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# Stable isotope markers differentiate between mass-reared and wild Lepidoptera in sterile insect technique programs

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**Abstract**

In this study we identified a number of moth (Lepidoptera) species that are potential targets for the sterile insect technique (SIT), and we assessed the feasibility of using stable isotope signatures as markers to distinguish mass-reared from wild moth species. Large natural differences in the isotopic signatures of commercially available sugars render them novel markers for mass-reared insects. Sugar beet (*Beta vulgaris* L.; Caryophyllales: Amaranthaceae), a $C_3$ plant, has a stable isotopic signature (a measure of the ratio of the stable isotopes $^{13}C$:$^{12}C$) of around −27‰ relative to Vienna Pee Dee Belemnite (VPDB; the international C isotope standard for the stable isotopes, $^{13}C$ and $^{12}C$), and sugarcane (*Saccharum* spp.; Poales: Poaceae), a $C_4$ plant, has an isotopic signature of around −11‰. Thus by means of such a distinct isotope ratio in the sugar in the diet, mass-reared insects can be easily distinguished from wild insects with a high degree of certainty. It was shown that the method could be extended using a multiple isotope approach, with $^{15}N$ or a full suite of C, N, S and O isotopes. Intrinsic isotope marking of mass-reared moths proved to be an accurate means of distinguishing wild from mass-reared populations, based on isotopic differences between the wild host plant species and the diets used in mass-rearing, which where possible, had been manipulated to contain the isotopically divergent sugar type. This intrinsic labeling using stable isotopes could be useful in the assessment of the quality of mass-reared moths, because a stable isotope is a marker that does not affect the insect in any detrimental manner.

Key Words: elemental analysis-isotope ratio mass-spectrometry (EA-IRMS); sterile insects; release/recapture; isotopic signature; sugar; larval diet; markers; moths

**Resumen**

En este estudio se identificaron una serie de especies de polillas (Lepidoptera) como objetivos potenciales para la técnica del insecto estéril (TIE), y se evaluó la viabilidad del uso de firmas isotópicas estables como marcadores para distinguir especies de polillas salvajes de las polillas criadas en masa. Las diferencias naturales mayores en las firmas isotópicas de los azúcares comerciales disponibles los hacen nuevos marcadores para los insectos criados en masa. La remolacha azucarera (*Beta vulgaris* L .; Caryophyllales: Amaranthaceae), una planta $C_3$, tiene una firma isotópica estable (una medida de la proporción de los isótopos estables: $^{13}C$:$^{12}C$) de alrededor de −27 ‰ relativa a Viena Pee Dee Belemnite (VPDB; el estandar internacional para el isótopo C de los isótopos estables, $^{13}C$ y $^{12}C$), y la caña de azúcar (*Saccharum* spp .; Poales: Poaceae), una planta $C_4$, que tiene una firma isotópica de alrededor de −11 ‰. Por lo tanto, por medio de esta relación de isótopos distinta en el azúcar en la dieta, los insectos criados en masa se pueden distinguir fácilmente de los insectos salvajes con un alto grado de certeza. Se demostró que el método podría ampliarse mediante un enfoque múltiple de isótopos, con $^{15}N$ o un grupo completo de isótopos C, N, S y O. El marcado de los isótopos intrínsecos de las polillas criadas en masa demostró ser un medio preciso para distinguir entre las poblaciones salvajes y las poblaciones criadas en masa, basado en las diferencias isotópicas entre las especies de plantas hospederas silvestres y de las dietas usadas en la cría en masa, que cuando sea posible, habían sido manipuladas para contener el tipo de azúcar isotópicamente divergente. Este marcador intrínseco

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utilizando isótopos estables podría ser útil en la evaluación de la calidad de las polillas criadas en masa, porque un isótopo estable es un marcador que no afecta el insecto de cualquier manera perjudicial.

