475	21856	21773	328311	258	196	4441	13462	187	11722	39	10387	63	154	181	186	11752		127
Mar 16 2010	Batch 13A	missing Prim Std values	secondary std B_17	40	2	36878	954685	41	13397		290	87	185	22175			242	627	4033	12744	162		22	9584	15	37	116		1		34
Mar 16 2010	Batch 13A	wrong method used	secondary std B-17	38	1	37303	912532	24	12029		269	83	169	20929			222	524	3809	11832	156		21	9018	14	35	109		1		34
Mar 16 2010	Batch 13A	missing Prim Std values	secondary std B-18	39	1	32077	1011678	35	12127		409	74	215	21774			222	564	3825	12497	161		19	9079	18	46	111		2		41
Mar 16 2010	Batch 13A	missing Prim Std values	Secondary Std B-18	35	2	28250	934981	16	10910		286	67	198	19843			205	513	3623	11463	148		18	8306	16	41	102		2		35
Mar 24 2010	Batch 13B		Moth std a1_24	43	1	53371	1000441	33	13636	5513	615	106	373	22090	22478	408533	219	506	3990	12731	204		26	9368	20	46	107	107	1		109
Mar 24 2010	Batch 13B		Moth std a2_24	37	1	46121	853618	22	11946	4726	506	88	323	18912	19252	348987	198	456	3601	10826	171		23	7943	16	37	91	93	1		89
Mar 24 2010	Batch 13B		Moth std b1_25	38	2	65546	840573	20	13222	6105	451	114	318	18724	19033	357842	184	421	3750	10733	142		29	8139	15	36	90	92	1		37
Mar 24 2010	Batch 13B		Moth std b2_25	34	2	56293	733083	12	11834	5388	391	100	274	16387	16680	315833	166	367	3454	9452	125		26	7204	13	31	81	81	1		32
Jun 22 2010	Batch 13C3		Moth Std	52	0	78510	856378	43	11410	3975	417	73	231	19286	19595	352355	191	435	3525	10911	131		19	7972	17	44	89	88	-2	57	57
Jun 23 2010	Batch 13C3		Moth Std 2	50	0	43815	874945	44	11485	3815	440	73	227	19651	19906	353118	195	425	3577	11115	137		19	8130	18	43	97	95	-2	57	58
June 21 2010	Batch 14		Moth Std 1	50	2	49161	908620	75	14225	4305	456	80	237	19763	19928	375609	196	457	3598	11321	143		20	8365	19	48	97	96	1	66	62
June 21 2010	Batch 14	may have sucked air	Moth Std 2	55	2	55038	1017747	61	15682	4832	515	92	272	22686	23043	372613	222	568	4137	12923	158		23	9426	21	52	117	116	1	70	68
Aug 11 2010	Batch 15		Moth Std A1	59	2	63253	1018387	73	22687	5524	345	109	237	21018	20997	419148	230	556	4026	12431	159		25	9350	16	42	101	99	1	47	49
Aug 11 2010	Batch 15		Moth Std A2	48	2	50554	935709	26	19172	4790	312	99	216	19489	19488	349513	214	484	3671	11562	152		22	8717	14	33	90	91	1	37	38
Feb 4 2011	Batch 16		Moth Std A	60	3	85242	903675	41	19633	5555	331	97	239	18365	19027	385486	201	571	3653	10987	143		29	8054	28	68	90	91	2	68	64
Feb 4 2011	Batch 16		Moth Std B	59	3	82195	900712	35	19719	5537	325	100	243	18266	18943	381154	197	562	3666	10949	141		28	8042	28	67	89	88	2	68	63
Feb 4 2011	Batch 16	Dil series 1:2	Moth Std 1:2	63	4	87100	877880	48	19290	5672	330	101	245	19131	19913	391485	192	514	3805	11390	144		29	8138	29	69	93	91	4	70	65
Feb 4 2011	Batch 16	Dil series 1:3	Moth Std 1:3	62	3	86961	882506	43	18999	5623	348	98	248	19646	20334	398183	195	528	3813	11409	144		30	8240	29	72	89	87	2	70	63
Feb 4 2011	Batch 16	Dil series 1:4	Moth Std 1:4	63	2	90617	878643	48	19185	5304	398	99	251	19568	20253	396851	194	500	3767	11390	143		29	8193	29	70	88	85	2	80	75
Mean				46	2	53975	916110	38	14570	5005	397	90	247	19960	19864	368349	206	502	3746	11530	152		23	8544	18	44	99	95	1	59	54
SD				9	1	17348	76637	19	3691	713	97	14	54	1710	1684	28300	19	71	204	945	19		4	689	5	11	11	9	1	12	22
%CV				20	62	32	8	50	25	14	24	16	22	9	8	8	9	14	5	8	12		16	8	25	25	11	10	130	20	40

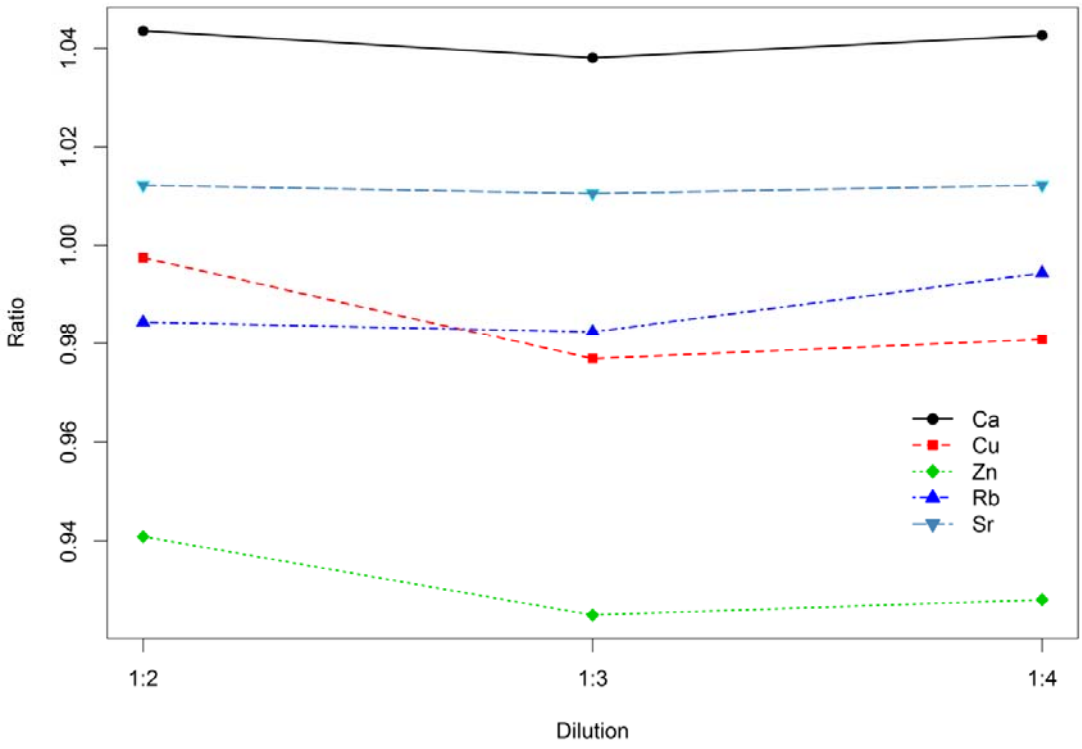


Figure 2.2 Dilution series. Ratios of selected elements' concentrations of diluted solutions of PH-armig moth standard (1:2 – 1:4) over the long term averages of the non-diluted PH-armig moth standard (1:1).

2.6.2.8 Soil trace element concentration analysis: ICP OES

The soils' trace element aliquots were analysed on a Varian 720 Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES) at Lincoln University. The general instrument parameters are given in Table 2.6. The samples, in 6 M HNO_3 were introduced into the ICP OES, via a SPS-3 auto-sampler. The emitted light was spectrally resolved by diffractive optics, where the concentration of a specific element is related to the intensity of lines in its optical spectrum, and the intensity of light measured. Wavelength coverage was from 167 to 785 nm, with a resolution of 7 picometre, and all wavelengths were captured in one simultaneous reading. Element concentrations in solution were calibrated using serially diluted Merk ICP calibration standards and internal standard solutions, and the corresponding sample (dry weight) concentration calculated using the sample mass and aliquot dilution volume. Standards and a calibration blank were run for every 15 samples. The external standards were ISE-921 soil standard, and BHVO-2. Precision and accuracy were assessed by comparison with the internal and external standards.

Table 2.6 ICP-OES instrument settings used for soil trace element analysis

ICP OES system	Varian 720 - axially aligned torch instrument
Power	1.20kW
Plasma gas (Ar) flow rate	15.0 L/min
Aux	1.5 L/min
Nebuliser pump	not used
Elements measured	Li, Be, Al, Ca, Sc, Cr, Co, Ni, Cu, Zn, As, Rb, Sr, Cd, Ba, Ce, Pb,

2.6.2.9 Moth $^{87}Sr/^{86}Sr$ ratio analysis: TIMS

For moth strontium isotope ratio analysis ($^{87}Sr/^{86}Sr$), both MC-ICP-MS and ICP-MS analyses were trialed. Although MC-ICP-MS is the most sensitive mass spectrometric technology available in New Zealand, the single moth samples did not have enough mass to overcome the carrier gas Rb interference for the small amount of Sr present (F. C. Ramos et al., 2004). Therefore ICP-MS $^{87}Sr/^{86}Sr$ ratio analysis using the method described by Almeida & Vasconcelos (2001) was subsequently trialed, using the same Agilent 7500CS as used in the previous section. However, poor accuracy and precision were achieved using proxy ≈ 300 ng Sr (to match the average moth sample Sr abundance) BHVO-2 samples (average value = 0.706848 ± 0.001972 2SD, n = 4, versus the accepted value 0.703469 ± 0.000017), as well as low internal precision (average 2SE = 0.004397). Therefore it was necessary to utilize the greater sensitivity of Thermal Ionization Mass Spectrometry (TIMS) to get $^{87}Sr/^{86}Sr$ data.

Sr isotope ratios for the moth samples were measured on a Thermo-Finnigan Triton TIMS at the Laboratoire Magmas et Volcans, Clermont-Ferrand, France. The Sr samples were taken up in 1 M H_3PO_4 mixed with tantalum salt as an activator. Solutions were then loaded onto single Re filaments that were previously outgassed at 4.0 ampere (A) for 30 min. The sample was then dried down slowly at 1 A. Once dry, samples were loaded onto the mass spectrometer magazine. Filaments were heated up to a temperature of 1400 to1500ºC, until a high enough ion beam was reached. Measurements were made in multidynamic mode with 2 cycles, ion beams being shifted one collector down during the 2nd cycle and samples were run until signal started to drop off in order to maximise internal error. Internal mass bias was corrected for internally using a $^{86}Sr/^{88}Sr$ ratio of 0.1194 and a linear law.

Performance was measured against repeated in-house moth standard and BHVO-2 preparations of ≈ 300 ng Sr that were both processed at the same time as the samples, under the same conditions (Table 2.7). The repeated in-house moth standard $^{87}Sr/^{86}Sr$ values, agreed to the 4th decimal place and the average internal precision for all of the moth analyses is 0.000143 (2SE). The repeated BHVO-2 average value was 0.70350775 ± 0.000035 2SD, which is close to the accepted reference value, and had an average internal precision of

0.000015 (2SE). These levels of $^{87}Sr/^{86}Sr$ measurement precision and accuracy is similar to, or exceeds that of other provenance determination studies using Sr isotopes (Font et al., 2007; Sellick et al., 2009) and allowed for the confident use of the $^{87}Sr/^{86}Sr$ data generated in insect provenance determination.

Table 2.7 $^{87}Sr/^{86}Sr$ values from standards assessed during this study (TIMS, Clermont-Ferrand Magmas et Volcans laboratory)

Standard	Batch/ sample label	$^{87}Sr/^{86}Sr$	2SD
BHVO2	13_26	0.703527	0.000024
BHVO2	13_26B	0.703517	0.000015
BHVO2	13_59	0.703498	0.000007
BHVO2	13_59B	0.703489	0.000014
Moth Std, PH_armig	13_24	0.721017	0.000066
Moth Std, PH_armig	13_25	0.721936	0.000027

2.6.2.10 Soil $^{87}Sr/^{86}Sr$ ratio analysis: MC ICPMS

The $^{87}Sr/^{86}Sr$ ratio analyses for the soil samples were carried out on the Nu Plasma-HR MC-ICP-MS (Nu Instruments Ltd., UK) at the Centre for Trace Element Analysis, Otago University, Dunedin. The samples, in 0.5 wt% Seastar HNO_3, were introduced via self-aspiration with a micro-concentric glass nebuliser (Glass Expansion) and a Cetac ASX100 autosampler. The data acquisition parameters optimized for Sr isotope ratio measurements are summarized in Table 2.8. Data collection was made using three blocks with 25 measurements each. The strontium isotopic standard (NIST SRM 987 Sr) was analysed repeatedly throughout the measurement session to assess the accuracy of the analyses. Nineteen measurements of this standard yielded an average $^{87}Sr/^{86}Sr$ ratio of 0.710274 ± 0.000023 (1σ) consistent with that found in Shaw, et al. (2011) and in agreement with the accepted value of 0.71034 ± 0.00013 (1σ) from L. J. Moore, et al. (1982).

Table 2.8 Typical instrument operating conditions of the Otago University Nu MC-ICP-MS used for soil Sr isotope analysis

(Otago Uni.) Multi-collector ICP-MS System	Nu Instruments MC-ICP-MS
High voltage	6000 V
RF power	1350 W
Coolant Ar flow	13 L/min
Auxiliary Ar flow	1.2 L/min
Variable slit width	LR
Sample uptake rate	130 µL/min
Nebuliser pressure	34.6 PSI
Sensitivity for Sr	~23 V / ppm

2.6.2.11 $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$ ratio analysis: MC ICPMS

All moth corn and soil sample Pb isotope ratios analyses were carried out on the Nu Instruments MC ICPMS at the Geochemistry laboratory, Victoria University, Wellington. The samples, in 0.5 wt% Seastar HNO_3, were introduced via a desolvating nebulizer. The typical data acquisition parameters optimized for Pb isotope ratio measurements are summarized in Table 2.9. Data collection was made using two blocks with 25 measurements each. NBS 981 calibration standards bracketed each three samples.

Three separate standards that matched the various experimental sample type matrices were used for the $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ measurements. These were repeated preparations of JB-2 ≈ 4 ng Pb (to match the average moth sample Pb abundance), the in-house moth standard and NCS ZC73010 (maize). The values are given in Table 2.10. The repeated in-house moth standard values agreed to the 2nd decimal place, and had external precision ± 0.0078 2SD for $^{208}Pb/^{206}Pb$ and ± 0.0062 2SD for $^{207}Pb/^{206}Pb$. This precision is less than other recent ecological studies of Pb isotopes (Berglund et al., 2009; Notten et al., 2008). However, this is suspected to be due to heterogeneity in the in-house standard, as the average internal precision for the actual moth sample Pb isotopes analyses were ± 0.00098 2SE for $^{208}Pb/^{206}Pb$ and ± 0.00049 for $^{207}Pb/^{206}Pb$, and the repeated NCS ZC73010 values agreed to the 3rd decimal place with external precision of 2.1179 ± 0.0009 2SD for $^{208}Pb/^{206}Pb$ and 0.8650 ± 0.0006 2SD for $^{207}Pb/^{206}Pb$. The average internal precision for the corn samples analyses were ± 0.00024 2SE for $^{208}Pb/^{206}Pb$ and ± 0.00010 for $^{207}Pb/^{206}Pb$. The repeated JB-2 average was 2.08718 ± 0.00012 2SD for $^{208}Pb/^{206}Pb$ and 0.848593 ± 0.000056 for $^{207}Pb/^{206}Pb$ (n = 7), within 0.03% the accepted values of 2.0868 ± 0.0001 and 0.8484 ± 0.00004, respectively (Baker et al., 2004).

Total procedural Pb blanks in this study yielded <15 pg Pb, which represents < 0.55% and < 0.06% of the average moth and corn samples Pb abundance respectively, and were considered not-significant.

Table 2.9 Typical instrument operating conditions of the Victoria University Nu MC-ICP-MS and the DSN-100 parameters used for Pb isotope analysis

(Victoria Uni.) Multi-collector ICP-MS System	Nu Instruments MC-ICP-MS
High voltage	4000 V
RF power	~1300 W
Coolant Ar flow	~13 L/min
Auxiliary Ar flow	0.9 L/min
Variable slit width	0.03 mm
DSN-100 parameters	
Membrane temperature	110 ºC
Membrane gas flow	3.0-3.5 L/min
Hot gas flow	23 PSI
Spray chamber temperature	110ºC
Sample uptake rate	100 µL/min
Nebuliser pressure	31 PSI
Sensitivity for Pb	~300 V / ppm

Table 2.10 $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ values from standards assessed during this study (MC-ICP-MS, Victoria University Geochemistry laboratory)

Standard/Batch/label	$^{208}Pb/^{206}Pb$	2SD	$^{207}Pb/^{206}Pb$	2SD
JB2_10_1	2.086849	0.000135	0.848478	0.000055
JB2_10_2	2.088275	0.000144	0.849197	0.000054
JB2_10_2B	2.088060	0.000113	0.849080	0.000045
JB2_10_3	2.087338	0.000145	0.848609	0.000064
JB2_10_3B	2.087259	0.000178	0.848533	0.000070
JB2_10_3C	2.087164	0.000158	0.848582	0.000069
JB2_13_60	2.087345	0.000173	0.848760	0.000081
JB2_13_27	2.086632	0.000083	0.848359	0.000036
JB2_13_Corn JB2	2.087009	0.000113	0.848493	0.000054
Moth Std, PH_armig_9_M1	2.020309	0.000280	0.807551	0.000110
Moth Std, PH_armig_9_M2	2.029225	0.000248	0.815182	0.000099
Moth Std, PH_armig_9_M3	2.019773	0.000283	0.808224	0.000100
Moth Std, PH_armig_9_M4	2.020736	0.000084	0.808832	0.000040
Moth Std, PH_armig_9_M5	2.023582	0.000114	0.811371	0.000050
NCS_9_1	2.117934	0.000168	0.865007	0.000074
NCS_9_2	2.117672	0.000152	0.865270	0.000091
NCS_9_3	2.117904	0.000156	0.865283	0.000079
NCS_9_4	2.117267	0.000183	0.864573	0.000075
NCS_9_5	2.118562	0.000139	0.864845	0.000059

2.7 Chapter conclusion

A model system and the methods required to meet the research needs of this PhD project have been described. This has required the development of an analytical method so that δ^2H, trace element concentration, and Sr and Pb isotope ratios can be read from single insects. The suite of elements evaluated in this study were based on the elements that were theorized would provide signatures for New Zealand different from our major trading partners – from both geological features and anthropogenic activities.

The methods developed now enable proof of concept studies to critically assess a wide range of biogeochemical markers for forensic insect geo-location using *H. armigera* and *Zea mays* model system.

Chapter 3
Uncertainty in biochemical geo-location marker expression in insects: The influence of input signal variation

3.1 Introduction

Fundamental uncertainties in regard to biochemical geo-location marker expression in insects currently limit our ability to use these markers for point-of-origin determination (Section 1.3). The experiments described in this chapter address the most significant of these uncertainties.

Several principles and assumptions underlie the ability to use biogeochemical signals as effective indicators of geographic origin. Hobson (2008) and Langin, et al. (2007) summarized the basic principles as:

1. The biogeochemical signals of consumers are assumed to reflect those of the food web that supports them. Individual members of a population are expected to display minimal variation in fractionation or elemental discrimination (Hobson & Wassenaar, 1997; Norris et al., 2004). In addition, the effect of inter-annual and seasonal variation in input signal is assumed to be negligible within any given location (e.g., Rubenstein et al., 2002).
2. Different tissues retain spatial information over different periods of time. Metabolically active tissues will reflect the biogeochemical inputs over recent periods. This can be weeks to months for hair (Tieszen et al., 1983) and bone collagen (Hobson & Clark, 1992b), and days for blood (Podlesak et al., 2005). Whereas for metabolically inactive tissues it is assumed that the spatial information will be locked in indefinitely and will only reflect the location the consumer occupied during the growth of that tissue.
3. The processes involved in the transfer and possible alteration of the environmental signal to the consumer are identified and taken into account. This includes the effect of fluctuating environmental and physiological variables on stable isotope fractionation and the distortion of other biogeochemical markers.

In reality these principles are usually only partially satisfied or uncertain in most studies (Hobson, 2008). Aspects that contribute to these uncertainties need to be identified, and ideally quantified in each case (e.g., Wunder et al., 2005). Essentially, the application of biogeochemical signals as meaningful geographical markers needs to be supported by a sound

understanding of the manner in which such markers are expressed in the organism of interest (Barnes et al., 2008; Hobson, 2008; Rubenstein & Hobson, 2004).

Despite the vast potential of biogeochemical markers, the use of such signals in the study of insect movement has been limited to date to a handful of papers (Section 1.3.4), and the above principles have been minimally researched in entomological science. Thus, there is inadequate premise on which to rely with respect to input signals being faithfully reproduced in insects.

Accordingly, a principle objective of this research has been to enable an understanding of the most significant sources of uncertainty in location-to-insect marker imprinting in phytophagous insects, and ideally quantify to what extent the 'input noise' is expressed in the insects' signal. This will enable a more confident interpretation of biogeochemical marker data in insect provenance studies.

The highest priority sources of uncertainty are considered experimentally in this chapter. Specifically:

1. For δ^2H alone (Sections 3.2 and 3.3):

- It is necessary to appreciate the fractionation relationships within the system of interest, including the possibility of irregular water to host-plant fractionation. However, plant to insect fractionation has thus far only been quantified for two insect species.
- The effect of fluctuating precipitation δ^2H input on insect δ^2H is not quantified.
- It is unknown if insect access and express the highly δ^2H variable plant water pool, or the plant solid δ^2H.

2. For multivariate biogeochemical markers:

- The effect of polyphagy on insects biogeochemical signal is unknown (Section 3.4), and
- The effect that chemical turnover in adult moth tissue will have on endogenous geo-location markers is unknown (Section 3.5).

3.2 The influence of precipitation ^2H variation on the δ^2H expression in insects.

Precipitation δ^2H in New Zealand can be highly variable over short and medium time frames. For example, the monthly average precipitation δ^2H over a two month period in late summer 2007 varied by approximately 50‰ in Auckland, and in Christchurch average precipitation δ^2H in Feb 2007 was -18.2‰ compared to -82.4‰ in May of the same year (both months had similar rainfall – about 20 mm) (Frew et al., 2011).

The isotopic composition of leaf water is also highly variable. This variation includes changes in leaf water δ^2H of ≈ 10‰ in response to fluctuating humidity, and irregular influences, including temperature (Yakir, DeNiro, & Gat, 1990) and wind (Bariac et al., 1989). Further isotopic variation in leaf water of up to 40‰, within a given leaf, occurs due to matching variation in the source water (Bariac et al., 1994; Luo & Sternberg, 1992; Yakir et al., 1989). Similar daily isotopic variation has been shown to occur in fruiting bodies (Cernusak et al., 2002) – the site of feeding for the model insect in this study.

It is unknown how these types of short term isotopic variation in the input signal are expressed in herbivorous insects. Generally, the immature feeding life stages of insects cover short periods of time, often as little as a few weeks. Uncertainty arises because it is unknown if the 'metabolically fixed' adult tissues will express the precipitation δ^2H high variation over the short-term i.e., the immature feeding period, as suggested by Brattström, et al. (2008), or the longer term average signal. It is also unknown if larval insects will express highly variable leaf water or the more consistent plant solid δ^2H. The pathways of δ^2H signal expression in insects is therefore unknown, and potentially highly variable. This non-delimited uncertainty impedes the confident application of δ^2H as a geochemical provenance determination in entomology.

Large within-population variation in δ^2H is well documented in the avian ecology literature. For example, mountain plover (*Charadrius montanus*) chick feathers grown in the same year were found to have within site δ^2H variation of ≈ 10 to 60‰ by Wunder, et al. (2005), while Rocque, et al. (2006) reported within-population δ^2H ranges for up to 110‰ in adult American golden plovers (*Pluvialis dominica*). In this respect, Farmer, et al. (2008) considered how the confidence of provenance assignment (primarily in regard to birds) is affected by input δ^2H precipitation inter-annual variation – and concluded provenance assignment is not possible for samples originating less than 7° latitude apart. Such broad variation brings the precision of intrinsic δ^2H as a geographic marker into question (McKechnie et al., 2004). However, the works listed here, and others, do allow useful inferences to be made with respect to previous provenance of individuals based on their δ^2H signatures. Although, the variation need not be absolutely quantified, the potential error(s) need to be able to be approximated (Hobson, 2008) for the species and tissue of interest "before geospatial interpretations of origin are attempted" (Wassenaar & Hobson, 2006). To this end Langin, et al. (2007) has measured δ^2H variation within individual birds and within populations in American redstarts (*Setophaga ruticilla*) of known origin. Similarly, Wassenaar & Hobson (2006) catalogued δ^2H ranges within individual feathers, birds and

populations. Likelihood-based provenance assignment models can be constructed that incorporate the understanding of the sources of variation or error gained from these empirical studies (Royle & Rubenstein, 2004; Wunder & Norris, 2008b). These models have been used to correctly assign the origin of species to discrete regions within broad ranges of potential destinations in North America (Wunder & Norris, 2008b) and Europe (Hobson et al., 2009). However, these avian models do not address the uncertainties regarding the effect of environmentally driven H isotope variation on the signals in insects. The first of these uncertainties to be tested in this project was the influence of precipitation δ^2H variation on the δ^2H expression in adult moths. This was examined through a source water δ^2H switching experiment, and had the following objectives:

1. To determine if the δ^2H values of host plants and phytophagous insect will reflect either the average rainfall δ^2H value or the high short-term variation in rainfall δ^2H.
2. To determine whether the intrinsic δ^2H signal of the adult insect will be derived from the δ^2H of the plant leaf water (and hence highly variable), or the longer term δ^2H averages expressed in plant solids.

3.2.1 Methods

Overview

This trial was carried out using sweet corn plants (*Zea mays*), raised on waters of known isotopic composition, in a glasshouse at Lincoln University. *Helicoverpa armigera* larvae were reared individually on cobs harvested from these plants. There were five source water δ^2H regime treatments, comprising two controls of constant waters and three treatments involving water δ^2H switches to mimic precipitation δ^2H variation. All treatments were run simultaneously.

Plant watering regimes

The plants for all the treatments were sown on 23 Feb 2009 and reared in Planter Bags (6.75 litres of standard potting mix) irrigated with Lincoln University bore water (δ^2H -49.7‰) until development of the reproductive structures (as swelling on the 6^{th} node of the stems) was first seen in the 2^{nd} week of May. On 13 May (Time 0) the separate watering regimes (including the control treatments) were started, with 15 plants for each treatment arranged in a 15 X 5 replicated Latin Square layout, to avoid bias from location in the glass house. The water regimes were supplied via a network of 8 ml/min dripper lines. These lines were fed from tanks of water of appropriate isotopic composition, made up from doses of 99.9% ^2H_2O (Cambridge Isotope Laboratories Inc) calculated via the Delta Equation (Equation 1.1) (Frew,

2008), added to the tank volumes to achieve the desired water δ^2H values. The measured water δ^2H values were: -52.0; -39.7; -10.0; 0.0; 13.6; and 485.0‰. The rationale for these values is as follows.

- The control treatments, Treatments (T) 1 and 2, were constant -39.7‰ and +485‰ water respectively. The -39.7‰ water approximated the average precipitation δ2H value of Auckland; the extreme 485‰ enriched water was used to ensure treatment effects were observable, in case the within treatment variation in the natural range was too high to achieve significant results.
- Treatments 3, 4 and 5 were designed to replicate the fluctuating precipitation δ^2H patterns of Auckland and Brisbane, which are both centres of biosecurity importance. These treatments were used to evaluate both the effect of variation in precipitation δ^2H and if moths from these two locations, reared under controlled and monitored condition, can be distinguished. Further, to examine whether the intrinsic ^2H signal of the adult insect is derived from the ^2H of the plant leaf water, or the longer term δ^2H averages expressed in plant solids, the final value for all the switching treatments (T3 – 5) was +485‰.
- Treatments 3 and 5 mimicked the δ^2H pattern observed in Auckland rainfall over Feb and March 2007, which ranged from -4 to -52‰ (Frew et al., 2011). T3 was varied to follow the weekly precipitation δ^2H pattern in Auckland; to contrast T5 which was an approximation of the average precipitation δ^2H over the same period. At Time 0 (13 May 2009) T3 was stepped from the Lincoln water to -39.7‰ for four weeks, then to 0‰ for three weeks, -52‰ for two weeks, and then +485 for two weeks. T5 plants were given -39.7‰ for 7 weeks from Time 0, then switched to +485‰ for the final four weeks of the experiment. Auckland in summer was chosen as a location and season template, as this region location of the majority of new incursions in New Zealand (Biosecurity New Zealand PPIN data 2007) and includes the site of the PAM response (Section 1.1.2). Feb and March were used as the model period as this is the time of maximum plant and insect growth in Auckland.
- Treatment 4 mimicked δ^2H rainfall variation over the same period in Brisbane, which was the most likely point of origin of PAM. From Time 0, Treatment 4 was given -10‰ for seven weeks then stepped to +13.6‰ for two weeks, and then 485‰ for the last two weeks of the experiment.

All treatments were under the same environmental conditions of natural day length, glass house humidity and a continuous 22°C.

To study solid turn-over times in the plant compared to the insects' δ^2H, a sample of kernels was taken every two days following the switch to $\delta\ ^2H$ +485‰ in Treatment 5. This collection continued until the trial end. Corn kernel samples were also collected from the other treatments at two separate times during the period the larvae were feeding on them. The kernel samples were stored frozen (-20°C) for subsequent analysis (Sections 2.4 – 2.6).

Insect feeding

H. armigera eggs were sourced from the Plant & Food Research (New Zealand Crown Research Institute) disease free colony at Mount Albert Research Centre. They were reared on a lima bean (*Phaseolus lunatus*) based artificial diet (McManus & Burgess, 1995) with Auckland tap water of -32‰ until 2nd instar (3 – 5 days)[13]. At Week 8.7 (10 July), 25 to 33 2nd instar larvae were placed individually onto sterilised pieces of recently harvested corn cobs from the treatment plants. Single cobs were harvested every 2 – 3 days, and the cob chunks fed to the larvae were replaced daily. The 'ex-plant' feeding regime was used as a proxy for 'on plant' feeding and exposure to real time plant δ^2H variation, as prior attempts to rear *H. armigera* on the plants in the glasshouse had been unsuccessful. When the larvae pupated, the pupae were placed on vermiculite in solo rearing cups and emerged under constant 25°C 16:8 light dark. Most larvae had pupated by July 25, and all by July 27 and nine to ten moths per treatment successfully completed development. Emerged moths were killed and stored by freezing at -20°C, until sample preparation and ^2H analysis (Sections 2.4 – 2.6).

3.2.2 Results and discussion

Fractionation

The corn kernel and source water δ^2H data of the non-fluctuating controls (T1 and 2) were used to establish the water-to-plant fractionation relationship (Figure 3.1) A linear relationship was assumed, as has been previously demonstrated (Epstein et al., 1977; Hobson, Wassenaar, et al., 1999). The water-to-corn fractionation observed in T1 was a relative enrichment[14] of about 20‰, at the natural ^2H abundance δ^2H_{WATER} (δ^2H_W) of-39.2‰. However, in the δ^2H_W = +485‰ treatment (T2) the relationship trended to a relative depletion of about 225‰. These results are opposite to the depletion of 10 to 20‰ found in maize stem and root tissue by Ziegler, et al. (1976) at natural water ^2H abundances, which indicates that

[13] It was assumed that any signal from the 1st and 2nd instar artificial diet is completely replaced by the subsequent larval moults and feeding on the target plant material. This assumption was applied for this and the other manipulated feeding artificially cultured experiments in this chapter.
[14] The terms 'enrichment' and 'heavier' are used in this thesis as meaning samples with a higher % of the heavy isotope. Less of the heavy isotope = 'depleted' = 'lighter' (Fry 2006).

there is a between plant part fractionation difference (G. J. Martin et al., 1992). The results herein also contrast with the water-to-plant depletion of 43‰ at δ^2H_W-33‰ and -80‰ shift at δ^2H_W +70‰ reported by Hobson & Wassenaar, et al. (1999) for milkweeds (Asclepidae).

Table 3.1 Treatment and results summary of a series of source water δ^2H switches to corn plants upon which *H. armigera* larvae were reared. Average values ± 1 standard deviation (1SD). Values within a row that are followed by a different letter are significantly different (Fishers Restricted LSD = 5%). The average marked with * includes a value believed to be an outlier; the value in parentheses is with the assumed outlier removed.

Treatment number	1	2	3	4	5
Treatment description	-39.7‰ control	+485‰ control	'Auckland fluctuating'	'Brisbane fluctuating'	'Auckland average, early enrichment'
Plant watering regime	-39.7‰ 11 wks	+485‰ 11 wks	-39.7‰ 4 wks; 0‰ 3 wks; -52‰ 2 wks; +485‰ 2 wks	-10‰ 7 wks; +13.6‰ 2 wks; +485‰ 2 wks	-39.7‰ 7 wks; + 485‰ 4 wks
Corn kernel average δ^2H‰ (n = 2)	-19.4±0.2 [a]	+258.9±4.0 [b]	-42.0±18.7* [c] (-28.8[a])	-2.1±6.8 [a, d]	+2.4±10.4 [d]
Moth wing average δ^2H‰	-41.7±4.3 [a] (n = 9)	+122.9±6.2 [b] (n = 10)	-55.1±9.2 [c] (n = 10)	-32.5±8.0 [d] (n = 9)	-30.7±10.6 [d] (n = 10)

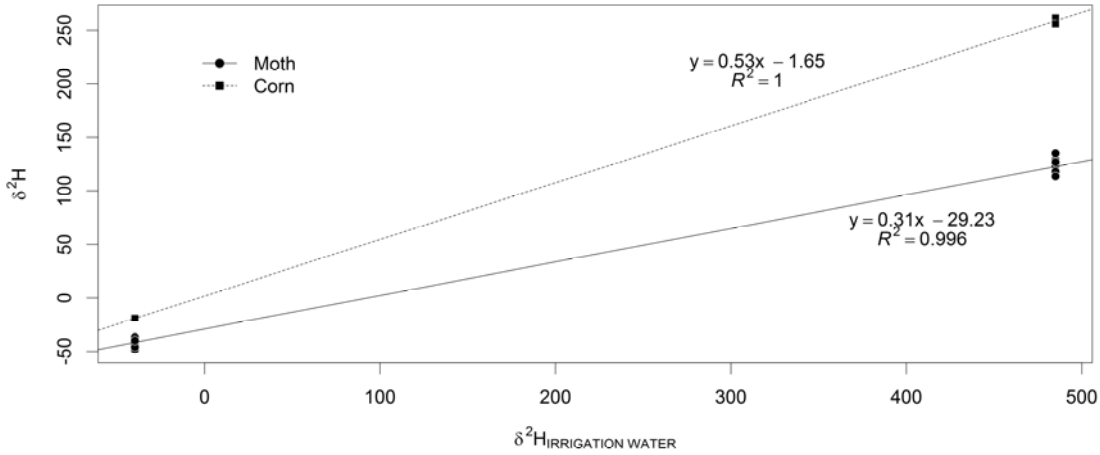


Figure 3.1 The fractionation relationships between source water δ^2H and δ^2H expression in the model host-plant (corn) and insect (*H. armigera,* 'moth'). The values are contrasting source water δ^2H control treatments, showing δ^2H of the kernel solids and wings of adult moths reared on the kernels.

The averaged plant-to-moth fractionation was a depletion of about 20‰, at the -39.7‰ δ^2H_W treatment (T1), and a depletion of 136‰ at δ^2H_W = +485‰ (T2) (Figure 3.1). Therefore, at the natural abundance range of Auckland precipitation δ^2H (-40 to -30‰) the $\delta^2H_{MOTH\ WING}$

(δ^2H_M) was approximately equal to that of δ^2H_W; while at more positive natural abundance precipitation δ^2H values, it is expected that δ^2H_M will be relatively depleted. The function given on Figure 3.1 can be used to predicted expected moth wing values at different δ^2H_W ($y = 0.31x - 29.23$; where $x = \delta^2H_W$ and $y = \delta^2H_M$). It was assumed the same water-to-corn, corn-to-moth fractionation relationship occurred in the manipulated water experimental treatments (T3-5).

This plant-to-moth fractionation relationship was also different to the few other entomological ^2H fractionation relationships that have been described elsewhere. Hobson & Wassenaar, et al. (1999) found a plant to monarch butterfly δ^2H fractionation of -0.7 – +4 ‰ over a 100‰ δ^2H_{PLANT} (δ^2H_P) range, i.e., a more-or-less direct plant-to-target relationship. Similarly, the unpublished Husheer & Frew (2006a) report showed a low δ^2H_P fractionation shift for *Epiphyas postvittana* moths (Tortricidae) reared on broad beans (*Vicia faba*) at natural ^2H abundances, but their model system trended to a 25.5‰ enrichment at δ^2H_P 15‰.

The mechanism for the fractionation differences between the different insect/ host-plant systems is unknown, and warrants futher investigation. Potential sources of the dissimilarity include physiological/ digestive differences associated with the leaf feeding mode of monarchs and *E. postvittana*, as opposed to the fruit feeding of *H. armigera*. Alternatively, water stress differences due to both the different water content of the diets and the exterior larval habit of the former two insects versus the interior niche used by *H. armigera*, with widely differing osmo-regulation requirements, are likely to affect fractionation. Regardless of the cause, the different fractionation relationships demonstrate that all insect systems probably have unique source-to-host, host-to-insect δ^2H relationships. As a consequence, to use δ^2H as a marker, the ^2H fractionation relationship will be need to be quantified in each insect system of interest, rather than relying on a single generic model. Future research may reveal predictable fractionation patterns within feeding guilds.

The effect of precipitation δ^2H variation

An ANOVA overall F-test (GenStat 12.2) confirmed that the treatment effects on δ^2H_M were highly significant ($F_{4, 43} = 832.67$; $p < 0.001$). Pair-wise comparisons of the δ^2H_M means revealed that, although they had overlapping ranges, Treatments 3 and 4 δ^2H_M were significantly different (Fishers Restricted LSD 5%). This supports the potential to distinguish moths from Brisbane from those from Auckland.

