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Towards incorporating insect isotope analysis using cavity ring-down spectroscopy into area-wide insect pest management programs

Rebecca Hood-Nowotny[1,], Leo Mayr[2], Nabil Saad[3], Rakesh K. Seth[4], Goggy Davidowitz[5] and Gregory Simmons[6]*

Abstract

Measurement of natural stable isotope signatures of insects has proven to be a useful method in determining their natal origin, feeding strategies and mating behavior, and has spawned a new discipline of insect isotope forensics. However the complexity of measuring isotope signatures using elemental analysis-isotope ratio mass-spectrometry (EA-IRMS) has encountered significant technical barriers, which together with costs, have hindered the advancement and wide spread adoption of this potentially useful approach. In the last decade simpler technologies to measure stable isotope ratios in samples based on laser spectroscopy have been developed. We foresee that these instruments will fuel the advancement and adoption of insect isotope forensics into pest management programs because they are easy to use, more affordable, and complement existing marking tools. However prior to the adoption of these new technologies it is necessary to test their fitness for purpose and explore their limitations. In this paper we set out to test the suitability of using combustion module-cavity ring-down spectroscopy (CM-CRDS) for measuring isotope ratios in an entomological context, covering marking of insects, mating behavior and dietary assessment. We carried out a series of experiments to compare the isotope measurements made with the CM-CRDS with the traditional method of EA-IRMS. Initially we compared measurements of carbon isotope values of a variety of common diet components used in insect rearing, which covered a wide range of carbon isotope values. There was good agreement in the isotope values obtained, using the CM-CRDS and the EA-IRMS with similar accuracy and precision. Secondly we compared the 2 methods to measure carbon isotope values of the common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) samples, and again there was good agreement in the values obtained. Thirdly we tested whether the CM-CRDS could be used to determine the paternity of spermataphores, based on the differential isotope signatures of the paternal and maternal moths, as a result of differences in their rearing diets. The CM-CRDS proved to be fit for the purpose of measuring isotope values in the spermataphores of common cutworm and suggested that the CM-CRDS technology would be suitable for these isotope based mating studies in moths if the spermatophores were of sufficient mass. Finally we explored a number of the issues surrounding CM-CRDS and isotope technologies in general, e.g., logistical considerations, economics of operation, sample size, etc. We conclude that CM-CRDS is a suitable instrument for measuring stable isotope carbon signatures in moths, and most probably other sufficiently large insects and that CM-CRDS could be easily used in both operational and research contexts.

Key Words: marking; labelling; enrichment; natural abundance; mass spectrometry; nuclear application; laser; Lepidoptera; moths

Resumen

La medición de las firmas de isótopos estables naturales de los insectos ha demostrado ser un método útil para determinar su origen natal, estrategias de alimentación y su comportamiento de apareamiento y ha dado lugar a una nueva disciplina de la ciencia forense de isótopos de insectos. Sin embargo, la complejidad de la medición de las firmas isotópicas usando el análisis de espectrometría de masas de ratio elemental de isótopos (AEM-REI) se ha encontrado con obstáculos técnicos importantes, que junto con los costos, han obstaculizado el avance y la adopción generalizada de este enfoque potencialmente útil. En la última decada se han desarrollado tecnologías más simples para medir las proporciones de isótopos estables en muestras basadas en espectroscopia láser. Prevemos que estos instrumentos se alimentan el avance y la adopción del uso de forense de isótopos de insectos en los programas de manejo de plagas, ya que son fáciles de usar, menos costos, y complementan las herramientas para marcar ya existentes. Sin embargo, antes de la adopción de estas nuevas tecnologías es necesario probar su aptitud para el uso y explorar sus limitaciones. En este trabajo nos propusimos evaluar el apropiado del uso de la espectroscopia de combustión de cavidad de módulo de anillo-abajo (ECC-MAA) para medir el ratio de los isótopos en un contexto entomológica, incluyendo el marcar de los insectos, el comportamiento del apareamiento y la evaluación de la dieta. Realizamos una serie de experimentos para comparar las medidas de isótopos realizadas con la ECM-CAA con el método tradicional de AEM-REI. Inicialmente se compararon las medidas de los valores de los isótopos de carbono de una variedad de componentes comunes utilizados en la dieta en la cría de insectos, que cubría una amplia gama de valores de los isótopos de carbono. Hubo buen acuerdo en los valores de isótopos obtenido, utilizando el ECM-CAA y el AEM-REI con exactitud y precisión similar. En segundo lugar se compararon los 2 métodos para medir los valores

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de isótopos de carbono en muestras del gusano cortador común, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), y de nuevo había un buen acuerdo en los valores obtenidos. En tercer lugar hemos probado si el ECM-CAA podría ser utilizado para determinar la paternidad de espermatoforos, sobre la base de las firmas diferencial de isótopos de las polillas paternas y maternas, como resultado de las diferencias en sus dietas de cría. El ECM-CAA demostró ser apto para el propósito de medir los valores de isótopos en los espermatoforos del gusano cortador común y sugirió que la tecnología ECM-CAA sería adecuada para estos estudios de apareamiento de isótopos con sede en polillas si los espermatóforos fueron de suficiente masa. Finalmente, hemos explorado algunas de las cuestiones relacionadas con ECM-CAA y las tecnologías de isótopos en general, por ejemplo, las consideraciones logísticas, economía de funcionamiento, tamaño de la muestra, etc. Concluimos que el ECM-CAA es un instrumento adecuado para medir las firmas de carbono de isótopos estables en polillas y muy probablemente otros insectos suficientemente grandes y que el ECM-CAA podría utilizarse fácilmente tanto en contextos operacionales y de investigación.