Palabras Clave: elemental análisis de isótopos relación de espectrometría de masas (EA-IRMS); insectos estériles; liberación/recaptura; firma isotópica; azúcar; dieta larval; marcadores; polillas

Sterile insect technique (SIT) embedded within area-wide integrated pest management (AW-IPM) programs has proven to be a successful strategy for the suppression, containment, prevention or eradication of a number of insect pest species (Hendrichs et al. 2005; Vreysen 2005; Vreysen et al. 2007; Tobin et al. 2014). Progress in x-ray sterilization (Mastrangelo et al. 2010) has lowered the technological barriers to the commercialization of the SIT provided reliability issues with x-ray irradiators are overcome. With the advent of commercialization there is an increased focus on overall program management and profit margins. Program efficiency and effectiveness could be improved by strengthening the feedback from field to the factory in terms of improved sterile insect identification, achieving optimum over-flooding ratios, and evaluating mating success. However, despite progress made in the last decade, there is still a lack of adequate tools and methods to assess performance of sterile insects in comparison with wild insects (Dyck et al. 2005; Simmons et al. 2010) making the completion of the essential quality evaluation loop challenging. Stable isotope tools offer an opportunity to contribute to the completion of the quality control loop, as they allow us to study the essential biology and ecology of insects (Hood-Nowotny & Knols 2007), including the distribution and movement of populations across the landscape, and estimation of population size (Hood-Nowotny & Knols 2007)—all of which are factors critical in an insect control program (Hamer et al. 2012). Stable isotopes are non-radioactive and ubiquitous; for example $^{13}C$ with its 1 extra neutron constitutes 1 percent of all C atoms globally, posing no threat to public or environmental safety. In addition, stable isotopes have no impact on insect quality, behavior or fecundity, which allows the unhindered assessment of moth movement and performance in complex environments often encountered in insect pest management programs integrating the SIT (Helinski et al. 2007).

In area-wide eradication or suppression programs with an SIT component it is imperative to be able to distinguish sterile mass-reared individuals from wild populations, particularly when eradication is the strategic approach and the program nears its final goal of eradication. Misidentification of mass-reared individuals as native wild insects can lead to expensive response actions, reducing program efficiency and effectiveness (Tomic-Carruthers et al. 2013). The mixing of Calco red (Oil Red 2144, Royce International) to the larval diets of some Lepidoptera species, which imparts a red color to the fatty tissues and produce easily identifiable red moths, has proved to be a practical and appropriate identification strategy at early stages of release programs (Graham & Mangum 1971; Hagler & Jackson 2001; Dyck 2010). However as a program nears its final stages, additional independent markers would be very useful to verify the identity of ambiguous samples. Stable isotope markers are potentially robust and reliable independent markers which incur minor implementation costs (Hood-Nowotny et al. 2009).

The isotopic signature of an organism is dependent on what it eats (DeNiro & Epstein 1978; Hood-Nowotny et al. 2012). The inherent difference in the isotopic signatures of mass-rearing diets compared with wild diets provides us with low-cost natural markers for mass-reared insects (Hood-Nowotny et al. 2009). Terrestrial vascular plants 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 their relative discrimination against the heavier $^{13}C$ atoms. Nearly all fruit trees and other dicotyledonous plant species are $C_3$ plants, e.g., apple, banana, citrus, mango, etc.; 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. $C_3$ plants have a typical isotopic range of values between −25‰ and −35‰ with an average around −27 ± 2‰; hence, in every 1,000 atoms there are 27 fewer $^{13}C$ atoms than in the international standard Vienna Pee Dee Belemnite (VPDB). The latter value is the delta value (δ value; pronounced "delta thirteen cee"), which conventionally is referred to as parts per thousand, per mille, per mil or ‰. All δ values herein are reported according to the internationally accepted scale.