Pair-wise comparisons also showed that T1 moths (δ^2H_M -41.7 ± 4.3‰) were significantly heavier than T3 (-55.1 ± 9.2‰). This indicates that the δ^2H values of host-plants and insects

will express the value of δ^2H_W during a discrete period, i.e., express short-term variation in rainfall δ^2H, and not the average over the whole development time of either the host-plant or the insect. Specifically, the average input δ^2H_W over the entire development time of the plants was -44.5‰ for T1 and 5.8‰ for T3, and the input δ^2H_W value over the feeding period of the larvae was -39.7 and 485‰ for T1 and T3 respectively (Figure 3.2). Whereas, T3 δ^2H_M (-55.1‰) reflects δ^2H_W uptake over the two week period before the larvae went onto the plant (Weeks 7 – 9) when δ^2H_W = -52.0‰. The T3 average δ^2H_P concurs with that same period of input δ^2H_W, with the water-to-plant fractionation described above taken into account. (Note: The light T3 δ^2H_P value is considered to be an outlier, as it is inconsistent with the pattern of all the other treatments. Although this cannot be empirically tested in this case, this value was discounted for the preceding interpretation). Similarly, discrete isotopic rain events have also been found to be expressed in grassland plant species (Schwinning et al., 2002).

The water-to-moth relationship function determined from the control treatments (given in Figure 3.1, $y = 0.3139x - 29.227$) was then re-arranged to solve for x [$x = (y + 29.227)/0.3139$] and used to generate predicted source water δ^2H values – and hence estimate the period that the source water is being imprinted – using the adult moth wing δ^2H values. The predicted source water δ^2H interpolated from T4 δ^2H_M was -10.5‰, which is very close to the value of the source water over Weeks 0 – 7, i.e., until two weeks before the larvae went onto the plant (Figure 3.2). The relationship in the T4 δ^2H_P was similarly interpreted. The average value observed for T4 δ^2H_P was -2.1‰, which gave an expected input δ^2H_W of -7.1‰ (generated using the water-to-plant fractionation equation ($y = 0.5304x + 1.6532$)), which is very similar to the actual δ^2H_W over the Weeks 0 – 7 period (-10‰). This suggested period of δ^2H_P (and hence δ^2H_M) sensitivity to precipitation δ^2H variation over Weeks 0 – 7 covers an extended phase of corn plant development, and is an earlier period than interpreted from the T3 moths and plants δ^2H.

The relationship was subtly different again in the moth and corn δ^2H values from Treatment 5, in which the high water ^2H enrichment was two weeks earlier than Treatments 3 and 4. The average T5 δ^2H_M value was 9‰ more positive than the -39.7‰ source water over the Weeks 0 – 7 period and the predicted δ^2H_W value generated from the δ^2H_M was -4.8‰. Both of these more positive values indicate δ^2H_M partially expresses the highly enriched δ^2H_W input (485‰) applied two weeks before the larvae commenced feeding and/or during larval development. Similarly, the δ^2H_P values clearly show a gradual enrichment over the larval feeding period (Figure 3.3). However, the plant shift was larger at the end of the time series

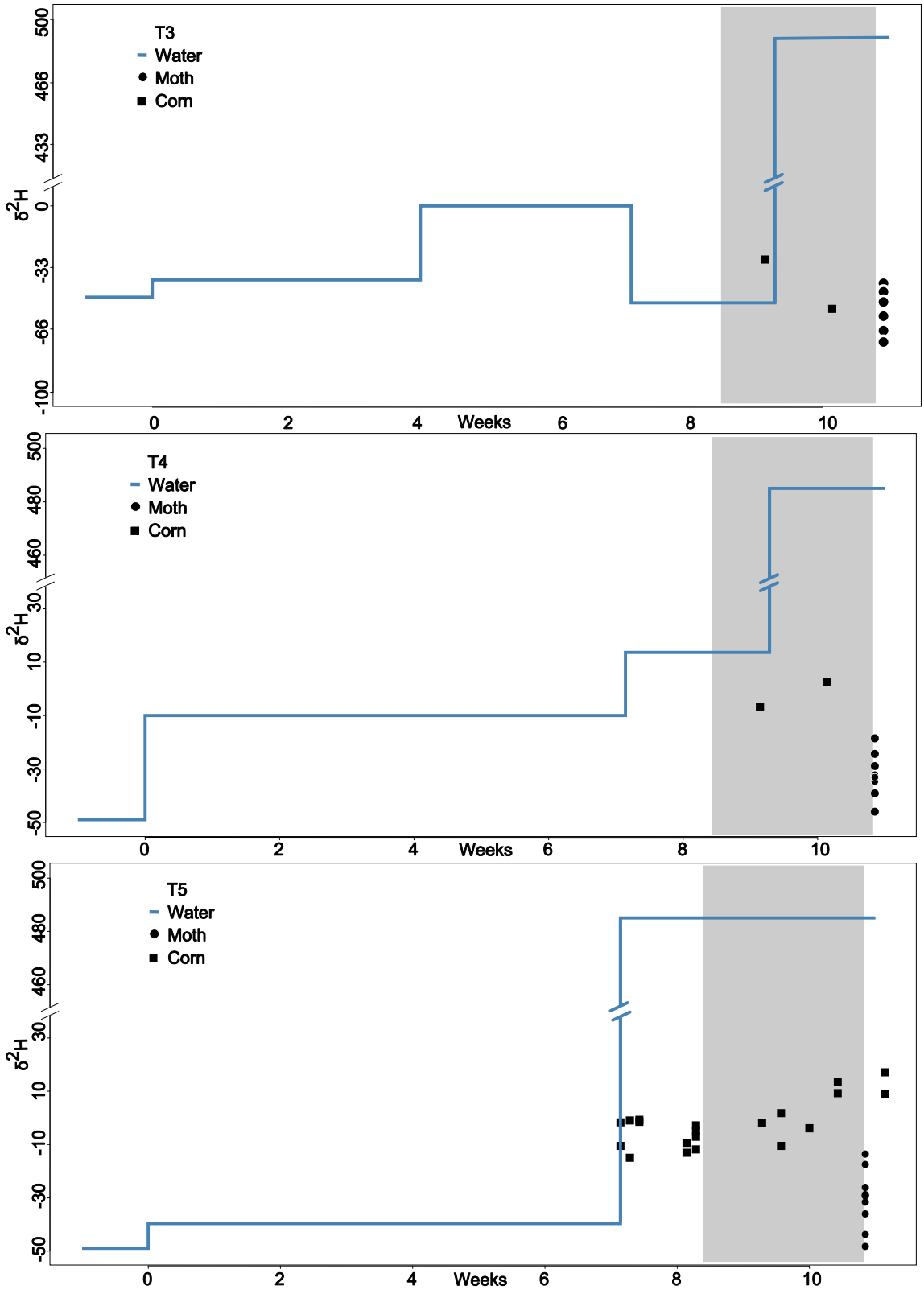


Figure 3.2 The influence of varying precipitation δ^2H on the δ^2H expression in insects and their host plant. Corn kernel solids δ^2H and adult *H. armigera* wing δ^2H reared on the kernels from a series of manipulated source water δ^2H treatments. Larvae were fed over the period marked in grey.

than that observed in δ^2H_M values, with the average for the latter being -30.7‰. This suggests that the H ultimately incorporated into the adult moth wing tissue was assimilated over the first half of larval development, when corn δ^2H values were around -10‰. This value corresponds with the δ^2H_P in over weeks 7 – 10; and the overlapping period of larval feeding was the Weeks 8.7 – 10 (Figure 3.2, T5).

This indicates that the plant development stage immediately before the larvae commenced feeding and/or during larval development was still partially subject to the effects of precipitation variation. However, the shift in T5 δ^2H_M values was relatively modest compared to both the magnitude of the enriched water spike and the δ^2H_M of T2. This gradual nature of the enrichment in T5 $\delta 2H_P$ shows that 1) the transition to a new input water is slowly expressed at the later stages of corn fruiting body development, and 2) earlier plant developmental stages will be more significantly affected by precipitation variation.

These results show that the corn kernels reflect the δ^2H_W at a discrete period during the development time of the plant. The data also suggests that the insects' δ^2H reflects the same period. This supports the theory that the δ^2H values of host-plants and phytophagous insect will express the high short term variation in rainfall δ^2H, rather than reflect the average rainfall δ^2H (Brattström et al., 2008). However, the results are not wholly consistent as to the specific time(s) and/or the stages of plant development when the system is most sensitive to precipitation δ^2H variation, and additional experimentation undertaken in this regard (Section 3.3).

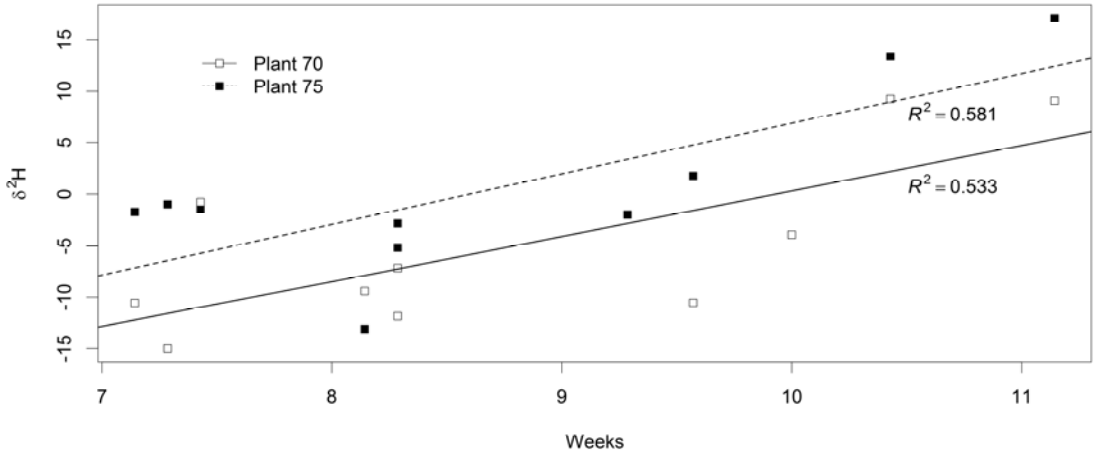


Figure 3.3 Detailed view of the effect of a source water δ^2H -39.7 to +485‰ switch (Treatment 5) on the δ^2H expression in corn kernel solids. Kernel δ^2H values are shown from the time of the enrichment switch, which occurred when the cobs were nearing maturity - over kernel milk to dough growth stages.

The effect of plant water H

An assumption of this experiment was that the stem water is turned over within 24 – 48 hours and that the water is replaced in cobs in two to seven days (based on Ehleringer & Dawson (1992), Schwinning, et al. (2002) and unpublished data (Barbour & Phillips, 2009)). For Treatments 3 and 4 the input waters were switched to +485‰ at the same time as the larvae were placed onto the host plant material. As the average larval feeding time was 14 days, it was also assumed that the 'plant water' in the cobs would be approaching 485‰ at the maximum feeding period (last two larval instars (Anon., 2011a)). For Treatment 5 the input water was switched to +485‰ 12 days before the larvae were placed onto the host plant material and it was assumed that the plant water would be completely equilibrated with the enriched source water before the larvae started feeding.

If these assumptions are correct, the results of the previous section indicate that the δ^2H of the adult moths follow a direct relationship with the plant solid δ^2H, and appear to be independent of the plant water δ^2H. However, the conclusion that there is no or little effect of plant water H on the adult insect intrinsic δ^2H is unconfident, as this is inconsistent with the findings of Hobson, Atwell, et al. (1999) and Husheer & Frew (2006a) – from which it was expected that while δ^2H_M would be mostly from plant solids, approximately 20% would be from plant water. Further, attempts to measure the δ^2H of the plant water in the kernels over the feeding period of the larvae, via captured and in-line analysis of transpired water vapour, were not successful. Therefore, without direct measurement of the plant water, this aspect of the trial was not considered conclusive, and this parameter was also re-examined in the following experiment (Section 3.3).

Within treatment variation

A significant feature of the data for this trial, along with the other experiments in the project, was the high degree of within treatment variation. As with extrinsic contributors to isotopic variation, it is necessary to have an appreciation of intrinsic variation, in order to confidently apply isotopic data in ecological studies (Lancaster & Waldron, 2001; Langin et al., 2007; Wunder & Norris, 2008a). The within treatment range of δ^2H_P was as high as 25‰, and 35‰ for δ^2H_M. It is assumed that the within treatment variation arises from physiologically linked fractionation responses to subtly different micro-environmental conditions that the individual plants and insects are exposed to. In addition, the treatments that had oscillating input water δ^2H had within treatment δ^2H_M variation that was twofold greater (average range = 30.9‰) than the variation in the constant control treatments (average range = 14.7‰), indicating an additional effect of variable precipitation δ^2H will be higher within insect population δ^2H

variation. However, given the generally consistent outcomes between the treatments, it was assumed that the within treatment variation does not swamp δ^2H patterns using average values.

3.3 The influence of precipitation ^2H variation on the δ^2H expression in insects: The timing of variation and within plant mixing

The preceding trial demonstrated that the relationship between precipitation δ^2H and the isotopic signal of herbivorous insects is complex. The relationship is dynamic in time, such that the effect of precipitation δ^2H variation alters relative to the plant's developmental stage of maturity, as well as possibly the insect life-stage (instar) of the larval feeding. For the model host-plant and insect system used here, the plant reflects precipitation δ^2H over a critical period. However, the specific growth phase and the length of maximum sensitivity to precipitation δ^2H change were not able to be deduced from the first trial – although it was considered most likely that this critical stage would be the period of maximum cell division and growth during fruit development and maturation. In addition, the possible influence of plant water on δ^2H_M remained inconclusive.

Therefore, a second manipulated source water δ^2H experiment was conducted to further examine the effect of these parameters. This experiment had the following objectives:

1. To determine which growth phase of the plant is most sensitive to precipitation δ^2H variation.
2. Determine if the isotopically sensitive plant growth phase is expressed in the insect's δ^2H signal.
3. To determine whether the intrinsic δ^2H signal of the adult insect will be derived from the ^2H of the plant water, and hence be highly variable, or the longer term δ^2H averages expressed in plant solids.

3.3.1 Methods

Overview

As with the previous trial, this experiment also raised sweet corn plants on varying isotopic water treatments, in a glasshouse at Lincoln University. The same method of feeding *H. armigera* individually on cobs harvested from the experimental plants was also used. There were two control and four experimental treatments. The experimental treatments only differed with respect to the point in time of the switch from -49.7‰ δ^2H source water to +475‰ water. The switches were staged around the stages of corn maturity, as described by

Nielsen (2001), and *H. armigera* larval development. All the treatments were run simultaneously.

Treatments

The plants were sown on 14 Oct 2009 and reared in Planter Bags (6.75 litres of standard potting mix), initially on Lincoln University bore water (-49.7‰).

- Treatment 1 was a control of constant Lincoln bore water, i.e., constant -49.7‰.
- Treatment 2 was the second control, of 'constant' +475‰. The plants were switched from -49.7‰ to +475‰ water at Time 0 (18 Dec 2009), three weeks before Silking plant development stage.
- Treatment 3 stepped up from -49.7‰ to +475‰ at the beginning of Silking Stage (at Week 3), 4 weeks before the larvae went onto the corn.
- Treatment 4 was stepped up from -49.7‰ to +475‰ at Week 7.4, which was at the beginning of kernel milk stage. This is the stage *H. armigera* commonly start feeding on corn in the field.
- Correspondingly, also on Week 7.4, 30 2nd instar larvae were placed on sections of harvested cobs of all the treatments. The larval rearing method is given below
- At Week 8, Treatment 5 was stepped up from -49.7‰ to +475‰. The larvae were at 3rd and 4th instars and estimated to have completed approximately half their development. The corn was at early kernel dough stage at that point.
- At Week 8.6, the larvae had achieved or were entering the 5th instar, and Treatment 6 was switched from -49.7‰ to +475‰. The corn was at mid kernel dough Stage. This switch was timed to coincide with the period of maximum larval feeding and development, over the last two larval instars (Anon., 2011a).

Other than the timing of the water δ^2H switch, all treatments were subject to the same environmental conditions of natural day length, glass house humidity and temperature regulated around 22°C. The plants were divided into six sets of 12 samples per treatment in a replicated Latin Square, and labelled with individual numbers and treatment number. Water tanks were set up with sufficient water for the entire trial. The enriched water was made up by adding ^2H_2O (Cambridge Isotope Laboratories Inc) to the bore water, which has a δ^2H value of -49.7‰. The plants were watered daily.

Cob samples were collected from plants in each treatment at the trail end, and stored frozen for later δ^2H analysis (Sections 2.4 – 2.6.1). Plant water was extracted from a sub-sample of

kernels via the cryogenic extraction method of Ehleringer, Roden, et al. (2000) and A.G. West, et al. (2006) (Section 2.6.1.1).

Insect feeding

H. armigera eggs were sourced from the Plant & Food Research disease free colony and reared on a lima bean artificial diet until 2nd instar (3 – 5 days). The larvae were then placed individually onto pieces of corn cobs that had been harvested from the treatments. Single cobs were harvested from each treatment every 2 – 3 days, and the cob chunks were replaced daily from the cobs most recently harvested. All the larvae entered the final instar on Week 9.6 +/- 1 day, and all had stopped feeding and were beginning to pupate at Week 10 (26 February). As the larvae pupated, the pupa were collected into vermiculite in Solo rearing cups and emerged under constant 25°C 16:8 light dark. Emerged moths were euthanized and stored for later ^{2}H analysis by freezing (-20°C) (Sections 2.5 – 2.6.1).

3.3.2 Results and discussion

As with the previous trial, the fractionation relationships were quantified using the control, constant source water $\delta^{2}H$ treatments. The water to plant fractionation relationship in this experiment was an enrichment in the natural ^{2}H abundance range, with the average $\delta^{2}H_{P}$ value being approximately 18‰ heavier than the -49.7‰ source water. The relationship trended to a depletion of approximately 200‰ at the artificially enriched source water of 475‰[15] (Figure 3.4). This relationship was very similar to that observed in the previous experiment, (Section 3.2). In contrast, the corn-to-moth fractionation observed in this experiment was larger than that observed in the previous experiment. In the current natural ^{2}H abundance control (T1, $\delta^{2}H$ -49.7‰ water) $\delta^{2}H_{M}$ values (average -70.2‰) being approximately 50‰ lighter than the corn kernels, and hence ≈30‰ lighter than the water that was fed to the plants; trending to a depletion of about 200‰ at the $\delta^{2}H_{W}$ = 475‰ treatment (T2). (In the previous experiment, the natural ^{2}H abundance plant-to-moth fractionation was -20‰, and $\delta^{2}H_{M}$ was within 0 – +10‰ of the irrigation water). The difference between the two plant-to-insect fractionation relationships may be due to the discrete dissimilarities in environmental parameters under which the separate experiments were conducted e.g., temperature

[15] Other researchers have reported similar reduced expression of ^{2}H as the source water values are increased (Hobson, Wassenaar, et al., 1999; Husheer & Frew, 2006a). The mechanism for this lower ^{2}H discrimination by plants at increasing enrichments appears to be unknown. As there is negligible fractionation in the plant uptake of water (Yakir, 1992), the phenomenon may be the result of intra-cellular enzymatic reactions increasingly discriminating against ^{2}H with increasing source water $\delta^{2}H$ values (Meier-Augenstein & Frew, 2011, pers. comm.). Another contributing factor could be the direct exchange of atmospheric water with metabolic water and/ or the reactions in the heterotrophic component of photosynthesis, both of which would result in a dilution of ^{2}H and the dilution would become more pronounced as the $\delta^{2}H$ of the source water increased.

(Grossman & Ku, 1986; Høie et al., 2004). However, given the magnitude of the inconsistency in fractionation between the experiments, it is considered more likely that the difference reflects biochemical differences linked to the different water content and/or diet quality that the insects experienced in the different trials. The cobs were mature in the previous experiment and the kernels relatively dry and doughy; in contrast, in the current trial the larvae went onto the cobs at the beginning of kernel milk stage, when the kernels were still watery. The differences in water content (Nielsen, 2001) would have placed distinct osmoregulation requirements on the larvae in the separate experiments, as well as potential differences in energy expenditure due to physical differences in the kernels. Additionally, the different stages of kernel maturity will have concomitant differences in protein content (lower in more mature corn) (Pukrushpan et al., 1977) and potentially other nutrients, which may effect ^{2}H expression in insects, as has been shown in vertebrates (Podlesak et al., 2008).

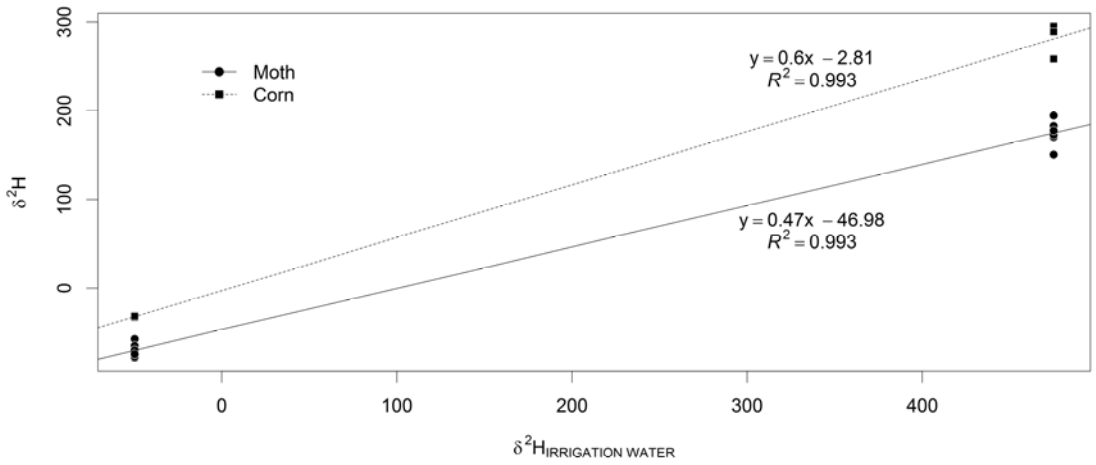


Figure 3.4 The fractionation relationships between irrigation water δ^2H and δ^2H expression in corn and *H. armigera*, from Expt. 1.3. The values are contrasting source water δ^2H control treatments.

With regard to the effect of precipitation ^{2}H variation on the host plant, the contrast between δ^2H_P of T2 and T3, and the δ^2H_P values of the treatments switched later (T4 –6) (Figure 3.5) shows that corn kernels reflect the δ^2H value of the source water during the plant development stages from at least three weeks before silking (i.e., when T2 switched) until before the beginning of kernel milk physiological stage (Table 3.2). Treatment 4 was switched at the beginning of kernel milk stage, and had a δ^2H_P value that was 48‰ more enriched than the T1 control, which indicates partial H turnover in the kernels at that plant developmental stage. In contrast, there is no evidence of H turnover in the kernel solid in the treatments switched after the start of the kernel milk phase, including T5 which was switched just 4 days later. It is probable that similar ^{2}H relationships occur in other herbaceous plant systems, where the δ^2H value of the input water at the beginning of the plants' physiological developmental – i.e.,

before and at early flowering – will, with fractionation offsets, determine the δ^2H of the fruiting structures of the plant, and that water and H turnover in fruiting structures after that point is very slow.

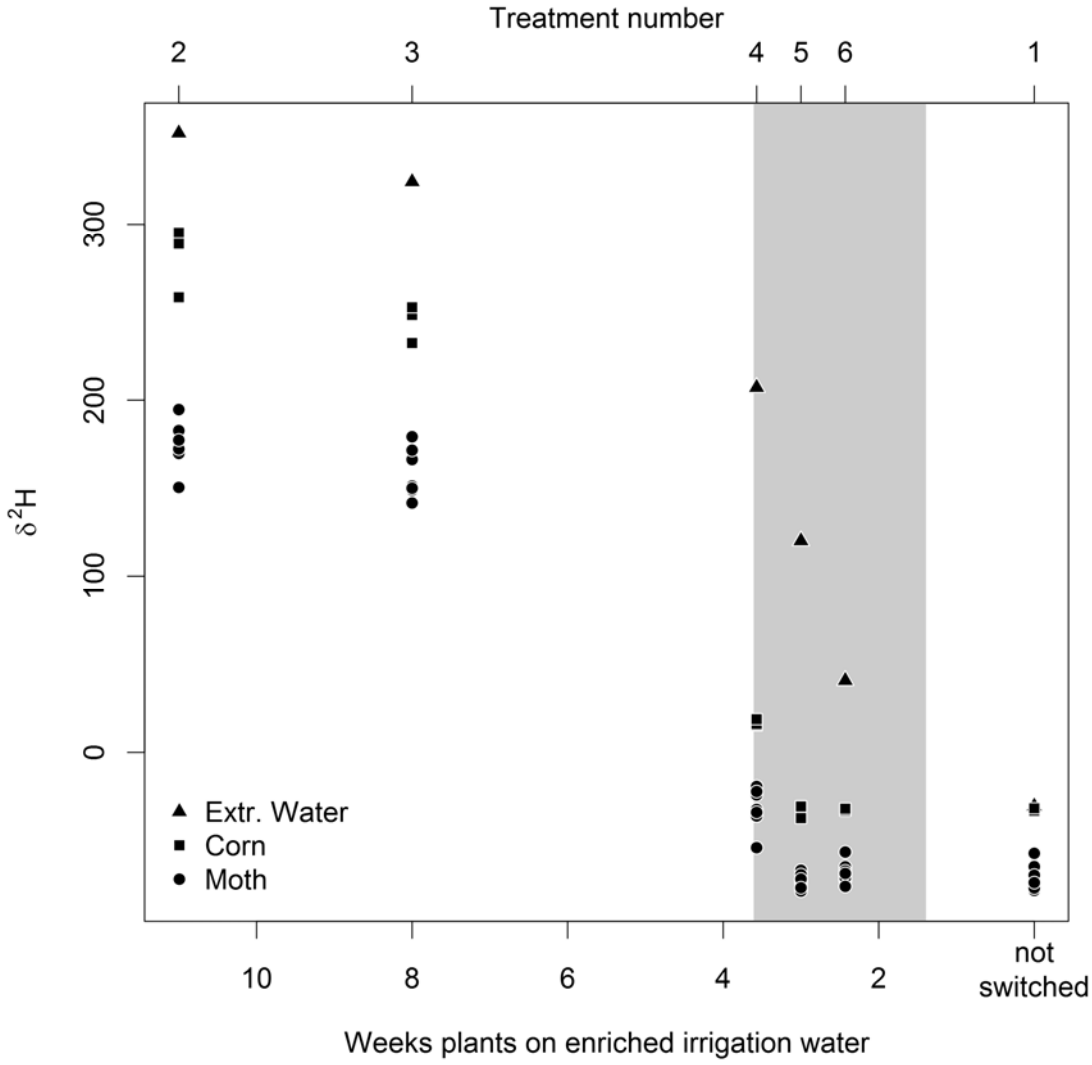


Figure 3.5 The effect of timing in precipitation ^2H variation and within plant mixing on δ^2H expression in insects. Adult *H. armigera* wing, host-plant (corn) solid and extracted plant water δ^2H from a sequence of irrigation water switches from -49.7 to +475‰. The switches were synchronized to host-plant maturity stages and *H. armigera* larval development (given in the preceding table). Larvae feed over period marked in grey.

An ANOVA was applied to the moth wing δ^2H data across the treatments, which confirmed that the overall treatment effects were highly significant ($F_{5, 47} = 1061.83$; $p < 0.001$). Pair-wise comparisons of the means revealed the insect δ^2H values (δ^2H_M) for Treatments 1, 4 and 5 were not significantly different (Fishers Restricted LSD = 5%); with the averages for these treatments all falling within a 5‰ range. As with the kernel data, T2 δ^2H_M values were dramatically higher that the δ^2H_M of treatments that were switched at later plant developmental stages. T3δ^2H_M average values were 227‰ greater than T1 (the -49.7‰

control), but only 17‰ less enriched than T2 (the 'constant' +475‰ control). In contrast, T4 had δ^2H_M values that were on average approximately only 40‰ more enriched than Treatments 1, 5 and 6 δ^2H_M.

Table 3.2 Corn kernel solid, extracted 'plant water' (from kernels), and *H. armigera* wing δ^2H values from a sequence of irrigation water enrichment switches (from δ^2H -49.7 to +475‰). The *H. armigera* larvae were reared on the kernels. Average δ^2H values are ± 1SD. Values within a row that are followed by a different letter are significantly different (Fishers Restricted LSD = 5%).

Treatment	1	2	3	4	5	6
Time and date water switched	constant -49.7‰.	Week 0 (18 Dec 2009)	Week 3 (8 Jan 2010)	Week 7.4 (8 Feb 2010)	Week 8 (12 Feb 2010)	Week 8.6 (16 Feb 2010)
Plant stage that water δ^2H switched	control	control 2: pre-flowering	@ beginning of silking	@ beginning of kernel milk	early kernel dough	mid kernel dough
Extracted plant water δ^2H‰	-30.6	352.0	324.3	207.1	120.1	40.9
Corn solid av. δ^2H‰ (n = 3)	-32.5±1 [a]	280.9±19.7 [d]	244.5±10.7 [b]	16.8±1.8 [c]	-32.9±3.8 [a]	-32.7±0.6 [a]
moth av δ^2H‰	-70.2±6.8 [a] (n = 9)	174.6±14.7 [d] (n = 10)	157.0±13.6 [b] (n = 8)	-32.3±11 [c] (n = 9)	-73.0±3.5 [a] (n = 10)	-68.6±9.4 [a] (n = 6)

The δ^2H_M data in this trial complement the findings of the previous experiment, confirming that 1) the δ^2H_M values will not reflect the average precipitation δ^2H over the whole development time of the host-plant, and 2) the δ^2H_M reflects the value of δ^2H_W during the feeding period of the insect to a relatively small extent. However, contrary to expectations, the critical period (maximum sensitivity to precipitation δ^2H variation) was not one of the developmental stages of the fruit, for which this experiment was structured to test. Rather, the δ^2H_P and δ^2H_M values primarily express the δ^2H_W value during the pre-flowering period of plant development. The oscillating source water method of the previous experiment suggested that there is a discrete period of maximum sensitivity; however, this subsequent experiment has failed to delimit that phase. A superior experimental design would be to pulse enrichments spikes only for specific periods of time and/or larval developmental stages.

The relationship between the plant water and δ^2H_M was examined by comparing the divergence between the extracted water, corn kernel and moth wing δ^2H values, and the differences in this relationship between the treatments. The dissimilarity between Treatments 4, 5 and 6 is the most informative in this regard. Treatments 4 and 5 were switched to the enriched water 8 and 4 days, respectively, before the maximum feeding period of the larvae

and T6 switched at the start of this period. The differences between the plant and moth δ^2H values for these treatments are similar; whereas the extracted water-to-moth differences varied widely between the treatments. The T4 extracted water δ^2H to δ^2H_M difference was ≈ 230‰; ≈ 190‰ in T5; and ≈ 109‰ in T6. The extracted water values of T4 and T5 indicate that the plant water had partially turned over during the feeding periods of the larvae, noting that the entire larval feeding period in Treatment 4 was over the period of enriched water input. These results strongly suggest that in phytophagous Lepidoptera, the δ^2H of the wing (and possibly other regions of the cuticle)[16] are either entirely, or almost entirely, determined by the plant solid δ^2H and not that of the leaf or plant water. This agrees with the analysis of the previous experiment.

This interpretation contrasts with Gröcke, et al. (2006), where they state, without reference, that insects' chitin H is derived from both ingested water and food H, and is hence an isotopic expression of both these sources. (These authors were possibly referring to carnivorous beetles only and not making a general statement; however, this caveat was not stated). Further, no literature has been found that shows how food-derived water influences δ^2H values of terrestrial insect exoskeleton, for either predatory or herbaceous insects (cf. Wang et al., 2009). It appears that this process is not actually understood, and remains to be empirically determined.

In addition, the results here are not consistent with those given for other categories of organism – both invertebrate and vertebrate – which have consistently been shown to have ≈ 30% of structural H derived from free water, despite the widely different creatures and tissues examined. For example Hobson, Atwell, et al. (1999) found that 26 – 32% of the H in quail feathers and nails were derived from drinking water. Similarly, Kreuzer Martin, et al. (2003) showed that 30% of the H in *Bacillus subtilis* spores was derived from the broth water; Wang, et al. (2009) demonstrate that 31% of H in chironomid (Diptera) larval head capsules is from their habitat water; and estimates of the drinking water contribution to H in human hair range from 27% to 35% (Ehleringer et al., 2008; O'Brien & Wooller, 2007).

As the findings given herein conflict with that reported for other classes of organisms, a follow-on question is highlighted: What is the source of H in insect cuticle? The fundamental building blocks of chitin are trehalose and glucose (Cohen, 2001), which are presumably derived from plant sugars in herbivorous insects (Becker et al., 1996). These substrate molecules are the beginning of a complex biochemical and physical transformation cascade which produces the N-acetyl-D-glucosamine polymer that constitute chitin (Cohen,

[16] Insect wings are a specialized region of the cuticle.

2010). On the other hand, the origin of the H in cuticular proteins is not clear. Although the cuticle proteins of immature Lepidoptera life stages, including wing buds, have been examined in some detail (Magkrioti et al., 2007; Mita et al., 2003), the proteins of adult wings appear not to have been specifically examined nor characterised (Willis, 2007, pers.comm.). There is an array of over 300 different cuticular proteins, and several different proteins are commonly present in one cuticle location (Willis et al., 2005). This array of proteins has a wide diversity of structural components, with some containing carbohydrate and others not; plus a variety of amino acid components (S. O. Andersen, 1979). As a result, the structural components of the cuticle proteins can arise from several potential, but unknown pathways. Additionally, the contribution of H derived from water to protein synthesis cannot be predicted, although it is reasonable to assume that the majority of organically bound H to be sourced from organically bound H and primarily carbohydrates, not water.

This question cannot be further investigated with the experimental design employed in this project. As the relationship between water (both plant and free or atmospheric water) and the δ^2H of insect cuticle is important for both geo-location and paleo-climatic studies, additional research in this area is warranted. The 'same diet, two separate waters' method used by Hobson, Atwell, et al. (1999) may serve as a template.

Within treatment variation was similar to that observed in the previous experiment.

3.4 The influence of insect polyphagy on δ^2H and trace element marker imprinting

Polyphagous insect species are those in which the larval stages can successfully complete development on host plants from a number of taxonomic groups. Polyphagy is made possible by these species having 'broad-spectrum' detoxification enzymes, which imparts the ability to digest or detoxify the defensive plant compounds from a broad range of host plant (Gullan & Cranston, 2011).

The range of plant species used by polyphagous insects can have widely different biogeochemical signatures, even at the same location. This includes within C_3 group δ^2H differences of 20 – 30‰ (Chikaraishi et al., 2005), and broad differences in trace element marker concentrations (Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Bonnin, 1983; Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Kuennen, 1983). Correspondingly, it is expected that a population of a polyphagous species will express a range of biogeochemical signals similar to the range found in their host plants. The preliminary research of (Menozzi et al., 2007) supports this notion, as they reported the δ^2H of adult moths from different hosts

at the same location being 20‰ different. However, this was only an incidental observation in a pilot study, and the effect of polyphagy on entomological expression of δ^2H has never been specifically tested and quantified. The effect of polyphagy on insect trace element markers was examined in the 1980s, but the results were not conclusive (Bowden et al., 1984), and the range of elements considered at that time was smaller than can be determined with current technologies.

Conversely, the effects of diet heterogeneity on other isotopes systems, especially ^{13}C and ^{15}N, has been demonstrated for many insect groups (Grey et al., 2004; Ikeda et al., 2010; Lancaster & Waldron, 2001; O'Grady et al., 2010; Spence & Rosenheim, 2005; Vanderklift & Ponsard, 2003).

Unfortunately, the majority of high impact biosecurity insect pests are polyphagous (N. Martin & Paynter, 2010; Stephenson et al., 2003). It is therefore necessary to quantify the effects of herbivorous insect polyphagy on the expression of δ^2H and other biogeochemical markers values that may be useful in insect provenance assignment, in order to ascertain if the effect of polyphagy will negate the location specific signal.

In this experiment, *H. armigera* were reared on a range of common host plants of this species. The δ^2H and trace element markers values from the adult insects were then determined and compared. The objectives of this experiment were to:

1. Establish if there are differences in the δ^2H values of herbivorous insects reared on different plant species at the same location.
2. Determine if the H fractionation relationships are different for insects reared on different host-plants.
3. Establish if there are differences in the trace element concentration signals of herbivorous insects reared on different plant species at the same location.

3.4.1 Methods

Plants were grown under natural conditions on a research farm at Lincoln University, Canterbury, New Zealand. Four host plants ('treatments') were selected from the preferred hosts of *H. armigera* (Firempong & Zalucki, 1989) and represented both ground water and surface water feeding plant species. The deep rooted species were sunflower (*Helianthus annuus*) and sweet corn (*Zea mays*); surface water feeding plants were green beans (*Phaseolus vulgaris*) and 'roma' tomatoes (*Lycopersicon esculentum*). Twenty four plants of each species were reared from seed (in seedling cells with standard potting mix) then transferred to the prepared natural soil beds. Sowing of the different plant species was

staggered over early spring 2009, so the fruiting bodies – the part of the plant consumed by *H. armigera* – were all developing over the same period, and the 'fruits' (seed bodies in the case of sunflower) were simultaneously available for all hosts. The plants were arranged in a 4 X 6 replicated Latin Square design. All treatments were under the same unregulated environmental conditions of natural day length, precipitation, humidity, and temperature. The mean temperature over the duration of the experiment was 14.8°C (temperate range: 9.9 – 20.3°C) (Anon., 2011b). The plants were irrigated lightly with Lincoln bore water (δ^2H -49.7‰) twice daily.

Fruit samples were collected (Section 2.2.2) at trial end, frozen to allow the plant water to be extracted from a sub sample, and analysed later for δ^2H only.

H. armigera eggs were sourced from the Plant & Food Research disease free colony and reared on lima bean based artificial diet until 2nd instar (3 – 5 days). Then, for each plant species, approximately 30 of these larvae were placed individually on recently harvested pieces of fruit under sterile laboratory conditions.

The most mature fruit or flower in the field was harvested every 2 – 3 days and the pieces of food being fed to the larvae were replaced daily. As the larvae pupated, the pupae were placed on Vermiculite in solo rearing cups and emerged under constant 25°C 16:8 light dark. Only five to nine moths per treatment successful completed development. Emerged moths received no food or water and were euthanized by freezing (-20°C) 4 days after emergence, and stored for subsequent sample preparation and analysis (Sections 2.4 – 2.6). All insect samples were subjected to ^{2}H and trace element analyses.

PERMANOVA+ (version 1.0.3) (PRIMER-E version 6.1.13) permutational multivariate analysis of variance (PERMANOVA) and principle co-ordinate (PCO) analysis[17] were used to interpret the trace element data. For this the data was log (x+1) transformed, normalised and the analyses carried out using a Euclidean distance resemblance matrix. (MANOVA test was not appropriate as the basic assumptions are violated).