Palabras Clave: marcar; etiquetar; enriquecimiento; abundancia natural; espectrometría de masas; aplicación nuclear; láser; Lepidoptera; polillas

Area-wide integrated pest management (AW-IPM) approaches, especially when these programs have a sterile insect technique (SIT) component (Hendrichs et al. 2007) require a full understanding of the ecology and biology of the target pest insect species to efficiently implement these area-wide strategies. Marking tools are invaluable in addressing the scientific knowledge gaps in the ecology and biology of insects such as distribution, movement, larval host origin, as well as certain behavioral aspects of the released sterile insects. However, there is currently a lack of widely available, simple and ethically acceptable methods for marking and tracking small insects and economically important pest species, including moths in situ. Stable isotopes could be used as markers, to naturally or synthetically label the vast numbers of insects required for studying population dynamics and in a SIT context whilst fulfilling the marking criteria set out by Hagler & Jackson (2001) of retention, not affecting the insect's fecundity or behavior, durability, non-toxicity, ease of application, clear discernibility and affordability. The latter criterion could be contested in the case of current stable isotope analysis using mass-spectrometry, however, here we present and explore the economics and feasibility of a new system, which could significantly reduce the cost of analysis, and therefore expand the scope and applicability of this technology. The reasons why isotope techniques have not been routinely adopted in pest management are clear: isotope analyses often needs to be outsourced to a commercial isotope laboratory, isotope analysis requires access to expensive, complex, elemental analysis-isotope ratio mass-spectrometry (EA-IRMS) instruments that are generally only fully operational in developed countries. These instrumental constraints and the requirements for highly skilled operators represent significant access barriers for the technological adoption into an operational insect management program.

As already explained by Hood-Nowotny et al. (2016) in this issue, terrestrial vascular plant species differ in their $^{13}C/^{12}C$ ratios because of their photosynthetic and enzymatic pathways, C_3, C_4 or crassulacean acid metabolism (CAM), and the greater discrimination against heavier ^{13}C atoms by C_3 than by C_4 and (CAM) species. Sugar beet, *Beta vulgaris* L. (Caryophyllales: Amaranthaceae), and nearly all fruit tree species and other dicotyledonous plant species are C_3 species, whereas most C_4 plant species belong to the Poaceae or Graminaeae and Cyperaceae, e.g., grasses and sedges, and desert-adapted species in various families. C_3 plant species typically have an isotopic ($\delta^{13}C$) range of values between −25‰ and −35‰ with an average around −27 ± 2‰; that is, in every 1,000 atoms there are 27 fewer ^{13}C atoms than in the international standard Vienna Pee Dee Belemnite (VPDB). In contrast C_4 species have $\delta^{13}C$ values ranging between −7 and −18‰. The $\delta^{13}C$ values of sucrose from sugar beet and sugarcane (*Saccharum* spp.; Poales: Poaceae) are around −27‰ and −11‰, respectively. Thus the incorporation of sucrose from either of these 2 sources into insect mass rearing diets results in adult insects that have a stable isotopic signature similar to the sucrose in the diet, and this signature may be used to distinguish them from their wild counterparts.

Stable isotope tools offer the opportunity to safely trace the source, distribution and movement of populations of economically important insect species and deliberately released sterile insects across the landscape posing no environmental threats or hazards. Earlier work using stable isotopes in insects focused on understanding basic ecological questions such as the natal origin of the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae) (Wassenaar & Hobson 1998) or the upstream movement of mayflies, *Baetis* spp. (Ephemeroptera: Baetidae) (Hershey et al. 1993). Recently a number of papers have focused on the use of isotopes in an AW-IPM context, specifically to track and trace insects and to study mating patterns. Malaria-mosquitoes, *Anopheles arabiensis* Patton (Diptera: Culicidae) were labeled with stable isotopes and the label could be detected. This led to marking of mosquitos in the field to study for example the movement and distribution of mosquitoes in the context of West Nile Virus research (Hood-Nowotny et al. 2006; Hamer et al. 2012, 2014). Natural abundance and labelling techniques have also been used to distinguish wild tsetse flies (Diptera: Glossinidae) from flies reared in mass-rearing facilities (Hood et al. 2011). Furthermore, the naturally different isotope signatures of mass-reared and wild fruit flies enabled researchers to differentiate the mass-reared individuals from their wild populations (Hood-Nowotny et al. 2009) and in this special issue we show that this method also works well for Lepidoptera (Hood-Nowotny et al. 2016). From these data it is clear that isotopic differences can be easily detected using mass-spectrometry and can be used to determine the origin of an insect in an SIT program context. Moreover it has been previously demonstrated that simple natural abundance marking techniques could also be used as an effective method for determining the paternity of moth species through coupled female leg and spermatophore carbon isotope analysis (Stringer et al. 2013).