Plants such as sugarcane have a $C_4$ metabolism and have $\delta^{13}C$ values which range between −7 and −18‰. The $\delta^{13}C$ of maize is around −11‰. Desert-adapted plants have CAM photosynthesis, characteristically opening their stomata and collecting $CO_2$ at night, and have a $\delta^{13}C$ signature similar to $C_4$ plants, around −11‰. Although in this paper we concentrate on using $\delta^{13}C$ as the isotope marker—because it is the easiest to measure and reliable to manipulate—there are instances where the differences in $\delta^{13}C$ are not sufficient between the mass-reared and wild populations; and we explore whether other alternative isotope markers could be used. This is based on the idea that every region and production process is isotopically unique because the isotope ratios of elements are influenced by environmental factors and the natal production process (Rossmann 2001). For example the spatial patterns of H and O isotope ratios in precipitation are the result of discrimination against the heavier deuterium (D) and O atoms in a number of exchange and kinetic processes in the global hydrological cycle and will vary systematically according to distance from the sea, latitude, altitude and climatic factors (Meier-Augenstein & Fraser 2008; Galimov 2005). Plant N isotopes vary according to the source of N fertilization, and S isotopes vary with distance from the sea and the influence of pollution (Rossmann 2001). Isotopic signatures are independent of the conventional marking techniques such as Calco red. Moreover the isotope signatures of C, O, N and S in mass-reared insects are generally independent of each other, i.e., sugar source, water source used to make up the diet, fertilization of the primary N source in the diet and the geographical origin of the S source. This means they can be treated as independent markers in a naïve Bayesian framework, with each isotopic measurement adding weight of evidence to the nature of the origin of the moth in question (Girón & Ríos 1980).

Isotope markers have proved to be a suitable population marker for mosquitoes (Hood-Nowotny et al. 2006; Hamer et al. 2012), fruit flies (Hood-Nowotny et al. 2009), and tsetse flies (Hood-Nowotny et al. 2011). In this paper, we set out to determine whether stable isotope signatures could also be used as secondary independent markers in lepidopteran species, based on the natural signature differences between wild host plants and laboratory artificial diets. The majority of moth species feed on $C_3$ plants in the wild that have a $\delta^{13}C$ of around −28‰ (O'Leary 1988). However, almost all Lepidoptera mass-rearing facilities and laboratories that keep experimental colonies use a high concentration of sugar in the larval diet (Fig. 1), which is usually a $C_4$ sugar source (with a signal of around -11‰) (O'Leary 1988), and therefore this could provide an easy signature to differentiate mass-reared released from wild moths. It should be noted that the host plant of some moth species is either a CAM species or a $C_4$ species, both of which have a $\delta^{13}C$ signal around −11‰; therefore, rearing on $C_3$-based sugar beet (*Beta vulgaris* L.; Caryophyllales: Amaranthaceae; $\delta^{13}C = -27$

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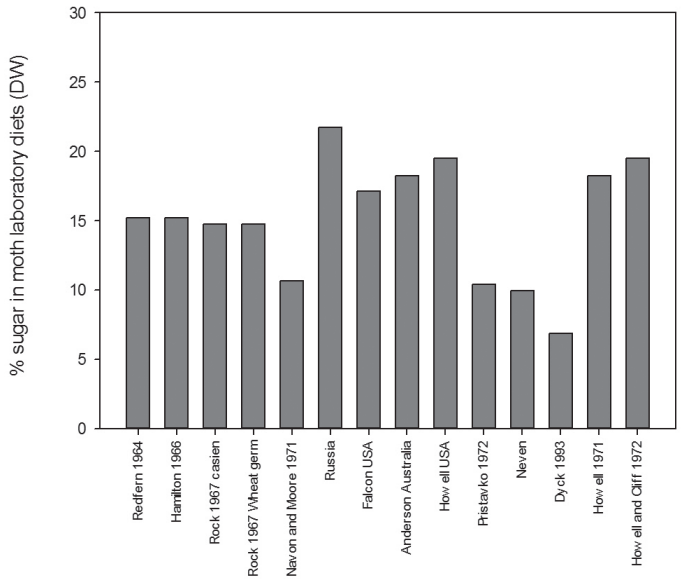



Fig. 1. Percentage of sucrose based on dry weight (DW) in popular artificial diets for mass rearing various moth species, as described by Dyck (2010).