[17] In a PERMANOVA, a pseudo-F value is calculated for *a priori* groupings from a resemblance (distance, dissimilarity or similarity) matrix; and a p-value is obtained using permutations of the matrix, according to the experimental design (M. J. Anderson, 2001; Marti J. Anderson, 2001). Principal co-ordinate analysis visualizes group dissimilaritties by fitting main axes through the matrix and calculating and ranking a series of eigenvalues for the data points (Legendre & Legendre, 1998)

Results and discussion

δ^2H interpretation

As clearly illustrated in Table 3.3, the host plant solid δ^2H (δ^2H_P) values were significantly different from one another (ANOVA, δ^2H_P $F_{2, 6} = 499.26$, $p < 0.001$). Sunflower was the lightest (-102.8‰), corn the heaviest (-48.0‰), and bean δ^2H was intermediate between these. The tomato host-plant material was lost in an equipment failure, and therefore solids and extracted water δ^2H values cannot be provided for this host.

Table 3.3 The effect of polyphagy on δ^2H expression. Plant solid and extracted plant water δ^2H values, along with adult *H. armigera* wing values from laravae reared on different host-plants grown simultaneously in a single plot. Averages δ^2H values ± 1SD. Summary table attached; values within a row that are followed by a different letter are significantly different (Fishers Restricted LSD = 5%).

	Corn	Tomato	Bean	Sunflower
extracted plant water δ^2H‰	-28.5		-11.5	-20.7
plant solid av. δ^2H‰	-48±1.5 [a] (n = 3)		-64.8±3.2 [b] (n = 3)	-102.8±2.5 [c] (n = 3)
moth av. δ^2H‰	-62.7±4.5 [a] (n = 6)	-70.7±3.6 [b] (n = 6)	-79.9±5.8 [c] (n = 9)	-87.5±3.5 [d] (n = 6)

As expected, the differences in the host-plant δ^2H values were reflected in the adult moth δ^2H values (δ^2H_M) (Table 3.3). An ANOVA of the δ^2H_M values confirmed that the overall treatment effect (i.e., host) was significant ($F_{3, 22} = 32.24$, $p < 0.001$); and pair-wise comparisons of the means revealed the δ^2H_M values for all the host treatments were significantly different from each other (Fishers Restricted LSD = 5%). The δ^2H_M average values for the four treatments covered a range of approximately 25‰, and the results confirm that different host-plant species significantly contribute to intra-population δ^2H variability in polyphagous herbivorous insects. Menozzi, et al. (2007) previously reported δ^2H_M variation in *H. armigera* collected from two host-plant species at the same locality. However, their results are the inverse of the relationship presented here, with their corn reared moths average δ^2H (-79.8‰) being lighter than tomato reared moths (-61.5‰) (host plant δ^2H values and varieties not given).

The δ^2H values of corn, the only C_4 host-plant in the trial, were expected to be heavier than the C_3 hosts' δ^2H (Sternberg et al., 1984; Ziegler et al., 1976). However, the ≈ 35‰ difference within the C_3 hosts' δ^2H is slightly higher than anticipated (Chikaraishi et al., 2005). This may possibly be due to either the plants accessing water of different δ^2H at different depths in the soil (fig. 4-12 in Clarke & Fitz, 1997), and/or physiological differences

between the plant species (e.g., differences in biochemical reactions or evapotranspiration) (Chikaraishi et al., 2005; Sternberg & DeNiro, 1983; Sternberg et al., 1984). The different plants used in this trial are not intended to demonstrate the entire possible inter host-plant species range, but merely demonstrate the effect of polyphagy in principle. Data sets showing the extensive potential host-plant biogeochemical differences, especially for δ^2H, are available from the literature (Chikaraishi et al., 2005; Luo & Sternberg, 1991; Sternberg, 1988; Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Bonnin, 1983; Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Kuennen, 1983; Yakir, 1992).

The different host-plant treatments also gave rise to different host-to-moth fractionations. The moth wing δ^2H reared on corn and bean were approximately 15‰ lighter than the host δ^2H, but the sunflower moths were approximately 15‰ heavier than their host plant δ^2H value. The mechanism for the fractionation differences between the different treatments is unknown, but may be caused by different physical stress and/or different metabolic processes required of the insects on the different host plant. For example, the primary site of feeding in the sunflower treatment is the developing seeds on the basal plate of the flower. The seeds are relatively dry and hard compared to the fleshy parts of the plant consumed in the corn and bean treatments. The different hosts therefore are assumed to present significantly different physical stresses. Alternatively, the unequal diet quality of the different plants the different hosts may require differences in the metabolic processes involved in assimilation, such as unequal energy demands or higher excretory demands due to unequal proportion of water in the host-plants and/or the host-plants specific defensive compound may require unequal de-toxification effort for the insect consumers. Another indicator of divergent stresses associated with the different host-plants was the different larval development times observed. The development rate on beans was slightly faster than sunflower, and 4 -5 days faster than corn (results now shown).

The extracted plant water δ^2H values covered a 17‰ range, centred at ≈ -20‰ and they were not correlated with the plant solid or insect δ^2H values. This supports the conclusion drawn in the previous experiments, in that the plant water does not appear to directly affect the insects' H isotopic composition. Further, the differences between the extracted plant water δ^2H and the δ^2H_P are widely divergent between plant species – ranging from 19.5‰ difference in corn to 82.3‰ in the sunflower treatment. This divergence may be due to metabolic differences between the plant species or daily leaf water variation being unequal between hosts (Flanagan et al., 1991).

As in the previous experiments, the intra-treatment variation in the moth δ^2H values were larger than the ranges observed in the δ^2H_P. The δ^2H_P intra-treatment variation ranges spanned from ≈ 2‰ (sunflower) to 7‰ (bean). The moths reared on corn, tomato and sunflower had intra host-plant treatment ranges of ≈ 10‰, and those reared on sunflower ≈ 17‰.

Trace element data interpretation

A PERMANOVA analysis applied to the trace element data derived from the moth bodies showed that the larval host-plant treatments had a significant effect on the overall elemental composition of the adult *H. armigera* (Pseudo-$F_{3, 12}$ = 3.2101, p(perm) = 0.0033). Pair-wise PERMANOVA tests were run in an attempt to distinguish between the treatments, however, there were insufficient samples to allow confident appraisal of multivariate statistical significance (indicated by p(perm) ≠ p(monte-carlo)). The POC graph (Figure 3.6) suggests that that the moth multivariate data from tomatoes were different to the other host treatments, and the corn, sunflower and bean treatments appear not to be different, although sunflower and bean treatments only over lap on a single point.

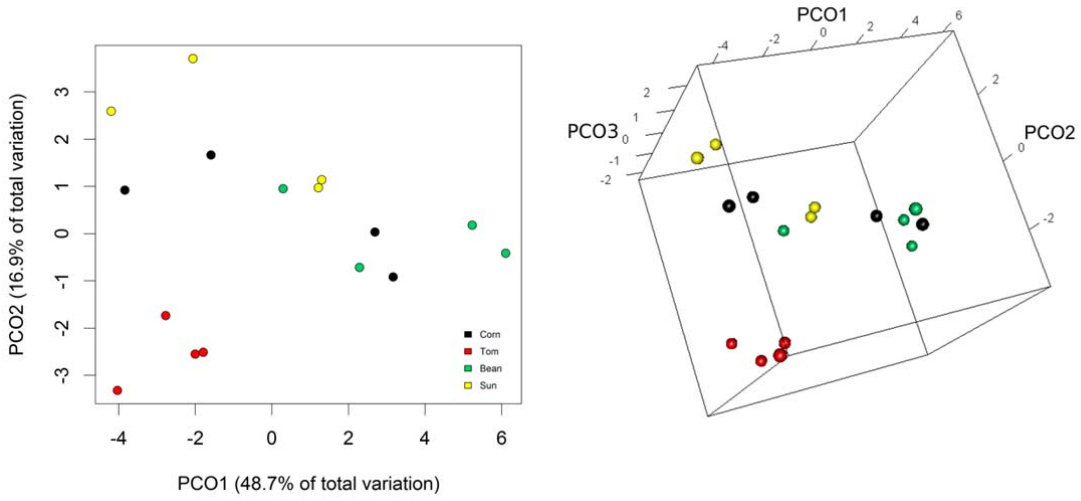


Figure 3.6 The influence of insect polyphagy on adult moth trace element concentration. A Principle co-ordinate plot (from a Euclidean distance resemblance matrix) of trace element concentration data (ng/g) of *H. armigera* adults reared on different host-plants.

A series of univariate analyses reveals which elements were individually significantly different between the moths from the different host-plant treatments (Table 3.4). These elements were Co, Ni, Cu, Zn, Cd (all essential[18] elements), as well as Al, Sr, Ba, La, and Ce.

[18] Essential trace elements are defined according to Mertz (1981): F, Na, Mg, Si, P, S, Cl, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd? Sn? I.

The elements not affected by host-plant were Li, Ca, Cr, As, Rb, W and, importantly, Pb. These latter elements therefore, have potential as geo-location markers not confounded by host type. Sc and Cs were different by host, but not significantly different overall ($F_{3,12}$ p = 0.058 and 0.054, respectively). (Note 1: these analyses do not take into account any interaction between the elements, e.g., Cd is known to affect Ca uptake by plants (Malkowski et al., 2005)). (Note 2: as Table 3.4 is a series of separate analyses, there is an additive chance of a spurious result (Type I error)).

Table 3.4 Trace element concentration data (ng/g) of adult *H. armigera* from larvae reared on tomato, sunflower, corn and bean host-plants. Only the elements considered to have geo-location potential are shown. Univariate analysis statistics of the individual elements are also given: ns = not significantly different; † = p <0.10; * = p <0.05; ** = p <0.01. SD = 1SD. n = 4 per treatment.

	Li	Al	Ca	Sc	Cr	Co	Ni	Cu	Zn	As	Rb	Sr	Cd	Cs	Ba	La	Ce	W	Pb
Tom Av	17	2083	1217717	34	78	5	37	26934	200811	8	1204	97	62	1	120	1	2	6	30
Tom SD	5	499	370617	10	14	3	11	2719	21731	1	831	43	20	0	45	0	1	9	5
Corn Av	21	8647	1102825	22	88	22	67	24046	366476	12	1618	893	34	1	986	23	86	5	36
Corn SD	10	7904	216919	12	40	19	45	4451	104908	2	474	468	15	1	385	23	88	4	41
Sun Av	28	6735	1214144	11	73	22	56	28574	443531	10	1702	668	598	1	673	19	70	3	20
Sun SD	3	5186	452737	5	31	12	31	5567	138432	7	685	387	430	1	214	16	58	4	17
Bean Av	29	23064	1273086	34	138	59	155	19259	265410	16	1914	851	39	3	1743	70	253	6	56
Bean SD	18	16234	364627	17	59	34	93	2443	72338	5	1351	177	9	2	810	55	199	5	19
p (df=3, 12)	0.414	0.042	0.924	0.058	0.129	0.017	0.042	0.031	0.016	0.083	0.728	0.014	0.007	0.054	0.003	0.044	0.047	0.916	0.251
signif	n.s.	*	n.s.	†	n.s.	*	*	*	*	n.s.	n.s.	*	**	†	**	*	*	n.s.	n.s.

Of the elements that were significantly different, Sr and Cd were only different in one of the four hosts. Sr concentrations were not different in the moths from corn, sunflower and bean, but were approximately 10 times lower in the tomato treatment moths. In contrast, Cd concentrations were more or less the same in the moths reared on tomato, corn and beans, but approximately 10 times higher in the sunflower moths. Sr and Cd are two elements that have high geo-location potential.

The differences observed in the moths from the various host treatments are assumed to reflect the host species element concentration profiles, as data is not available for the actual plant material. This assumption follows

1. Differences in concentrations of the essential trace elements are well known for crops such as those used here (Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Bonnin, 1983; Wolnik, Fricke, Capar, Braude, Meyer, Satzger, & Kuennen, 1983), with the differences between plant species arising from plants regulating the uptake of these

elements to meet their own physiological requirements (Tangahu et al., 2011), often using species specific ion uptake mechanisms (Kabata-Pendias, 2004). And

2. Variation in the expression of trace elements resulting from the diet of consumers has been shown for many classes of organisms, including insects (Gu et al., 2001; Wanner et al., 2007; Z. Zhang et al., 2009).

The uptake of 'non-essential' elements in plants is less directly regulated (Chaney, 1980). As a result, these many of these elements are believed to occur in plants in similar, low concentrations, regardless of the plant species. This may explain the similarity of the concentrations of Li, Rb, W and Pb in the moths from the different host-treatments. However, the essential element concentrations (Ca, Cr, As) in moth that were not affected by host species and the non-essentials that were affected (Al, Sr, Ba, La, and Ce) are exceptions to this assumption that cannot be explained without the plant element data.

Of the the essential elements considered here, the finding that larval host-plant is a significant factor in the elemental composition of adult *H. armigera* is consistent with earlier research regarding the other polyphagous Lepidoptera *Gonepteryx rharnni* (Pieridae) (Dempster et al., 1986) and *Agrotis segetum* (Noctuidae) (Sherlock et al., 1985).

In contrast, data regarding the less abundant elements has not previously been achieved obtained from insect samples, and these have geo-location potential and appear not to be affected by host type. Therefore, these markers may offer significant potential as tracking markers in entomological science. Additional testing of the place-to-insect transmission integrity of these elements is a research priority for future research (Holder & Armstrong, in prep). The influence of multiple host-plants on the Pb and Sr isotope ratios of insects is also to be investigated in this future research project.

3.5 The influence of adult feeding on δ^2H and trace element marker imprinting

The pioneers of stable isotope biogeochemistry, Michael DeNiro and Samuel Epstein, elegantly showed that the isotopic profiles of consumers are determined by their diet, which led to the maxim 'you are what you eat' or, more accurately, 'you are what you assimilate, plus a few per mil' (DeNiro & Epstein, 1978, 1981; Fry & Sherr, 1984). In more recent times the turnover of H, C and N, and the concomitant change in isotopic composition of various tissues during the life time of the consumer have also been studied (Ayliffe et al., 2004; Cerling et al., 2007; Hobson & Clark, 1992a; Logan et al., 2006; Ogden et al., 2004; Tieszen et al., 1983; A. G. West et al., 2004).

Interest in isotopic turnover includes the turnover of C and N in adult insect tissues, primarily in regard to food web studies. For example, Ostrom, et al. (1997) demonstrated ^{13}C and ^{15}N turnover in 'whole insect' *Coleomegilla maculata lengi* (Coleoptera: Coccinellidae). This was refined by Gratton & Forbes (2006) who showed the $\delta^{13}C$ value of the fat and reproductive tissues of *Harmonia axyridis* and *Coccinella septempunctata* (Coleoptera: Coccinellidae) changed by 10‰ within 5 days of a dietary $\delta^{13}C$ switch from C_3 to C_4 food, and was completely equilibrated to the new diet $\delta^{13}C$ value in 8 – 10 days; whereas the abdominal and thoracic integument changed by only ≈ 4‰ over 15 days and the flight wing ≈ 2‰. Gratton & Forbes (2011) then studied crop colonization by the same cocinelids, using the difference in turnover rates between forewing and the fat + reproductive tissues.

Adult diet has also been shown to be expressed in adult moth wing $\delta^{13}C$ values (Ponsard et al., 2004). However, it is unknown to what extent that adult diet $\delta^{2}H$ is expressed in adult moth wing, or if trace element markers in the adult diet are expressed in whole insect digestions. This is of primary interest to this study given that this turnover could obscure the larval origin signal for potentially informative markers. In addition, the rate of metabolic activity of the adult insect appears to affect the turn-over rate (Haubert et al., 2005). European corn borer moths (*Ostrinia nubilalis,* Crambidae) with high metabolic rates have been shown to have wing $\delta^{13}C$ values that closely reflected the larval diet $\delta^{13}C$ value, indicating they used a greater proportion of their larval resources in the adult wing; whereas adults with low metabolic rates had wing $\delta^{13}C$ values that less accurately expressed the larval diet $\delta^{13}C$ value by 0.5 – 0.9‰, suggesting a lower proportion of larval resource use in the wing (Ponsard et al., 2004). These deviations in larval diet to adult wing stable isotope value are possibly due to the ongoing development of the cuticle during teneral adult phase (Hayes & Wall, 1999; Neville, 1983).

Hence, the final parameter of uncertainty to be evaluated in this chapter is the influence of adult feeding on $\delta^{2}H$ and trace element imprinting in adult moth tissues. An experiment was conducted wherein adult moths were subject to a range of different feeding treatments, including starvation, with the specific objective(s) of determining:

1. Is there turnover of H in adult moth wing, as known to occur with C, and hence a corresponding alteration of $\delta^{2}H$? If so, to what extent?
2. To what extent do trace elements turnover in adult insect tissue, especially in the 'whole insect' context? (As it was necessary to use whole insects for the trace element and heavy metal isotope analysis, Section 2.3.1.1).

3. Does any turnover of δ^2H and trace element markers occur to the extent that inputs from adult feeding will obscure larval point of origin signal?

3.5.1 Methods

H. armigera adults were sourced from larvae reared on a homogeneous lima bean based artificial diet, made with tap water of δ^2H -32‰. The newly emerged adults were exposed to either one of three δ^2H drinking water treatments, to examine the δ^2H response; or one of two sucrose solutions to examine the effect of adult feeding on trace element profiles.

The δ^2H treatments were:

- Treatment 0 (T0) consisted of newly emerged moths, that were not fed and killed < 12 hr after emergence.
- The controls for the whole time series
    - Treatment 1: nil water, nil food starvation (T1); and
    - Treatment 2: nil sucrose, -32‰ water (i.e. no δ^2H shift from water in the larval diet).
- Treatment 3 nil sucrose, +485‰ water (T3).

The trace element treatments were different adult food sources

- Treatment 4: a 10% Auckland honey solution; and
- Treatment 5 was a 10% Queensland cane sugar solution.

To examine gender linked physiological differences, within and between treatments; male and females were evenly assigned to each treatment.

The experimental design, including the time series and number of samples per treatment, are given in Table 3.5. Ten moths per gender per treatment were set up. However, moths died naturally at a more-or-less a even rate from day 4 onwards, so the time series that was intended to have samples on days 5, 10 and 20 only, yielded samples on most days from natural mortality, and the experimental period was reduced to 16 days. The natural mortality moths were supplemented with euthanized moths only on days 0 – 4. All moths were killed and/or stored for later analysis by freezing (-20°C) (Sections 2.4 – 2.6).

Table 3.5 An examination of the influence of adult feeding on δ^2H and trace element marker expression in adult tissue: experimental design and number of samples per treatment. Note: High natural mortality disrupted the intended distribution of replication of moths older than 4 days.

Treatment no.	T0		T1		T2		T3		T4		T5	
Treatment description	nil water, no sucrose/ starvation		nil		-32‰ δ^2H water		+485‰ δ^2H water		Honey soln.		Cane sugar soln.	
Gender / **Days**	m	f	m	f	m	f	m	f	m	f	m	f
0	10	10										
0.5							10					
1							10					
2							10		2		1	
4			10	10	10	10	8	8	8	9	8	11
5							10		2	2	3	2
6								2	1	1	5	2
7					2	4		8	1	1	1	5
8						4		2		2		4
9						2	2	2	2	1	1	3
10					8	8	8	2	6	9	4	2
11						4					1	1
12					6			2	2			
13					4		8	2				
14							2		1	1	4	
15							2		3	3		
16									1	1		
17										1		

The *H. armigera* larvae were reared at the Plant & Food Research disease free rearing facility, Auckland. The pupae were sorted into gender before emergence and individually caged in ventilated solo cups, with access to a wick soaked in the appropriate treatment.

This trial was carried out in a controlled temperature room. All treatments were run simultaneously and subject to the same environmental conditions of artificial 16:8 diurnal cycle and a constant 22°C, and RH of approximately 50%.

Moth wing samples (≈200 µg) from all T0 – T3 replicates were subjected to ^2H analyses. The ICP-MS trace element analyses used the bodies of four male and four female 4 day old moths

from each of T1, T4 and T5. Unfortunately, there were insufficient resources to directly analyse the moths for heavy metal isotopes. The effect of adult feeding on these markers was inferred from comparing the Sr and Pb concentrations in the different treatments.

Univariate ANOVA were performed using GENSTAT (12.2 and 14.1). PERMANOVA+ (version 1.0.3) (PRIMER-E version 6.1.13) PERMANOVA and PCO analyses were used to interpret the trace element, using a Euclidean distance resemblance matrix derived from the normalised ng/ g dataset.

3.5.2 Results and discussion

Influence of adult feeding on δ^2H

A comparison of newly emerged adults (T0) with 4 day old adults that were also starved (T1) and those that received water of the same δ^2H as the larval input (T2), demonstrated an age linked δ^2H enrichment in the moth wings over the first four days post emergence (Figure 3.7). The T0 adult wing had, on average, δ^2H_M values 6.5‰ lighter than T1 moths and 9.2‰ lighter than four days old T2 moths. Despite overlap between data sets, the treatments were significantly different (T0 vs. T1 $F_{1, 38} = 24.5$, $p < 0.001$).

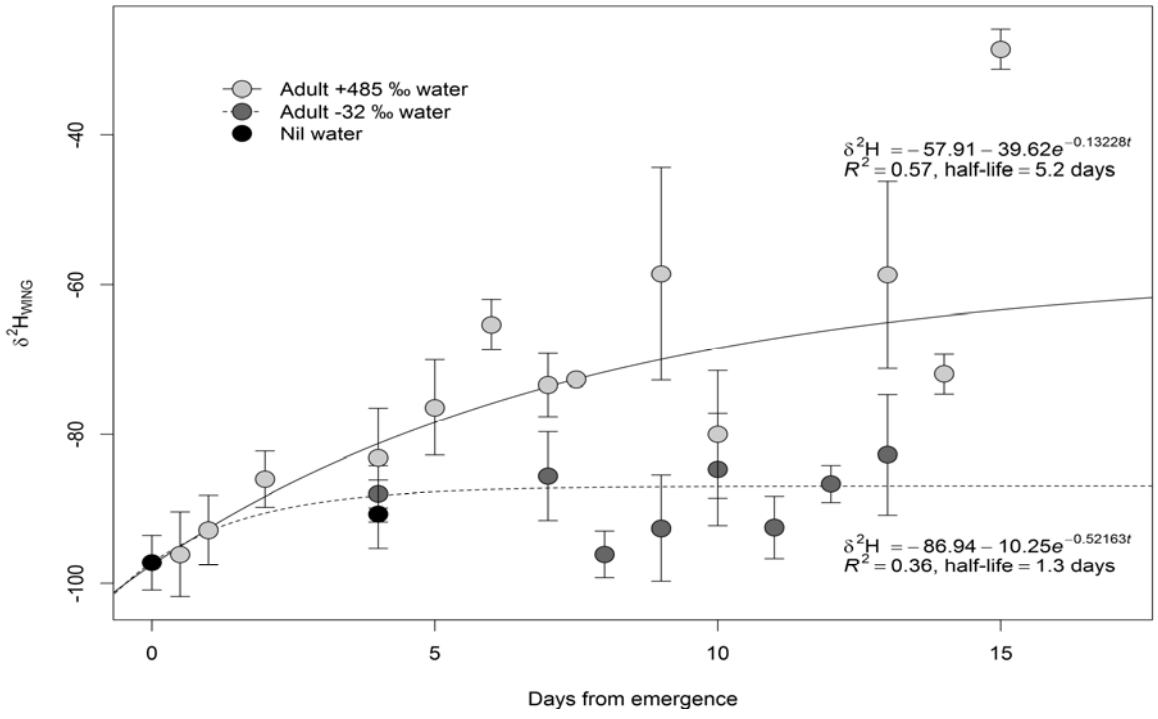


Figure 3.7 The influence of adult moth feeding on wing tissue δ^2H turnover. Changes in moth wing δ^2H over time from emergence for *H. amigera* adults exposed to contrasting δ^2H drinking water. The points displayed are means ± 1SD. The fitted curves are -32‰ δ^2H constant treatment (dashed); and +485‰ δ^2H treatment (solid) and were generated using Equation 3.1, below.

This age-related enrichment may potentially be caused by the displacement of some H (including as OH^-) during protein synthesis and/or the cross linking of the proteins (the later step involves hydrolysis (Xu et al., 1997)) and/or protein (including enzymes) migration into the cuticle (Chapman, 1998; Kramer et al., 1995) during post-emergence sclerotization of the cuticle.

From the 4th day post emergence, the δ^2H values of the moths under the constant -32‰ water treatment (T2), follow a flat linear trend (Figure 3.8). This suggests, that on average, there is no or very little further age related fractionation in moth wing cuticle from that point.

The turnover rate of the -32‰ δ^2H**,** non-enriched treatment was calculated using the exponential model of Hobson & Clarke (1992a)

Equation 3.1

$$\delta\ ^2\mathrm{H} = a + b\mathrm{e}^{ct}$$

Where *a* is the new value approached asymptotically, *b* is the total change in value after source water switch, *c* is turnover rate, and *t* is time (days) since the source water switch. Turnover rate is expressed as a half-life, the time it takes for the isotopic composition of the tissue to reach a midpoint between the initial and final values:

Equation 3.2

$$t_{1/2} = (\ln 0.5) / c$$

GenStat (12.2) was used to estimate the function for this fractionation relationship using the raw data. The parameters and depicted values (as curve) are given in Figure 3.7.

Four day old adult moths that had undergone starvation (T1) were also contrasted against moths of the same age that had received a liberal supply of water (T2), to determine if the nil adult water treatment affected the adult δ^2H signal. The 4 day old T2 moth average was 2.7‰ heavier than that of T1, and an ANOVA suggests that these treatments produced significantly different results ($F_{1, 38} = 4.19$, $p = 0.048$). However, the p value trended to non-significance and some data points had large residual errors. Therefore it was concluded that the treatments could not be confidently distinguished, and the four day old moths from both starved (T1) and -32‰ water (T2) treatments were considered to show more or less the same pattern of post-emergence enrichment.

In addition to the age-related sclerotization enrichment, the +485‰ feed moths (T3) show that H from the adult diet is incorporated into the H of the adult moth wing. In contrast to the

sclerotization enrichment, the expression of the adult dietary H occurred both over the first four day post-emergence period, and continued beyond that period. The T3 average δ^2H_M changed from -97.5‰ on day 0.5, to -83.2‰ on day 4, to -65.6‰ averaged over days 10 – 15. The four day old moths from T3 (mean -83.2) were significantly more enriched than the four day old moths from the larvae-to-adult constant -32‰ treatment (T2, mean -88.0‰) ($F_{1, 35}$ = 7.39, p = 0.010). The data from the -32‰ adult water treatment (T2) and the adult diet +485‰ enrichment (T3) were also compared from the 4th day post emergence, using non-linear regression analysis (GenStat 12.2). Fitting a single exponential curve to all the data, explained only 31% of the variation in the δ^2H_M data; however, fitting separate parallel exponential curves for both treatments significantly improved the model (F = 59.01, p<0.001). This confirmed that T2 and T3 were significantly different, and there was H turnover in the adult wing tissue which reflected the adult diet. The Y-intercepts for the curves for T2 and T3 were -89.68 and -76.16‰ respectively (i.e., T3 δ^2H_M curve was approximately 13.5‰ heavier than that of T2). (The model was not significantly improved by fitting separate curves for each treatment (F = 0.81, p = 0.37)).

To quantify the effect of H turnover in the adult moth wing, it is necessary to separate out the contributions of the two processes identified above, i.e., the sclerotization enrichment and the expression of the adult dietary H. A series of different mixing mode approaches were considered in this regard.

A negative exponential curve was fitted to the enrichment observed in the T3 δ^2H_M results using Equation 3–1. The parameters generated estimated that the total H turnover was only 0.13‰/day, and predicted that equilibration to the δ^2H of the new diet was not achieved until >10 days. However, as the relationship observed in T3 δ^2H_M contained both the sclerotization and the adult dietary input processes, it is not possible to quantify the influence of adult diet assimilation on the total H turnover with the single exponential model (Hobson, 2011). In an attempt to separate out the effects of the different mixing pools, the H turnover relationship in the T3 data was also modelled using the Reaction Process Variable (RPV) given by (Cerling et al., 2007, equ. 11 and 15):

Equation 3.3

$$(F-1) = \frac{\delta^t - \delta^{\text{Equil}}}{\delta^{\text{Initial}} - \delta^{\text{Equil}}}$$

This describes the fractional approach to equilibration, where F = 0 at the beginning of the exchange reaction, and F = 1 at equilibration (t = ∞); δ^t is the delta value at time *t*; δ^{Equil} the

delta value achieved when the substrate of interest is in balance with the new diet (i.e., a from Equation 3—1); and $\delta^{Initial}$ is the initial δ value (at $t = 0$). The RPV is 'linear-ized' using

Equation 3.4

$$\ln(F - 1) = -\lambda t$$

Where λ is the turn over constant. The quicker and slower turnover pools are plotted separately, i.e., sclerotization enrichment and the adult feeding input respectively. The slopes of the plots give the λ, and the y-axis intercepts give the fractional contributions to the total turnover.

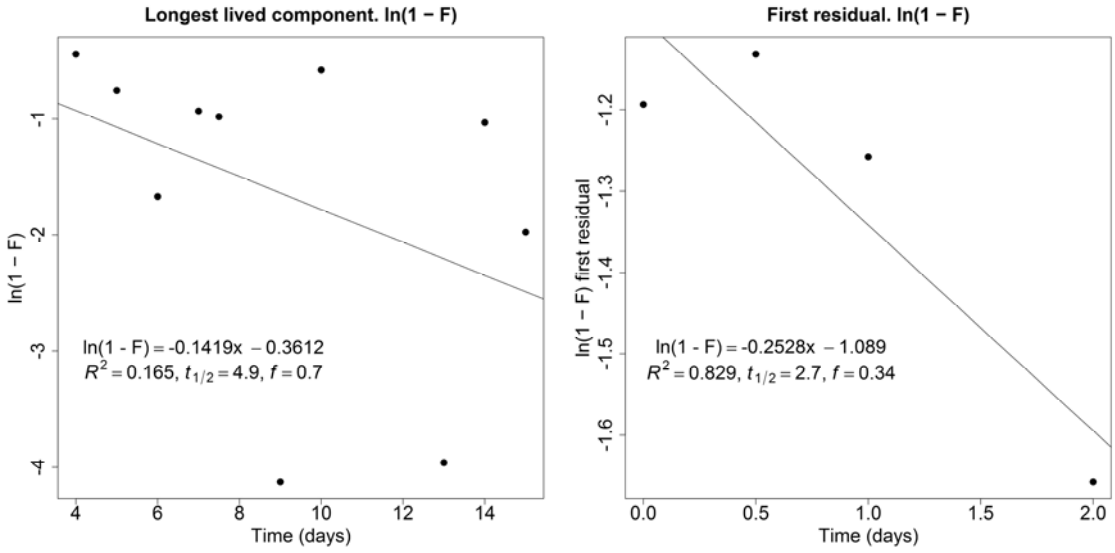


Figure 3.8 Reaction process plot [ln(1 − F)] of δ^2H_M data from adults given 485‰ drinking water. The longest lived component is an estimate of the adult diet contribution to the total H turnover and the 1st residual is the sclerotization enrichment. Estimated parameters for each component are given in the appropriate plot.

Although the data is a relatively poor fit to the predicted function (see R^2 values), the estimated parameters (f) suggest that the adult diet turnover was responsible for 70% of the total H turnover in the moth wings, and the sclerotization enrichment approximately 30%, with the half-life of the two processes being 4.9 and 2.7 days respectively (Figure 3.8). This is the predicted status at time = equilibration. However, as equilibration was approached at 10 days post emergence – by which time most of the moths were dead – and the two mixing pools were operating simultaneously within the first 4 days (Figure 3.7), the two processes were probably not correctly separated using the RPV approach. Moreover, the RPV estimated parameters were potentially inaccurate due to the large degree of variance in the data (Cerling, 2011, pers. comm.), and may have been compromised by the limited number of samples past day 13. This inaccuracy is indicated by the sum of the two f values being > 1. Future

research into the relationship between dietary H and its expression in insects tissues will benefit from having a higher number of samples in the 0 to 4 days period, as well as in the later stages of the time series (Cerling et al., 2007).

To further investigate the effect of adult diet H on δ^2H_M expression, predicted values for δ^2H_M were interpolated/ extrapolated for a series of adult drinking waters, over a range of natural ^2H abundances (as opposed to the extreme enrichment applied in T3). This was achieved by assuming a linear relationship between the high and low treatments and using the differences between the curves for T2 and T3 (Figure 3.7), such that

Equation 3.5

$$\delta^2 H^t = \delta^A + (\delta^B - \delta^A) * [X - (-32)] / [(485 - (-32)]$$

Where $\delta^2 H^t$ is the predicted δ^2H_M at time t; δ^A and δ^B are the δ^2H_M values at time t for Treatments 2 and 3 respectively; and X is the δ^2H value of the adult drinking water. This equation was then used to generate predicted δ^2H_M series for adult moths' drinking waters of -50, -32, -10, and 10‰, values based on precipitation averages of the same places of biosecurity interest referenced in Sections 3.2 and 4.2, namely Queensland, Auckland and Canterbury (Figure 3.9).

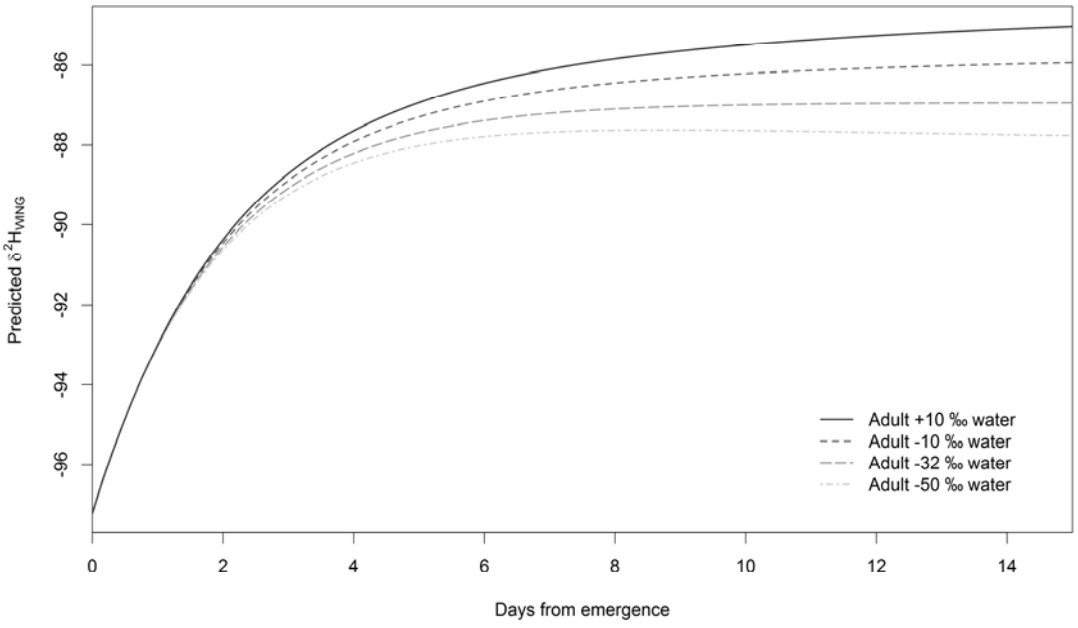


Figure 3.9 Extrapolated moth wing δ^2H values derived from the difference between high and low δ^2H adult drinking water treatments. The extrapolations are for a series of potentiual adult drinking waters.

The extrapolated δ^2H_M values suggest that the expression of the adult diet in moth wings will be modest. With a δ^2H 20‰ change from larval to adult diet, the predicted values show a δ^2H_M shift of 0.6 – 0.8‰ at 10 days post emergence, and about 1‰ at 14 days. It can therefore be concluded that the δ^2H value of moth wings is driven primarily by the larval diet input. The adult diet driven turnover is a relatively small parameter. Whereas, the sclerotization enrichment, resulting in a shift of approximately 7‰ in the wings of the model insect over the first four days post emergence, is of greater importance in the 'diet H-to-insect' relationship. This parameter needs be taken into account went applying the diet to consumer H fractionation relationship in geo-location or ecological studies – i.e., the age of the insect may need to be determined in wild caught insects.

This experiment is the first time H turnover in insect tissue has been demonstrated. Moreover, it has been shown that the cuticle of moth wings contains both larval and adult input signals. However, the effect of adult diet is small and less than the within treatment errors observed in previous experiments where moths all had identical larval and adult treatments. Thus adult diet input should not compromise the geo-location information in δ^2H from adult Lepidoptera wing samples.

Indeed, the turnover in H was less than anticipated, given the previously reported $\delta^{13}C$ shift in adult wings of *Ostrinia nubilalis* from the larval signal by up to 4.8‰ toward that of the adult diet (Ponsard et al., 2004). This relative difference may be due to different biochemical pathways affecting the separate elements at diverging rates, or inter-species difference in wing/ cuticle physiology. Alternatively the difference may be a consequence of Ponsard et al.'s method not removing the lipids before analysis. The hind wing of adult cocinellid beetles (analogous to moth flight wings) has similarly been shown to be subject to C turnover, giving a $\delta^{13}C$ shift of ≈2‰ in a C3 to C4 based adult diet switch (Gratton & Forbes, 2006). In contrast Webb, et al. (1998) found a smaller ^{13}C turnover in purified chitin (from the cuticle) *Locusta migratoria* (Orthoptera: Acrididae) during adult maturation, giving a ≈1‰ in a similar C3 to C4 diet switch. The difference observed in cuticle C behaviour in these various studies may be due to the removal of the cuticular protein in Webb's samples, supporting the proposition (given above, page 83) that the turnover of H in adult cuticle is due to protein activity. Alternatively, the difference observed may be due to physiological differences in the cuticle formation of hemimetabolous insects (those with incomplete metamorphosis), as used by Webb, et al. (1998), compared to the holometabolous subjects of Ponsard, et al. (2004) and Gratton & Forbes (2006). Other studies regarding isotopic turnover in specific tissues in insects have primarily used $\delta^{13}C$ and $\delta^{15}N$ to explore resource allocation to reproductive

tissues (Fischer et al., 2004; Min et al., 2006; O'Brien et al., 2003, 2004; O'Brien et al., 2000), or C turnover in whole insects, including cocinellids (Ostrom et al., 1997), *Anopheles arabiensis* (Diptera: Culicidae) (Hood-Nowotny et al., 2006) and med-fly (*Ceratitis capitata*, Tephritidae) (Hood-Nowotny et al., 2009).