Despite the many successful examples, laboratory-based EA-IRMS for isotope analysis still presents itself as a significant adoption barrier for these isotope-based techniques. Here we present an alternative method of isotope analysis that could overcome a number of constraints and therefore has potential for wider adoption of isotopic techniques as part of AW-IPM programs. Since a few years there has been a viable alternative to EA-IRMS for isotope analysis, i.e., laser-based instruments for high-precision isotope ratio measurement that link samples to high-throughput elemental analyzers. The strategic advantages of the laser-based technology of combustion module cavity ring-down spectroscopy (CM-CRDS) systems are that they are compact, relatively low cost, easy to use and they require only standard carrier gasses, making them ideal for use in field studies, operational programs and in developing countries (Gupta et al. 2009). The main cost saving comes from the fact that these CM-CRDS systems require minimal technical training and infra-structural support, however, currently only single element devices are available.

In this paper we make direct comparisons of insect samples measured on the EA-IRMS and the combustion module (CM) connected to the CRDS system (CM-CRDS). We also measured a range of common mass-rearing dietary components in both systems, which allowed us to test the system over a range of natural isotope values. We then set out to determine whether the CM-CRDS method would be suitable for

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conducting the isotope analysis of moths using the approaches that we had developed with EA-IRMS; the details of which are discussed in this issue (Hood et al. 2016). We therefore carried out exploratory isotope studies of 2 important moth species that are considered major threats to production agriculture, i.e., the common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), a widely distributed polyphagous pest found in India, and the light brown apple moth (LBAM), *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), a polyphagous pest with over 500 known host species (Brockerhoff et al. 2011). Moreover we examined a number of operationally important implementation factors, such as whether trap glue interfered with the isotopic signatures measured. Finally, we discussed future applications, opportunities and constraints of using CM-CRDS and also considered some practical and managerial issues for implementing such an isotope approach.

Materials and Methods

In a first study, the carbon isotopic values of common dietary components used for insect rearing were measured using the EA-IRMS and the CM-CRDS systems. These components covered a broad range of carbon isotope values allowing us to compare the 2 isotope measurement systems. In a second study, populations of wild and mass-reared moth samples were analyzed and values compared using both EA-IRMS and the CM-CRDS and finally isotope analyses of moth spermatophores, trap glue and moth diet was made using the CM-CRDS to determine whether it would be feasible to analyze these samples using this system and explore opportunities for using the system in an operational context.

COMPARISON OF FOOD COMPONENTS USING EA-IRMS AND CM-CRDS

Sixteen diet components were tested in this experiment: AP100 (Zeilgler USA, a commercially available shrimp larval diet), bean powder, bovine liver powder (BLP), brewer's yeast, carrot powder, chick pea, corn, rice, soy hydrolysate, spirulina, squid liver powder (SLP), tuna, vitamin-mix, wheat, wheat bran and yeast hydrolysate.

Sample Preparation. Ten mg of dried (60 °C for 24 h) ground diet was placed into 8 by 5 mm tin cups. Duplicate samples were then either measured using EA-IRMS or CM-CRDS.

MOTH EXPERIMENTS

To test the CM-CRDS method of analysis for moth samples, 3 experiments were conducted using the common cutworm and LBAM. In the common cutworm experiments CM-CRDS isotope measurements were compared with values obtained using EA-IRMS. Having established measurement comparability, we conducted an independent data analysis of the LBAM samples based on the isotope measurement results from the CM-CRDS, to test whether it would be possible to determine the source of the population using this based method. Finally, in a preliminary study we used the CM-CRDS to determine the paternity of the spermatophores based on their isotopic signatures. The latter aimed at detecting the nuptial gift of the differentially labeled laboratory-reared males in the female spermataphore that is dissected out of the female and measured for isotopic signature (Stringer et al. 2013).

COMMON CUTWORM REARING

Laboratory-reared common cutworms were maintained on a synthetic meridic diet containing chickpea seeds and sinigrin as a phagostimulant. All insects were reared in an insectary under ambient environmental conditions of 26.8 ± 1 °C, 75 ± 5% RH and a photoperiod of 12:12 h L:D. Wild moths were captured in the castor fields in India.

COMMON CUTWORM SAMPLE PREPARATION

Leg and head samples of the same insect were analyzed using either CM-CRDS or EA-IRMS as described below and data from both the field collected and laboratory-reared samples compared with regression analysis.

Preliminary Sperm transfer experiments in the common cutworm

In the sperm transfer studies laboratory-reared female common cutworms (reared as described above) were mated with field-collected males. Spermatophores were dissected out of the females under a binocular microscope and dried for isotope analysis. An additional leg sample from the female was also taken for isotope analysis to determine the background value of the mated female.