± 2‰) diet would give the isotopic distinction required. In addition, where we could not achieve this required distinction, we tested a multiple isotope approach. It uses the isotopic signature of 4 independent isotope signatures, i.e., C, N, O and S, to separate the populations. These hypotheses were tested using samples of moths from several colonies and wild populations. We focused on a number of species that are considered major threats to fragile ecosystems or production agriculture and for which a number of control strategies have been initiated or planned.

## Materials and Methods

### INSECT SPECIES

### Description and History of Each Species Used in the Experiments

*Cactus moth.* The cactus moth, *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae) is well known for its role as a biological control agent for weedy *Opuntia* spp. in several countries such as Australia and South Africa. However, its introduction into the Caribbean and subsequent unintended arrival in North America has resulted in an economic and ecological threat to native and cultivated *Opuntia* spp. in the USA, Mexico, and other countries throughout the world (Zimmermann et al. 2004). To mitigate the economic and ecological threat of *C. cactorum* to native *Opuntia* spp. in North America, *C. cactorum* have been reared in the laboratory to support the development of survey and control tactics including the SIT (Carpenter et al. 2001; Hight et al. 2005). The SIT was one of the tactics used in eradication of 2 incursions of this species from the Yucatan Peninsula of Mexico (Carpenter et al. 2008).

*African sugarcane borer.* The African sugarcane borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae) (Eldana) is indigenous to Africa, where it feeds on a variety of wild host plants including wetland sedges (Cyperaceae, Juncaceae, Typhaceae) and native grasses (Poaceae) (Polaszek & Khan 1998; Conlong 2001; Mazodze & Conlong 2003). First described from sugarcane in Sierra Leone over a hundred years ago, *Eldana* has been reported throughout much of sub-Saharan Africa (Betbeder-Matibet 1981; Conlong 1997, 2000, 2001; Polaszek & Khan 1998; Mazodze & Conlong 2003). It has been of concern in South Africa since 1939 (Dick 1945) having achieved major pest status in all South African sugarcane growing areas since the 1970's (Carnegie 1974; Paxton 1982; Webster et al. 2005).

*Common cutworm.* The common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is an economically important widely distributed polyphagous pest found in India and Australia. It is reported to attack more than 100 host species (Lefroy 1908; Moussa et al. 1960; Thobbi 1961; Chari & Patel 1983) including $C_3$ plants such as cotton, flax, groundnuts, jute, lucerne, rice, soybeans, tea, tobacco, and vegetables, and $C_4$ plants such as taro (*Colocasia esculenta* (L.) Schott; Alismatales: Araceae) and maize. The SIT has been investigated and proposed as a suitable method of control, as the common cutworm has developed resistance to a number of insecticides (Seth & Sharma 2001).

*Light brown apple moth.* The light brown apple moth (LBAM), *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) was originally a native pest of Australia but has since been detected in New Zealand, the UK, and Ireland (Suckling et al. 2010). It was reported in Hawaii in the late 1800s; however, the first USA mainland detection of LBAM was in Alameda County, California on 22 Mar 2007. Since then its spread has been limited through intensive control activities carried out by the United States Department of Agriculture (USDA) and the California Department of Food and Agriculture (CDFA) (Suckling et al. 2010). LBAM is of particular concern because it can damage a wide range of crops and other plants, over 500 species in total (Brockerhoff et al. 2011), including redwoods, oaks and a number of agricultural crops such as grapes, citrus, stone fruit (peaches, plums, nectarines, cherries, apricots), and many others. Elements of the SIT have been developed against this species, and limited field releases have been made in New Zealand, Australia and the USA (Kean et al. 2011; Suckling et al. 2014; Soopaya et al. 2011; Suckling 2011).

*Diamondback moth.* The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is an important pest of cruciferous crops worldwide, causing an estimated annual loss of US$ 1,000 million due to damage and control costs (Gryzwacz et al. 2010). The host range of this pest is limited to plants belonging to the family Brassicaceae. Intrinsic isotopic labelling would be useful in studies of diamondback moth because it is small and fragile making it difficult to label using other techniques.