Hydrogen turnover in various tissues of other organisms has been catalogued. For example, O'Brien &Wooler (2007) demonstrated different H turnover rates in human hair and urine, and Podlesak, et al. (2008) has shown a similar relationship in woodrat (*Neotoma* spp., Cricetidae) breath, blood plasma, hair and teeth enamel δ^2H. It is assumed that a similar relationship will occur in insects, where the most metabolically active tissues – e.g., hemolymph, body fats and reproductive tissues – have a faster H turnover than the relatively fixed tissues like the wing cuticle. Therefore, mixed tissue or whole insect analyses will probably not be suitable for provenance studies.

The relationship between gender and the δ^2H values in the moth wings was also analysed (data not shown). The results were contradictory, and difficult to interpret. The newly emerged males were significantly lighter than the females ($F_{1, 18} = 8.96$, $p = 0.008$), with the means for males and females being -99.28‰ and -95.11‰ respectively; but there was no significant difference between the genders in the starved four day old moths (T1) (($F_{1, 18} = 2.89$, $p = 0.106$). Similarly, the analysis of the gender assigned data for moths older than four days was also ambiguous. A non-linear regression analysis applied to T3 δ^2H_M data revealed no significant gender difference ($F = 0.32$, $p = 0.575$); whereas the same statistical method found a significant difference between T2 (from days 4+) gender δ^2H_M values ($F = 8.99$, $p = 0.004$), with the curve fitted to the males values being on average 6‰ heavier.

Influence of adult feeding on trace element markers

A PERMANOVA analysis applied to the trace element data derived from the moth bodies showed that the different adult food sources (T1, T4 and T5) had a highly significant effect in multivariate data space (Pseudo-$F_{2, 21} = 6.4034$, p(perm) = 0.001). A two way PERMANOVA confirmed that there is also a significant difference between the genders across all treatments (Pseudo-$F_{1, 18} = 7.1619$, p(perm) = 0.006), as is visualised in Figure 3.10, yet there was no interaction between treatment and gender (Pseudo-$F_{2, 18} = 1.1405$, p(perm) = 0.2998).

The differences in the trace element profiles of the genders were also examined within the separate treatments. There was a significant difference in the gender specific trace element data (Pseudo-$F_{1, 6} = 6.1678$, p(perm) = 0.038) of the no food or water treatment, T1.

However, the genders in Treatment 5 were different, but not significantly different (Pseudo-$F_{1,6} = 1.8816$, p(perm) = 0.094). The gender-response differences observed between Treatments 1 and 5 were probably manifestations of differing physiological stresses experienced by the two genders under the starvation treatment. Varying gender responses to starvation was similarly noted in ^{13}C turnover and expression in *Ostrinia nubilalis* by Ponsard, et al. (2004).

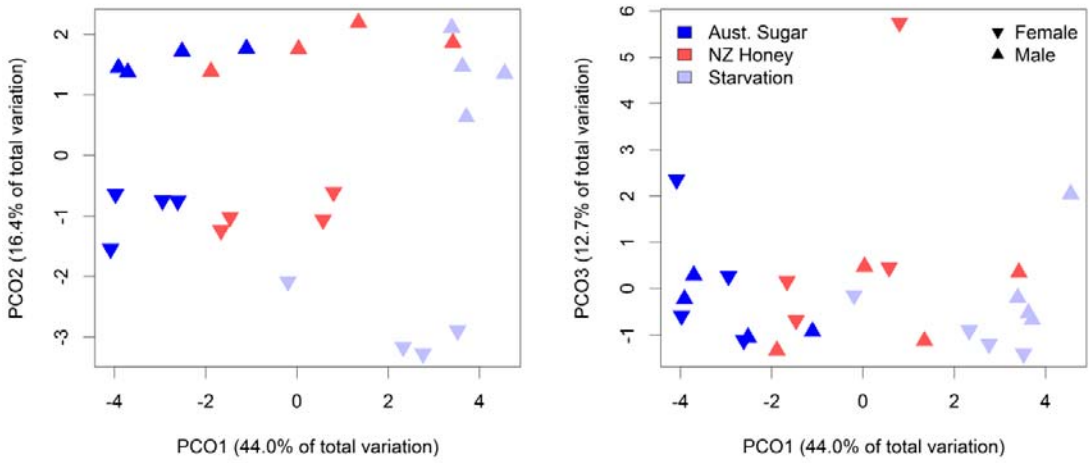


Figure 3.10 The influence of adult moth feeding on whole moth trace element concentration signal. Principle co-ordinate plot of trace element concentration data (ng/g) of *H. armigera* adults given differing adult diet treatments.

The differences in the expression of individual elements between the honey (T4) and sugar (T5) fed adults were also examined (Table 3.6). The univariate analyses show that, with whole moth sample preparations, the concentrations of the majority of the elements were significantly different between the two treatments, despite the fact that the larvae had identical diets. This included As, Rb, Cs and Ba, which are elements of geo-location potential (Chapter 4). It is therefore assumed that the larval point of origin profile for these elements will be masked by the elemental concentration variations of the adult diet. Conversely, Li, Al, Cr, Ni, Sr, Cd and Pb, appeared not to be affected by the cane sugar versus honey adult diet treatments. Of these, Cr, Ni, Sr, Cd and Pb have previously been shown to be elements of geo-location potential (e.g., Poesel et al., 2008).

There were also significant differences in the concentrations of several elements between the genders, as Dempster, et al, (1986) Bowden, et al. (1984) and Sherlock, et al. (1985)had found for essential elements in other Lepidoptera. Of the essential elements in *H. armigera*, the T4 versus T5 univariate comparisons (Table 3.6) indicated that the females had significantly higher concentrations of Ca, Co, lower Zn, and lower, but not significantly different Cu across

both treatments. Of the non-essential elements, the females had higher concentrations of Rb and Sr, and lower Ba. Given that these non-essential elements are of geo-location potential (Chapter 4), it was necessary to separate the effect of gender expression from that of adult diet. Accordingly, ANOVA of the nil adult food (T1) data was also carried out (Table 3.6). These analyses confirm that there was a gender effect, independent of adult diet for Ca, Cu and Zn concentrations, but not for Co. Rb, Sr and Ba were also significantly different between genders in T1, but As and Pb are not affected.

Table 3.6 Trace element concentration data (ng/g) of *H. armigera* adults reared on identical larval diet but given three separate adult diets: no food or water (T1), cane sugar (T4) and honey (T5). $n = 4$ for each gender in all treatments. Univariate ANOVA F-test statistics from the data are also given: n.s. = not significantly different; † = $p < 0.10$; * = $p < 0.05$; ** = $p < 0.01$.

	Li	Al	Ca	Ti	Cr	Co	Ni	Cu	Zn	As	Se	Rb	Sr	Cd	Cs	Ba	W	Pb
T1 male av	13	3797	770676	19099	81	62	33	17477	688149	138	438	5164	466	269	11	1116	83	26
SD	3	998	68870	989	12	3	5	1145	19456	5	14	612	93	25	4	188	7	25
T1 female av	10	580	1184146	20002	75	72	36	12903	482164	138	351	8118	1064	223	15	761	65	31
SD	2	56	172533	2522	14	13	3	984	72914	38	30	873	139	36	4	119	13	13
T1 M vs. F p values (df=1,6)	0.149	<.001	0.004	0.553	0.575	0.186	0.272	<.001	0.002	0.999	0.002	0.001	<.001	0.08	0.245	0.019	0.045	0.711
Significance	n.s.	**	**	n.s.	n.s.	n.s.	n.s.	**	**	n.s.	**	**	**	†	n.s.	*	*	n.s.
T4 male av	13	624	577653	14917	79	40	29	14730	576474	120	376	2568	778	290	5	1007	74	25
SD	3	219	123865	3331	12	10	11	3048	126210	36	80	274	211	81	2	195	10	10
T4 female av	20	2413	1008796	15859	88	54	40	12257	473981	98	305	4566	1447	604	5	879	64	29
SD	4	3953	128230	1292	9	2	8	1635	135159	5	55	469	194	807	1	129	17	13
T5 male av	20	443	426588	10517	90	33	266	11025	434882	86	270	1766	841	226	2	855	53	15
SD	10	368	50926	1213	14	7	475	1375	47455	18	49	493	87	81	1	130	5	7
T5 female av	27	559	771355	11991	96	40	35	9549	272334	82	222	3046	1143	185	4	656	50	29
SD	22	301	53992	454	20	8	12	529	40202	8	28	268	313	53	1	102	8	13
T4 vs. T5 p values (df=1,12)	0.289	0.328	0.002	0.001	0.214	0.015	0.349	0.005	0.004	0.03	0.006	<.001	0.287	0.26	0.008	0.022	0.008	0.368
Significance	n.s.	n.s.	**	**	n.s.	*	n.s.	**	**	*	**	**	n.s.	n.s.	**	*	**	n.s.
T4 + T5 M vs. F p values (df=1,12)	0.248	0.358	<.001	0.962	0.301	0.015	0.373	0.062	0.019	0.237	0.057	<.001	<.001	0.517	0.197	0.041	0.25	0.124
Significance	n.s.	n.s.	**	n.s.	n.s.	*	n.s.	†	*	n.s.	†	**	**	n.s.	n.s.	*	n.s.	n.s.
Treatment: Gender interaction p values (df=1,12)	0.947	0.418	0.389	0.248	0.833	0.318	0.332	0.609	0.55	0.39	0.677	0.091	0.115	0.403	0.075	0.626	0.469	0.406
Significance	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	†	n.s.	n.s.	n.s.

The effect of adult diet on Sr and Pb isotopic ratios, markers of high geo-location potential (Section 1.3.5), can only be inferred from this data. As the concentration of Sr was not affected by adult diet, it may be assumed that the isotopes of this element will similarly not be affected by adult diet. Conversely, the gender effected elements included Sr, which indicates that the gender of the sample may need to be taken into account when using Sr concentration

and isotope ratios in wild caught moths. The lack of adult diet or gender effect on Pb concentration indicates that the Pb isotopic ratio may also not to be affected by these two variables. These relationships are intended to be researched further in a future project (Holder & Armstrong, in prep).

In summary, the trace element signal in whole moth sample preparations may be confounded by adult diet, nutritional status (i.e., starvation) and gender of individual adult moths, but this could be surmounted for provenance determination by judicious use of specific markers that are not affected, particularly Li, Cr, Ni, Pb and possibly Cd; and potentially Pb and Sr isotope ratios.

3.6 Conclusion

The purpose of the experiments described in this chapter has been to address fundamental uncertainties in biochemical geo-location marker expression in insects – primarily related to hitherto unknown effects of variation in input signal – so that the appropriate level of confidence can be applied in interpretation of such markers in the provenance assignment of insects, particularly in a biosecurity context.

It was found that short term precipitation δ^2H variation did get expressed in the host-plants and the variable plant δ^2H value, with fractionation off-sets, is then expressed in the insect consumer. For the corn+*H. armigera* model system, the period that was 'recorded' may be as short as a discrete two week window. It was also shown that the H fractionation relationship in the corn+*H. armigera* system differs in scale to other insect H fractionation relationships reported in the literature (Hobson, Wassenaar, et al., 1999), and is affected by the maturity of the host fruit, possibly due to an associated change in diet quality effecting δ^2H fractionation in the insect, as has been shown in vertebrates (Podlesak et al., 2008). The results also suggest that in phytophagous Lepidoptera, the δ^2H of the wing is mostly or entirely co-related with the solid component of the moth diet, and not the δ^2H of the plant water they are ingesting. If correct, this interpretation is at variance with a previous theory regarding insects (Gröcke et al., 2006), and the accepted relationship in other categories of organisms, which all have been shown to have ≈30% of their structural H derived from free water (e.g., Hobson, Atwell, et al., 1999).

It was confirmed that different plant species have widely different δ^2H values at the same site (e.g., Chikaraishi et al., 2005). These differing host-plant inputs, as well as varying plant-to-insect fractionation relationships on the different hosts, resulted in intra-population δ^2H variability of 25‰ in *H. armigera*. The confounding effects of polyphagy and gender

(individually) on essential trace element profiles in insects were also confirmed (Bowden et al., 1984; Sherlock et al., 1985). However, several minor elements that have geo-location potential appeared not to be affected by host type nor gender, and some elements were only different in one host of four (Table 3.7), indicating that these minor elements have positive potential as natural abundance markers geo-location and other ecological tracing studies.

Table 3.7 Summary table showing the influence of polyphagy, gender and adult feeding on the expression of variables considered for insect provenance determination. Expressed as ANOVA F-test statistics: n.s. = not significantly different; † = p <0.10; * = p <0.05; ** = p <0.01. The regional discrimination potential of the variable is indicated by rank 1 (largest regional discrimination potential) – 4 (see Chapter 4 for details).

Factor	Li	Al	Ca	Sc	Ti	Cr	Co	Ni	Cu	Zn	As	Rb	Sr	Cd	Cs	Ba	La	Ce	W	Pb	δ^2H	$^{208}Pb/^{206}Pb$	$^{207}Pb/^{206}Pb$	$^{87}Sr/^{86}Sr$
Polyphagy	n.s.	*	n.s.	†		n.s.	*	*	*	*	n.s.	n.s.	*	**	†	**	*	*	n.s.	n.s.	**	n.s?	n.s?	?
Polyphagy, only 1 T affected				√									√	√										
Gender (starved)	n.s.	**	**		n.s.	n.s.	n.s.	n.s.	**	**	n.s.	**	**	†	n.s.	*			*	n.s.	n.s.	n.s?	n.s?	?
Gender (fed)	n.s.	n.s.	**		n.s.	n.s.	*	n.s.	†	*	n.s.	**	**	n.s.	n.s.	*			n.s.	n.s.	?	n.s?	n.s?.	?
Adult feeding	n.s.	n.s.	**		**	n.s.	*	n.s.	**	**	*	**	n.s.	n.s.	**	*			**	n.s.	n.s.	n.s?	n.s?	n.s?
Regional discrim	4			4						4	4	3	2		3	3	4			3	2	2	2	1

It was shown that that the wing tissue of adult *H. armigera* undergoes H turnover, with an age-linked enrichment over the first four days post emergence, as well as H turnover that reflects the adult diet. The age-linked enrichment was attributed to cuticle sclerotization, and resulted in a δ^2H shift of approximately 7‰. The adult diet affected turnover was a smaller effect, with only a partial expression of the water assimilated by the adult moth, e.g., a 40‰ enrichment in the adult diet resulted in a modelled change of < 1.8‰ from the natal δ^2H values. The trace element profile in the whole moth sample preparations was also confounded by adult diet, as well as the nutritional status and gender of the individual adult moth. Therefore, potentially informative markers in the relatively fixed tissues are obscured by the signals of the metabolically active tissues, which reflect an insect's adult feeding and hence recent geographic region. Although some geo-location informative markers may not be always affected (Table 3.7), future research is warranted regarding micro-analytical methods that will enable markers to be sourced from the relatively permanent body structures.

A significant theme in the experiments was the within treatment δ^2H variation in the moths. Such variation needs to be quantified and taken into account for confident insect provenance determination (Wunder & Norris, 2008b). Further, as the variation in individual moths may be greater than that between region or treatment δ^2H average differences, it is recommended

that the number of insects analysed for each region (or data point) be appropriate to the level of variation (Langin et al., 2007). This inherent variation in δ^2H_{MOTH}, when combined with the variation from additional hosts and precipitation δ^2H variation described above, can potentially limit the regional discriminatory power of δ^2H. Therefore, the consideration of additional complementary geo-location markers in entomological provenance studies (Section 1.3.5) appears to be appropriate.

Chapter 4
Biogeochemical markers for provenance assignment of insects: a test of geographical resolution

4.1 Introduction

The purpose of this chapter is to test the potential for multiple biogeochemical markers to determine the point of origin of insects. In contrast to the study of larger organisms, there are few natural abundance insect geo-location studies, and none have provided spatial resolution sufficient to meet the objectives of this thesis (Section 1.3.4). Insect provenance research in the late 1970s to the early 1990s explored the use of trace element profiles. Provenance determination was not achieved in these studies primarily because the technological capacity at the time was restricted to elements that are confounded by physiological processes and concomitant variation (Dempster et al., 1986). More recently δ^2H and $\delta^{13}C$ were successfully used to reveal the natal origins of migrating adult monarch butterflies over eastern North America (Hobson, Wassenaar, et al., 1999). However, there is a profound lack of understanding regarding the imprinting of these markers, and applicability of them for insect provenance determination remains unknown for other places and insect species. The few subsequent insect tracking studies that have used natural abundance biogeochemical markers are all limited by being either preliminary and/or not conclusive (e.g., Menozzi et al., 2007; Tigar & Waldron, 2002), or not validated with respect to linking the profile of the insect samples back to the input signal (i.e., host) of the of insect, therefore fractionation relationships remain unknown (Brattström et al., 2008).

Following a review of the literature (Chapter 1), it was hypothesised that the degree of spatial resolution available with δ^2H will be too coarse to distinguish New Zealand samples from those of other regions, and that $\delta^{13}C$ was unlikely to provide geographical information in a biosecurity context[19]. To overcome these limitations, it is proposed that greater spatial resolution could be achieved by combining Sr and Pb isotope and trace elements profiles with δ^2H from single insect samples; and that this improved geographical resolution will provide the ability to distinguish insects of New Zealand origin from insects of overseas origin.

[19] $\delta^{13}C$ is not considered further in this work

As described in Chapter 1, the $^{87}Sr/^{86}Sr$ ratio in a given bedrock is primarily a function of the age and type of geological substrate (Capo et al., 1998; Sun & McDonough, 1989). Geological heterogeneity then imparts $^{87}Sr/^{86}Sr$ heterogeneity to associated soil and water bodies, and enables $^{87}Sr/^{86}Sr$ to be used as a geochemical marker in several fields including geology (Faure & Mensing, 2005; USGS, 2007), palaeontology (Hoppe et al., 1999), archaeology (Ericson, 1985) and ecology (Kawasaki & Oda, 2005). Terrestrial vegetation takes up Sr from the 'soil exchange complex' reservoir of ions and the soil solution aqueous mixing pool. Thus, the Sr isotopic composition of plants is predominantly that of the labile cations in the soil (Capo et al., 1998), with varying input from precipitation and/or atmospheric Sr (Price et al., 2002). The Sr isotope ratio is ultimately transferred from the plants into the herbivores and subsequent trophic levels (Aberg, 1995; Capo et al., 1998; Price et al., 2002), also with varying, lesser input from precipitation and atmospheric Sr (Frei & Frei, 2011). The $^{87}Sr/^{86}Sr$ ratio has distinct advantages as a marker. It has good soil-target organism expression (Aberg, 1995; Bentley, 2006), does not have large seasonal variations that occur in the stable isotopes of biologically abundant elements, and is robust against biological fractionation (Blum et al., 2000; Smith et al., 1985), anthropogenic processing and specimen deterioration (Rossmann et al., 2000). Thus, $^{87}Sr/^{86}Sr$ is a key tool in provenance determination and migration studies e.g., for ancient mammoths (Hoppe et al., 1999) and humans (Hodell et al., 2004; Montgomery, 2010). The application of Sr isotope tools in hominid research was reviewed by Bentley (2006), and reviews of natural $^{87}Sr/^{86}Sr$ ratio as an environmental maker have been given by Aberg (1995) and Capo, et al. (1998). The potential of $^{87}Sr/^{86}Sr$ for modern natural system tracing has been promoted in review articles (Aggarwal et al., 2008; Hobson, 1999; Holzl et al., 2004; Hood-Nowotny & Knols, 2007; Rubenstein & Hobson, 2004). Bird migration studies using $^{87}Sr/^{86}Sr$ ratio include Chamberlain, et al., (1997 - bone), and Sellick, et al. (2009 - feather). The $^{87}Sr/^{86}Sr$ ratio in fish otoliths has also been used to discriminate migratory patterns (B. P. Kennedy et al., 2000; B. P. Kennedy et al., 2002) and ecological linkages (Blum et al., 2001; Blum et al., 2000; Zitek et al., 2010). In addition, the validity of Sr isotope ratios for provenance determination has been demonstrated in several food tracing studies (Crittenden et al., 2007; Fortunato et al., 2004; Rossmann et al., 2000; Voerkelius et al., 2010; J. B. West et al., 2009). Although examples of natural strontium abundance in insect tracing studies have not been found, the trophic linkage studies of Blum, et al. (2001; 2000) have demonstrated that the use of $^{87}Sr/^{86}Sr$ is possible in insects samples (Chapter 2).

Similarly, heterogeneity in the type and age of rocks enables Pb isotope data to be a highly informative marker in the study of earth and astrological sciences (Halliday, 2004; USGS,

2007; Wood & Halliday, 2010). Pb isotopes are also frequently used to identify point sources of Pb pollution in both soil and atmosphere (Komarek et al., 2008; Rabinowitz, 1995) e.g., Bollhofer & Rosman (2000); Hamester, et al. (1994). Although, Pb isotopic information is less commonly used than Sr in tracking applications, the value of Pb isotopes for provenance determination has been demonstrated in anthropological studies (e.g., Aggarwal et al., 2008; Olofsson & Rodushkin, 2011). The high-precision measurements of isotope ratios allow for a high discriminatory power with minimal statistical manipulation, and it is generally held that it is possible to provide definitive answers from a small number of samples (Cheng & Hu, 2010). This present study is believed to be the first application of Pb isotopic data from insects.

The reviews of Hobson (2005) andRubenstein & Hobson (2005) and S.D. Kelly, et al. (2005) have all stressed the geo-location potential of trace elemental analysis, particularly in combination with stable isotope data. However, although there are many studies that have used artificially enriched trace element marking for insect trophic-interaction and movement determination (Hagler & Jackson, 2001), there have been no successful applications of natural abundance trace element analysis for insect geo-location. In contrast, there are many examples of successful trace element tracking in avian and forensic science (Oulhote et al., 2011). For example, signal fidelity to the feeding grounds of several bird species has been shown (e.g., Parrish et al., 1983; Szep et al., 2003), although, ecological complexities and geological heterogeneity of expression within populations need to be understood and taken into account (Bortolotti & Barlow, 1988; Bortolotti et al., 1990; R. Ramos et al., 2009). However, the geographical discriminating potential of trace element data in ecological applications is not universally accepted. Donovan, et al. (2006) and Torres-Dowdall, et al. (2010) reported unreliable or ineffective provenance discrimination with trace-element abundance for various shorebird species, and Font, et al. (2007) suggested that trace elements in feathers are of limited usefulness as tracers because physiological processes of individual birds determine the uptake and expression of these variables.

These conflicting reports underline the requirement to examine the location to organism signal expression for the system of particular interest (i.e., quantify the 'discrimination factor' – e.g., ^{2}H fractionation), and identify the variables that will provide both geographic resolution and integrity of location-to-sample expression. Disappointingly, such validation of signal expression is frequently not done in ecological biogeochemical studies. Entomological examples where such validation *has* been presented include Blum, et al. (2001; 2000); Bowden, et al.(1984; 1985) and Hobson & Wassenaar, et al. (1999).

The hypothesis that these biogeochemical markers can distinguish New Zealand insect specimens from those of overseas, in a biosecurity context, is tested in this chapter using wild populations of *H. armigera* and contrasts adult moths from several different regions in New Zealand and eastern Australia. The objectives of this trial were to:

1. Determine if the multivariate combination of δ^2H, heavy element isotope ratios and trace element concentration data of New Zealand insects are measurably different and can be reliably distinguished from those of our main trading partner, Australia.
2. Identify the elements that have the potential to contribute to a New Zealand point of origin signature, by querying the intra-region versus inter-region variation of single variables, and thus:
   - Assess if δ^2H can independently provide a unique New Zealand signal
   - Assess the diagnostic value of the isotope ratios of Sr and Pb
   - Assess if trace element concentrations can independently provide a unique New Zealand signal, or a signal that is complementary to stable isotope analysis
3. Evaluate the integrity of 'location' (i.e., soil and precipitation) to insect geo-location marker expression under natural conditions

4.2 Methods

Study design

This trial was undertaken on commercial *Zea mays* ('corn') growing properties. Wild *Helicoverpa armigera* specimens, corn cobs and soil samples were collected from three regions in New Zealand and two in Australia. The collection regions in New Zealand were Mid-Canterbury (MC), Bay of Plenty (BP) (Tauranga), and Auckland (AK) bio-geographical regions (Crosby et al., 1998), and in Australia they were the corn growing areas around Toowoomba (Queensland – QLD) and Wagga Wagga (New South Wales – NSW). These regions were selected because they represent geological contrasts and similarities, the model insect and plant species occur together, and they are important areas regarding New Zealand biosecurity. Auckland and the Bay of Plenty are the New Zealand bio-geographical regions that present the highest risk for entry and establishment of pests and diseases arriving from Australia and Asia, as these regions have New Zealand's busiest international shipping ports, they have climate and niche availability that is amenable for exotic species establishment (e.g., Derraik, 2004), and these regions are where most biosecurity incursions in New Zealand are detected (Biosecurity New Zealand PPIN data 2007). Further, these regions are relatively close geographically, thus representing a challenge for separating δ^2H data by region. Mid-Canterbury was selected to give a geologically and geographically distinct New Zealand

region to contrast to AK and BP, and includes New Zealand's second largest urban centre and is hence also a biosecurity risk point of entry. The Toowoomba region was selected as it is the nearest area to Brisbane – the probable origin of the PAM incursion (Kumarasinghe, 2007, pers. comm.) – where corn and *H. armigera* were able to be widely sampled. Similarly, Wagga Wagga represented a reliable source of corn and *H. armigera*, and a region of climatic and geological contrast with the other Australian region.

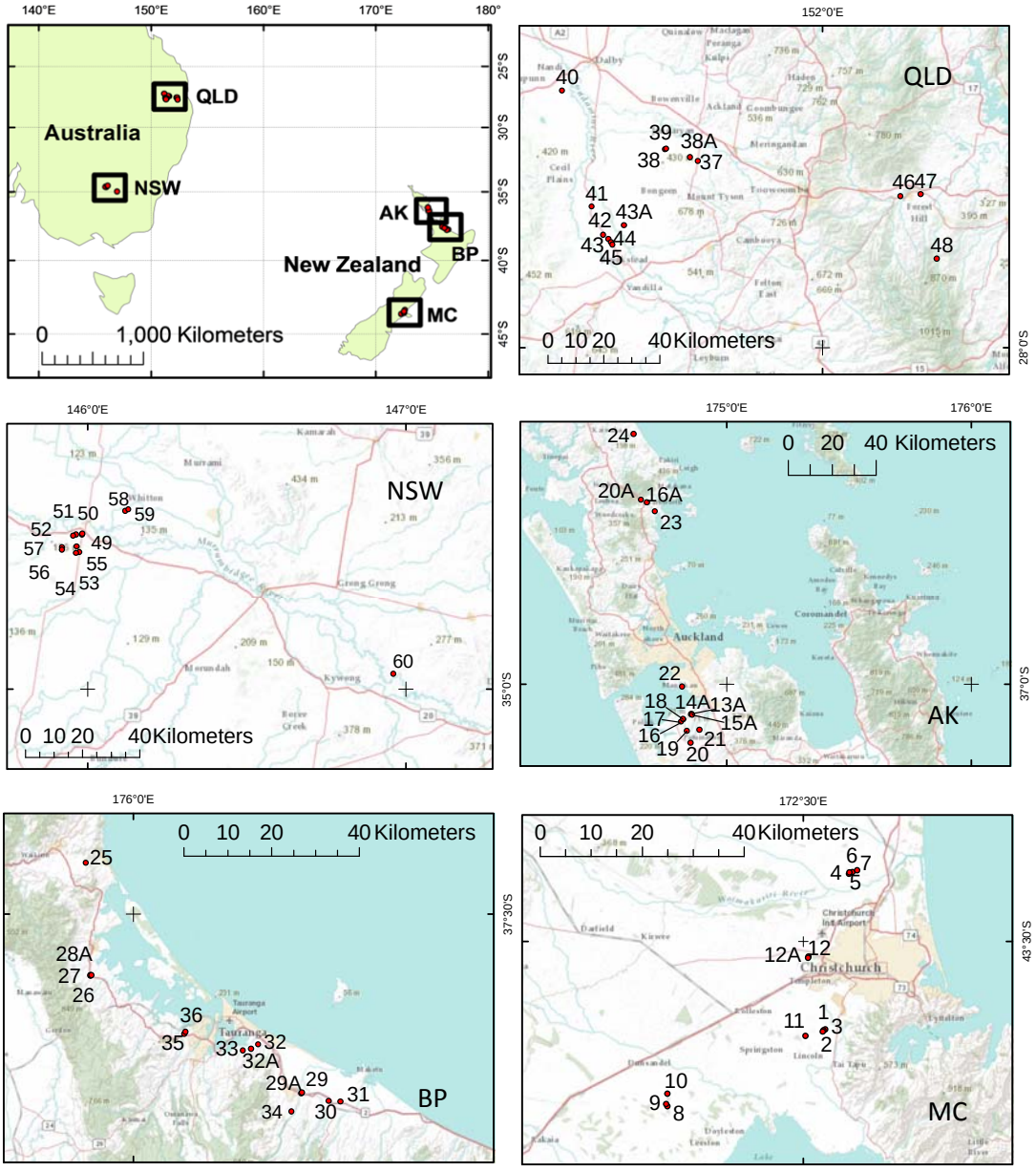


Figure 4.1 Distribution of collection sites (1–60) used for a field test of δ^2H, Sr, Pb isotope and trace element markers for provenance assignment of *H. armigera*. QLD = Toowoomba, NSW = Wagga Wagga corn growing areas, Australia; MC = Mid Canterbury; BP, Bay of Plenty; and AK, Auckland biogeographically zones, New Zealand.

These regions also included locations where precipitation δ^2H data was expected to be collected over similar collection periods (co-ordinated with the National Isotope Precipitation Map of New Zealand CDRP collection effort (Frew et al., 2011)). The distribution of the collection sites within these regions is given in Figure 4.1.

Material was collected during late summer of two consecutive years, 2008 and 2009, so as to also examine inter-year variation. The collection periods are given in Table 4.1. It was not possible to conduct the two collection periods at exactly the same period in the two years, due to differences in the development times for both the corn crop and *H. armigera* between the two summers.

Table 4.1 Collection dates for *H. armigera,* as well as associated host plant and soil samples. (dd/mm).

	MC	BP	AK	NSW	QLD
2008	6/3 – 7/4	19/3 – 21/3	17/3 – 18/3	29/5 – 30/5	26/5 - 275
2009	20/2 – 25/3	19/1 – 26/2	15/2 – 2/3	8/2 – 11/2	5/2 – 6/2

The experimental design used regions as the primary element for comparison, but to enable intra-region variation to be assessed, at least *H. armigera* sample, along with soil and plant samples – from 12 separate sites (paddocks) at each of the five different regions was collected. However, some sites did not yield any adult moth samples, and others provided several. The actual number of samples achieved is given in Table 4.2.

Table 4.2 Number of *H. armigera* ('moths'), associated host plant and soil samples.

			MC	BP	AK	NSW	QLD
2008	Soil (sites)		12	12	12	12	12
	Corn (sites)		12	12	12	12	12
	Moths	*n*	17	8	24	22	26
		sites	9	6	11	7	9
2009	Soil (sites)		12	12	12	12	12
	Corn (sites)		12	12	12	12	12
	Moths	*n*	44	38	61	58	36
		sites	8	13	12	11	9

Field sampling

Helicoverpa armigera adults undergo long distance migration in some years (Scott et al., 2006). Therefore, trapping adults was not an appropriate collection method to represent

geographic source populations. To ensure reliable location provenance and to avoid the influence of multiple host plant sources as well as adult feeding (Chapter 3), late instar larvae were collected from corn cobs for subsequent rearing, or as pupae from the soil under the host plants at each site. The field collected larvae were reared individually on their original cob, until they had pupated. Pupae were held in vermiculite in Solo rearing cups and emerged under a constant 25^{o}C, 16:8 light: dark regime. Emerged moths were held without food or water for four days to allow the wings to complete sclerotization (Section 3.5), then euthanized and stored frozen (-20^{o}C), dry in individual 50mm polycarbonate specimen tubes, for later identification and analysis (Section 2.6).

The identification of the collected moths was confirmed by screening to genus by fore-wing patterns; and to species or species group by hind-wing markings. For specimens where species determination was not possible using exterior morphological examination; the identification was confirmed using characteristics of the genitalia (Common, 1953; Pogue, 2004), and DNA bar-coding (Armstrong & Ball, 2005).

The corn and soil samples were collected from the same locations as that of the insects. As *H. armigera* larvae feed primarily in the tips of the corn cobs, and almost exclusively on the kernels, only apical corn kernels were collected. The kernels were collected into numbered polycarbonate tubes and then frozen as soon as possible – usually within 8 hours.

Soil was collected and stored following the TRACE soil sampling protocol (TRACE 2005). Approximately 50 g of topsoil was collected below the litter layer, at each sample location. The soil was stored in the dark at room temperature until processing.

The position of each collection event was recorded using a hand-held GPS unit. Each collection site was given a unique site identifier number, and all the collected material was labelled with the appropriate site identifier.

Processing and Analysis

The samples were sectioned and processed as described in Chapter 2 (Sections 2.4, 2.5). In brief, the insect samples were partitioned prior to the processing required for the various analyses – a set of wings was dissected for $\delta^{2}H$ analysis; the remainder of the moth bodies were used for trace element and heavy element isotope analyses. Sub-samples of the corn were not dried and subject to cryogenic plant water extraction for plant water $\delta^{2}H$ analysis. The remainder of the kernels were cleaned and dried for plant tissue ('solid') $\delta^{2}H$, trace element and heavy element isotope analyses. The soil samples were 'cleaned' of visible plant debris before drying, grinding and analyses.

Analysis sub-sampling regime

All insect and corn samples were subjected to H isotope analyses. However, logistical constraints necessitated the number of samples be reduced to only six moth, plant and soil samples per region for the other biogeochemical marker analyses. Further, due to analytical error, several of the trace element results for the 2008 moths were unavailable, and the number of sample analysed for trace element per region was four for MC, AK, NSW and QLD and only two for BP. In addition to δ^2H, the markers from the 2008 samples were elemental concentrations for Li, Al, Sc, Cr, Mn, Ni, Zn, Ga, As, Rb, Sr, Cd, Cs, Ba, W, Pb; and the Pb isotope ratios $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$. The selection of variables was refined for the 2009 material, and concentration profiles were obtained for Li, Al, Ca, Sc, Ti, Cr, Co, Ni, Cu, Zn, As, Rb, Sr, Cd, Cs, Ba, La, Ce, W, Pb, as well as the H, Sr and Pb isotope ratios (Section 2.2.5).

Statistical analyses

It was necessary to used non-parametric methods for the multivariate datasets (i.e., combined δ^2H, $^{87}Sr/^{86}Sr$, $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$ and trace element concentration data), as the samples sizes were restricted compared to the number of variables, and statistical assumptions regarding the distribution of heavy metal isotope ratios (Pb in particular), could not be sustained (Baxter, 2008; Baxter & Gale, 1998; Oulhote et al., 2011). All data were log (x+1) transformed and normalised, and the multivariate statistical analyses carried out using Euclidean distance resemblance matrices. The moth multivariate datasets were assessed for regional difference using PERMANOVA+ (version 1.0.3) (PRIMER-E version 6.1.13) analysis of variance main test (i.e., overall Pseudo-F) and pair wise comparisons. The moth multivariate datasets were then assessed for regional grouping and discrimination using a Canonical analysis of principal coordinates (PERMANOVA+ CAP analysis). CAP is a resemblance matrix based constrained statistical method for maximising the separation between *a priori* groups in multivariate data space (M. J. Anderson & Willis, 2003).

A dimension reduction process (N. H. Timm, 2002) was assessed for the potential to achieve a combination of isotope and trace element values that maximised the separation between the regions by removing non-informative variables. This was accomplished by first ranking the variables according to their relative contribution to the original CAP regional grouping (assessed using the linear correlations between the variables and the CAP ordination axes) for CAP axes 1 – 3. The least informative variables were eliminated by nominally selecting and discounting those that had a correlation coefficient less than half the largest correlation coefficient on all of the 3 CAP axes (N. H. Timm, 2002). The CAP analysis was then re-run

with both years' datasets, without the least informative variables. The regional assignment of the moth samples was then tested by 'Leave-one-out Allocation of Observations to Groups' cross-validation and re-run pair-wise PERMANOVA tests.

The regional discrimination potential of the individual variables was also analysed using univariate ANOVA overall F-tests (GenStat 14.1). For the most informative variables, pair-wise differences between the regions were tested using the unrestricted LSD test ($\alpha = 0.05$). Moth δ^2H sample sizes were uneven at each site and region, and therefore unbalanced ANOVA were employed. Regression analyses were also conducted on the un-grouped (i.e., not-mean values) these data to test goodness of fit versus latitude.

The relationship between sample sizes and confidence of discrimination was examined using power analyses for the δ^2H and $^{87}Sr/^{86}Sr$ datasets, by calculating the differences between the means of the regions and then the minimum sample size required for each comparison to be 5% significantly different, with a power[20] of 90%, was determined. Power analyses were not carried out for the other markers, and this needs to be considered in future work, along with research into the provenance discrimination potential of small samples size using multivariate datasets.

The integrity of geo-location marker expression from 'location' (i.e., soil and precipitation) to insect signal was assessed for precipitation-corn-moth (for δ^2H) and soil-corn-moth (for all other variables) by correlating the levels in the moth, corn and soil (in a pairwise fashion).

Precipitation δ^2H modelling

Values for precipitation δ^2H at the New Zealand collection sites were predicted from a multiple linear regression model driven by location specific and daily climate variables (Equation 4-1). This models daily δ^2H values by references to the nearest VCSN climate station (Virtual Climate Station Network (NIWA, 2011)). To develop the model, daily climate variables were weighted by daily precipitation amounts to generate monthly-weighted climate means corresponding to precipitation samples measured for δ^2H (Frew et al., 2011). The weighted means of climate variables were then regressed against all available monthly δ^2H (Baisden et al., 2011).

[20] The power of a statistical test is the probability that the test will correctly reject the null hypothesis when the null hypothesis is false (i.e. the probability of not committing a Type II error)

Equation 4.1

$$\delta D = (-1069.01680825756 + 1.56807456075515 * \text{Latitude} - 0.0166287061634354 * \text{Elevation} + 1.06318099644303 * \text{MSLPress} + 1.72028791394397 * \text{TempEarth10cm} + 1.25380241450627 * \text{Radn} - 1.69843289084924 * \text{Tmin} + 1.17920104372668 * \text{WindSpeed} * \text{Rain})$$

Where latitude is expressed in degrees; elevation is in m; MSLPress = Mean sea level pressure at 9am local day (hPa); TempEarth10cm = Earth temperature at 10 cm depth at 9 a.m. local day (°C); Radn = 24 h global solar radiation total from midnight local day (MJ/m^2); Tmin = Minimum temperature over 24 hours to 9am local day (°C); WindSpeed = Average wind speed at 10 m above ground level over 24 hours from midnight local day (m/s); Rain = 24-hour rainfall total from 9 a.m. local day (mm). A similar model for North American precipitation δ^2H has been published by Meehan, et al. (2004).