LIGHT BROWN APPLE MOTH (LBAM) REARING

LBAM were reared on a standard pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) mass-rearing diet using the standard rearing protocols described by Stewart et al. (1984). The dry mix for the artificial diet was made up from the following ingredients per 267 kg: 50 kg soy flour, 25 kg wheat germ, 4 kg torula yeast, 690 g corn oil, 11.33 kg agar, 9.81 kg cane sugar, 16.8 g alphacel, 0.646 g methylparaben, 0.82 g potassium sorbate, 683 g fumidil B, 4.3 g chlortetracycline, 50.76 g D calcium pantothenate, 25.92 g nicotinic acid, 12,96 g riboflavin, 12.96 g folic acid, 6,48 g thiamine hydrochloride, 6.48 g pyridoxine hydrochloride, 0.52 g biotin, 0.03 g vitamin B 12, 680 g choline chloride, 2.5 L acetic acid, 84 g red dye (made up from 336 g of Calco red No 1700 dye (American Cyanamid Corp)), 300 g sugar, and 5700 g wheat germ

For rearing purposes, mature pupae were loaded into the emergence system, which consisted of emergence boxes, collection lines with ultra-violet fibre-optic light sources, cyclone knockdown traps and an adult collection chamber maintained at 3 °C. Teneral LBAM adults were collected at this point in the production.

Wild LBAM were trapped in Scentry® Delta traps (Scentry Biologicals Inc., Billings, Montana, USA) baited with 2 mg of the synthetic female sex pheromone, gossyplure, in California and removed on a weekly basis.

The trap glue was analysed by removing a small amount of the substance from the trap and putting it into a tin cup and running it through the CM-CRDS. All samples were dried at 60 °C for 24 h, and weighed into 8 by 5 mm tin cups.

ISOTOPE ANALYSIS WITH EA-IRMS AND CM-CRDS

Samples were analyzed using an EA-IRMS, Carlo Erba (Milan, Italy) carbon nitrogen elemental (CN) analyzer, linked to an Optima automated IRMS (GV Instruments, Manchester, United Kingdom).

Isotope analysis with the CM-CRDS system was carried using the Picarro CM-CRDS based at the University of Arizona, Tucson, Arizona and the diet components at the Picarro Head Quarters, Santa-Clara, California. The CM-CRDS system basically consists of an elemental analyzer combustion module to produce a gaseous sample from the solid sample, an interface for collection and transfer of the gaseous combustion product and a CRDS analysis chamber. This technique measures the decay rate of light intensity at different wavelengths in order to determine gas concentrations and isotope compositions. Briefly, single frequency light is introduced into the sample gas chamber, when the light reaches a threshold value it is switched off and

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the decay of the light is measured over a vast path distance using a series of highly polished mirrors and detectors. In the absence of an absorber, path distance can reach up to 20 km. In the presence of an absorber the ring down times are considerably shorter and dependent on the presence and isotopic composition of the gaseous absorber targeted.

A full complement of internal and external standards was run with both sets of samples in order to calculate the isotopic ratios and % C values. The isotope ratios were expressed as parts per thousand per mille (‰) or $\delta^{13}C$ deviation from the internationally recognized standards Vienna Pee Dee Belemnite (VPDB).

ANOVA or Student's *t* tests were used to distinguish populations and standard regression analysis to compare methods. A program officer wants to know the probability of a sampled insect being derived from one population or the other, e.g., a wild or laboratory or mass-reared insect, and therefore we used a cut off of 2σ. This means that if we take a 2σ upper and lower limit of both the mass-reared and wild moths we can say with 95% certainty that we are correctly assigning each of the moths to the correct population. This issue is discussed in more detail by Hood-Nowotny et al. (2016) in this special issue. All statistical analyses were carried out using Microsoft Excel or using the Analysis function in Sigma Plot 12.

RESULTS

We set out to test the suitability of the CM-CRDS system for determining isotopic signatures of moths in ecological research or programmatic settings. There were highly significant correlations between the values for common mass-rearing dietary components measured using EA-IRMS and the CM-CRDS (Fig. 1). The 99% confidence interval of the intercept included the value 0 and the 99% confidence interval of the slope included the value 1 suggesting that the results from the 2 methods did not differ significantly (Fig. 1). The offset of the absolute isotope value was only 0.2 ‰.

MOTH SAMPLES

For the common cutworm there were highly significant correlations between the values obtained using EA-IRMS and the CM-CRDS (Fig. 2). The 95% confidence interval of the slope included the value 1 for the samples measured and again this suggests that the results from the 2 methods did not differ significantly.

It was possible to distinguish the wild field-caught and mass-reared populations of common cutworm based on the separation of $\delta^{13}C$ signatures measured using the CM-CRDS (Fig. 3). This would allow for identification of the diet-reared moth with 99.7% confidence (no overlap of individual values even at 3σ) and this was a comparable result with that obtained using the EA-IRMS system.

The legs and spermatophores of mass-reared female common cutworm mated with wild male moths had $\delta^{13}C$ signatures of −22‰ (legs) and −27‰ (spermatophores) (Fig. 4). This demonstrated that there was a nuptial gift to the females from the field-caught males, which had an isotopic signature of around −30‰. It was possible to detect this nuptial gift using these isotopic techniques. The sample was sufficient to get a reliable reading from the CM-CRDS, i.e., it was above the detection limit of the CM-CRDS, which is given as 250 µg of carbon by the manufacturer and it was demonstrated that CM-CRDS could be used for this type of paternity tracing experiment where there is sufficient sample mass.