*European grapevine moth.* The European grapevine moth, *Lobesia botrana* (Denis & Schiffermuller) (Lepidoptera: Tortricidae) is a significant pest of grapes, berries and berry-like fruits in Europe, countries surrounding the Mediterranean Sea, Russia, Japan, and more recently in Chile and Argentina (Avidov & Harpaz 1969; CIE 1974). It was first found in the Napa Valley of California in Oct 2009, the first record in the USA. Due to its small size (10–12 mm) isotopic methods are an ideal method of identification should conventional marking methods fail, when it is controlled using the SIT or if the determination of its area of origin was important.

*Pink bollworm.* The pink bollworm, *Pectinophora gossypiella* (Saunders) was a major pest of cotton in the southwestern USA (Henneberry & Naranjo 1998). The moth is native to Asia, but has become an invasive species in most of the world's cotton-growing regions (Henneberry & Naranjo 1998). In 2001, an area-wide eradication campaign was launched against pink bollworm and it has driven this pest to extinction across the south-western cotton belt in 4 USA states and northern Mexico (Tabashnik et al. 2010). The success of the eradication campaign was in part guaranteed by the use of multiple tactics, such as the use of *Bt* cotton combined with the release of up to 200 million sterile moths per week.

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## MOTH FIELD SAMPLE COLLECTION AND REARING

*Cactus moth. Cactoblastis cactorum* was reared on excised *Opuntia ficus-indica* (L.) Miller cladodes and an artificial diet following the methods described by Marti et al. (2008). A walk-in environmental chamber maintained at 29 °C with a ± 2 °C daily variation, 14:10 h L:D photoperiod, and 60–80% RH was used to hold all rearing containers. The artificial medium used in the study (Carpenter & Hight 2012) was composed of the following: 2.5 L water, 45 g agar, 15 mL of mold inhibitor (made from a stock solution consisting of 418 mL propionic acid, 42 mL phosphoric acid, and 540 mL water), 9.6 g ascorbic acid, 4.2 g sorbic acid, 100 g sucrose (derived from sugar beet, *Beta vulgaris* L.), 6 g nipagin (Methyl *p*-hydroxybenzoate), 186 g brewer's yeast, 15 g malic acid, 5 g citric acid, 5 g oxalic acid, and 630 g of pulverized white kidney beans (*Phaseolus vulgaris* L.). Diet was poured into a tray, allowed to solidify, and then cut into blocks (50 × 50 × 20 mm). Diet blocks were dipped into molten beeswax to provide a thin waxy coat simulating a wax-covered cactus cladode (Marti et al. 2008).

*African sugarcane borer.* Live stem borer larval stages were collected from one of its indigenous hosts, *Cyperus papyrus* L. (Cyperaceae) located on a farm (29° 54′ S; 30° 31′ E; 704 masl) in the Eston area of KwaZulu-Natal (KZN), South Africa, and from infested sugarcane at 2 different locations, Tinley Manor (29° 27′ S; 31° 15′ E; 17.1 masl) on the north coast of KZN and another farm (29° 54′ S; 30° 39′ E; approx. 700 masl) in the Eston area. They were placed individually into 30 mL plastic vials containing a piece of sugarcane stalk or *C. papyrus,* depending from which plant the insect stage was collected, and returned to the laboratory. Adults were allowed to emerge and subsequently collected.

Mass-reared moths were produced according to methods described by Graham & Conlong (1988). The artificial diet composition was as follows: dried crushed sugarcane 22 g, ground chickpea 120 g, glucose 20 g, casein 12 g, brewer's yeast powder 12 g, ascorbic acid 4 g, sorbic acid 2 g, agar 109 g, water 1000 mL, nipagin (methyl paraben) 1.6 g, benomyl 0.03 g, formaldehyde 40% 1.2 mL, methanol 50 mL.