In Sections 3.2 and 3.3 the critical period that precipitation input is expressed in corn and *H. armigera* was indicated to be a four week period, three weeks prior to field collection. Modelled precipitation data from these periods (specific to each region) were used to assess the correlation (regression) between δ^2H_M and precipitation δ^2H). However, the precipitation δ^2H data from the Australian regions was only available as monthly averages from single main centre collection points in each region. This coarse level of sampling was not sufficient to allow for site specific interpolation, and hence the 'nearby, actual' precipitation δ^2H is used for the Australian correlation assessments. Other sources of predictive Australian precipitation data (IAEA, 2002; Liu et al., 2010) are based on 1962 – 2002 data, and were not used given that long term averages are unlikely to be relevant to the collected material (Sections 3.2 and 3.3).

4.3 Results and discussion

The multivariate data is examined in the initial sub-section, immediately below. In the subsequent sub-sections, the δ^2H, Sr and Pb isotope ratios and trace element concentration data are individually assessed both for regional assignment potential and for integrity of geo-location marker expression.

4.3.1 A multivariate test of provenance assignment

The multivariate datasets from the moth bodies (i.e., δ^2H, $^{87}Sr/^{86}Sr$, $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$ and trace element concentrations) were assessed for regional discrimination for both 2008 and 2009 data sets. PERMANOVA analyses confirmed that there was a regional difference for

both years (2008 Pseudo-$F_{4, 13}$ = 2.4051, p(perm) = 0.0033; 2009 Pseudo-$F_{4, 25}$ = 2.79, p(perm) = 0.0001). Post-hoc PERMANOVA pair-wise tests further discerned differences between the regions (Table 4.3). In the 2008 moth dataset, BP moths were not significantly different from moths from any of the other regions. This was probably due to the very small number of samples from BP in that year (n = 2) – at least for the contrast with the Australian moths. The NSW and QLD 2008 moths were also not significantly different from each other. However, the AK and MC moths were distinguishable both from the Australian moths and each other. In contrast, with the 2009 moth dataset, pair-wise tests showed all the regional comparisons to be significantly different, except for the BP versus MC, and BP versus AK tests.

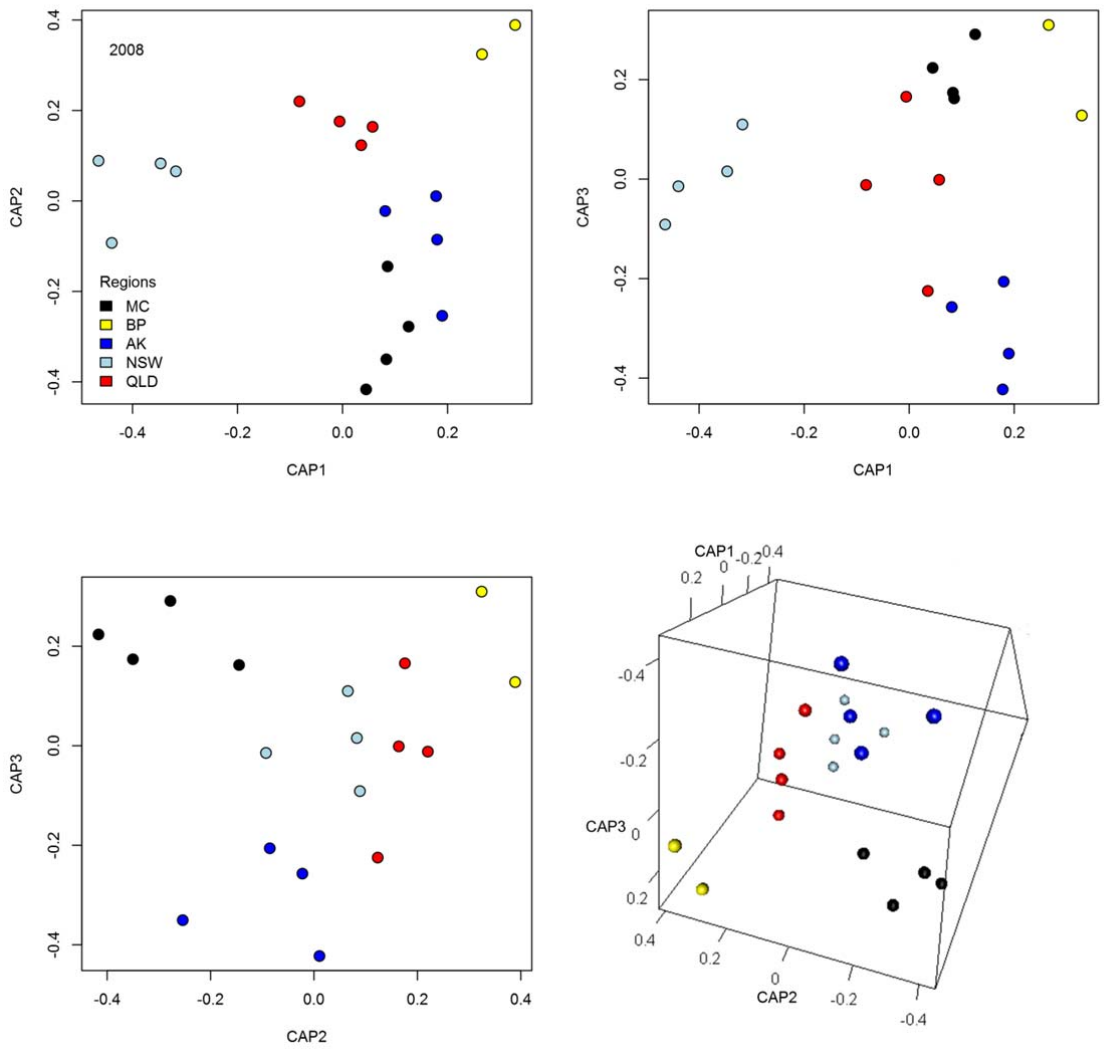
Table 4.3 Pair-wise tests of regional differences for *H. armigera* populations show significant differences between the collection regions. Generated by PERMANOVA analyses of the multivariate datasets (using Euclidean distance resemblance matrices). † = p <0.10; * = p <0.05; ** = p <0.01.

	Regions compared	T	P(perm)	sig
2008	MC, BP	1.2667	0.1329	ns
	MC, AK	**1.5664**	**0.0869**	**†**
	MC, NSW	**1.9746**	**0.0278**	*****
	MC, QLD	**2.2951**	**0.0271**	*****
	BP, AK	1.0209	0.2681	ns
	BP, NSW	1.3102	0.1354	ns
	BP, QLD	1.4058	0.1317	ns
	AK, NSW	**1.5538**	**0.0271**	*****
	AK, QLD	**1.5137**	**0.0278**	*****
	NSW, QLD	1.2158	0.1445	ns
2009	MC, BP	1.2996	0.1372	ns
	MC, AK	**1.8044**	**0.0269**	*****
	MC, NSW	**1.5723**	**0.0299**	*****
	MC, QLD	**1.7403**	**0.0215**	*****
	BP, AK	1.2023	0.1894	ns
	BP, NSW	**1.7564**	**0.0101**	*****
	BP, QLD	**1.6599**	**0.0182**	*****
	AK, NSW	**1.8677**	**0.0023**	******
	AK, QLD	**2.0125**	**0.004**	******
	NSW, QLD	**1.8334**	**0.0029**	******

The more powerful geographical separation in the 2009 dataset was primarily due to the inclusion of the $^{87}Sr/^{86}Sr$ data, which provided robust separation between the moths from the Australian regions, as well as a lesser but still significant difference between the Australian

regions and the New Zealand regions (Section 4.3.3). In addition, the 2009 dataset also included a greater number of informative trace elements variables than the 2008 dataset, including Co, Ce and La (Appendix B.1), all of which contributed to the improved regional separation. The individual variables are considered in further detail below.

The canonical analysis of principal coordinates (CAP, Figure 4.2) gives a visual indication of the geographical resolution achieved between the moths from the different study areas, including the relative differences in the confidence of separation between various regions in multivariate data space. For example, in the 2009 graphs, NSW is most distant from AK and QLD, mirroring the smallest p-values (i.e., the largest statistical significance and highest confidence provenance assignment achieved). In contrast, the regions for which there is less confidence of geographical resolution have contiguous scatter of points, and the regions that are not significantly different have overlapping scatters-plots.



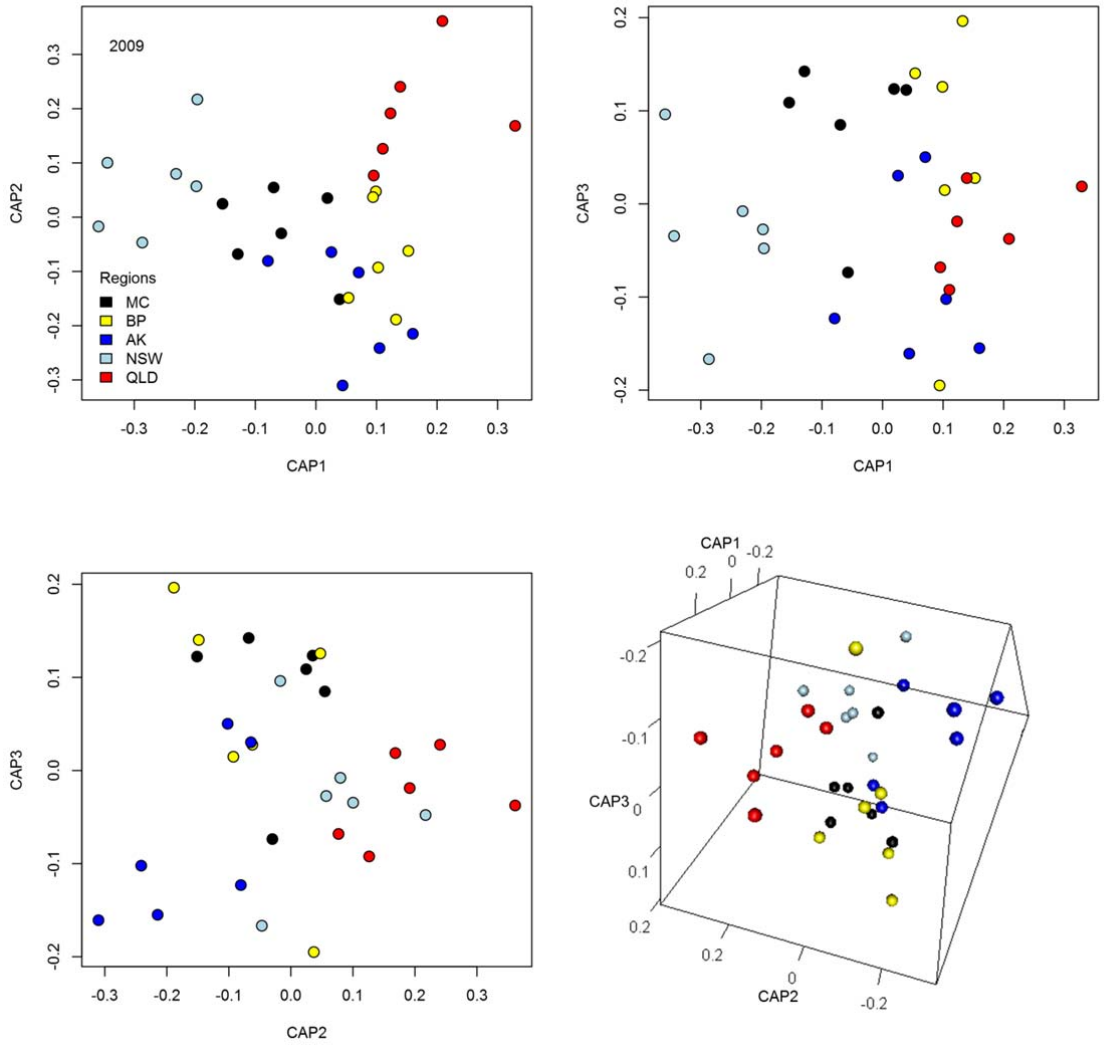


Figure 4.2 Canonical analysis of principle co-ordinates plots of δ^2H, trace element concentration data (ng/g) and ^{206}Pb/^{208}Pb, ^{207}Pb/^{208}Pb data from *H. armigera* adult specimens, reared from Australian and New Zealand sites, March – May 2008 and Jan – March 2009. The 2009 moth data also includes ^{87}Sr/^{86}Sr value; and the 2009 data was optimised to remove non-informative variables. MC = Mid Canterbury; BP, Bay of Plenty; and AK, Auckland biogeographically zones, New Zealand; NSW = Wagga Wagga; QLD, Toowoomba corn growing areas, Australia.

Results for the 'Leave-one-out Allocation of Observations to Groups' cross-validation of the CAP regional assignment are given in Table 4.4. With the 2008 data, this test gave a mis-classification error of 22.2%. The same cross validation process of the larger 2009 dataset had a misclassification error of 26.7%, following the application of the linear dimension reduction process to optimise the dataset (N. H. Timm, 2002). The latter dataset responded positively to the dimension reduction process, with the misclassification error being reduced

from 36.7%. In contrast, with the 2008 data, attempts at 'optimisation' increased misclassification error to 33.3%.

Table 4.4 Cross Validation tests of CAP generated regional assignment using 'Leave-one-out Allocation of Observations to Groups' method for individual moth samples. 2008 scored 14/18 correct, misclassification error = 22.2%; 2009 scored 22/30 correct, misclassification error = 26.7%.

		Classified						
2008	Original Group		MC	BP	AK	NSW	QLD	Total	%correct
		MC	4	0	0	0	0	4	100
		BP	1	0	0	0	1	2	0
		AK	0	0	3	0	1	4	75
		NSW	0	0	0	3	1	4	75
		QLD	0	0	0	0	4	4	100
		Classified							
2009	Original Group		MC	BP	AK	NSW	QLD	Total	%correct
		MC	4	1	1	0	0	6	66.667
		BP	1	4	1	0	0	6	66.667
		AK	2	1	3	0	0	6	50
		NSW	0	0	0	6	0	6	100
		QLD	0	1	0	0	5	6	83.333

A significant finding regarding provenance assignment for biosecurity is that all the moths from the New Zealand regions are distinguished from the Australian moths using the 2009 dataset (see both Tables 4.3 and 4.4). The misclassification of a single QLD moth (as a BP moth) is attributed primarily to the $^{87}Sr/^{86}Sr_M$ value for site 42 ($^{87}Sr/^{86}Sr = 0.708$, Section 4.3.3) being high relative to the other QLD moths. The misclassification between the New Zealand collection regions is attributed to the similarities in the mean values and over-lapping ranges of several of the variables. The mostly dependable New Zealand versus Australia resolution in the 2009 dataset contrasts with the 2008 dataset, where one moth from both AK and BP are misclassified as of QLD origin. As with the pair-wise tests above, the superior inter-country allocation resolution in the 2009 dataset is attributed primarily to the inclusion of $^{87}Sr/^{86}Sr$ as a variable.

Another significant observation is that 100% accurate re-allocation was achieved in only one in five regions with the 2009 dataset and two out of five regions with the smaller 2008 dataset. These misclassification rates indicate that single insect samples will be difficult to

reliably assign to place of origin. Conversely, the discrimination between regions appears to be more reliable when the sample-sizes are larger, e.g., $n = 6$ (Table 4.6).

As the grouping derived by CAP analysis is essentially "hypothesis generating", it was prudent to subsequently test the group separations independently (M. J. Anderson, 2012). This was attempted via canonical variates analysis (CVA, GenStat 14.1). CVA gave a more distinct regional separation by greater vector loadings on the most informative variables for both 2008 and 2009 datasets than the CAP analysis. However, the CVA results (not shown) are not considered further as conditions regarding samples sizes versus the number of variables are potentially violated (Stevens, 1996). Further, although the requirement for multivariate space normally distributed data, concomitant with metric methods (Baxter, 2008), was addressed via data transformation, the potential for outlying data points leading to over-emphasised groups (StatSoft_Inc, 2011) cannot be discounted; especially given there was extreme Pb isotope values (Section 4.3.4). Validity of the separation of the groups provided by the CAP analyses is considered further in the subsequent discussion regarding the individual variables (Sections 4.3.2 – 4.3.5).

Table 4.5 Relative contribution of the individual variables to CAP regional grouping (expressed as Pearson's correlation coefficient between the variables and the CAP ordination axes). 2-sided significance test expressed as different shades (10%, 5%, 1% ; 2008 data df = 16, 2009 df = 18. 2009 data is an optimized suite.

2008	Li	Al	Sc	Cr	Mn	Ni	Zn	Ga	As	Rb	Sr	Cd	Cs	Ba	W	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr	d^2H	$^{208}Pb/^{206}Pb$	$^{207}Pb/^{206}Pb$
CAP1	-0.515	-0.144	0.129	0.176	-0.149	-0.414	0.092	0.239	-0.323	0.462	-0.462	0.109	0.404	0.239	0.407	0.168	0.283	0.492	0.405	0.482	-0.295	-0.646	0.743
CAP2	0.134	0.422	0.247	0.536	0.301	0.038	-0.062	0.576	0.258	0.490	0.450	-0.386	-0.170	0.581	0.537	-0.159	-0.318	-0.151	0.022	-0.557	0.669	-0.022	-0.084
CAP3	-0.019	-0.310	-0.530	-0.310	-0.212	-0.091	-0.638	-0.100	-0.295	-0.079	0.107	0.226	-0.017	-0.091	0.397	0.709	-0.263	-0.487	-0.423	0.377	-0.478	-0.031	-0.041
CAP4	0.046	0.065	0.181	-0.162	0.145	0.217	0.026	-0.088	0.032	0.280	-0.178	-0.152	0.612	-0.084	0.176	0.245	0.295	0.489	0.025	0.288	0.020	0.521	-0.197

2009	Ti	Co	Ni	Cu	As	Rb	Sr	Cd	Cs	Ba	La	Ce	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr	d^2H	$^{208}Pb/^{206}Pb$	$^{207}Pb/^{206}Pb$	$^{87}Sr/^{86}Sr$
CAP1	-0.306	-0.281	-0.097	0.303	-0.088	0.271	0.089	-0.053	0.100	0.083	0.174	0.150	0.435	0.288	0.256	0.163	0.320	0.690	-0.280	0.194	-0.848
CAP2	0.126	0.255	-0.091	-0.336	-0.413	-0.368	0.630	-0.353	-0.511	0.037	0.006	-0.036	-0.044	-0.535	-0.410	-0.427	-0.472	0.298	0.140	-0.082	-0.096
CAP3	0.401	0.525	0.554	0.196	0.275	0.369	0.046	0.235	0.020	0.729	0.707	0.725	0.189	-0.069	0.314	0.478	-0.092	-0.021	0.418	0.306	0.088
CAP4	-0.120	-0.019	0.421	0.296	0.639	-0.460	0.384	0.411	-0.350	0.021	0.403	0.409	0.512	-0.358	-0.545	-0.533	-0.044	0.054	-0.139	0.402	-0.160

The relative contribution of the variables are indicated by CAP Pearson's correlations (i.e., linear, Table 4.5). These values are measures of the association of the individual variables (ignoring all others) with the CAP ordination axes. The essential, biologically linked elements (Mertz, 1981) are not reliably geo-location informative: Li and Zn had some discrimination power in the 2008 dataset, but these were uninformative in the larger 2009 data set, and the other essential elements did not contribute significantly to geographical

assignment in either year. The most informative variables in the 2008 dataset were: the Pb isotopes, the elemental ratios Rb/Sr, Ba/Sr, Pb/Sr, concentrations of Rb and Sr, δ^2H, and Li, Cr, Ga, Ba and Pb concentrations. In the 2009 dataset, the most informative variables were: $^{87}Sr/^{86}Sr$, δ^2H, concentrations of Pb, As, Sr, Ba, Cs, all the elemental ratios considered, Pb isotopes, as well as the CAP3 informative variables Ti, Co, Ni, La and Ce.

Clearly, the relative discriminating power of the individual variables was not consistent between the two years that were sampled. The most noteworthy examples of inter-year variation are the NSW Pb isotope ratios (discussed in Section 4.3.4 below) and the As and Pb concentration data. Arsenic concentration contributed to the regional separation in the 2009 dataset yet had no significant difference in the moth values in the 2008 dataset; and the Pb concentrations of AK and QLD moths were twice as high in the 2009 moths than the 2008 samples (probably as a result of individual site variable in bio-available Pb). Variables that gave significant regional discrimination in both years were: δ^2H, concentrations data of Sr, Rb, Cs and Ba and Pb, and Pb/Sr ratio, (see also Figure 4.8 and Appendix B.1). Across both years, the variables with the strongest individual discrimination power were $^{87}Sr/^{86}Sr$, $^{207}Pb/^{206}Pb$, δ^2H, $^{208}Pb/^{206}Pb$ and Sr concentration (see Sections 4.3.2 – 4.3.5). Although, on average, the W concentrations were significantly different between the moths in the 2008 dataset, W was not used in the analysis of the 2009 data as several moths (all regions) had W concentrations at or below detectable limits, and hence returning unreliable ng/g values.

The potential for provenance discrimination between insect populations presented herein builds on earlier insect biogeochemical provenance studies conducted in the late 1970s to early 1990s (see Chapter 1). The earliest attempts at natural abundance geo-location markers in insects used a small series of common elements, and demonstrated inter-regional profile heterogenity that could enable discrimination. The principal examples include the ambosia beetle (Scolytidae) (McLean & Bennett, 1978); western spruce budworm (Tortricidae) (McLean et al., 1979); brown planthopper, (Delphacidae) (Turner & Bowden, 1983), and *Noctua pronuba* (Noctuidae) (Bowden et al., 1979). However, the common, biologically active elements able to be analysed with the X-ray spectrometry techniques available at the time were confounded by physiological processes which masked the point-of-origin signals (Bowden et al., 1984; Dempster et al., 1986). The only previous successful provenance determination in entomology using natural abundance biogeochemical markers was the geographical resolution achieved by Hobson & Wassenaar, et al. (1998) over continental scales using light element stable isotopes.

The regional separation achieved in this study indicates that multiple biogeochemical markers can provide superior geographical resolution for entomological studies than the light elements isotopes in isolation (see discussion in Sections 1.3 and 4.3.2 and Farmer, et al. (2008)), or the concentrations of the essential elements (Section 4.3.5; Bowden, et al. (1984); Dempster, et al. (1986)). This improved resolution includes being able to distinguish moths from two areas that have close precipitation δ^2H ranges. For example, the moths from AK were distinguished from those of NSW in both 2008 and 2009 datasets. This was achieved primarily due to the addition of the optimised suite of elements between As – Pb as variables that complement δ^2H, as well as $^{87}Sr/^{86}Sr$ in the 2009 dataset.

No other ecological studies using a similar range of variables and data-interpretation have been found for comparison with the provenance assignment results achieved above. The most closely aligned studies are in the more actively researched agricultural commodity provenance arena. The multivariate studies of Pillonel, et al. (2003) and Rossmann, et al. (2000) both reported that dairy products from regions with similar climatic-linked markers (i.e., $\delta^{13}C$, $\delta^{18}O$) may be subdivided further according to their $^{87}Sr/^{86}Sr$ values, and Pillonel, et al. (2005) reported the necessity of multivariate analysis, including multiple trace elements analyses, in commodity provenance studies. Generally, the accuracy of geographical resolution achieved is greater in those studies that have regarded plant based commodities than achieved herein. For example, K.A. Anderson & Smith (2005) demonstrated combined elemental and stable isotope analysis was able to identify the point of origin for Pistachio nuts with 100% accuracy. Similarly, 97.6% correct point-of-origin and temporal classification was reported by Pilgrim, et al. (2010) regarding the provenance of tea using δ^2H, $\delta^{13}C$, $\delta^{15}N$ and trace element profiles.

The following sections report on integrity of expression of the individual variables used above, along with a more detailed examination of their individual contribution to geographic resolution.

4.3.2 Geo-location potential of δ^2H

Further to the examination of within insect-host system δ^2H signal expression in Chapter 3, the potential of δ^2H as a geographical marker for moths is examined in this subsection, with respect to:

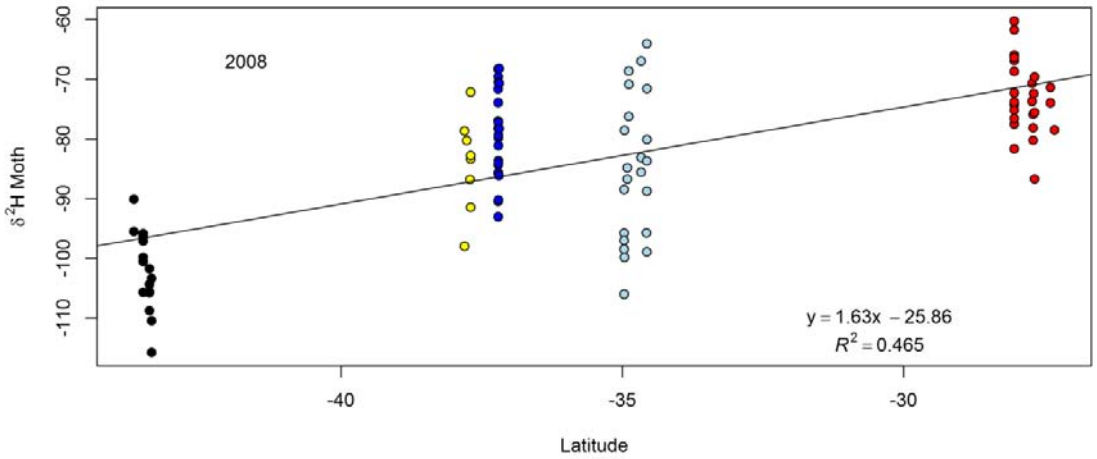
- The capacity for δ^2H, as a single variable, to discriminate moths from different regions.
- The relationship between precipitation δ^2H and the δ^2H values expressed in wild moths (i.e., the integrity of location to moth imprinting).

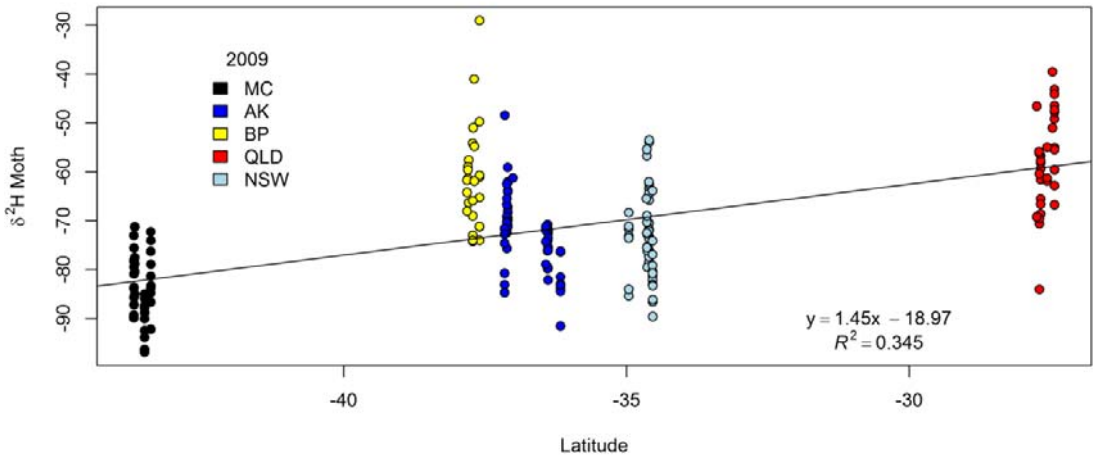
- Consistency in the moth δ^2H signal between years.

The plot of wild *H. armigera* wing δ^2H values (δ^2H_M) against latitude confirms a latitudinal continental scale cline in both years (Figure 4.3). The 'moth δ^2H ‰ per degree latitude' relationship observed is 1.6 and 1.5‰ per degree in the 2008 and 2009 datasets respectively. This linear regression is slightly less than the ≈ 2‰ per degree described by Hobson & Wassenaar, et al. (1999) for monarch butterflies over eastern North America. An overall F-test determined there was significant variation between the regional δ^2H_M means, in both the 2008 and 2009 years (2008 $F_{4, 92}$ =33.67; $p < 0.001$; 2009 $F_{4, 210}$ 56.93, $p <0.001$). Pair-wise comparisons of the δ^2H_M means reveal that, on a population level, the moths from the most southern region, MC, were able to be distinguished from the moths from the more northerly regions, being significantly lighter than all the other regions in both years ($\alpha = 5\%$). However, δ^2H_M values of the other regions were too similar (the 2008 means for BP, AK, NSW and QLD all fall within 12‰) and/or have too much overlap to be reliably distinguished.

Further, BP, AK, NSW and QLD cannot be reliably distinguished over successive years, as the δ^2H_M values for the 2009 dataset were significantly heavier than the 2008 dataset ($F_{1,4}$ = 27.87, $p = 0.006$) and the relative differences between the regions were inconsistent for the two years. For example the 2009 AK mean was 6.4‰ heavier that the AK 2008 value, whereas the 2009 BP value was 22.7‰ heavier than 2008. Within the 2008 dataset BP was significantly lighter than QLD and was not significantly different from AK or NSW; and AK, NSW and QLD δ^2H_M means were all significantly different. In contrast, the 2009 BP mean was only 3.5‰ lighter than QLD, and AK and NSW means were only 0.7‰ different (both non significant differences). Although it is important to appreciate that the collections were made at different weeks in each year, the inter-annual variation in δ^2H_M observed indicates that researchers using insect δ^2H need to correct or specifically calibrate the data for each period of interest (Farmer et al., 2008).

Figure 4.3 *H. armigera* wing δ^2H values from specimens reared from Australian and New Zealand sites, March – May 2008 and Jan – March 2009. The summary table gives the regional δ^2H averages ± 1SD; values within a row that are followed by a different letter are significantly different (Fishers Restricted LSD = 5%).





	MC	BP	AK	NSW	QLD
2008 av	-102.0±6.5 [a] (n=17)	-84.2±8.0 [bc] (n=8)	-79.3±7.3 [b] (n=24)	-85.0±12.0 [c] (n=22)	-72.8±6.1 [d] (n=26)
range	25.7	25.9	24.8	42.0	26.5
2009 av	-84.2±7.0 [a] (n=43)	-61.5±10.6 [b] (n=26)	-72.9±7.4 [c] (n=59)	-73.6±8.4 [c] (n=54)	-58.0±9.5 [b] (n=33)
range	25.7	45.2	43.2	36.1	44.6

The minimum sample sizes required to achieve significant differences for the comparison of the regional δ^2H_M means are given in Table 4.6. These estimations show the potential of δ^2H to discriminate moths from different regions where the means are distinctly different; hence MC can be distinguished from all the other regions by sample sizes of 12 or fewer moths and some comparisons required *n* of only 3 or 4. Conversely, a major limitation of δ^2H as a

marker was also illustrated – where the δ^2H_M means are close and and/or variation is high, the required samples sizes are impractically large. For example, some of the comparisons with NSW require > 1300 samples, and all the NSW comparisons require sample sizes greater than typically collected in biosecurity incursions (commonly 2 – 6 insects). Further, unlike the multivariate test, small sample sizes have unacceptably high misallocation errors (= low power), e.g., with $n = 2$ moths, the most distinct region, MC, returns power values ranging from 0.13 – 0.41 (GenStat 14.1, 2-sided, 2-sample t-Test, significance 0.05). (Single insect samples were not able to be tested).

Table 4.6 Power analysis for moth δ^2H. To detect significant differences between the regional means (Δ), at a two-sided significance level of 0.05 with a power of 0.90 using a two-sample t-test, replication of the calculated sample size (*n*) is required for each sample.

			MC	BP	AK	NSW	QLD
2008	MC	Δ‰		18	23	17	29
		n		**5**	**4**	**9**	**3**
	BP	Δ‰			5	-1	11
		n			**50**	**2166**	**10**
	AK	Δ‰				-6	7
		n				**59**	**21**
	NSW	Δ‰					12
		N					**15**
	QLD						
2009	MC	Δ‰		23	11	11	26
		n		**5**	**11**	**12**	**4**
	BP	Δ‰			-11	-12	4
		n			**16**	**15**	**134**
	AK	Δ‰				-1	15
		n				**1325**	**8**
	NSW	Δ‰					16
		N					**8**
	QLD						

Furthermore, consideration of only the means disguises a large degree of both intra-region and intra-site variation in the δ^2H moth values. Moth δ^2H versus latitude regressions indicates that at least 46% of the variation was due to latitude (R^2=0.45; p<0.001) for the 2008 dataset, and 35% for the 2009 data (Figure 4.3). Thus suggesting that biological and/or localized environmental variation within regions is of equal or greater influence than latitude. Although, unexpected regional wide environmental conditions in BP (possibly unusually high temperatures) are likely to have caused the distribution of the 2009 BP δ^2H_M values to run counter to the latitudinal trend, and thereby contributing to the low R^2 value for the 2009 regression.

The δ^2H_M variation within the regions spanned 24.8 – 44.6‰ (Figure 4.3), with the average variation being 38‰ in 2008 and 38.9‰ in the 2009 dataset. This is higher than the differences between all the regions means. This variation is also greater than found in other studies; specifically the ≈ 26‰ (interpolated) intra-region heterogeneity of the Hobson & Wassenaar, et al. (1999) monarch butterfly dataset; the ≈ 25‰ range observed by Husheer & Frew (2006b) in *Epiphyas postvittana* (Lepidoptera: Tortricidae) in Auckland; and the intra-site variation of ≈ 28‰ reported in *Inachis io* (Lepidoptera: Nymphalidae) (Brattström et al., 2008). However, the results herein have similar variability to that reported by Schimmelmann, et al. (1993), who found a 40‰ within site variation in an unidentified "insect" species (possibly beetle) chitin. Thus the importance of quantifying within population δ^2H heterogeneity (Wassenaar & Hobson, 2006) is confirmed as necessary for insects as it is for birds (e.g., Langin et al., 2007). Such within population heterogeneity needs to be taken in account when using insect δ^2H information in paleoclimate reconstruction (cf. Gröcke et al., 2011), as well as geographical assignment (Holder & Frew, 2010), and is used to propagate error in predictive geographical assignment modelling (Wunder & Norris, 2008b).

Intra-site variation was on average 7.0‰ in the 2008 dataset (heavily skewed by sites where $n = 1$, i.e., range = 0), with a maximum within site range of 29.0‰, and 9.5‰ and 27.8‰ respectively in 2009. Thus, within site variation made up the largest component of the within region variability. As the plants at each site had very similar growing conditions, the intra-site δ^2H_M variation may be attributed to the influence of other biological parameters on individual moth physiological reactions that consequently affect H fractionation. The varying relative contributions of the different physiological paths in exoskeleton development, including sclerotization processes (S. O. Andersen, 1979; S.O. Andersen, 1991) (as discussed in Section 3.5.2) are considered the most likely source of the observed intra-site and within treatment δ^2H_M variation (cf. Miller et al., 1988). Similar intra-site fractionation variation has been found in *Arhopalus ferus* beetles (Cerambycidae) (Holder & Frew, 2010).

In addition, the average precipitation-to-moth fractionation relationships were inconsistent between the years (Figure 4.4, Appendix A), which further suggests that these relationships and hence ^2H_M expression are influenced by environmental factors. The inconsistency in precipitation to δ^2H_M relationship is driven by the 2009 fractionation for BP, NSW and QLD being 10, 6.5 and 17‰ less than 2008. As three of the five points were all skewed in the same direction, these differences are not considered random error. The differences in fractionation are at the plant trophic level (possibly due to drought stress – 2009 was a

drought year) as well as the plant-to-moth trophic interface, mentioned above. Figure 4.4 shows that none of the components of the fractionation relationships were consistent between either the collection years or regions. For example, the corn δ^2H was significantly heavier than the extracted water in 2008 ($F_{1,4}$ 19.09, $p = 0.012$), yet these components were not significantly different in 2009 ($F_{1,4}$ 0.59, $p = 0.486$). The corn-to-moth fractionation was the most highly variable, with an average depletion of -76.4‰ in 2008 and only -37.5‰ in 2009.

The influence of environmental factors other than precipitation δ^2H on the fractionation relationships within the model system is further revealed by contrasting the fractionation results from the different experiments in this project. The water-to-corn fractionation (at natural abundance ranges) was ≈ -20‰ in both the two glass house trials (Sections 3.2 and 3.3), which contrasts to the -2 and +10‰ in the two field trials (Section 3.4 and this chapter)[21]. The differences indicate that that the water-to-plant ^2H fractionation responded to the contrasting environments – with the former trials being conducted under controlled conditions, including optimised watering regimes, whereas the plants from the latter experiments were grown under highly variable field conditions (see Zhou et al., 2011). There was even larger corn-to-moth fractionation differences between the experiments 1.2 (average -22‰), 1.3 (-38‰) and Experiment 4 (-29‰, BP 2009 to -87‰, QLD 2008), which, as discussed is assumed to be due to the physiological responses by the developing moths to varied diet quality and/or other environmental parameters. A similar difference in the monarch butterfly fractionation relationships was described by Hobson, Wassenaar, et al. (1999), where they found a water-to-monarch fractionation of ≈ -40‰ under lab conditions versus ≈ -65‰ with wild caught material.

However, despite the inconsistent fractionation relationships and δ^2H_M variation, the inter-annual variation in δ^2H_M appears to be primarily a function of annual difference in precipitation δ^2H (see Table 4.7). As expected, the relationship between precipitation δ^2H and δ^2H_M had a stronger correlation that the δ^2H_M versus latitude (Figure 4.3). The 2008 dataset had an almost linear relationship. The correlation in the 2009 dataset was also significant ($R = 0.768$), but weaker possibly due to the high variance in the precipitation δ^2H over the months before collection being reflected in δ^2H_M. (As discussed in Section 1.3.1.1, the complex and variable climate of New Zealand (MetService, 2008; NIWA-Science, 2007)

[21]Only the natural abundance δ^2H fractionation observations from Sections 3.2 and 3.3 are used here, as the plants' diminished ^2H discrimination at the artificially enriched source water (page 66) invalidates contrast here.