Although the mean isotopic signature of the 2 populations of LBAM were significantly different from on one another ($P < 0.001$ Student's *t* test), the wild population had a $\delta^{13}C$ value of −28.6‰ (+/− 2.4 SD) and the mass-reared population a value of −25.0‰ (+/−0.2 SD). This was not a sufficient difference to be able to confidently assign an individual moth to one population or the other, as they were separated by only 1.3 standard deviations allowing us to predict the population member with only an 80% confidence not the 95% confidence we would normally require. Although the standard diet was used, it had a sugar content less than 4%, which was lower compared with other moth mass-rearing diets and this was reflected in its isotopic signature of −23.8‰, we had expected a less negative value and we are confident that a C_4 plant-derived sugar was used in its formulation.

The diet used for the rearing of LBAM was measured repeatedly with the CM-CRDS and had a $\delta^{13}C$ value of −23.8‰ and the reproducibility was ± 1 SD ± 0.2‰ ($n = 9$), which is similar to the manufacturers reported values for machine error and values reported for organic rich shales (Balslev-Clausen 2013).

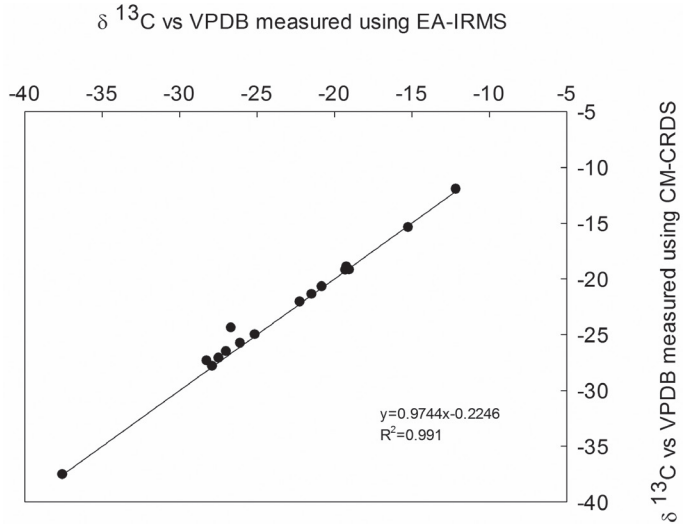

Fig. 1. Carbon isotope ratios of 16 different common dietary components measured using either elemental analysis isotope ratio mass spectrometry (EA-IRMS) or combustion module cavity ring down spectrometry (CM-CDRS).

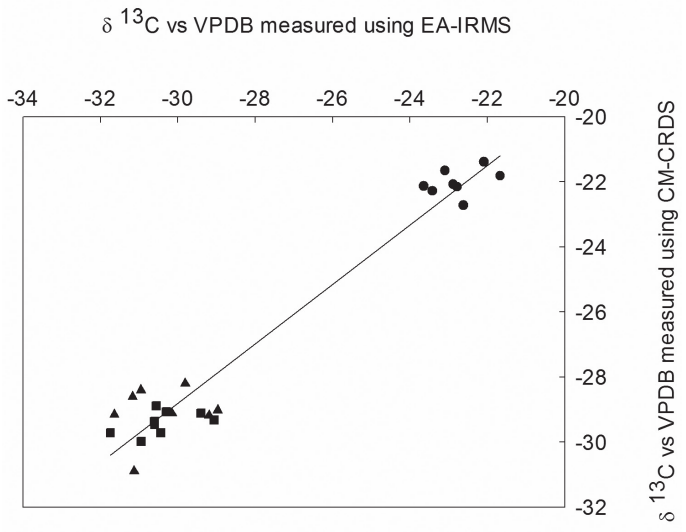

Fig. 2. Carbon isotope ratios of 3 populations of the common cutworm measured using either elemental analysis isotope ratio mass spectrometry (EA-IRMS) or combustion module cavity ring down spectrometry (CM-CDRS): Field-caught moths: squares; synthetic diet-reared moths: circles and laboratory-reared on castor diet moths: triangles.

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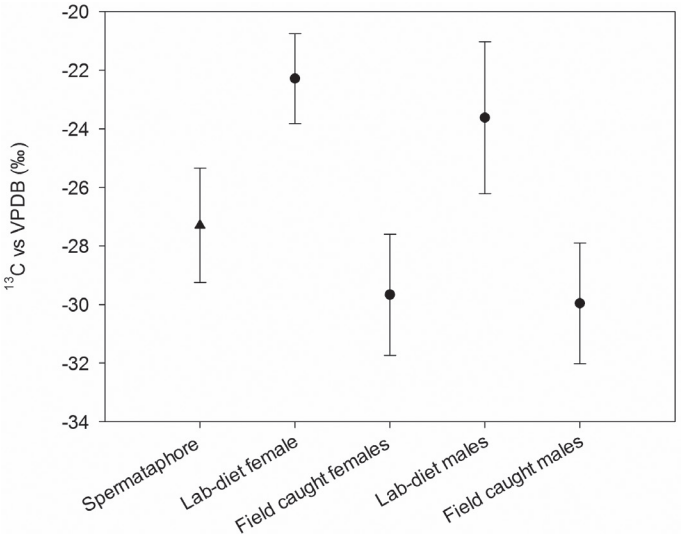

Fig. 3. Carbon isotope signature of common cutworm leg samples from different moths reared on the artificial laboratory diet or caught in the wild (circles, *n* = 5, Bars +/- 3 SD). The spermatophore data point (triangle) is the carbon isotope signature of spermatophores dissected from laboratory-reared females mated with field-caught males (*n* = 5, Bars +/- 3 SD). All samples measured using CM-CRDS.