*Light brown apple moth.* A dry mix for the artificial diet was made up from the following ingredients per 12 kg mix: cellulose powder 1321.68 g, lactic casein, 12% moisture content 427 g, agar 203.32 g, Wesson's salts mix 122 g, fine wheat germ (milled health food wheat germ) 366 g. Then 2440 g of the dry mix was mixed with 6 g cholesterol, 8640 mL water, and 30 g linoleic acid, stirred and autoclaved. After cooling, the following ingredients were added: (i) 240 g of Vanderzant's mixture (360 g sucrose, 60 g glucose, 1.8 g streptomycin, 1.8 g penicillin, 1200 mL water), (ii) 180 mL of mold inhibitor consisting of 150 g Nipagin™, 200 g sorbic acid, 1700 mL of 95% ethyl alcohol, and (iii) Prochloraz™ (fungicide) 0.48 g. Larvae collected from the glasshouse were fed on potted kumquats (*Fortunella japonica* Thunb.; Sapindales: Rutaceae).

*Common cutworm. Spodoptera litura* was reared on 2 diets, i.e., on castor leaves (*Ricinus communis* L.; Malpighiales: Euphorbiaceae) which is the natural food, and a synthetic meridic diet containing chickpea seeds and sinigrin as a phagostimulant. Synthetic diet ingredients consisted of: agar 25 g, deionized water 750 mL, casein 44 g, ground chickpea seeds 93.5 g, Wesson's salts 12.5 g, cholesterol 1.25 g, yeast (dried, brewer's) 19 g, methyl-p-hydroxybenzoate 1.25 g, sugar 39 g, sorbic acid 2 g, deionized water 400 mL, 4 M KOH 6.25 mL, corn oil 2.50 mL, linseed oil 2.50 mL, formaldehyde (10%) 5.50 mL, sinigrin (1%) 3.53 mL, antibiotic and vitamin mixture 7.50 g [made up from a mixture of chloramphenicol (2 g), streptomycin (4 g), tetracycline (36 g), ascorbic acid (80 g), Evion (vitamin E; 0.2 g; Merck Co.), vitamin mixture (2 g; Roche Co.), choline chloride (1.25 g)]. All insects were reared under ambient environmental conditions in the insectary at 26.8 ± 1 °C, 75 ± 5% RH and a photoperiod of 12:12 h L:D. Larvae developing on the castor leaves were allowed to pupate in moist, loose soil. Larvae developing on the chickpea diet pupated in the diet container.

*Diamondback moth.* Wild diamondback moths were hand collected from an organic farm as second or third instar larvae infesting kai-lan (*Brassica oleracea* L.; Brassicales: Brassicaceae) (Ladybird Organic Farm Semenyih, Malaysia) and reared in the laboratory on plant materials (transplanted kai-lan growing in pots). Adult moths were separated by gender upon emergence and placed in a screened cage. Laboratory-strain moths (more than 36 generations) were obtained from the Malaysian Agricultural Research and Development Institute, Serdang, Malaysia. Adults were allowed to oviposit in the creases of corrugated aluminum foil. A strip of foil containing eggs was placed in a disposable cup containing artificial diet. Eclosed neonates were reared on artificial diet and completed their life cycle within the cup.

The diet consisted of 96.0 g agar, 2,900 mL water, 126.0 g casein, 30.0 g cabbage leaf powder, 175.0 g wheat germ, 26 mL linseed oil, 36.0 g vitamins B-complex, 0.9 g vitamin C, 25.0 g cellulose, 36.0 g Wesson's salt, 46.0 g ascorbic acid, 5.4 g benzoic acid and 135.0 g sugar (modified from Hou 1985).

*European grapevine moth.* Eggs hatched in a Petri dish, half filled with a premix (38-0600, WARD'S, New York www.wardsci.com; unfortunately, despite inquiry, composition information is proprietary). Before pupation, larvae left the food, in search of a dry place to pupate, and thus the Petri dish was opened, allowing the larvae to migrate to and pupate in crumpled paper towels, which lined the rearing box. Emerged adults were removed and placed in a net cage with oviposition substrates (wax paper strips) and a 5% sugar solution. Vineyard-collected larvae were reared in net cages within the collected grapes and the emerged adults were immediately sampled on emergence.