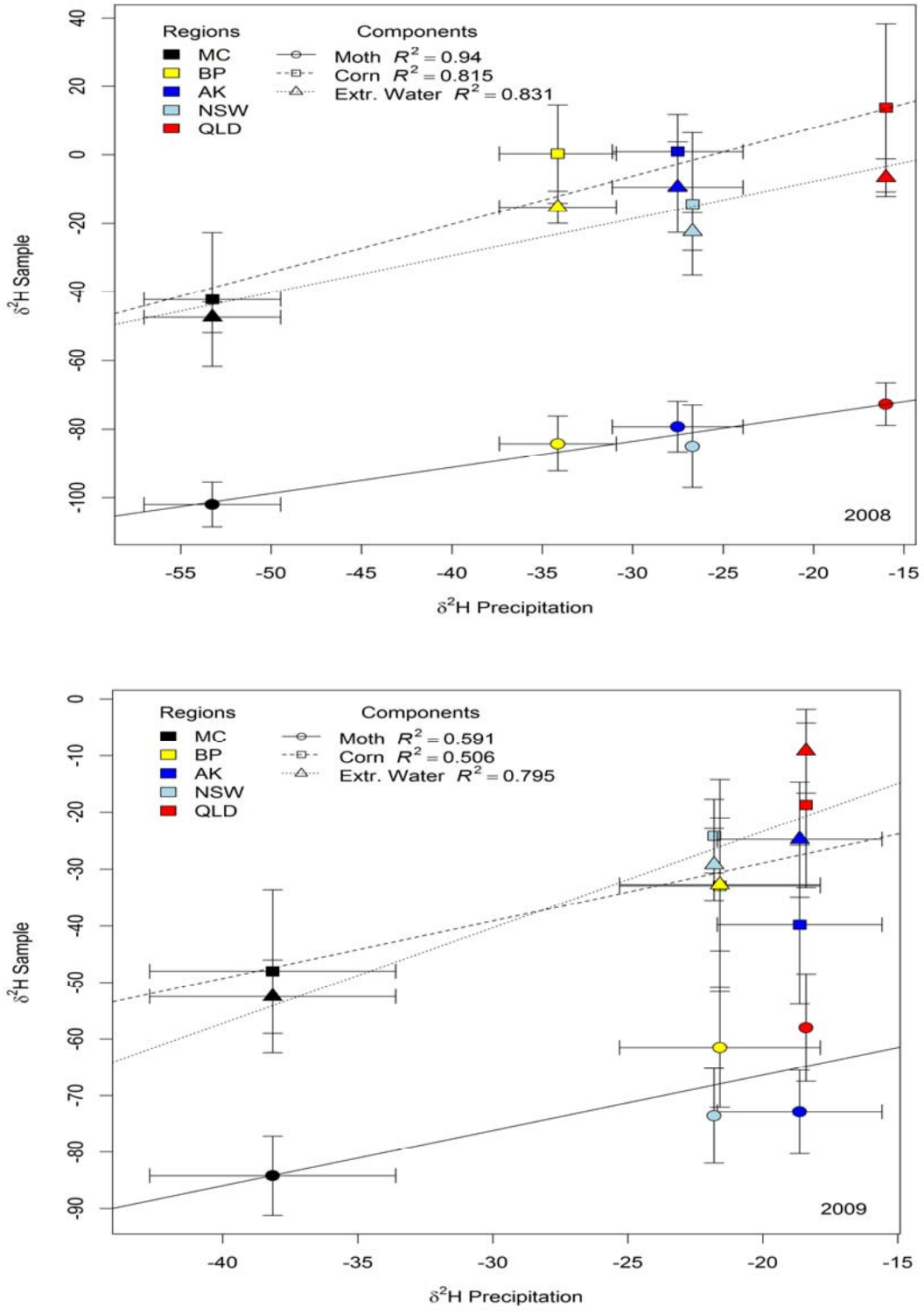


Figure 4.4 δ^2H fractionation relationships relating to wild *H. armigera*, host plant solid, extracted plant water (from corn kernel) and precipitation from the Australian and New Zealand regions. Error bars = 1SD. Samples collected March – May 2008 and Jan – March 2009.

presents a highly variable δ^2H/ latitude relationship[22]). These positive covariations are similar to the relationship found in monarch butterflies ($\delta^2H_{monacrh} = 0.62*\delta^2H_{precipitation}$; $R^2 = 0.69$) (Hobson, Wassenaar, et al., 1999), as well as those reported in several species of birds (Chamberlain et al., 1997; Hobson & Wassenaar, 1997; Wassenaar & Hobson, 2000b). The results thus strengthen the hypothesis that the δ^2H profiles of terrestrial insects, like birds, *generally* follow the growth season precipitation δ^2H values. Although the intra-region variationbetween individual moths, discussed above, diminishes the power of H as a marker for small sample sizes; the overall positive correlation, above, indicates δ^2H is an informative entomological post-hoc geo-location marker at the population level. This also supports the relative contribution assigned to δ^2H in the multivariate analysis (Section 4.3.1).

Table 4.7 Precipitation δ^2H for the periods relevant to the collection of wild *H. armigera* and the growth seasons of the host corn used in this experiment. The precipitation δ^2H values for the New Zealand regions are the average for all sites within the region and were generated using a multiple linear regression model; the precipitation δ^2H values for the Australian collection regions are monthly averages from nearby main centres. The precipitation data that pertains to the larval diet growth periods is highlighted in light blue, and is the values used for correlation assessments.

	Nov-07	Dec-07	Jan-08	Feb-08	Mar-08	Apr-08	May-08
MC	-36.4	-26.3	-49.8	-53.2	-54.1	-49.5	-48.6
SD	1.9	1.3	4.5	3.8	2.2	2.8	0.7
BP	-18.9	-18.0	-28.6	-34.2	-27.2	-35.4	-40.4
SD	3.4	2.3	3.1	3.2	1.8	1.4	2.4
AK	-24.4	-21.3	-26.2	-27.5	-34.2	-32.5	-37.2
SD	1.1	2.2	2.0	3.6	1.4	1.3	0.9
NSW	2.6	-7.7	-44.2	-26.7	-6.5	-65.0	-12.4
QLD	-7.1	-2.6	-29.1	-16.0	-1.9	-7.2	-12.2

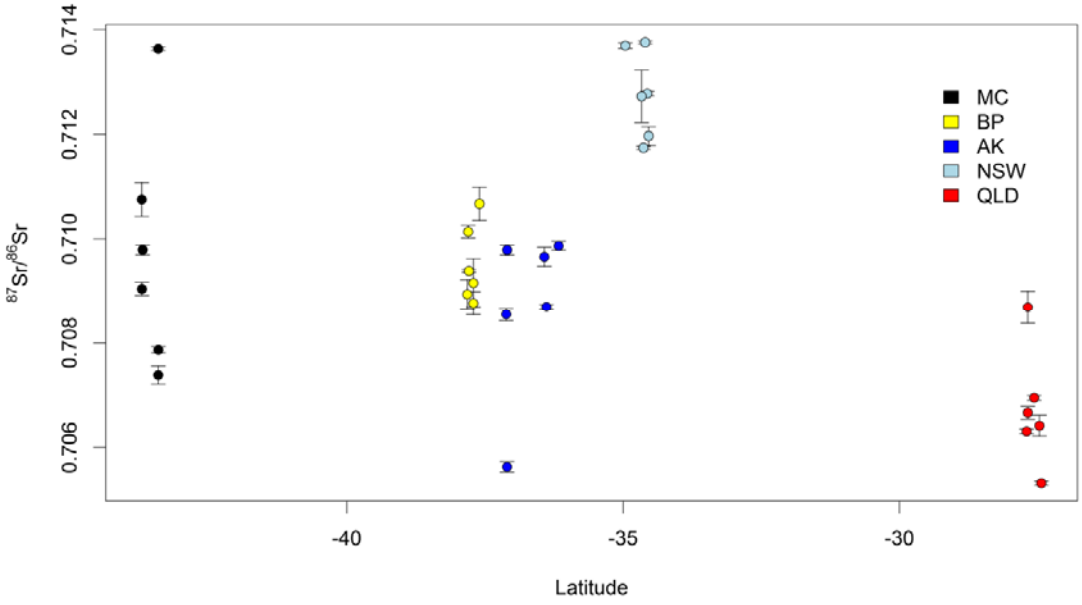
	Sep-08	Oct-08	Nov-08	Dec-08	Jan-09	Feb-09	Mar-09
MC	-43.8	-48.8	-49.6	-50.7	-38.2	-43.5	-33.7
SD	2.6	1.9	1.7	1.6	4.6	2.0	1.4
BP	-30.6	-26.3	-28.5	-21.6	-18.8	-31.9	-27.8
SD	1.7	1.6	2.1	3.7	1.6	1.7	2.0
AK	-24.0	-27.6	-27.0	-18.6	-18.6	-28.8	-21.9
SD	2.3	2.5	1.3	3.1	3.1	1.3	1.4
NSW	-9.9	18.9	6.25	-32.9	-21.8	-5.5	-36.0
QLD	-18.7	11.0	-3.2	8.6	-18.4	-11.9	-9.9

[22] Other drivers for inter-annual isotopic variation in precipitation, including amount and geographical complexities, are discussed by Cole, et al. (1993) and Gleixner & Mügler (2007)

4.3.3 Geo-location potential of strontium isotope ratios

The *H. armigera* $^{87}Sr/^{86}Sr$ results (Figure 4.5) illustrate that there are significant differences between the regions in the moth $^{87}Sr/^{86}Sr$ values ($^{87}Sr/^{86}Sr_M$) ($F_{4, 25} = 14.04$, $p < 0.001$). The NSW moths had the highest $^{87}Sr/^{86}Sr$ ratio (mean $^{87}Sr/^{86}Sr = 0.71278$), and QLD the lowest (mean $^{87}Sr/^{86}Sr = 0.70673$). The New Zealand moth $^{87}Sr/^{86}Sr$ values were intermediate to the Australian regions, with all the NZ regional means falling around 0.709. Pair-wise comparisons confirmed that the New Zealand moth specimens are significantly different from both NSW and QLD moths (Fishers Restricted LSD = 5%). However, the NZ regions were not distinguishable from one-another, with the median $^{87}Sr/^{86}Sr$ values being separated by only 0.0003. The average internal precision of the moth $^{87}Sr/^{86}Sr$ analyses was 0.000143 (2SE); additional quality assurance parameters are given in Chapter 2.

Figure 4.5 *H. armigera* $^{87}Sr/^{86}Sr$ distribution from specimens collected in Australian and New Zealand, relative to degrees latitude south (error bars = analytical 2SD). Summary statistics are given in the attached table: averages ± 1SD; values within a row that are followed by a different letter are significantly different (Fishers Restricted LSD = 5%). *n* = 6 for each region.



Region	MC	BP	AK	NSW	QLD
Average	0.709747 ± 0.00227 [a]	0.709513 ± 0.00074 [a]	0.708701 ± 0.00161 [a]	0.712776 ± 0.00084 [b]	0.706727 ± 0.00111 [c]
Median	0.709420	0.709276	0.709180	0.712750	0.706543
Range	0.0062	0.0019	0.0042	0.0020	0.0034

The significance differences in the regional $^{87}Sr/^{86}Sr_M$ values were strong contributors to the separation shown in the multivariate analysis (Section 4.3.1). Furthermore, the capacity of

$^{87}Sr/^{86}Sr$ to separate the most vulnerable biosecurity regions in New Zealand from the highest risk regions in Australia indicates that strontium isotopes, on a population level, are a potentially powerful tool for provenance determination of biosecurity specimens. However, given that the New Zealand moths were not able to be assigned to region using $^{87}Sr/^{86}Sr$ without impractically large sample sizes (see below), the regional discrimination potential of strontium isotopes as a single variable cannot be assumed, even for places that are geologically distinct and geographically widely separated.

A power analysis with the strontium isotope data showed that the minimum sample size required to achieve significant differences between the Australian and New Zealand regions with $^{87}Sr/^{86}Sr$ alone was 12 or fewer insects (Table 4.8). The power associated with sample sizes $n = 2$ for Auckland (the highest biosecurity risk centre in New Zealand) versus NSW and QLD is 0.46 and 0.14 respectively. The power associated with $n = 2$ for NSW versus QLD is 0.85 (GenStat 14.1, 2-sided, 2-sample t-Test, significance 0.05).

Table 4.8 Power analysis for the moth $^{87}Sr/^{86}Sr$ data. To detect significant differences between the regional means (Δ), at a two-sided significance level of 0.05 with a power of 0.90 using a two-sample t-test, replication of the calculated *n* for each sample is required.

		MC	BP	AK	NSW	QLD
MC	Δ		-0.000234	-0.001046	0.003029	-0.003020
	n		**1096**	**76**	**9**	**9**
BP	Δ			-0.000812	0.003263	-0.002786
	n			**52**	**3**	**4**
AK	Δ				0.004075	-0.001974
	n				**4**	**12**
NSW	Δ					-0.006049
	n					**3**
QLD						

A prominent characteristic of the $^{87}Sr/^{86}Sr_M$ data is the within region heterogeneity. The MC, AK and QLD moths all had $^{87}Sr/^{86}Sr$ ranges of 0.0034 or greater. MC had the most diverse range, probably reflecting geological heterogeneity of an alluvial flood plain (GNS, 2011), }; with the range spanning from 0.7136 – similar to the values that have been reported for both Rhyolite volcanic rock (Barley, 1987) and Metasiltstone metamorphic rock (Adams & Maas, 2004b) in mid-Canterbury, to 0.7074 – which is consistent with Miocene volcanic rock on banks peninsular (C. Timm et al., 2009). The AK $^{87}Sr/^{86}Sr_M$ heterogeneity variety is also consistent with the geological diversity of the region – with a single "low" value, 0.7056 laying within the range of values reported for nearby Greywacke (Adams & Maas, 2004a); and the other five data points cluster around 0.7091 – even though from different parts of the

Auckland isthmus – possibly reflecting Metapelite metamorphic rocks (Adams & Maas, 2004a) and/or input from ocean spray (Faure & Mensing, 2005) (both around 0.709). In regard to the QLD $^{87}Sr/^{86}Sr_M$, no geographically close rock or soil $^{87}Sr/^{86}Sr$ values have been found in the literature, however the $^{87}Sr/^{86}Sr_M$ may reflect local Trachyte (approx. 0.706) or Rhyolite rocks (0.7077) (GeoRoc, 2012). BP and NSW had the lowest $^{87}Sr/^{86}Sr$ ranges – 0.0019 and 0.002 respectively. The degree of within population $^{87}Sr/^{86}Sr$ variation found in the *H. armigera* populations were greater than generally reported for human populations (typically 0.001) (Bentley, 2006; Schweissing & Grupe, 2003), although, the *H. armigera* ranges are consistent with human population samples from geologically diverse area, as in Hodell, et al. (2004). Terrestrial ecology references also report similar within population $^{87}Sr/^{86}Sr$ ranges as found in the *H. armigera*. Chamberlain, et al. (1997) found ranges up to 0.0018 in black-throated blue warblers (but *n* only 2), Sellick, et al. (2009) reported 1SD up to 0.00113 (actual values or range not given) in tree swallow, and Frei & Frei (2011) found $^{87}Sr/^{86}Sr$ values in snail (Pulmonata, family indet.) populations vary by 0.0025. In indet. species of Geometridae and Notodontidae (Lepidoptera) caterpillars at single forest sites, Blum, et al. (2000) found a $^{87}Sr/^{86}Sr$ range of 0.00252, and Blum, et al. (2001) 0.00307, which are both similar to the within site $^{87}Sr/^{86}Sr$ variance found in *H. armigera* (0.00039 – 0.00203).

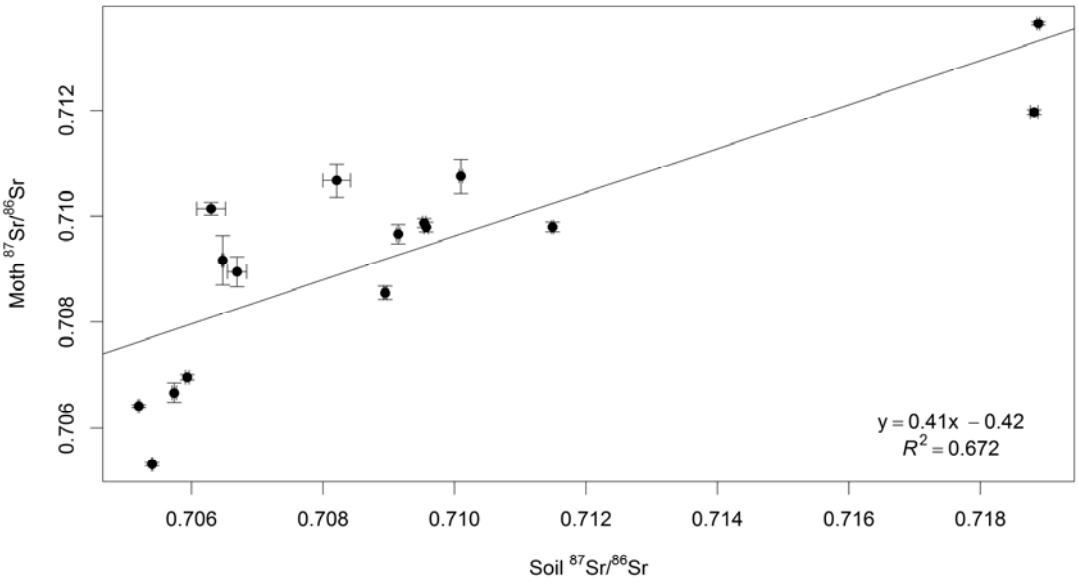


Figure 4.6 The relationship between the moth and soil $^{87}Sr/^{86}Sr$ values. Error bars = analytical 2SD, where error bars are not visible, the error is less than the width of the symbol.

The integrity of soil to moth strontium isotope expression is scrutinized in Figure 4.6. Although the $^{87}Sr/^{86}Sr$ values are highly correlated ($r_{dof=13} = 0.820$), the relationship has a

slope of ≈ 0.4 suggesting there is not a direct relationship. It is unknown if the divergence between the two components was due to either 1) the soil digestion method used accessing the total recoverable Sr and not the bio-available Sr[23]; or 2) sources of Sr in addition to the soil contributing to the moth $^{87}Sr/^{86}Sr$ values. As discussed in Chapter 2, the pool of Sr liberated by total-recoverable (i.e., aqua-regia) soil digestion method is probably significantly different to the Sr in the acid or water exchangeable fraction of the soil that is available to plants (Prohaska et al., 2005; Rao et al., 2008). Although this issue appears not to have been directly evaluated, Sillen, et al. (1998) reported that the total-total soil digestion $^{87}Sr/^{86}Sr$ of their study area was more variable than the labile, bio-available and plant $^{87}Sr/^{86}Sr$, supporting the first possibility. On-the-other-hand, both the intra-site variation (given above) and a highly distinctive $^{87}Sr/^{86}Sr$ value from one of the NSW collection points (Table 4.9; Site 59, 0.729654; value not used above, discussed further in Section 4.3.4) strongly suggest that exogenous sources of Sr can contribute to the moth $^{87}Sr/^{86}Sr$ values. Indeed, the apparently logarithmic shaped relationship in Figure 4.6 may be explained because of mixing with ocean spray (approx. 0.709), with the $^{87}Sr/^{86}Sr_M$ values from coastal sites in AK and BP having the soil values "diluted" toward the oceanic isotopic ratio.

Although a comprehensive examination of the relative inputs of the different Sr sources contributing to the moth $^{87}Sr/^{86}Sr$ values cannot be completed at this point, as the corn $^{87}Sr/^{86}Sr$ values are not available, the relationship(s) can be evaluated by considering the mixing models developed elsewhere for modern vertebrate and invertebrate herbivores (e.g., Frei & Frei, 2011). In human samples the $^{87}Sr/^{86}Sr$ values generally lie between two contributing sources of Sr (mixing model end-members), the local rock and rainwater (Montgomery et al., 2007). In contrast, Sellick, et at. (2009) reported that the $^{87}Sr/^{86}Sr$ of Tree Swallow feathers was influenced primarily by the geology in the area where feathers are grown. Similarly, Blum, et al. (2000) found that the Sr isotope signal of whole caterpillars very closely matched that of the bio-available Sr in the soil.

Given the above, along with the fact that the Australian moths reflected the underlying geological signal of their more distinctive regions (NSW moths are generally consistent with the Darling River clay sediments $^{87}Sr/^{86}Sr$ basalts (Revel-Rolland et al., 2006), the QLD moths with the local Trachyte and Rhyolite rocks (see above)); it was concluded that that the

[23] A method that provides bio-available Sr from soil samples, yet is free of Pb contaminants was not able to be accomplished in the time frame available.

Sr pool in the model insect was predominately that of the bio-available Sr in the soil[24], but at least some of the moths have Sr inputs from sources other than via the plant. Therefore, as biosecurity areas are typically agricultural or otherwise heavily modified areas – and will generally include sources in addition to that of the underlying geology, such as fertilisers and industrial pollution – there is likely to be a higher degree of $^{87}Sr/^{86}Sr$ heterogeneity in plants and animals in such areas – as well as potentially between them (Frei & Frei, 2011; Vitoria et al., 2004).

4.3.4 Geo-location potential of lead isotope ratios

The lead isotope results are given in Figure 4.7. The soil results were generally consistent between the years (2008 versus 2009 ANOVA $^{207}Pb/^{206}Pb$ $F_{1, 4}$ 1.31, p = 0.316; $^{208}Pb/^{206}Pb$ $F_{1, 4}$ 0.17, p = 0.7), despite the different soil extraction methods used[25]. The only significant exception was that the MC soils ranged from approximately 0.8355 to 0.890 on the $^{207}Pb/^{208}Pb$ axis in the 2008 dataset, yet all clustered around $^{207}Pb/^{208}Pb$ 0.835 in the 2009 dataset. Both Australian regions occupied less steep mixing lines than the New Zealand regions. The QLD soil Pb isotopic ratios were consistent with values given for Northern Queensland soils that are affected and partially affected by fertiliser application (Lottermoser, 2009).

In contrast, the corn kernel Pb isotope values were significantly different between 2008 and 2009 ($^{207}Pb/^{206}Pb$ $F_{1, 4}$ = 43.51, p = 0.003; $^{208}Pb/^{206}Pb$ $F_{1, 4}$ = 32.75, p = 0.005). In addition, the corn Pb isotope values were significantly higher in both $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$ than their corresponding soils in all the study regions, for both years (2008 $^{207}Pb/^{206}Pb$ $F_{1, 4}$ = 81.3, p = 0.001; $^{208}Pb/^{206}Pb$ $F_{1, 4}$ = 82.36, p = 0.001; 2009 $^{207}Pb/^{206}Pb$ $F_{1, 4}$ = 39.16, p = 0.003; $^{208}Pb/^{206}Pb$ $F_{1, 4}$ = 17.68, p = 0.014), with the exception of 2009 BP corn, which had values that occupy a similar range to that of their corresponding soils. Overall, the 2009 corn Pb isotope data was closer to the soil values than the 2008 corn values, with the exception of the QLD corn, which had a similar shift (to higher ratios) in both the 2008 and 2009 datasets. For all regions except MC, the corn Pb isotopic values all displayed higher interregional variation than the distribution in ranges found in the soils.

[24] By inference, it is therefore also concluded that the poor relationship observed between total-recoverable soil digestion and the moths' $^{87}Sr/^{86}Sr$, is most likely to be a function to the soil digestion being inappropriate and inaccurate method in regard to bio-available Sr.
[25] Approximate bioavailable Pb 2008, total recoverable 2009.

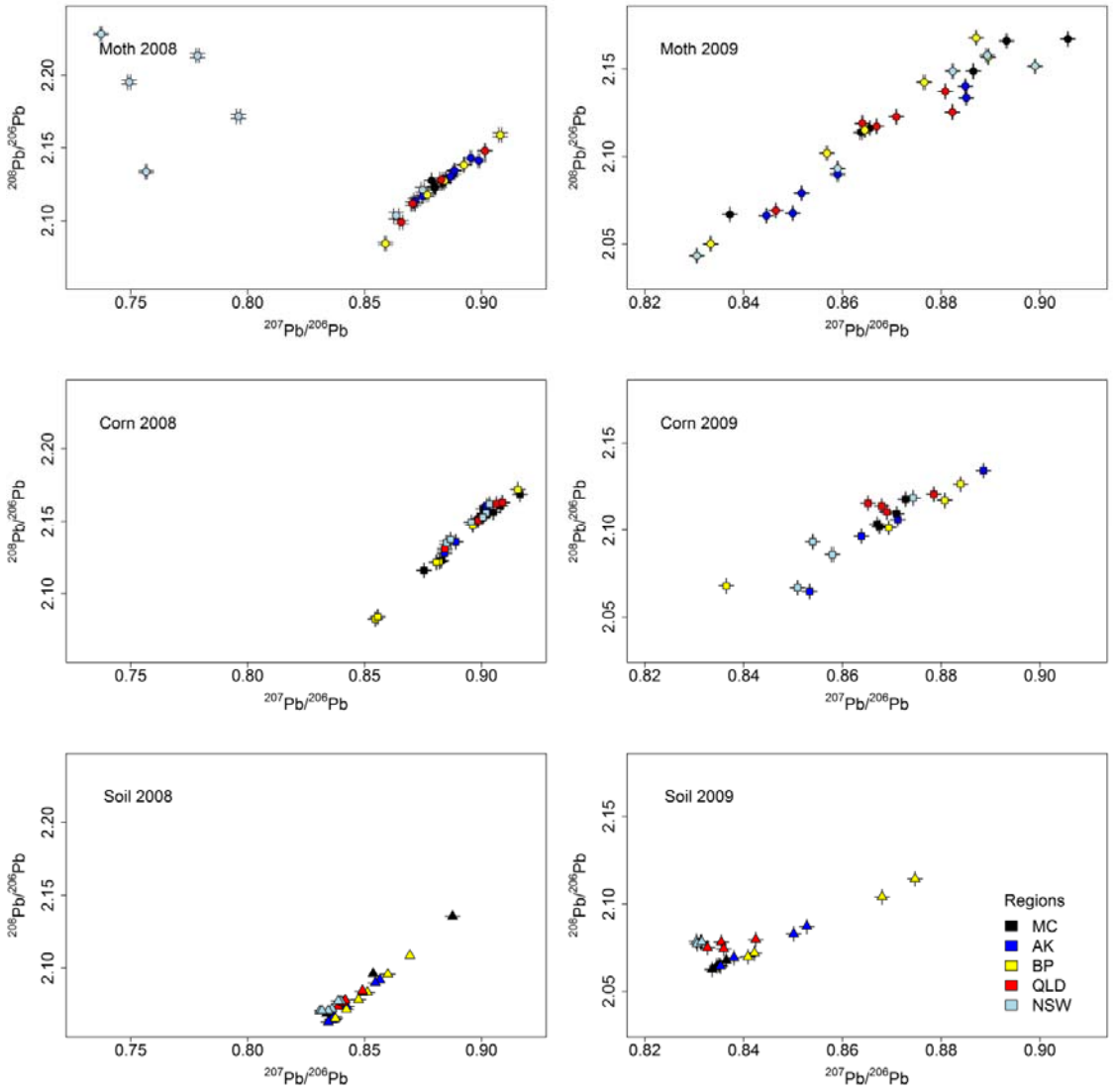


Figure 4.7 Pb isotope scatter plots of *H. armigera* adult specimens, from Australia and New Zealand. The deviation between the trophic levels is considered to reflect mixing with other sources. The analytical error bars (2SD) are smaller than the symbols. Note: the axes for the 2008 datasets are larger scale than 2009.

The discrepancy between the Pb isotope ratios ('shift') of the soil and the corn show that a source of Pb was expressed in the corn kernels that is in addition to that of the soil. These results are consistent with other studies that have shown the primary sources of heavy metal accumulation in plants are both the root uptake from soils and the leaf uptake from atmospheric aerosols (Berthelsen et al., 1995; Dollard, 1986; Harrison & Chirgawi, 1989a, 1989b; Klaminder et al., 2008). The transport of metals from leaves to the plant reproductive structures is also documented (Grusak, 1994; Patrick, 1997; Watmough et al., 1999), including the transport of Pb from corn leaves to the kernels (Cheng & Hu, 2010), which is of direct relevance to the model system used here.

This soil-to-plant shift is consistent with mixing from generalised anthropogenic Pb emissions for SE Australia and New Zealand ($^{207}Pb/^{206}Pb$ 0.909 - 0.917) (Bollhofer & Rosman, 2000). However, the relative contribution of atmospheric deposition of Pb in the study plants is not able to be accurately estimated without also establishing the isotopic compositions of the atmospheric Pb (Cheng & Hu, 2010), and is therefore not attempted here.

The 2008 moth Pb isotope data for MC, BP, AK and QLD were not significantly different to the host corn kernel Pb isotope values ($^{207}Pb/^{206}Pb$ $F_{1, 4} = 2.48$, $p = 0.191$; $^{208}Pb/^{206}Pb$ $F_{1, 4} = 1.78$, $p = 0.253$). In contrast, five out of seven 2008 moths analyzed from NSW expressed Pb isotope values very significantly shifted from the 'expected' NSW plant values (average $^{207}Pb/^{206}Pb$ approximately 0.898, $^{208}Pb/^{206}Pb$ 2.1515), to an 'extreme value group' cluster with the medians values of $^{207}Pb/^{206}Pb$ 0.7566 and $^{208}Pb/^{206}Pb$ 2.1951. To check these extreme values were not the result of systematic error, another pair of 2008 NSW moths were subject to separate analytical preparation runs and measured during separate multi-collector sessions. These had results that were consistent with those in Figure 4.7 – with similar extreme values and non-extreme values, and the repeated moth with the exotic Pb isotopic values was the same individual with the highly distinctive $^{87}Sr/^{86}Sr$ value (Table 4.9, site 59), which both support the validity of the earlier analyses. Also, no site bias was detected, with the extreme value group being from sites evenly spread over the entire NSW collection region (over a distance of approximately 100km i.e., Ganmain to Coleambally, NSW) and one site had both extreme and non-extreme samples.

Table 4.9 Sr and Pb isotopic analysis for repeated moth samples from NSW (collected 2008). The findings are consistent with the prior analysis (Fig. 4.7), in that the 2008/59 moth had both 'extreme' Pb and Sr isotopic values. These exotic isotopic values are considered to reflect mixing with a exogenous source of Sr and Pb. Values are rounded to six decimal places.

Moth ID	$^{87}Sr/^{86}Sr$	$^{208}Pb/^{206}Pb$	$^{207}Pb/^{206}Pb$
2008/59	0.729654	2.228182	0.737315
2008/60	0.712724	2.151718	0.899003

The marked dissimilarity between the NSW host plant and the moth Pb isotope values show that the affected moths had acquired Pb from sources in addition to the host plant. One possible pathway is via or rain irrigation water, however, it was earlier concluded that the larvae do not directly consume free water or plant water, or if so, only to a limited extent (Chapter 3). Similarly, the possibility for the exposure pathway to be via foliar absorption (Armes et al., 1989) is also rejected, as the corn Pb isotopic values were not distorted to the same extent. Further, ingestion of metals from the surface of the plant is considered

improbable, as *H. armigera* feeds inside the corn cob. It is therefore deduced that the additional transport path was respiratory inhalation, and the 'extreme' Pb source is aerosol or dust particulate. Invertebrate acquisition of Pb by inhalation has been shown in snails (*Cepaea nemoralis*) by Notten, et al. (2008). Notten, et al. (2008) also demonstrated that transfer routes of low concentration of contamination Pb can be accumulated and concentrated in invertebrates.

In most cases it is not possible to define the exact point of origin of deposited dust (De Deckker & Norman, 2010; Kamber et al., 2010). However, a comparison of the Pb isotope ratios of the 2008 NSW extreme value group, with the ratios of potential sources allows certain sources of the exotic dust signal in the NSW moth samples to be eliminated as possibilities and other transport paths to be tentatively hypothesized. The source does not appear to be anthropogenic contamination, as the plant-to-moth Pb isotopic value shifts were in the opposite direction to the average anthropogenic Pb emissions for Australia overall (approximately $^{207}Pb/^{206}Pb$ 0.917) and the more regionally specific emissions ($^{207}Pb/^{206}Pb$ 0.909 - 0.935) (Bollhofer & Rosman, 2000), as well as Broken Hill Pb ($^{207}Pb/^{206}Pb$, 0.961; $^{208}Pb/^{206}Pb$, 2.222) (Townsend et al., 1998) or SE Asia industrial Pb sources (Bollhofer & Rosman, 2000; 2001; Komarek, 2008). Very remote possible exceptions are Pb originating from the Mississippi Valley ore deposit ($^{207}Pb/^{206}Pb$ approx. 0.719 – 0.781) (refs in Komarek et al., 2008) and Doe Run Pb smelter, Missouri ($^{207}Pb/^{206}Pb$, 0.741 – 0.763) (Rabinowitz, 2002), both in the USA.

One potential transport path of the exotic Pb input is fine particle dust storms from the interior regions of Australia, as described by Hester & McTainsh (2003); Johnston (2001); McTainsh (1989) and Hesse (1994). The general plume pattern is for the dust to travel from the interior NW of Australia in SE direction (Revel-Rolland et al., 2006). Indeed De Beckker, et al. (2010, fig. 4) modelled a dust plume that passed over the NSW study area in early April 2008 – and the moths were collected in late May. However, the extreme value group have $^{207}Pb/^{206}Pb$ values that do not match values found in the Australian interior aerosols (Marx et al., 2010; Revel-Rolland et al., 2006; Vallelonga et al., 2010), nor are they consistent with mixing with Pb from these regions. Similarly, the extreme values do not indicate mixing with dust from the northern Murray-Darling basin (De Deckker et al., 2010; De Deckker & Norman, 2010).

The extreme Sr isotope ratio from the same collection period and region had a value that is consistent with mixing from the Lachlan Fold Belt (LFB) and Southern Highlands (SH) provinces where $^{87}Sr/^{86}Sr$ values of > 0.74 have been reported (Gingele & De Deckker, 2005).

The LFB/SH area is a significant component of the catchment of the Murrumbidgee River, which bisects the NSW collection region. Therefore the extreme Sr value supports the possibility that the NSW moths could include dust from local river borne sediments. However, the Pb ratios of the moth samples do not support this hypothesis; for although the dust from the Murrumbidgee River banks (i.e., LFB and SH sediment) have a unique geochemical signal, the Pb ratios from those places are probably not extreme enough to give rise to the extreme moth signals (De Deckker et al., 2010), especially when mixing with the Pb from the host-plant is included.

The origin of the exotic signal may be particulate dust from within few a hundred kms west of the collection area. The extreme group had $^{207}Pb/^{206}Pb$ values similar to the range known from Lake Frome, central South Australia soil ($^{207}Pb/^{206}Pb$, 0.7720; $^{208}Pb/^{206}Pb$, 2.066) (Kamber et al., 2010), and soils with similar $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$ values occur near Adelaide, South Australia (Gulson et al., 1981). These locations align with the general pattern of dust storms moving in a in SE direction (Revel-Rolland et al., 2006) and are considered as possible sources of the exotic signal.

A more speculative possibility is the extreme group's Pb values are the result of exposure to volcanic ash plume. On May 2, 2008, the *Chaitén* volcano in Chile erupted and there was a week-long main eruption period (Geology.com, 2011). This eruption period was associated with an extensive ash plume (Ewert et al., 2008; Guffanti et al., 2008) which was detected as atmospheric aerosol drifting over south-eastern Australia around May 10th, 2008 (Carn et al., 2009). The timing of this plume reaching Australia coincides closely with when the *H. armigera* samples collected in late May – as late instar larvae or early pupae – would have been mid-instar larvae. Although Sr and Pb isotope values for the 2008 *Chaitén* plume have not been found in the literature, $^{87}Sr/^{86}Sr$ and $^{206}Pb/^{204}Pb$ values of some volcanic rocks in central south Chile and Argentina are similar to those of the 'extreme group' moths (GeoRoc, 2012), tentatively supporting the possibility that dust of volcanic origin could be a major component of the extreme groups total Pb.

Regardless of the source to this exotic Pb isotopes aerosol, the input is assumed to have occurred over a short space of time coinciding with the development of the larvae. The corn Pb isotope values indicate that the exotic Pb aerosol input occurred too late to be absorbed into the corn via either soil or foliar routes, but that the plume event did occur over a temporal window long enough for the insects to acquire sufficient Pb *via* inhalation for the exotic Pb to make up a major component of the total Pb signal of the insects. The fact that not all of the 2008 NSW moths acquired the exotic Pb signal corroborates this suggestion. Unaffected

individuals may have been at the development stage susceptible to the Pb input (probably mid to late larvae) before the dust plume (and collected as later pupae) or after it (collected as earlier instar larvae). The extreme value group also have more variable Pb isotope values than the non-extreme samples – suggesting unequal exposure times and/or volumes. Further investigation of this phenomenon is warranted. Future researchers could also consider elemental concentration ratios, e.g., Pb/ Ba, that may identify the origin of the exotic Pb signal. Some authors have found that plotting total Pb concentrations versus Pb isotope ratios provides more specific information on mixing and transfer of potential pollution sources (Labonne et al., 2001; Monna et al., 2000). However, no such relationship was found in moth dataset: the moths in the extreme value group did not have unusual Pb or Sr concentration values.

The remaining minority of the 2008 NSW moths and all the 2009 NSW moth samples had $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{207}Pb$ ratios that are on the same broad mixing line of the plants Pb isotopic values, and they were consistent with that of the Murray or Darling sub-basins given in De Deckker (2010) and De Deckker & Norman (2010).

For the other regions, the moth Pb isotopic values for both years lay on the same mixing planes as the plants and the two sets of values were not significantly different (ANOVA for 2008 data given above; 2009 moth versus corn $^{207}Pb/^{206}Pb$ $F_{1, 4}$ 0.1, p = 0.764; $^{208}Pb/^{206}Pb$ $F_{1, 4}$ 4.17, p = 0.111). However, in all the study regions, the moth Pb isotopic values were more variable than the Pb isotopic values of the plants. These two observations indicate that the majority of the Pb in the insects was from the plants and this source was mixed with subtly varying amounts of locally generated environmental Pb –probably aerosol Pb. This is similar to the study of Notten, et al. (2008), where the Pb of the snails was accumulated from both their diet and environmental pollution.

In contrast to the corn data, the moth Pb isotope values for 2008 and 2009 were not statistically different, despite the large group of extreme values in the 2008 NSW moth dataset ($^{207}Pb/^{206}Pb$ $F_{1, 4}$ 0.0, p = 0.949; $^{208}Pb/^{206}Pb$ $F_{1, 4}$ 2.79, p = 0.17). This potentially spurious result is a consequence of the widely dispersed moth data. I.e., the range of the 2008 $^{207}Pb/^{206}Pb$ 2008 moth data is broadly scattered on either side of 0.850, whereas the 2009 moth $^{207}Pb/^{206}Pb$ data sits more tightly around 0.850.