The isotopic values of the different sticky trap glues were similar and in the range of −28 to −32‰ vs. VPDB (Table 1).

Discussion

It was previously demonstrated that the CM-CRDS system was suitable for measuring isotopic signatures in geological samples (Balslev-Clausen 2013). Here, we have shown that the system is also suitable for measuring isotopic signatures in insects, particularly moths. As in the above study we observed a slight offset in absolute isotope values between the CM-CRDS and the EA-IRMS system. The CM-CRDS system requires a minimal sample size of around 250 µg carbon whereas the EA-IRMS measures reliably with 150 µg carbon. Interestingly the offset for the feed samples between the 2 machines was low (0.2‰) and lower than those observed in other comparative studies with rock samples (Balslev-Clausen 2013), which is possibly due to the better combustibility of the diet sample compared with rock. The differences may be due to the slightly different procedures used to calibrate the machines and possibly due to issues with the front end combustion rather than the isotopic measurement itself (Balslev-Clausen 2013). However, the purpose of our study was to assess the suitability of the CM-CRDS system for measuring comparative differences in isotopic signature over a range of isotopic values rather than assessing absolute isotope values. Hence, these small differences would not affect the outcomes of the studies we propose. It should be stressed that the reference standard procedure needs to be implemented to yield scale normalized values which would allow for inter-laboratory analysis and overcome any instrument or sample conversion bias. This requires using at least 2 reference standards that bracket the measured values and require simple regression to obtain the actual sample values (Meier-Augenstein & Kemp 2011). Under normal circumstances a quality assurance, traceable C_3 and C_4 sugar internal standard would be suitable for the studies proposed. The only dietary components which were outside of this range were wheat and spirulina, *Arthrospira platensis* Gomont (Oscillatoriales: Phormidiaceae) which is an aquatic cyano-bacterium. So in the context of labelling mass-reared moths and distinguishing them from their counterparts, traceable C_3 and C_4 sugars should be adequate standards.

We showed that the CM-CRDS system is suitable for measuring isotopic ratios of moth samples and that moth samples are suited to the CM-CRDS due to the higher dynamic range of the CM-CRDS, such that whole individuals can be easily analyzed, thus reducing sample preparation time.

The wild and mass-reared leg samples of the common cut worm showed good separation as we had observed using the EA-IRMS. The larger error of measurement of the CM-CRDS analysis as compared

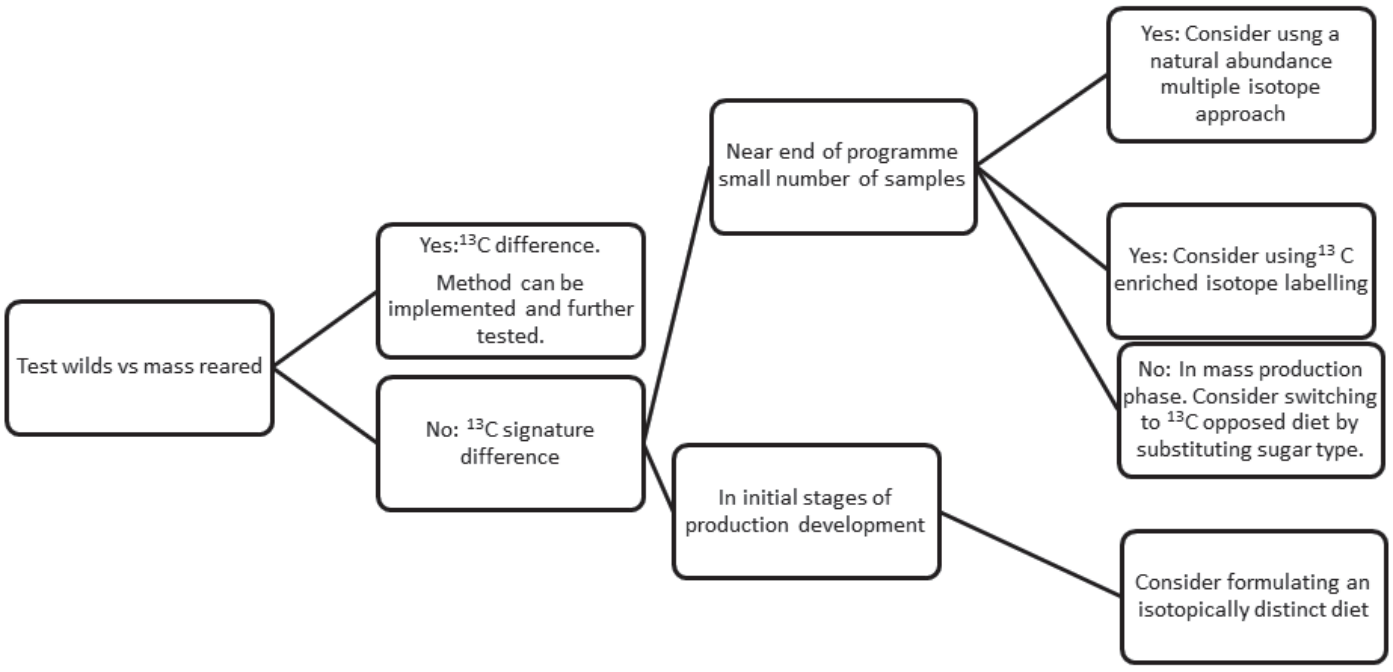

Fig. 4. Decision tree to select an option for using isotopes in an operational program.