*Pink bollworm.* All rearing procedures followed standard protocols for rearing pink bollworm (Stewart et al. 1984): egg collection cages with 35 g pupae and automated scale collection; egg pads from each cage were divided into 8 equal pieces (each holding approximately 4000 eggs) and used to infest 250 g of artificial diet. For rearing purposes, mature pupae were loaded into the emergence system, which consisted of emergence boxes, collection lines with ultra-violet fiber-optic light source, cyclone knockdown traps and a 3 °C adult collection chamber. Adults were collected from the Phoenix, Arizona mass-rearing facility at this point in the production. Wild moths were collected from infested cotton bolls from an untreated short staple cotton variety near Yuma, Arizona.

## METHODS FOR STABLE ISOTOPE ANALYSIS

All moth samples were collected in small plastic vials or Eppendorf tubes and dried at 60 °C, and a few grains of blue silica gel were added and vials were closed. The samples were then shipped to Vienna, Austria, for isotope analysis. Legs, wings or whole moths were sampled depending on the species size, but similar sample sizes and identical appendages, e.g., whole front right leg, from the same populations of the various species were always analysed for accurate comparison. The rationale was to take the simplest approach to sample preparation and to overcome possible contamination from capture glue (in which case comparable wings were sampled). For multiple isotope analysis, dried whole moth samples were ball milled and subsampled. For total N, C, $^{15}N$ and $^{13}C$ analysis, samples were placed into 8 × 5 mm tins and analyzed at SILVER (Stable Isotope Laboratory at the University of Vienna), using an isotope ratio mass spectrometer (Delta PLUS, Thermo Finnigan, Germany) interfaced with an elemental analyzer (Flash EA, CE Instruments, United Kingdom) (Hood-Nowotny et al. 2012)—this method is known by the acronym EA-IRMS. Sulphur and O isotopes were measured in silver cups using a Vario EL III Elemental analyzer (Elementar Analysensysteme GmbH, Hanau/Germany) combined with an isotope ratio mass spectrometer (Delta Plus X P, Themo Finnigan Brem-

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men Germany). Oxygen isotopes were measured by high temperature pyrolysis at 1,450 °C.

A full complement of internal and external international standards was run with the samples to calculate delta and % N and % C values. Analytical accuracy for the isotope measurements were ± 0.2‰ for C and N, and less than ± 0.5‰ for O and S.

The isotope values were expressed as parts per thousand or per mil (‰) or δ deviation from the internationally recognized standards, VPDB, atmospheric N, VCDT (Vienna Canyon Diablo Troilite), V-SMOW (Vienna-Standard Mean Ocean Water) (Gröning 2004) for C, N, S and O, respectively.

A lower-case delta value was defined as the isotopic ratio of a sample standardized to the isotopic ratio of a defined reference:

$$[(R_S - R_R) / R_R] \times 1000 = \delta, \text{ which can also be written as}$$
$$[(R_S / R_R) - 1] \times 1000 = \delta.$$

Where $R_S$ is the isotopic ratio of the sample and $R_R$ is the isotopic ratio of the reference standard.

### STATISTICAL ANALYSIS

Program officers wish to know the probability of a suspect insect caught being derived from one population or the other (wild or laboratory or mass-reared). Although standard *t* test-analyses, and their natural derivatives, ANOVA and MANOVA, determine the likelihood that 2 or more populations are statistically different from one another, with a standard probability of 95% certainty, they are not necessarily the test of choice to answer this question. In fact the confidence interval (2 standard deviations of $n/\sqrt{(n)}$) can overlap by as much 25%, but the populations can still be statistically different. Herein lies the issue, if we have an individual insect with a value within the 25% overlap we cannot say with any certainty from which population that individual came, although the populations may be significantly different. We want to know with what degree of certainty we can say an individual insect comes from one population or the other. Assuming a normal distribution we can say that one standard deviation σ about the mean encompasses 67 percent of the population values, 2σ encompasses 95%, 3σ encompasses 99.73%, 4σ encompasses 99.99%, etc. So 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 them to the correct population.

This double sided rigorous test is necessary to avoid both false negatives and positives. In the scenario of a wild false negative, there is still a wild population in the target area, but we have not detected it and so no response is initiated or continued; and the lack of response may lead to the resurgences of the wild population. In the reverse scenario false positives, a mass-reared individual is assigned as a member of the wild population, and this leads either to a costly program continuation or to the costly initiation of a response program.