4.3.5 Geo-location potential of trace element concentrations

Of the elemental concentrations considered in the multivariate test of provenance assignment (Section 4.3.1), the elements with atomic number ≤ Arsenic were generally less informative

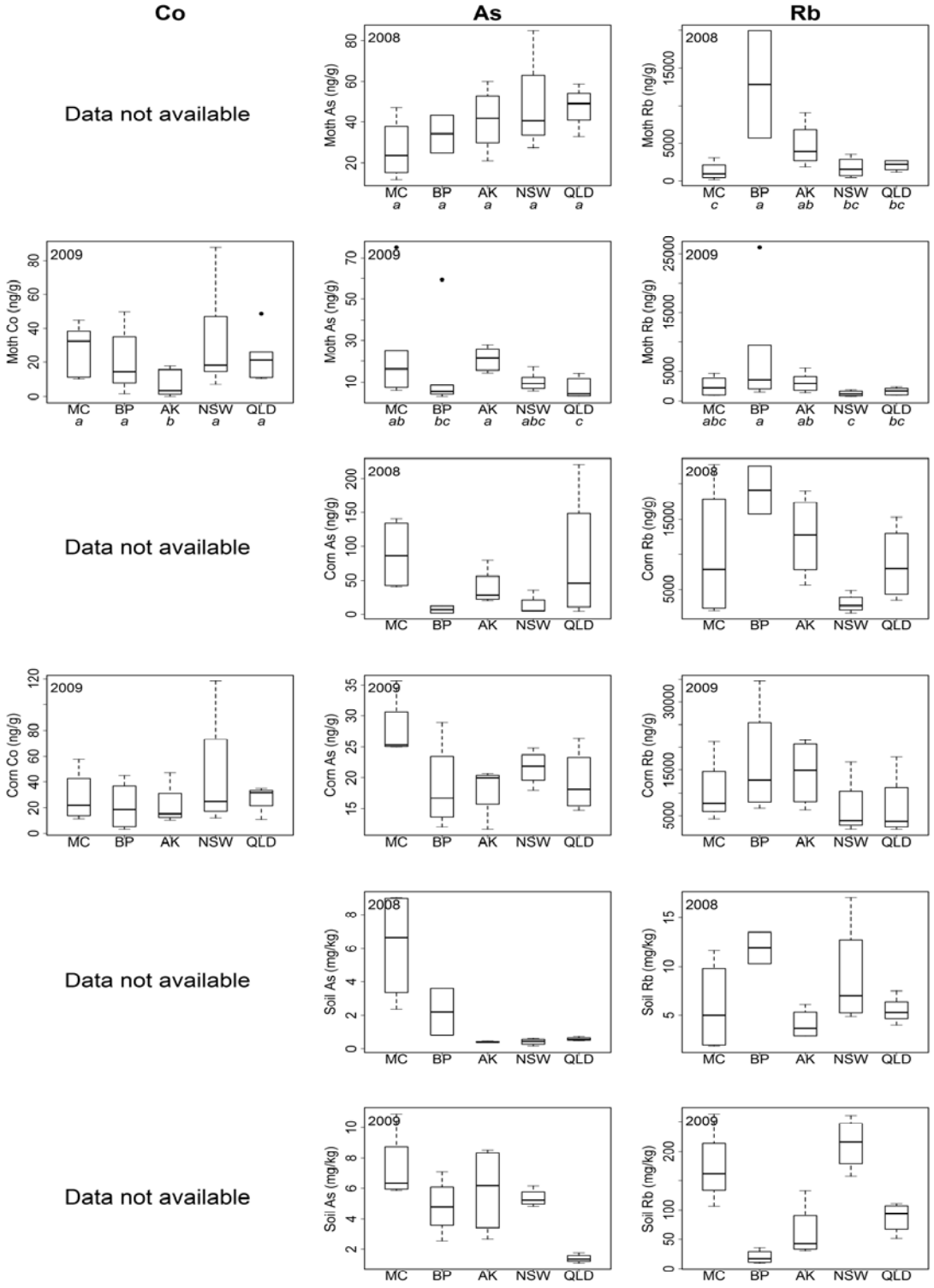
than the elements ≥ to the atomic number of Rb. The most informative trace element concentrations were those of Co, As, Rb, Sr, Cs, Ba; La, Ce and Pb. Except for Ba, all of these were significantly different, univariately, between the regions (results are summarized in Figure 4.8, full results are given in Appendix B.1). Trace element variables that were the most informative in both years are Sr, Cs, Ba and Pb, as well as the Pb/Sr elemental ratio.

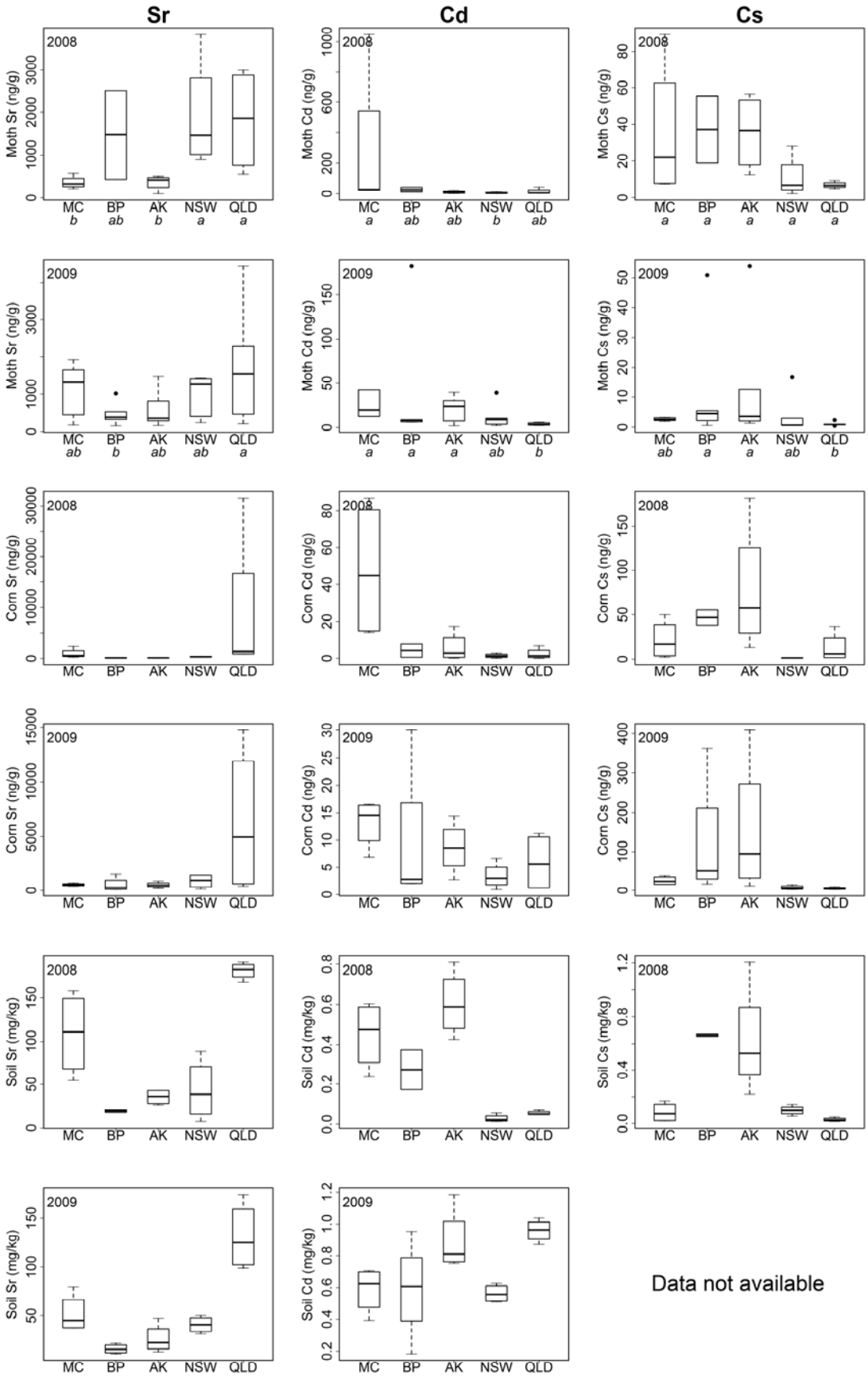
However, the values and the relative contributions of the elemental concentrations were not consistent between years. Further, none of the concentrations alone reliably discriminate moths from all of the different geographic regions, as the statistical differences were only between 2 or 3 of the five regions. For example, the BP and AK moths had the highest Rb and Cs mean concentrations in both years, and MC moths the highest Cd values, yet the other regions were not significantly or consistently different. The lack of a single geographical trace element marker is consistent with other ecological provenancing studies despite significant differences in regional mean values, (e.g., Kaimal et al., 2009). Never-the-less, elemental concentrations clearly contribute to geographical resolution. Strontium, Cd and Cs serve as examples and are described below.

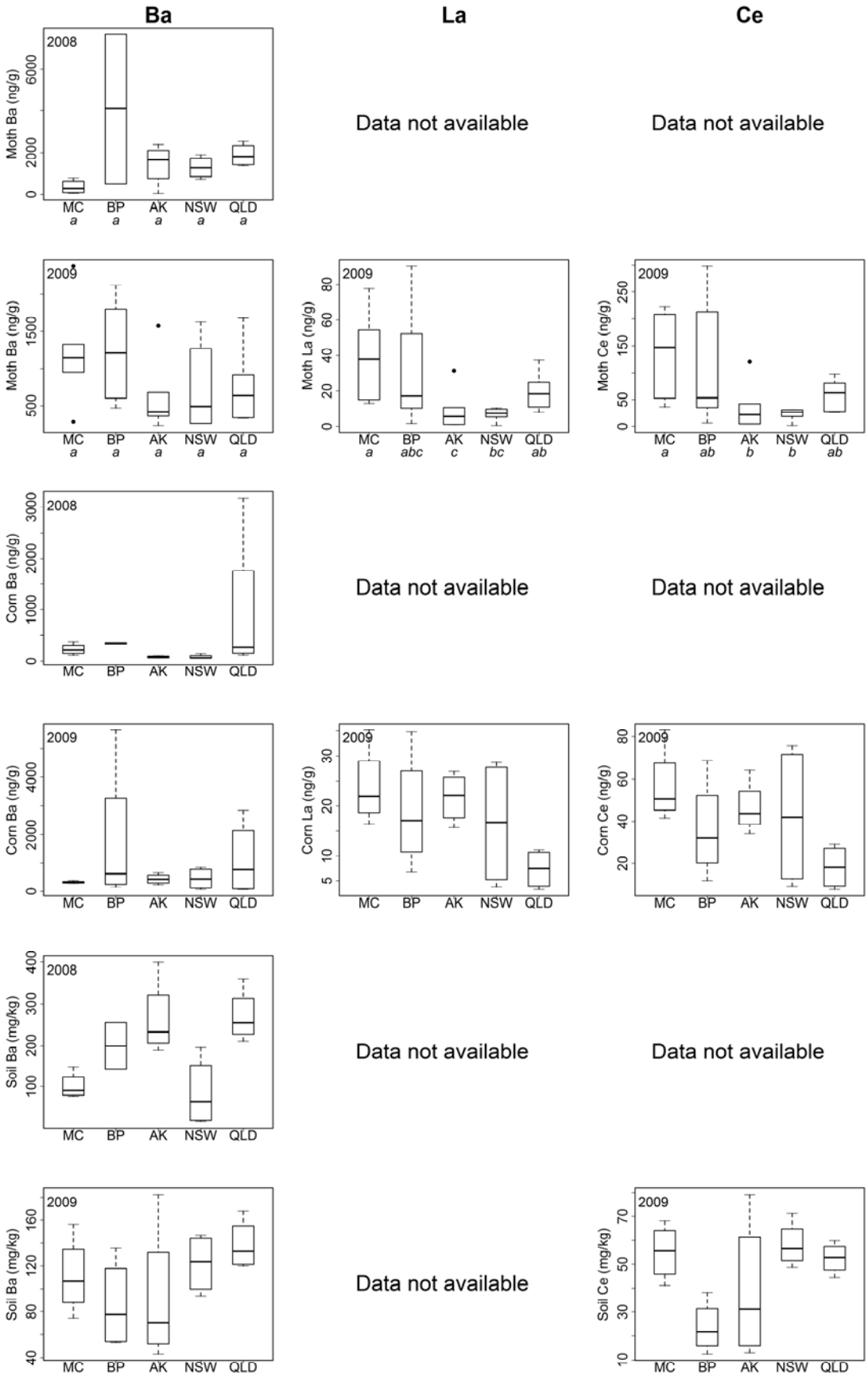
In the 2008 moth trace element data set, the Australian regions had significantly higher Sr concentrations than the AK and MC moths. In contrast, Sr concentration in the 2009 moth dataset were not significantly different overall ($F_{4,25} = 1.69$, $p = 0.183$), although BP and QLD were significantly different from each other in a pair wise test ($\alpha = 0.05$) (Figure 4.8). The geographical resolution potential of Sr identified here, agrees with the pistachio provenance study of K.A. Anderson & Smith (2005), who found that the Sr concentration was the most powerful discriminating element they analysed.

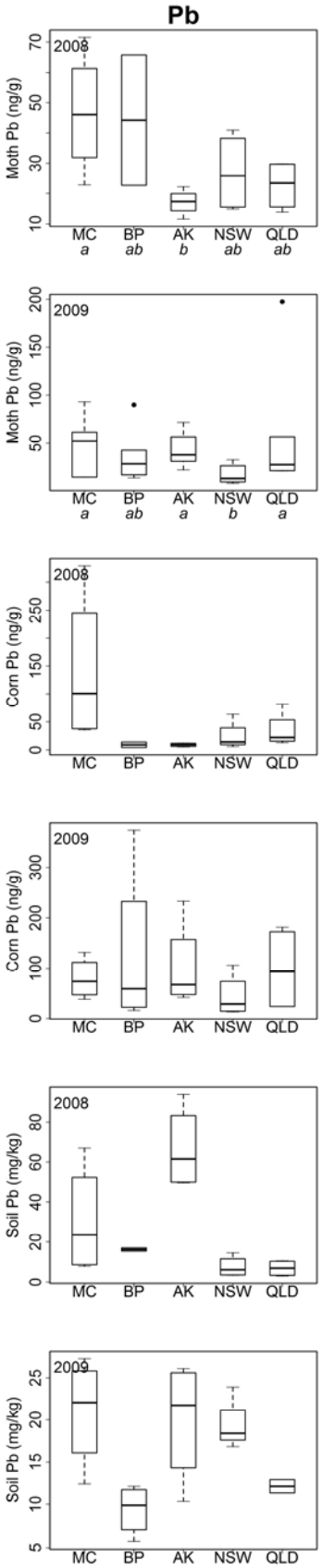
New Zealand moth samples had on average higher Cd levels than the Australian samples, consistent with the literature regarding the elevated levels of Cd in New Zealand agricultural soils (Longhurst et al., 2004; Schipper et al., 2011). However, despite some regional means being significantly different, moth Cd concentration was not a strong driver in the regional separation CAP analysis (Section 4.3.1). This is due to the large degree of intra-region variation in moth Cd concentration values in all the regions, giving poor allocation power on an individual moth basis.

Figure 4.8 Trace element concentration data for *H. armigera* adults, associated host plant (corn) and soil from Australian and New Zealand sites, collected March – May 2008 and Jan – March 2009. Only the most informative elements are shown. Data is displayed as median, quartiles and the minimum/ maximum value within 1.5 inter-quartile range; values outside 1.5 IQR are designated by a circle. Regions assigned a different lower case italic letters are significantly different (Fishers Restricted LSD = 5%). 'Data not available' = information for that element was not recorded or lost due to analytical error. The number of samples per treatment is given in Appendix B.




Sr
Cd
Cs
2008
2009
Moth Sr (ng/g)
Moth Cd (ng/g)
Moth Cs (ng/g)
Corn Sr (ng/g)
Corn Cd (ng/g)
Corn Cs (ng/g)
Soil Sr (mg/kg)
Soil Cd (mg/kg)
Soil Cs (mg/kg)
MC
BP
AK
NSW
QLD
Data not available


Ba
La
Ce
2008
Moth Ba (ng/g)
0
2000
6000
MC BP AK NSW QLD
a a a a a
Data not available
Data not available
2009
Moth Ba (ng/g)
500
1500
MC BP AK NSW QLD
a a a a a
2009
Moth La (ng/g)
0
20
40
60
80
MC BP AK NSW QLD
a abc c bc ab
2009
Moth Ce (ng/g)
0
50
150
250
MC BP AK NSW QLD
a ab b b ab
2008
Corn Ba (ng/g)
0
1000
2000
3000
MC BP AK NSW QLD
Data not available
Data not available
2009
Corn Ba (ng/g)
0
2000
4000
MC BP AK NSW QLD
2009
Corn La (ng/g)
5
10
20
30
MC BP AK NSW QLD
2009
Corn Ce (ng/g)
20
40
60
80
MC BP AK NSW QLD
2008
Soil Ba (mg/kg)
100
200
300
400
MC BP AK NSW QLD
Data not available
Data not available
2009
Soil Ba (mg/kg)
40
80
120
160
MC BP AK NSW QLD
Data not available
2009
Soil Ce (mg/kg)
10
30
50
70
MC BP AK NSW QLD



Moth average Cs concentrations were also consistently higher in the New Zealand samples, although the statistical distribution of the Cs values may limit the potential of Cs as a biosecurity marker – when sample sizes are typically < 6 insects. Most moths had Cs values < 10 ng/g, however the larger mean values were skewed by 2 – 5 moths with Cs values of > 50 ng/g in each years' dataset. However, all the Cs values > 30 ng/g occurred in New Zealand samples and the highest values were most common in BP and AK moths, thus Cs may be a useful geographical marker for New Zealand with larger sample sizes.

These trace element results were consistent with the avian studies of Norris, et al. (2007) and Szep, et al. (2003). They reported a similar suites of elements (Mg, Cd, Sr, Ba, Rb, Cd, Pb) to be the most informative, and similar degrees of intra-regional heterogeneity – resulting from between site differences (cf. within site variation). This intra-regional variation facilitates better near distance discrimination than light element stable isotopes, which typically separate populations on continental scales. However, the findings of Torres-Dowdall, et al. (2010) urge a cautionary interpretation of trace element data. They reported poor re-allocation accuracy for red knot shorebirds (*Calidris canutus*), due to both the lack of trace element marker resolution and because several elemental concentrations, including Sr and Pb, changed as the adult birds aged. Therefore, although the biochemical processes and changes will be different

between birds and insects[26]; as elemental profiles have been shown to also change during the moths' adult stadia (Section 3.5), trace element profiles from whole moths may not be a reliable indicator of point-of-origin.

An analysis of the integrity of trace element concentration expression from soil-to-plant-to-insect is given in Table 4.10, and the relationships of the most informative elements illustrated in Figure 4.9. The moth and the corn kernel elemental values were positively correlated for 6 of the 20 elements and elemental ratios (variables) in the 2008 dataset and 11 of 22 variables in the 2009 dataset. Thus, surprisingly half or more of the elements analysed did not have a significant relationship with the host, and some were negatively related. However, most of the positive correlations occurred in elements that are heavier than As, which are the most geographically informative elements (although in the 2009 dataset, Ti and Cu, which are lighter than As, were also positively correlated). Importantly, the geographically informative elements Rb, Cd, Cs and Pb and the Rb/Sr elemental ratio were positively correlated in both years. Although there was significant variation in expression by individual moth samples, which diminishes the confidence of trace elements as provenance markers when sample sizes are small, these generally consistent relationships impart confidence that trace elements can be geographically informative. The unexpected non-significance of the Sr corn-moth relationship in the 2008 dataset is driven by 3 outlying moth values that had Sr concentrations >2500 ng/g yet the corresponding corn and soil Sr concentrations were close to average. There was also a single outlier corn Sr value > 31000 ng/g (QLD), which may be the result of analytical error. However, removing this data-point did not improve the correlation coefficient, due to the dominant influence of the aforementioned outlying moth values. This variable Sr corn-moth relationship is not linked to the NSW samples with extreme Pb isotope values, as the outlying Sr values are distributed across the BP, QLD and NSW collecting areas.

Contrary to the moth-corn relationship, the moth elemental concentrations were not positively related to the measured soil values for any of the elemental variables, except for Cd in the 2008 samples, Arsenic and Sr in the 2009 dataset, and Ba/Sr ratio in both years. This low moth-soil correlation may be caused by several factors:

[26] The chemical profiles of feathers are believed to be affected by direct absorption from contaminants (Goede & Debruin, 1986), preening behaviour and chemical leaching (Bortolotti et al., 1988; W. R. Edwards & Smith, 1984)

Table 4.10 Correlation analysis for trace element concentrations from the separate components of the *H. armigera* model system. Expressed as Pearson's correlation coefficient values, calculated using logged concentration data (moth and corn ng/g, soil mg/kg). Results for 2-sided significance test indicated (10% = †, 5% = *, 1% = **).

2008 moth versus corn

	Li	Al	Sc	Cr	Mn	Ni	Zn	Ga	As	Rb	Sr	Cd	Cs	Ba	W	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr
correln coeff	-0.463	-0.429	0.122	-0.361	-0.339	0.123	0.099	0.057	-0.057	0.555	0.251	0.556	0.559	0.061	0.064	0.469	0.248	0.715	0.377	0.427
sig (df = 16)	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	**	**	ns	ns	*	ns	**	ns	†

2009 moth versus corn

	Li	Al	Ca	Sc	Ti	Cr	Co	Ni	Cu	Zn	As	Rb	Sr	Cd	Cs	Ba	La	Ce	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr
correln coeff	0.363	0.373	0.07	-0.287	0.462	0.07	-0.059	0.16	0.552	0.236	0.234	0.511	0.409	0.763	0.748	0.52	0.258	0.311	0.379	0.428	0.619	0.595	0.301
sig (df = 18)	ns	ns	ns	ns	*	ns	ns	ns	*	ns	ns	*	†	**	**	*	ns	ns	†	†	**	**	ns

2008 moth versus soil

	Li	Al	Sc	Cr	Mn	Ni	Zn	Ga	As	Rb	Sr	Cd	Cs	Ba	W	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr
correln coeff	-0.341	-0.165	0.245	0.078	-0.009	-0.13	0.049	0.051	-0.627	-0.11	-0.062	0.437	0.283	0.071	0.256	-0.279	-0.306	0.141	0.532	0.306
sig (df = 16)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	†	ns	ns	ns	ns	ns	ns	*	ns

2009 moth versus soil

	Li	Al	Ca	Sc	Ti	Cr	Ni	Cu	Zn	As	Rb	Sr	Cd	Ba	Ce	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr
correln coeff	0.017	0	-0.015	-0.354	0.024	0.071	0.081	0.275	0.246	0.528	-0.35	0.474	-0.048	0.265	-0.026	-0.175	-0.061	0.145	0.452	0.177
sig (df = 18)	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns	ns	ns	ns	*	ns

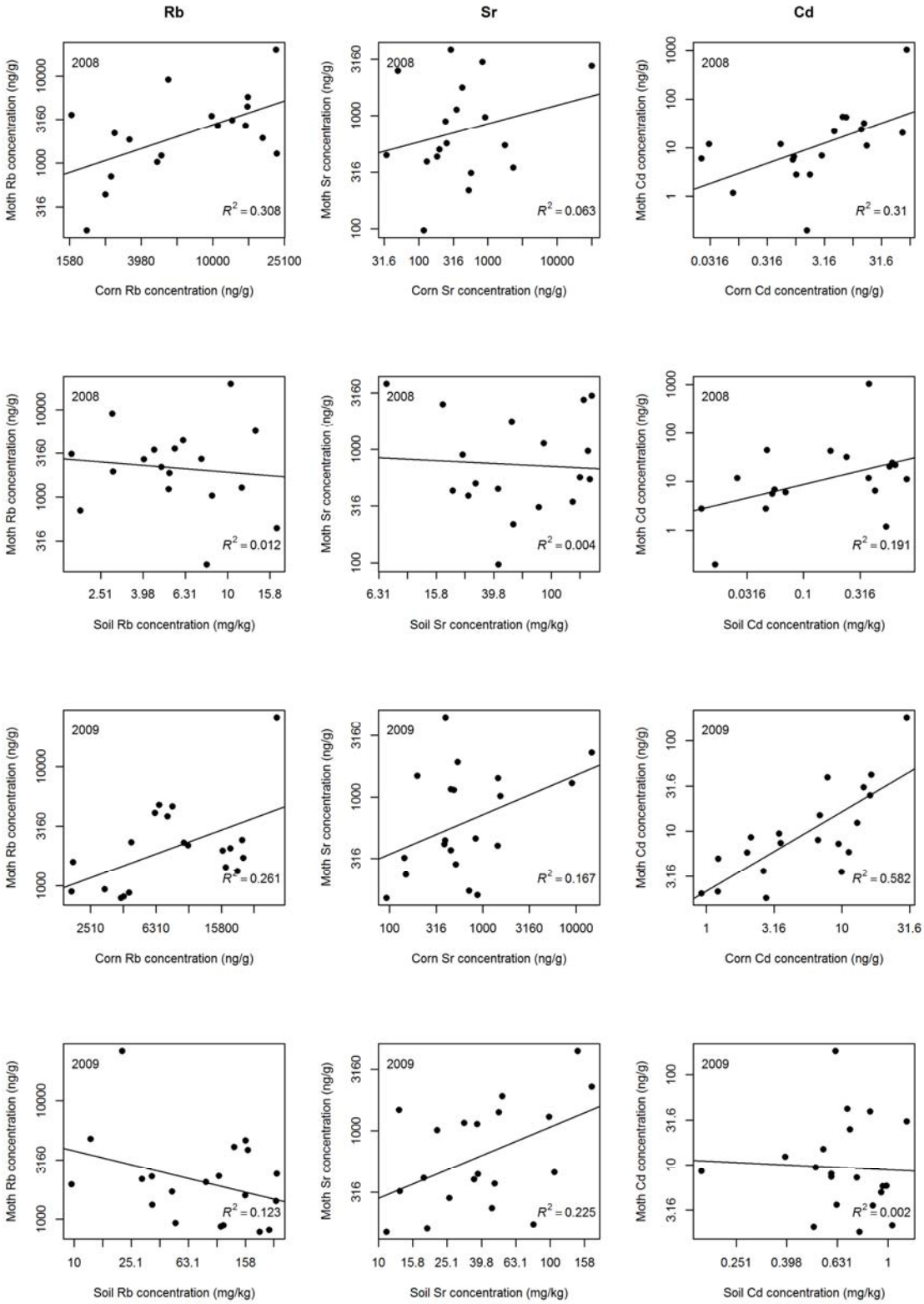
2008 corn versus soil

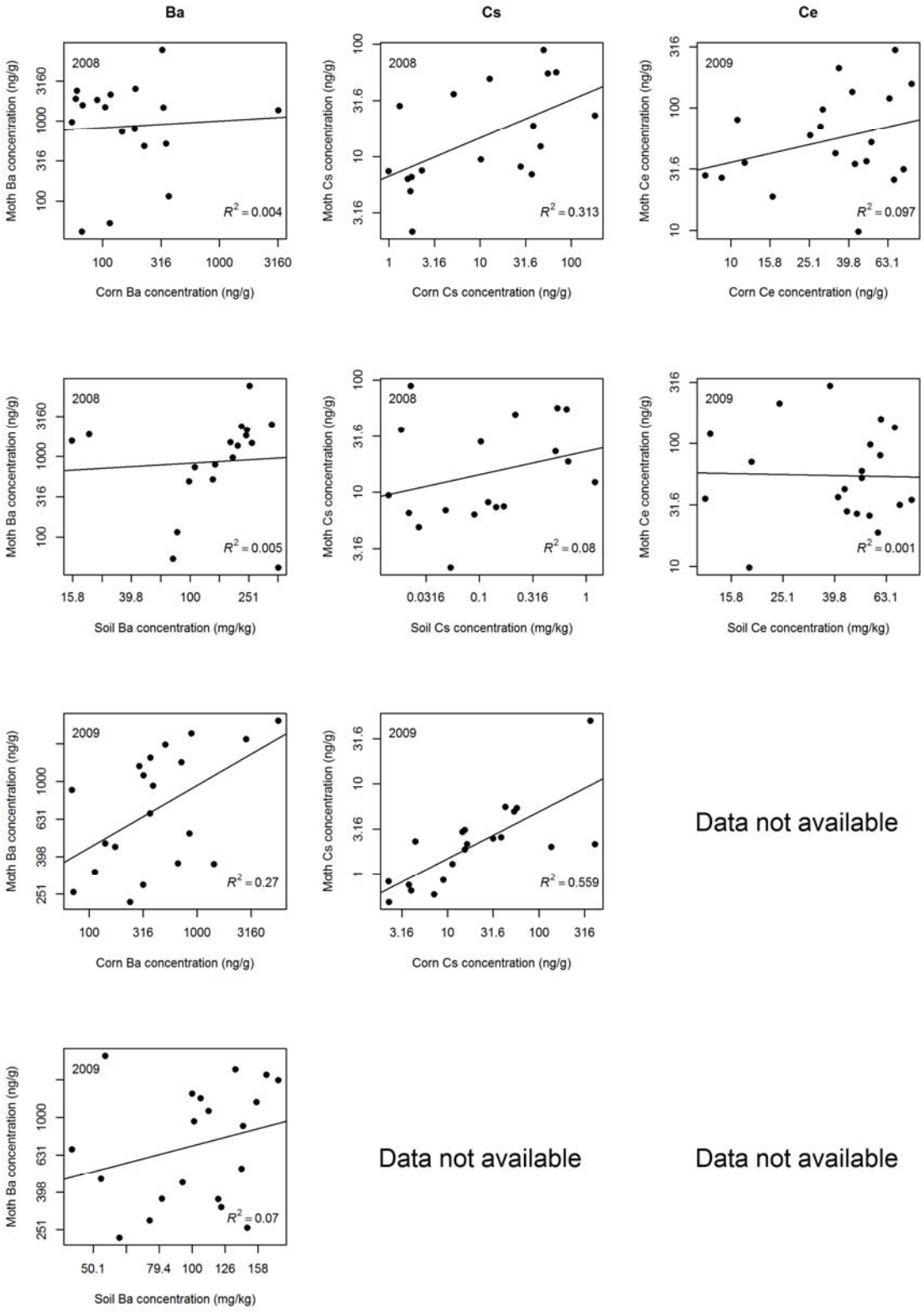
	Li	Al	Sc	Cr	Mn	Ni	Zn	Ga	As	Rb	Sr	Cd	Cs	Ba	W	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr
correln coeff	0.382	-0.03	0.104	0.323	-0.159	0.719	0.392	0.184	0.23	0.063	0.632	0.417	0.498	0.228	0.299	-0.122	-0.188	0.499	0.73	0.454
sig (df = 16)	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	**	†	*	ns	ns	ns	ns	*	**	†

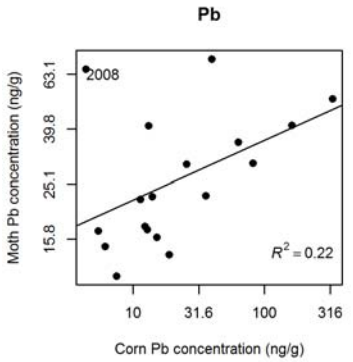
2009 corn versus soil

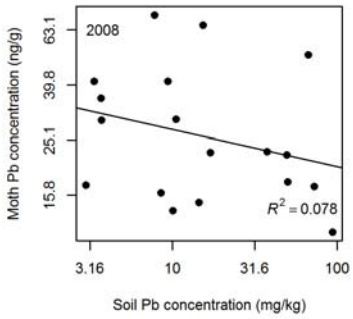
	Li	Al	Ca	Sc	Ti	Cr	Ni	Cu	Zn	As	Rb	Sr	Cd	Ba	Ce	Pb	Rb/Ba	Rb/Sr	Ba/Sr	Pb/Sr
correln coeff	0.422	-0.106	0.281	0.329	0.03	-0.065	0.634	0.177	0.394	0.284	-0.342	0.623	0.112	-0.043	0.069	0.086	0.049	0.319	0.769	0.674
sig (df = 18)	†	ns	ns	ns	ns	ns	**	ns	†	ns	ns	**	ns	ns	ns	ns	ns	ns	**	**

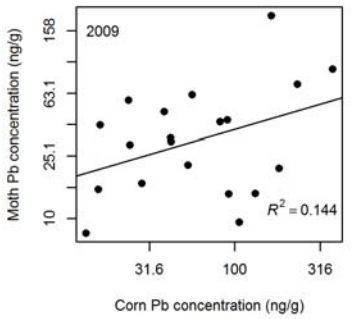
Figure 4.9 Scatter plots showing the trace element concentrations relationship between whole moth digests and their diet (corn kernels) and corresponding soil. Axes are $\log_{10}$-$\log_{10}$.

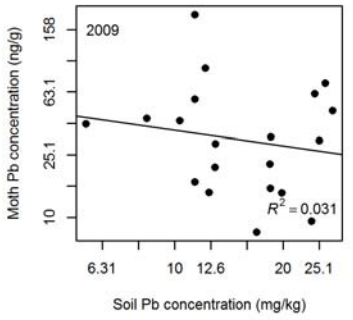



Ba
Cs
Ce
2008
2009
Moth Ba concentration (ng/g)
Moth Cs concentration (ng/g)
Moth Ce concentration (ng/g)
Corn Ba concentration (ng/g)
Corn Cs concentration (ng/g)
Corn Ce concentration (ng/g)
Soil Ba concentration (mg/kg)
Soil Cs concentration (mg/kg)
Soil Ce concentration (mg/kg)
$R^2 = 0.004$
$R^2 = 0.313$
$R^2 = 0.097$
$R^2 = 0.005$
$R^2 = 0.08$
$R^2 = 0.001$
$R^2 = 0.27$
$R^2 = 0.559$
$R^2 = 0.07$
Data not available
Data not available
Data not available









- Heterogeneity in trace element expression between individual moths may be distorting the relationships, as suggested by the soil-corn values being more correlated than the soil-moth values (Table 4.10), and the intra-treatment variation in expression demonstrated in Section 3.4 and elsewhere (Popham & Shelby, 2006). This variation is, in turn, probably caused by more than one influence. Elemental cofactor effect on trace element uptake is highly likely, as the effect of essential element cofactors in insect assimilation pathways has been shown in the closely related *Heliothis virescens* by Popham & Shelby (2006). Elemental cofactor effect on uptake has also been shown in humans (Tapiero et al., 2003), pelagic plankton (Morel & Price, 2003) and simple plants (Merchant et al., 2006). The heterogeneity in expression was probably also caused by subtle differences in biochemical processes in individual moths, which may in turn be genetically linked (Y. Zhang & Gladyshev, 2010) and/or the result of the unique environmental conditions experienced by the individuals.
- The extreme Pb isotope ratio values in the 2008 moth dataset show that exogenous elemental input does occur in the model system, as probably do the outlying moth Sr concentrations, and this phenomena occurs unevenly in some individual insects. However, the generally positive correlations in the corn-to-moth elemental expression in elements heavier than As suggest these irregularities are uncommon and not the cause of the low incidence of soil-to-moth correlations.
- The soil digestion methods used may not have given an accurate profile of trace element biological availability. As discussed in Section 2.5.3, soil extractions frequently

misrepresent the bio-available elemental profile for plants and herbivores (V. H. Kennedy et al., 1997; Rao et al., 2008).

It is assumed that the inconsistent soil-corn-moth relationships in the elements Li to As, were primarily caused by individual variation in biochemical processes, especially as many comparisons returned negative co-relation coefficients. For Ni there was a highly significant soil-corn relationship in both years (indicating the bioavailability of Ni was correctly determined by the soil extraction methods used); yet there was no discernible corn-moth relationship for this element. The 2009 soil Cu values may have been compromised by the extraction methods used, as there was a positive corn-moth Cu relationship, but not for soil-to-corn, contrary to the positive expression of Cu in corn reported elsewhere (Petruzzelli et al., 1989). Zn and As are not considered to be reliable provenance markers even though there were weak soil-corn relationships for these elements, because of the non-relatedness of the corn-moth relationship observed.

For the elements heavier than As, no general correlation trend has been established, and so only the most informative are discussed further in regard to integrity of expression and how this may effect geo-location discrimination potential. The lack of corelation between soil Rb values and both the corn or moth concentrations was likely to be the result of erroneous soil values caused by the over-aggressive extraction methods used to liberate Rb from the non-bio-available proportion of the soil[27] (Rao et al., 2008) e.g., A. White, et al. (2010). The Rb relationship observed here contrasts with S.D. Kelly, et al. (2002), who reported the Alkali metals, Rb and Cs, are easily transported into plant tissues and therefore good indicators of geographical origin.

The soil-to-corn Sr relationship is positively correlated for both years, as is the soil-to-moth relationship in the 2009 dataset. K.A. Anderson & Smith (2005) also found that the Sr concentration in pistachios was similar between years.

As mentioned above, the corn-moth expression of Cd was strongly correlated. In addition, for the 2008 dataset, there was also a positive relationship in the soil-corn and soil-moth transmission. This integrity of expression coupled with the intra-region heterogeneity observed, identify Cd as having high potential to be a near distance natural abundance geo-location marker. The non-significant soil-corn and soil-moth 2009 relationships are almost certainly due to the total-recoverable digestion liberating non-bio-available Cd (Rao et al., 2008).

[27] complex inorganic and biological binding

The positive soil-to-corn and strong corn-to-moth correlations for Cs suggest there is good integrity of expression. However, the weak relationship between soil and moth cannot be explained at this point (data not available for 2009 soil). Given the geographical discriminatory power of this element, along with the preliminarily positive integrity of expression, the use of Cs as a natural abundance geographical marker warrants further study. Cs is used as marker in geological sciences (e.g., McDonough et al., 1992), and has hitherto received only limited, but favourable focus as a geo-location marker (including as Rb/Cs ratio) (Arribere et al., 2006; Ribeiro Guevara et al., 2006).

The value of Ba as a geo-location marker is uncertain. Although Ba was one of the most provenance-discriminating trace element variables in both 2008 and 2009 datasets, the integrity of expression between host plant and the moth was inconsistent between the two years, with no discernible relationship in 2008 yet positively correlated in 2009. Further, the relationship between the soils and the corn was inconsistent with the soil-moth relationship – and neither of these relationships had significant correlations.

Similarly, the value of Ce as a geo-location marker is also uncertain. Ce contributed to the successful geographical resolution in the 2009 dataset, because the concentration in the MC moths was significantly greater than the AK and NSW moths (Figure 4.8). However, none of the soil-corn or corn-moth relationships were significantly correlated.

The relationship between the Pb concentration of the plants and insects, and that of the soil at the point of origin is also uncertain and potentially compromised. The soil-corn and soil-moth values were not significantly related in both 2008 and 2009 datasets. It is unknown if this lack of correlation was primarily caused by the soil extraction methods giving an inaccurate estimate of the bio-available Pb, or the effect of exogenous Pb. The variation in Pb isotopes between soil, corn and moth indicate that sources of Pb, in addition to that of the soil, are mixing in both the corn and the moth (Section 4.3.4). On-the-other-hand, the corn-moth concentration relationship is positively related in both years. Further, both the Pb concentration and isotopic values do provide good geographical discriminatory power – including the highly unique (in time and place) regional specific isotopic signal from exogenous Pb.