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Table 1. Description and mean isotope value of the glues used for capturing moths in various lepidopteran traps analyzed using CM-CRDS (*n* = 2).

Trap Type	Vendor/Description	Target pest	Origin trap with glue	$\delta^{13}C$
A) PBW program standard glue in Arizona.	Scentry® biologicals Inc. 3/12. White body trap, soft glue	Pink bollworm	Phoenix, AZ PBW lab	−31.0
B) Hard glue PBW trap, for possible future use.	AlphaScents™ Insect monitoring systems. PBW, red body trap	Pink bollworm	Phoenix, AZ PBW lab	−28.7
C) Trap insert for Large Plastic Delta trap, hard glue trap insert from AlphaScents	AlphaScents™, insert for large plastic delta trap	Used in CA-CPHST lab for LBAM, EGVM, codling moth and other lepidopterans	Salinas, CA	−30.4
D) Red paper delta trap, soft glue	Scentry® 4/10	EGVM and old PBW program traps	CDFA 2011 from CPHST stash	−31.1
E) White insert 8" x 7" soft glue insert for large plastic delta trap	Scentry® LPDT	Used in CA-CPHST lab for LBAM, EGVM, codling moth and other lepidopterans	2010, CPHST lab	−31.2

Abbreviations. AZ, Arizona; CA, California; CA-CPHST lab; California-Center for Plant Health Science and Technology Laboratory, US Department of Agriculture; EVGM, European grape vine moth; LBAM, light brown apple moth; PBW, pink bollworm.

with the EA-IRMS becomes irrelevant as it was often lower than the inherent variability in isotopic signature of the population.

Moreover in this preliminary study using CM-CRDS to analyze the isotope signature of spermatophores we were able to determine with a high degree of confidence whether the paternal male moth was mass-reared or wild. This method of determining parental identity of moths has been explored and been previously described by Stringer et al. (2013), and we merely demonstrate here that the CM-CRDS system could substitute the EA-IRMS system for spermtophore analysis, with all the associated benefits of low cost, minimal training required etc.

Although the isotopic signatures of the 2 LBAM populations were significantly different we were not able to confidently distinguish the 2 populations from one another. Normally we take an approach similar to analytical chemistry using a cut-off of 2 standard deviations of separation. If we get separation when we plot the 2 populations with 2 standard deviations of the mean and if there is no overlap in the error bars we can be 95% confident that if we assign an individual insect to a particular population it will be correctly assigned. If there is no overlap with 3 standard deviations of the mean then we can be 99.7% confident, etc. However in this case it was not possible to make that distinction although it should be stressed that this was not due to the isotope analysis method with CM-CRDS, but due to the lack of isotopic difference in the diet of the 2 populations. In this special issue, Hood-Nowotny et al. (2016) demonstrated that if it is not possible to get isotopic separation using $\delta^{13}C$ signatures alone, it is possible to use the additional isotopes of H, N, O or S to improve the prediction. This could be one of the limitations of using a CM-CRDS system compared to the EA-IRMS, i.e., that it does not allow measurement of additional N and O isotopes which the EA-IRMS systems do. There is a CM-CRDS system that also measures H in bulk material, but currently the machines cannot measure other isotopically interesting elements. Indeed one of the flexibilities and advantages of the EA-IRMS is that it can often analyze multiple elements simultaneously however this comes at a minimal addition cost per sample.

Depending on the juncture and target species in an area-wide management program it could be useful to design the mass-rearing diet to ensure isotopic separation based on the $\delta^{13}C$ signature, for example by adding C_4 based products in the diet formulations such as maize, sugar cane, sorghum etc. For illustration purposes; to get a target enrichment of the diet of around −24‰, which is usually sufficient to get sufficient differentiation from a C_3 host with a value of −28‰, approximately 25% of the diet needs to be of C_4 origin and vice versa if the host species is a C_4 or CAM plant. If this is not possible and it is a critical point in the pest management program, such as nearing eradication when managers may not want to change the diet formulation significantly, then it may be economical and useful to use synthetic isotope labelling with enriched ^{13}C sucrose or glucose added to the mass-reared diet. Here sufficient enrichments for separation can be achieved with isotope costs in the region of 50 USD per kg of diet, a small cost compared to the cost of continued program operation. A simple decision support tree is shown (Fig. 4) to emphasize this point and clarify the decision-making process.

One of the advantages of this isotope technique is that it is simple and cheap to test. The estimated variability of the wild population is usually captured within the first 12 or so samples if we assume normal distribution, because the standard deviation rarely increases above this number and would rarely change as we increased sample size, particularly for a specific habitat characterized by similar vegetation and host types (Fig. 5) (Altman & Bland 2005). In highly diverse habitats with insects of different feeding strategies and hosts this assumption may not hold, but in most of the samples that we have analyzed over the years this assumption appears to hold true. Under operational conditions this means that only 12 wild samples from a particular area are required to determine the spread of the wild population, and to determine whether this is a suitable method for distinguishing the wild population from the mass-reared population.

The implementation of this isotope-based technique in the field requires that samples are captured from traps. Some traps wash off surface markers and some traps have copious quantities of sticky glue. Indeed for isotope analysis the glue can be a major interfering factor. We have shown that the glue in common sticky traps of Scentry Biologicals has an isotopic signature of −30‰ and a high C content. Insects are about 50% C so even contamination of about 5–10% by weight with the glue can lead to a 1‰ shift in signature. Therefore it is advisable to sample strategically from the sticky traps. Often we will use a pre-defined appendage, either a wing or a front leg for sampling, and make certain that we take comparable samples from artificially reared populations, this overcomes the glue contamination problem by shear avoidance, ensuring that the insect sample is separated from the trap immediately and dispatched with minimal traces of glue on it. Another reason for selecting legs or wings is that there is minimal possibility of interference of variable fat concentrations, which lead to slight variations in the isotopic signal dependent on the lipid concentration. The reason is that lipids are generally lighter than proteins and carbohydrates (Park & Epstein 1961) because of discrimination against ^{13}C during lipid biosynthesis (DeNiro & Epstein 1977). If isotope meth-

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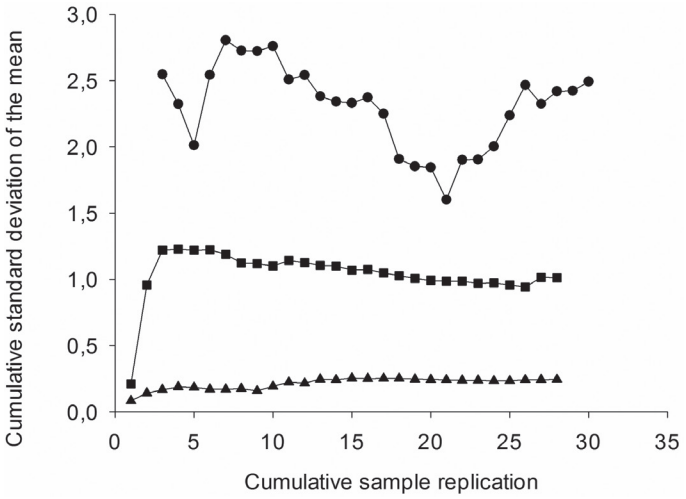

Fig. 5. Cumulative standard deviation of the mean carbon isotope signature of individual moths, field-caught LBAM (circles), mass-reared pink bollworm (squares) and mass-reared LBAM (triangles), analysed using the CM-CRDS module.

ods are to be used then the use of dry traps such as the AlphaScents™ traps maybe advised as these leave minimal amount of residual glue samples on the insects and are generally easier to handle. Moreover legs or wings are generally structural tissue and turn over at lower rates than the more metabolically active thoracic or abdominal tissues therefore retaining the dietary signal of the natal diet, which means that we do not have to place too much emphasis on adult feeding if it occurs, because the main signature measured comes from the larval diet. However it would be wise to test these factors in simple C_4 C_3 sugar switching laboratory experiments.

Our data demonstrate that it is possible to determine the paternal identity, mass-reared or wild, based on isotopic analysis of the spermatophores, as it has been shown there is sufficient C in the moth spermatophore for isotope analysis using CM-CRDS. It should be stressed though that there must be sufficient isotopic signal difference in the populations for this method to work and that the mass of spermatophores must be greater than about 250 µg of C to be above the detection limit for isotope measurement of the CM-CRDS. Moreover this CM-CRDS technology could easily be applied to other insects of interest.

In conclusion we showed that CM-CRDS could be used to measure isotope ratios of lepidopterans in an area-wide insect pest control operational context in the same way that they have substituted many of the current high-precision EA-IRMS machines used for isotopic CO_2 analysis in atmospheric studies, geological applications and human metabolic studies (Haviland et al. 2012; Hoke et al. 2013). The strategic advantages of CM-CRDS systems are mainly that they are easy to operate and technically robust, making them ideal for use in field studies and for operational programs. For example, the technician on the project was shown to be sufficiently competent and felt comfortable running the samples after just 2 days of training and operation. To get an idea of the capital investment required calculations were made based on current sales prices of the carbon CM-CRDS system. Based on a 148 daily sample throughput and using the machine 48 wk per yr, the 5 yr ameliorated capital cost per sample is around 0.36 € (0.50 USD). This is based on single insect sampling however if 3 samples were pooled, (i.e., 3 legs from different individuals) and this allowed for accurate distinction, the cost would come down to around 12/16 €/$ cents per sample. Running costs of the machine are relatively low requiring only N as the carrier gas. It should be noted that the overall technology costs are relatively low as this is now an off the shelf technology, which would require little methods development.

The data presented herein suggests that CM-CRDS systems could be used to determine the rearing origin of moths and similar-sized insects based on their $\delta^{13}C$ signature in an SIT context in addition that they could be used to resolve paternity issues in moths. Based on their affordability, ease of use and the quality of the data they produce CM-CRDS systems overcome a technical barrier to the adoption of stable isotope techniques in entomology.

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