The trace element component of this research has paralleled the results of other provenance studies (K. A. Anderson & Smith, 2002; Esechie, 1992). The concentration profiles of a select suite of trace elements in the corn kernels and the moths are an imprecise reflection of the trace mineral composition of the soil and environment in which the plant was growing.

These are, therefore, considered to have geographical discriminatory potential, and be useful for post-hoc predictions of point of origin. However, it remains to be determined whether these markers will be applicable in other insect systems. Species specific differences occur in the processes involved in trace element uptake and bioaccumulation. Thus, to use trace elements as markers, validation of expression is required for entire soil-to consumer food chain for all organisms of interest.

4.4 Conclusion

Using adult *H. armigera* as a model insect, the experiment described in this chapter has revealed that δ^2H values alone cannot discriminate between important biosecurity regions in New Zealand and Australia, although the moths from the most climatically distinct regions were able to be distinguished with samples sizes as small as four insects. For the other regions the by δ^2H mean values are not reliably or sufficiently different and the sample sizes required for reliable discrimination were impractically large. Never-the-less, δ^2H was a significant contributing variable to multivariate provenance separation.

Similarly, none of the other biogeochemical markers assessed were individually able to separate moths from the different experimental regions.

However, a multivariate combination of δ^2H, $^{87}Sr/^{86}Sr$, $^{208}Pb/^{206}Pb$, $^{208}Pb/^{207}Pb$ and selected element concentrations was able to distinguish the region of origin of *H. armigera* for approximately 75% of individual moths. Furthermore, all the moths from the New Zealand regions were distinguished from the Australian moths in the re-allocation validation assessment, indicating that multivariate natural abundance biogeochemical markers can be used to discriminate insects in biosecurity situations. These findings substantiate the superior provenance discrimination from multivariate analyses versus univariate analysis (S. D. Kelly et al., 2005; Luykx & van Ruth, 2008; Ogrinc et al., 2003; Oulhote et al., 2011; Rossmann, 2001).

In addition to advancing the application of insect tracking, this study has highlighted the value of using multiple *independent* variables for provenance assignment. Specifically, δ^2H, $\delta^{13}C$ and ^{18}O are all proxies for climate and therefore approximations of latitude, whereas the Sr and Pb isotopic ratios of the moths appear to be primarily that of the source point soils and underlying geology and are independent of climate.

Another significant finding of this work is that Pb isotopes can also represent a record of different exposure pathways in insects. The high precision of Pb isotope ratio analyses allows

the contribution of separate pathways, that are otherwise indistinguishable, to be determined. In the future, Pb isotopic composition may become a significant tracer in entomological science, and applied to the investigation of ecological linkages and pollution sources, which are hitherto not able to be elucidated. On the other hand, the expression of exogenous Sr and Pb in the isotopic profile of insects' tissues indicates that prudent interpretation of these markers remains paramount.

It was also shown that the integrity of location to moth imprinting is reduced by:

- the degree of variation between individual moth's expression – in all the markers considered
- inconsistent ^{2}H fractionation relationships; and
- variable assimilation of exogenous input influencing Pb isotopic profiles. (The integrity of expression remains uncertain for $^{87}Sr/^{86}Sr$ and most elemental concentrations.)

The within region heterogeneity of marker expression is commonly larger than the between region differences, for all the markers considered except $^{87}Sr/^{86}Sr$. A consequence of this heterogeneity is the strong relationship between confidence of provenance assignment, sample size and the degree of isotopic difference in the potential sources. Thus, both intra-regional variation and the extent of isotopic difference between regions needs to be taken into account when determining the number of replicates required in a sample.

These results provide empirical support for the necessity to quantify within region (or treatment) heterogeneity – for all markers of interest –for reliable spatial resolution (Wunder & Norris, 2008b). The value of soil or place to target organism validation is also underlined, and ideally all major potential exposure paths (i.e., atmospheric as well as food chain inputs) need to be considered when evaluating natural biogeochemical markers. Unfortunately, the majority of provenance studies only compare the article of interest, without including soil-to plant-to consumer validation.

The complexities in biogeochemical marker expression found in this experiment reflects the multifarious expression of univariate markers observed by other researchers e.g., $^{87}Sr/^{86}Sr$ can be influenced by both ecological and geological processes (Frei & Frei, 2011; Montgomery, 2010), and show that such complexity needs to be also addressed in multiple variable biogeochemical tracing. J.W. Moore and Semmens (2008) and Parnell, et al. (2010) have recently advocated the use of Bayesian isotopic mixing models, that reflect natural variation and uncertainty and generate robust probability estimates of the relative contribution of different sources for single isotope systems. Although, the applicability of this type of modelling has yet to be shown in multivariate provenance studies, the future use of such

models may enable more accurate forensic point-of-origin determinations, despite the complex distribution of isotopic signals.

Despite the limitations noted described above, the potential for accurate determination of the origin of insects that has been demonstrated here, using natural abundance biogeochemical markers, is likely to lead to significant further development and use of these methods within entomological science.

Chapter 5
Summary and Conclusions

The determination of the geographical point of origin of an organism or commodity is highly desirable in a wide variety of applications, including in ecological, forensic and commercial endeavours. Confident point of origin information imparts benefits of informing species management decisions (e.g., Sellick et al., 2009), protecting location linked commodity brands and/or food safety (S. D. Kelly et al., 2005) and revealing pathways for illicit trafficking of drugs (Ehleringer, Casale, et al., 2000) and people (Aggarwal et al., 2008). There is also an emerging requirement of point-of-origin discrimination for biosecurity incursions. The benefits of reliable information regarding point of origin in biosecurity activities include achieving appropriate response actions, which maximises the effective expenditure of biosecurity funds; and informed inform risk assessment and biosecurity policy.

Biogeochemical markers enable an understanding of spatiotemporal dynamics of the article or organism of interest, and have been successfully applied to track a wide range of dispersing organisms and items of forensic interest (Bowen, Winter, et al., 2005; Oulhote et al., 2011; Rubenstein & Hobson, 2004). These markers are intrinsically incorporated into the fabric of the object during its synthesis, and as such, the isotopic and elemental signature of the location of synthesis is 'recorded' within the object itself. Natural abundance biogeochemical markers have an advantage of being independent of artificial labelling – either 'marking', tagging or documentation – and thus free of the limitations of mark/ recapture error and document fraud.

However, the application of these methods to trace insects, man's most damaging competitors, has been under-utilised to date and our understanding in this field is still in a phase of basic development. Furthermore, no successful applications of biogeochemical markers in biosecurity have been published to date.

Stable isotope ratio analyses using H and C have given spatial resolution in a proof of concept, monarch butterfly, single host system in eastern North America (Hobson, Wassenaar, et al., 1999; Wassenaar & Hobson, 1998). Subsequently, the same geo-location methodology was employed in an attempt to determine the origin of two important biosecurity pests collected post-border in Auckland, New Zealand in 2005 and 2006 – painted apple moth and fall web worm. However, interpretation of the results was inconclusive (Husheer & Frew, 2005), as the accuracy and limitations of this methodology were unknown in a biosecurity

context, where, in contrast to the monarch butterfly study, the sample sizes are usually only one or two insects, accidentally introduced, and the species involved are generally polyphagous; i.e., biosecurity samples generally present low statistical confidence, and are from an unknown and unpredictable place, point in time and host plant, impeding the comparison and calibration of insects isotopic profiles to the known distribution of isotopes in nature.

This inability to determine the place of origin of biosecurity specimens was the rationale for this research program presented in this thesis. The overall research question was: "Can isotope analysis determine the geographic origin of potentially invasive insects collected in New Zealand?" Consequently, it was hypothesised that δ^2H, in isolation, cannot discriminate New Zealand bred insects from overseas reared insects. As a counterpoint, it was also hypothesised that reliable provenance assignment *can* be achieved through the combination of the global scale distribution patterns of δ^2H with the finer spatial scale of geological markers.

The fundamental unknown parameters were the focus of the research program; namely:

1. It was unknown if the highly variable spatial distribution of precipitation δ^2H in New Zealand over insect life-span time-scales would be expressed in the adult insects; and
2. The isotopic fractionation in insects was only partially quantified, and the extent of associated variation in the expression of this marker was unknown.

However, it was recognised that the variation from both these parameters was potentially large enough to overwhelm any New Zealand δ^2H signal. Further,

3. It was unknown if multiple biogeochemical variables of provenance potential, especially Sr and Pb isotope ratios, could be measured from insect samples, or if multiple markers would provide adequate spatial resolution.
4. It was unknown how additional host signals would be expressed in insects' δ^2H, trace elements and heavy element isotopic profile; and
5. The integrity of biogeochemical marker expression from place-to-host-to-insect was unexamined.

(The geo-location potential of $\delta^{13}C$ was considered uncertain, especially for polyphagous insects, and $\delta^{13}C$ values did not warrant further evaluation in this project).

To meet the objectives associated with testing the above hypotheses, it was first necessary to develop an integrated analytical method for reading natural abundance ^2H, $^{87}Sr/^{86}Sr$, $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{206}Pb$ isotope ratios and trace element profiles from single insect

specimens. The limited amounts of many biogeochemical marker elements[28] in insect samples, along with the constraints of restricted sensitivity of analytical methods and machines available, directed the development of a method that efficiently used the limited sample mass of one insect, minimised contamination and employed TCEA-IRMS, TIMS, MC-ICP-MS and ICP-MS mass spectrometry.

Using *Helicoverpa armigera* as a model insect, subsequent research has confirmed that none of the biogeochemical markers assessed, including δ^2H, can independently separate insects reared in New Zealand from those of overseas origin, with the small samples sizes typical of biosecurity interceptions. Most of the markers are insufficiently distinct between all of the study regions. In addition, signal distortion from both environmental variation and broad variation in individual moth expression was too high to enable reliable provenance assignment in biosecurity applications.

In contrast, it has been demonstrated that the region of origin is able to be distinguished for approximately 75% of individual *H. armigera* samples, using a combination of δ^2H, $^{87}Sr/^{86}Sr$, Pb isotopes and selected element concentrations. Furthermore, all the moths from the New Zealand regions were distinguished from the Australian moths. Therefore, the determination of whether a suspect sample has originated from its collection point, or not – i.e., in a biosecurity scenario – is likely to be successful. However, determination of a sample's actual point of origin is currently not possible without reference samples from the candidate areas.

This work provides empirical support for using the combination of stable isotope assessment with trace elemental analysis as proposed in the reviews of Hobson (2005); S.D. Kelly, et al. (2005) and Rubenstein & Hobson (2004), as surprisingly few researchers have empirically tested the combination of such markers (e.g., K. A. Anderson & Smith, 2005; S. D. Kelly et al., 2002; Szep et al., 2003). This study is the first completed evaluation of multiple isotope and trace elements to test for insect provenance assignment. It is also believed to be the first study that has considered Pb isotopic information from insects, and the first use of Sr isotopes for insect provenance assignment. This work has advanced the research into entomological trace element markers of Dempster, et al. (1986); McLean, et al. (1979) and Turner & Bowden (1983) and shown that natural abundance minor trace element markers can be applied in entomological provenance determination. In addition, this study is the first in-depth appraisal of biogeochemical markers for biosecurity.

[28] The suite of elements evaluated in this study were based on the elements that were theorized would provide signatures for New Zealand different from our major trading partners, Australia, and S.E Asia.

Conversely, it is well recognised that all natural abundance markers have their weaknesses (Hobson, 2005), and the advances described above need to be balanced against significant limitations that have also been revealed regarding biogeochemical marker expression in insects. These limiting factors need to be either taken into account or resolved; and therefore these limitations define the future research priorities. Although many uncertainties need to be resolved, the ongoing use and development of biogeochemical markers in entomology is warranted – with several caveats.

Firstly, not all of the markers were informative. Successful provenance determination requires a balanced appraisal of all available markers. The appropriateness of the markers and the statistical procedures used need to consider the potential for the signal to be confounded by biological processes, and the degree of overlap among and between the potential regions of incursion (Montgomery et al., 2007; Oulhote et al., 2011).

Second, and potentially the largest limitation of biogeochemical markers in entomology, is polyphagy – which introduces signals from more than one host species into the insect population. This phenomenon affects both light element isotope and trace element biogeochemical compositions. In biosecurity applications, we do not know the natal plant host of the insect (nor the water/ soil to host fractionation or elemental correlation), and so we need to at least appreciate the potential scale of effect that polyphagy may introduce. On-the-other-hand, it may be possible to mitigate the effect of polyphagy by using the potentially informative elements that were not affected by host type, or were only affected by one of the four host treatments. These elements, along with the possibility that Pb and Sr isotopes are similarly minimally affected, warrant further research. Additionally, some analytical methods have shown promise in larval host plant determination, albeit in limited circumstances (Dockx et al., 2004; Timmerman-Vaughan, 2011, pers. comm.). If these can be utilized, the application of biogeochemical markers in entomology will be greatly facilitated.

Thirdly, the turnover of insect cuticle has been shown to also significantly affect light element isotope and trace element biogeochemical signals. While it is concluded that the δ^2H value of moth wings is driven primarily by the larval diet input (with fractionation offset), a hitherto undetected, age-related sclerotization enrichment gave rise to an approximately 7‰ shift in wing tissue. Given this dynamic nature of arthropod cuticle (Richards, 1978), which has generally been under-appreciated in biogeochemical study (e.g., Hobson, Wassenaar, et al., 1999; Miller et al., 1988; Schimmelmann et al., 1986), the age of the insect needs be taken into account went applying a δ^2H fractionation correction in geo-location or ecological studies. And, more intuitively, many of the provenance informative trace element markers

from the whole insect preparations appear to reflect an insect's adult feeding – and hence recent geographic region, as well as the gender of the individual. This confirms that robust natal origin markers are most likely to be sourced from the relatively permanent body structures (IAEA, 2009a) such as the flight wings, and possibly the mouth parts and tarsus. Therefore, notwithstanding that several potentially informative markers may not be affected by adult feeding or gender, it is highly desirable that micro-analytical methods be developed so that researchers can 'read' a suite of biogeochemical markers – especially Sr isotopes – from the least metabolically active body structures, and thus mitigate these confounding effects. Ideally, such future methods will also be less expensive and more accessible than the analytical method that has been used herein, and so more readily available for real-world applications (Anon, 2010; Garcia, 2009; Spotl & Mattey, 2006). Further impetus for development in this area arises from the need to avoid analyses that consume the entire insect, as this is inconsistent with entomological convention that demands samples be preserved, or at worst minimally damaged, for future taxonomic enquiry and as the primary point for collection information to be catalogued (Mound, 1978). This is especially so for samples with voucher significance (e.g., quarantine material).

Fourthly, apart from *H. armigera* and to a lesser extent, monarch butterflies, it is unknown how accurately soil and precipitation signals are expressed in insects. Therefore, in order to use geological maps or isotope distribution databases for the determination of provenance, ecological linkages or paleo-climatic reconstruction by comparison with biogeochemical markers from insect samples, the 'discrimination factor(s)' (e.g., ^{2}H fractionation) needs to be quantified across several additional plant-insect systems. Currently, the confident application of biogeochemical signals to insect samples requires reference populations of the same species e.g., (e.g., Hobson, Wassenaar, et al., 1999; Holder & Frew, 2010).

However, correlations between many of the biogeochemical markers in the insects thus far studied appear to be weak or are uncertain. The weak correlations appear to be primarily caused by inconsistent ^{2}H fractionation relationships, variation in both the input signal and individual moth expression of the markers, and the accumulation of exogenous inputs. These are discussed in turn below.

The inconsistency observed in H fractionation relationships appears to be caused by fluctuating environmental conditions. In addition, these relationships are different between different plant-insect systems. It follows therefore, to facilitate the use of $\delta^{2}H$ as an entomological marker, the ^{2}H fractionation relationship needs to be quantified in each host + insect system of interest, rather than relying on a single generic model, and ideally the causes

of ^{2}H fractionation variation will be known or can be identified. This is especially so if the insect of interest utilises a mode of feeding for which H fractionation examples have not thus far been calibrated – i.e., the biology of the host and the insect needs to be taken into account.

The research presented in this thesis also shows that the $\delta^{2}H$ of chewing herbivorous insects will vary from the long term and annual precipitation average $\delta^{2}H$ values. The short term isotopic variation in precipitation $\delta^{2}H$ is expressed by the host-plant solid, and specific plant parts reflect the source water $\delta^{2}H$ at a specific growth phase, potentially over a time scale as short as a few weeks. The variable plant $\delta^{2}H$, with fractionation off-sets, is then expressed in the insect consumer. (On-the-other-hand, the insects appear not to express the even more highly $\delta^{2}H$ variable plant water pool). As a consequence of the irregular precipitation $\delta^{2}H$ values being reflected in the insects, the $\delta^{2}H_{MOTH}$ signal is not consistent between years. Therefore, if the samples cannot be referenced accurately to the appropriate period of precipitation $\delta^{2}H$ (precipitation $\delta^{2}H$ data is generally of too low resolution or not complete, (e.g., IAEA, 2002)), $\delta^{2}H_{MOTH}$ values are applicable in only a single time frame. Ideally, future researchers will have access to higher temporal and spatial resolution precipitation $\delta^{2}H$ data, as well as a well resolved understanding of the fractionation relationships.

Intra-treatment (or location) heterogeneity in the expression of $\delta^{2}H$ and trace element markers by individual insects is an additional complexity that also needs to be addressed in biogeochemical tracing. This source of variation is assumed to have several causes, most important of which are the differences in the biochemical processes within individual moths, and is the main factor distorting the place-to-insect correlations. $^{87}Sr/^{86}Sr$ appears to be less susceptible to the variation in biochemical processes between individual moths than are $\delta^{2}H$ and trace elements – as duplicate samples from the same site have similar Sr isotope ratios. However, the soil-to-insect correlation for $^{87}Sr/^{86}Sr$ is uncertain, as it is for several trace elements markers, and thus the integrity of expression of the most informative geological markers warrant further research using more specialized, sequential, soil digestion methods.

In addition, exogenous Sr and Pb were found to accumulate in the moths and obscure the soil and plant signals at one collection point. This finding contradicts the conventional thinking that states heavy-element isotope location fingerprints are assumed to be more-or-less accurately expressed from bedrock to soil to plant to animals (Aberg, 1995; Beard & Johnson, 2000; Bentley, 2006). On the other hand, although these results indicate that cautious interpretation is necessary, heavy-element isotope location markers, especially $^{87}Sr/^{86}Sr$, are still considered the biogeochemical variables most likely to give robust geo-location signature (Frei & Frei, 2011; Holzl et al., 2004). Furthermore, insect samples from biosecurity relevant

areas, such as seaports and heavily industrialised areas may acquire otherwise 'contaminating,' distinctive Pb isotope ratios from location specific pollutants, such as coal linked emissions. Such Pb isotopes values may provide additional provenance resolution from biosecurity samples. Additional research aimed at producing Pb isoscapes for insects at important biosecurity locations is therefore recommended, as is investigation into the applicability of Pb isotopes as recorders of ecological linkages and pollution pathways in insects.

A consequence of the heterogeneity of biogeochemical marker expression is the strong relationship between sample size and confidence of provenance assignment. Thus it is important to take into account both intra-region variation and the degree of isotopic difference in the potential sources when determining the required sample size. Predicting the required sample size is not possible, however, when the origin of the sample is unknown – as is the case with biosecurity incursions. Provenance assignment of biosecurity samples will not be reliable when sample sizes are <10 and using δ^2H and $^{87}Sr/^{86}Sr$ as single variables. The relationship between sample sizes and multiple variables in provenance determination is a research priority for a future program.

To conclude: this research presented in this thesis has addressed the unknown fundamental parameters that impeded the interpretation of the biogeochemical data and point of origin determination associated with the painted apple moth and fall web worm incursions in 2005 and 2006. The primary research objective has also been achieved; namely, integrated isotope and trace elements geo-location markers have been shown to be able to determine the origin of exotic pests intercepted in New Zealand.

Important limitations in the method have been assessed and the priorities for subsequent research identified. Further, these limitations have been quantified sufficiently to enable the application of biogeochemical markers to answer subsequent biosecurity point of origin questions (Holder & Frew, 2010) and the use of these methods for provenance determination for high impact biosecurity pests, such Tephritidae fruit flies and the potato/tomato psyllid (*Bactericera cockerelli,* Hemiptera: Triozidae) are currently being considered (Holder & Armstrong, in prep).

It is assumed that the understanding and methods established here can be applied to other systems, and exciting opportunities exist for the wider application of natural abundance biogeochemical markers in entomological science. The array of potential applications span from tracking continental scale immigration of highly economically damaging pests –

including various *Helicoverpa* species in Africa, Europe, Australia and the Americas, and thus informing pest management strategies, to improving the localised understanding of beneficial insects and host movement (Holden, 2006; Lavandero et al., 2004).

The maximum benefit of biogeochemistry in entomology will most likely emerge from enabling future development in the integration of probabilistic modelling with marker distribution databases (i.e., isoscapes), which, along with achieving the immediate research needs given above, will allow provenance determination of insect specimens without the need for time consuming and logistically demanding reference specimen collection.

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Appendix A

Experiment 4 ^{2}H fractionation relationships

δ^{2}H fractionation relationships relating to wild *H. armigera*, host plant solid, extracted plant (corn kernel) water and precipitation from the Australian and New Zealand regions used in Experiment 4. All units δ^{2}H‰. Values within a row that are followed by a different letter are significantly different (Fishers Restricted LSD = 5%).

		MC	BP	AK	NSW	QLD
2008	Precip	-53.2±3.8	-34.2±3.2	-27.5±3.6	-26.7	-16.0
	to-moth Δ	48.8	50.0	51.8	58.3	56.8
	Corn	-42.2±19.5 ^{a} (n=11)	0.3±14.3 ^{b} (n=11)	0.9±10.9 ^{b} (n=5)	-14.3±20.7 ^{c} (n=12)	13.8±24.5 ^{d} (n=10)
	to-moth Δ	59.8	84.5	80.2	70.7	86.6
	Extracted water	-47.4±4.5 ^{a} (n=11)	-15.2±4.7 ^{b} (n=11)	-9.4±13.1 ^{bc} (n=5)	-22.3±5.5 ^{d} (n=15)	-6.6±5.4 ^{c} (n=9)
	to-moth Δ	54.6	69.0	69.9	62.7	66.2
	Moth	-102.0±6.5 ^{a} (n=17)	-84.2±8.0 ^{bc} (n=8)	-79.3±7.3 ^{b} (n=24)	-85.0±12.0 ^{c} (n=22)	-72.8±6.1 ^{d} (n=26)
2009	Precip	-38.2±4.6	-21.6±3.7	-18.6±3.1	-21.8	-18.4
	to-moth Δ	46.0	39.9	54.3	51.8	39.6
	Corn	-48.0±14.4 ^{a} (n=6)	-32.9±18.7 ^{b} (n=6)	-39.8±14.0 ^{b} (n=5)	-24.2±6.5 ^{c} (n=6)	-18.7±14.5 ^{c} (n=6)
	to-moth Δ	36.2	28.6	33.1	49.4	39.3
	Extracted water	-52.5±6.5 ^{a} (n=7)	-32.7±11.7 ^{b} (n=6)	-24.8±10.1 ^{b} (n=6)	-29.2±6.3 ^{b} (n=6)	-9.2±7.4 ^{c} (n=4)
	to-moth Δ	31.7	28.8	48.1	44.4	48.8
	Moth	-84.2±7.0 ^{a} (n=43)	-61.5±10.6 ^{b} (n=26)	-72.9±7.4 ^{c} (n=59)	-73.6±8.4 ^{c} (n=54)	-58.0±9.5 ^{b} (n=33)

Appendix B

Experiment 4 trace element data

Trace element concentration data (ng/g) of adult *H. armigera*, and associated host plant (corn) and soil, collected from Australian and New Zealand sites, March – May 2008 and Jan – March 2009. Data is displayed as geometric means ± 1SD ranges. Overall F test p values given (1% = **, 5% = *, 10% = †). †† = Grouping information using Fisher LSD test (5%) revealed at least one mean was significantly different.

B.1 Moth trace element data

Moth 2008	MC (n=4)			BP (n=2)			AK (n=4)			NSW (n=4)			QLD (n=4)			p(df = 4,13)	sig
	Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range			
Li	3	1	7	8	5	14	5	2	12	13	3	63	7	6	8	0.348	
Al	2032	341	12103	12125	1358	108233	11041	1235	98750	22974	9458	55806	29141	24107	35227	0.187	
Ca																	
Sc	7	6	9	9	2	46	18	11	29	12	6	21	13	10	18	0.342	
Ti																	
Cr	69	16	290	180	6	5340	341	30	3885	385	145	1022	1234	901	1689	0.268	
Mn	23336	15412	35334	48140	43930	52753	37720	26161	54387	39055	20710	73650	39138	32867	46605	0.279	
Co																	
Ni	36	15	88	134	30	601	85	12	596	218	44	1092	214	175	261	0.346	
Cu																	
Zn	257706	163503	406185	191813	58348	630570	583712	303113	1124069	337782	198626	574431	325609	253515	418204	0.235	
Ga	30	9	103	256	35	1868	101	15	671	162	102	257	250	184	340	0.183	

As	23	13	42	33	22	49	38	25	60	44	27	71	46	36	59	0.262	
Rb	835	247	2826	10687	4423	25823	4072	2149	7715	1381	554	3444	2029	1393	2957	0.028	*
Sr	340	229	505	1044	302	3609	304	141	656	1622	855	3078	1446	632	3309	0.015	*
Cd	64[29]	10	417	22	9	56	7	2	23	1	0	32	8	2	26	0.102	††
Cs	21	6	70	32	15	69	30	15	61	7	3	21	7	5	9	0.081	†
Ba	222	63	775	1999	298	13393	724	107	4883	1209	783	1865	1822	1355	2450	0.174	
La																	
Ce																	
W	1	0	3	9	5	19	0	0	0	0	0	1	1	0	3	0.035	*
Pb	43	27	70	39	18	82	17	13	22	24	14	41	21	15	32	0.078	†

Moth 2009	**MC (n=6)**			**BP (n=6)**			**AK (n=6)**			**NSW (n=6)**			**QLD (n=6)**			**p(df = 4,24)**	**sig**
	Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range			
Li	8	3	25	10	2	36	4	2	8	3	1	11	5	1	20	0.446	
Al	11960	5289	27047	7118	2367	21408	14440	6101	34174	12150	2835	52072	8386	3150	22326	0.772	
Ca	723908	540181	970126	604893	437723	835908	530631	325979	863765	542477	355852	826978	481307	274878	842763	0.543	
Sc	24	19	29	23	10	55	15	11	23	33	13	82	24	13	43	0.445	
Ti	1679	1044	2701	1166	640	2122	667	421	1055	1424	566	3584	1114	620	2000	0.153	
Cr	140	90	219	118	100	139	136	103	178	151	94	243	121	86	170	0.731	
Mn																	
Co	24	13	46	13	4	44	2	0	21	23	9	56	20	11	36	0.017	*
Ni	122	51	289	69	24	198	47	31	70	60	28	131	57	32	102	0.289	
Cu	16479	12682	21413	17525	11799	26031	17722	13980	22467	12234	10375	14426	14257	10672	19047	0.141	

[29] High mean Cd concentration in 2008 MC due to unnatural abundance in one site

Zn	267315	234730	304423	236292	179748	310622	257834	187368	354801	216445	115941	404073	216977	130165	361689	0.853	
Ga																	
As	16	6	42	8	3	22	21	16	27	9	6	14	6	3	11	0.035	*
Rb	2009	996	4052	4441	1476	13356	2718	1569	4708	1107	730	1679	1470	965	2240	0.017	*
Sr	865	335	2228	394	210	739	436	196	969	821	374	1804	1146	372	3530	0.183	††
Cd	22	12	39	12	3	47	14	4	47	8	3	21	3	2	5	0.031	*
Cs	3	2	3	4	1	19	5	1	21	1	0	5	1	1	2	0.06	†
Ba	1017	502	2058	1077	591	1962	497	255	970	565	258	1237	621	314	1229	0.213	
La	33	16	66	16	4	69	4	1	18	5	1	17	17	10	30	0.019	*
Ce	111	52	237	58	14	233	18	5	69	16	5	55	53	31	92	0.025	*
W	2	0	10	3	0	24	0	0	5	2	0	12	1	0	11	0.294	
Pb	39	17	85	29	14	60	40	26	61	15	8	26	39	17	94	0.1	†

B.2 Corn trace element data

Corn 2008	**MC (n = 7)**			**BP (n = 7)**			**AK (n = 5)**			**NSW (n = 8)**			**QLD (n = 6)**			**p (df = 4,28)**	**sig**
	Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range			
Li	1	0	4	0	0	1	1	0	2	0	0	1	0	0	1	0.413	
Al	2096	969	4532	488	372	638	710	549	919	897	377	2133	709	315	1596	0.006	**
Ca																	
Sc	5	3	10	4	3	6	5	3	6	4	3	6	7	3	13	0.244	
Ti																	
Cr	44	22	88	11	6	23	11	6	21	23	14	38	6	0	72	0.05	†
Mn	19640	10431	36979	7475	4077	13703	10718	9035	12714	11617	6626	20368	14264	6365	31970	0.072	†
Co																	

Ni	327	173	616	92	59	144	93	57	149	351	289	426	471	337	660	<.001	**
Cu																	
Zn	52391	34624	79276	28205	19073	41708	41924	30310	57989	26759	18360	38999	24468	13177	45433	0.014	*
Ga	19	8	43	42	12	142	11	7	16	13	5	32	24	4	134	0.201	
As	61	25	150	9	3	26	31	18	54	7	2	23	26	6	105	0.004	**
Rb	9636	3526	26335	18996	8928	40419	11835	7333	19103	3866	1822	8205	6368	3599	11268	0.005	**
Sr	571	188	1734	158	37	667	131	55	315	400	213	750	1732	356	8436	0.005	**
Cd	27	9	87	3	1	9	2	0	22	1	0	5	1	0	7	0.004	**
Cs	19	6	64	42	27	67	45	16	123	2	1	4	4	1	15	<.001	**
Ba	167	76	370	386	120	1238	95	58	155	99	39	251	265	63	1109	0.077	†
La																	
Ce																	
W	14	8	27	3	0	35	5	2	9	3	1	9	6	2	15	0.177	
Pb	50	11	226	8	5	13	9	6	14	19	8	46	21	9	48	0.01	*

Corn 2009	**MC (n=4)**			**BP (n=4)**			**AK (n=4)**			**NSW (n=4)**			**QLD (n=4)**			**p (df = 4,15)**	**sig**
	Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range			
Li	33	25	45	18	8	44	28	19	42	14	7	31	9	5	15	0.049	*
Al	50078	38176	65689	29339	15105	56985	45109	34237	59433	26946	10179	71332	12697	8102	19899	0.036	*
Ca	144021	109639	189184	101787	30667	337844	154336	75089	317217	95103	37674	240074	174121	38421	789096	0.891	
Sc	14	8	26	16	7	34	26	16	43	15	7	33	20	6	67	0.795	
Ti	1894	1327	2703	1021	446	2338	1593	1216	2087	975	379	2508	895	388	2067	0.492	
Cr	206	174	244	206	95	450	173	132	226	176	109	284	142	115	176	0.746	
Mn																	

Co	23	12	47	13	4	45	18	10	35	31	12	80	25	15	44	0.693	
Ni	385	219	679	160	75	340	270	210	347	221	157	313	611	347	1074	0.024	*
Cu	3126	2172	4500	2751	1647	4594	2379	1423	3979	2135	1589	2869	2657	1252	5640	0.86	
Zn	29653	27304	32205	20804	14246	30380	22389	19062	26295	23395	20432	26789	25863	19555	34206	0.285	
Ga																	
As	27	23	33	18	12	26	18	13	23	22	19	25	19	15	25	0.126	
Rb	8566	4402	16669	13608	6607	28027	12736	7029	23078	4741	1916	11729	4656	1776	12201	0.193	
Sr	521	407	666	299	86	1037	449	243	831	616	207	1828	2582	441	15122	0.126	
Cd	12	8	19	5	1	16	7	4	15	3	1	6	4	1	13	0.226	
Cs	23	14	37	62	17	226	76	17	348	6	3	12	4	2	8	0.002	**
Ba	327	288	371	714	149	3425	413	266	641	295	90	962	422	67	2654	0.864	
La	23	17	31	16	8	32	21	17	27	12	4	33	6	3	12	0.076	†
Ce	54	40	73	30	15	62	45	35	59	29	10	85	16	8	30	0.137	
W	10	7	13	9	5	14	8	5	14	6	4	7	4	2	6	0.033	*
Pb	72	42	123	63	16	252	81	38	172	31	12	81	64	21	201	0.695	

B.3 Soil trace element data

2009 data is the elemental pool liberated by total-recoverable (pseudo-total, aqua-regia). mg/kg

Soil 2008	**MC (n=4)**			**BP**			**AK**			**NSW**			**QLD**			**p (df = 4,17)**	**sig**
	Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range			
Li	14.189	4.281	47.021	3.351	2.993	3.751	5.472	5.135	5.831	2.338	0.686	7.964	3.824	3.390	4.313	0.025	*
Al	15771	12290	20238	19652	13518	28570	29464	25775	33682	5639	2302	13809	17093	15846	18438	<.001	**
Ca																	

Sc	1.768	0.965	3.238	1.664	0.827	3.349	5.418	4.356	6.739	1.283	0.350	4.702	3.059	2.437	3.839	0.061	†
Ti																	
Cr	19.400	15.114	24.903	2.967	2.517	3.496	11.426	9.431	13.843	8.919	4.770	16.680	21.883	19.951	24.003	<.001	**
Mn	379	166	863	959	511	1797	7838	5603	10965	264	119	586	1168	837	1631	<.001	**
Co																	
Ni	15.155	11.479	20.008	0.899	0.657	1.230	4.672	3.848	5.672	6.101	2.023	18.400	40.906	37.785	44.285	<.001	**
Cu																	
Zn	74.205	33.455	164.593	36.803	25.540	53.031	63.211	49.245	81.138	17.149	13.872	21.201	42.544	35.431	51.086	<.001	**
Ga	15.722	11.639	21.236	29.400	23.547	36.707	37.262	27.545	50.407	7.488	2.361	23.747	35.678	29.263	43.499	0.002	**
As	5.387	2.837	10.231	1.429	0.686	2.977	0.396	0.360	0.436	0.367	0.204	0.661	0.560	0.478	0.657	<.001	**
Rb	4.299	1.669	11.072	9.987	7.900	12.627	3.876	2.665	5.637	7.927	4.518	13.910	5.415	4.338	6.760	0.064	†
Sr	99.665	60.786	163.411	16.539	13.799	19.822	34.664	27.041	44.437	29.971	10.031	89.548	182.907	173.536	192.785	<.001	**
Cd	0.419	0.273	0.645	0.310	0.171	0.562	0.586	0.446	0.770	0.023	0.012	0.045	0.058	0.046	0.073	<.001	**
Cs	0.054	0.017	0.168	0.619	0.492	0.779	0.519	0.258	1.044	0.092	0.061	0.139	0.026	0.017	0.041	<.001	**
Ba	97.683	72.635	131.369	199.441	155.483	255.826	252.496	182.822	348.723	50.977	14.816	175.394	261.078	214.174	318.254	0.002	**
La																	
Ce																	
W	0.049	0.023	0.103	0.024	0.013	0.045	0.004	0.002	0.010	0.016	0.003	0.086	0.012	0.007	0.020	0.02	*
Pb	20.759	7.269	59.286	14.356	10.837	19.018	64.245	47.202	87.442	6.253	3.103	12.598	5.139	2.706	9.758	<.001	**

Soils 2009	**MC**			**BP**			**AK**			**NSW**			**QLD**			**p (df = 4,15)**	**sig**
2009	Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range		Geomean	sd range			
Li	50.998	33.683	77.214	7.242	4.633	11.321	32.725	18.736	57.157	26.551	22.580	31.221	13.720	11.544	16.306	<.001	**
Al	1.140	0.856	1.520	0.384	0.231	0.638	0.351	0.152	0.810	1.299	1.206	1.400	0.994	0.854	1.157	<.001	**

Ca	23535	17535	31589	14189	9550	21081	41311	32393	52684	31287	25997	37654	37584	33925	41638	<.001	**
Sc	8360	5890	11866	5466	2191	13632	4528	2556	8022	2822	2655	3000	7550	4019	14185	0.389	
Ti	6.092	4.539	8.176	1.968	1.381	2.805	4.415	2.875	6.779	7.764	7.091	8.502	10.960	10.645	11.284	<.001	**
Cr	27.206	18.913	39.136	5.861	3.104	11.068	26.793	25.000	28.715	43.158	40.069	46.486	84.260	66.831	106.234	<.001	**
Mn																	
Co																	
Ni	12.201	7.515	19.809	4.170	1.353	12.851	9.730	6.213	15.238	24.829	23.907	25.787	66.579	51.651	85.821	<.001	**
Cu	13.376	9.964	17.955	12.684	6.326	25.432	17.392	9.190	32.913	18.821	17.390	20.369	29.921	26.956	33.212	0.035	*
Zn	72.224	40.623	128.410	50.965	30.817	84.288	59.000	37.502	92.822	64.197	54.499	75.621	95.517	85.884	106.231	0.172	
Ga																	
As	7.107	5.325	9.486	4.529	2.962	6.925	5.282	3.018	9.243	5.359	4.846	5.926	1.391	1.154	1.676	0.007	**
Rb	164.672	113.907	238.062	17.517	9.902	30.988	50.949	26.165	99.210	208.919	168.03	259.759	83.814	59.151	118.761	<.001	**
Sr	49.483	34.695	70.574	15.635	11.523	21.213	23.591	13.717	40.571	40.219	32.504	49.763	127.303	97.506	166.205	<.001	**
Cd	0.571	0.435	0.750	0.504	0.248	1.023	0.874	0.708	1.077	0.559	0.505	0.620	0.957	0.888	1.031	0.016	*
Cs																	
Ba	107.254	78.914	145.771	79.102	49.769	125.725	78.715	42.520	145.721	119.853	96.183	149.349	136.968	117.211	160.057	0.354	
La																	
Ce	54.095	43.431	67.379	21.725	13.651	34.577	30.277	13.398	68.420	57.629	49.080	67.668	52.176	45.990	59.194	0.043	*
W																	
Pb	20.097	14.208	28.428	9.010	6.373	12.736	18.766	12.230	28.796	19.202	16.500	22.347	12.130	11.260	13.067	0.011	*

Appendix C

Periodic table of elements