Theoretically we could also use a limit of detection, akin to methods used in analytical chemistry, which use 3σ deviations of the noise as the base line for detection. Laboratory or mass-reared insects will have an isotope signature that is dependent on their diet. The subsequent signature of the insect will be subject to variation dependent on 2 factors, i.e., (i) the isotopic continuity of the source of the dietary components and (ii) the natural variation in the isotopic signature of the individual insects. If we assume that most factories have consistent diets, then we can refer to the isotopic variation in the diet and individuals, as the noise level. Using the analytical chemistry analogy the detection limit is the concentration that is required to produce a signal greater than 3 times the standard deviation of the noise level (3σ). This maybe a more implementable approach for a project officer as it is always logistically easier to correctly estimate the standard deviation of the mass-reared population, and it could accommodate temporal variations in diets. However it would always be astute to estimate and assess the variation of the wild population before pursuing this approach, given the consequences of false negatives described above. This method may also be more implementable because the standard deviation of the mass-reared population is likely to be lower than that of the wild insects.

For the multiple isotope statistical analysis of both $\delta^{13}C$ and $\delta^{15}N$ signatures we used a multivariate analysis MANOVA SPSS. We have provided *P* values for all of the tests (Roy's Largest Root, Hotelling trace, Pillais trace and Wilk's lambda). To determine probabilities of wrongly designating a captured moth as either mass-reared or from the wild population, in the case of the pink bollworm, we used the simplest Bayesian approach based on the probability that these populations were distinguishable by means of the standard deviation (SD) values. That is, if we could separate the populations with 2 SD, then there was a 95% probability that the captured moth came from one population and only 5% probability it came from the other population. Designation of an insect to it population of origin would be done with even greater probability if the 2 populations could be separated by 3 SD (99.9%:0.01%), etc. Using these probabilities as our "prior distributions" in the classical Bayesian equation we sequentially calculated the probability of the sample coming from the same population based on the C, S, O and N isotope values (Gión & Ríos 1980). We assumed that each isotopic value was an independent variable, and we believe this to be a sufficiently valid assumption given that different environmental processes influence the isotopic composition of an organism.

## Results

### SOUTH AMERICAN CACTUS MOTH

As foreseen, the cactus moth reared on cactus gave a $\delta^{13}C$ signal of about −15‰ which is reflective of its host plant species' CAM metabolism, resulting in clear separation between the laboratory diet-reared population and the cactus-reared population (Fig. 2). This separation of isotope signatures could allow for identification of the diet-reared moth with 99.7% confidence based on the $^{13}C$ signature (no overlap of signature even at 3σ ($P < 0.01$)). Additional validation could come from the $^{15}N$ signature, as there also was a significant isotopic difference of about 4‰ in the $^{15}N$ signature, which could separate the 2 populations with 95% confidence ($P < 0.01$). There were no significant differences in isotope signatures between male and female moths. Multivariate MANCOVA analysis demonstrated the populations had significantly different isotope signatures (Table 1).

### SOUTH AFRICAN SUGARCANE BORER

Wild *E. saccharina* moths collected from sugarcane host plants yielded an isotopic signal of about −12‰$^{13}C$ reflecting that of the $C_4$ metabolism of its host species (Fig. 3). This resulted in clear separation between the reared (artificial diet) population and both the sugarcane (Eston and Tinley Manor) and *C. papyrus* (Eston) host plant populations, which allowed for identification of reared moths with 99% confidence based on the $\delta^{13}C$ signature ($P < 0.01$). There was no significant difference in $\delta^{13}C$ between the wild moths found in sugarcane and papyrus host plants. In addition, there was good separation based on the $\delta^{15}N$ signature between the wild Tinley Manor sugarcane population, the laboratory population and the wild Eston papyrus population, allowing for identification with 99% confidence ($P < 0.01$) using the $\delta^{15}N$ signature, with about 4‰ difference between the Tinley Manor sugarcane

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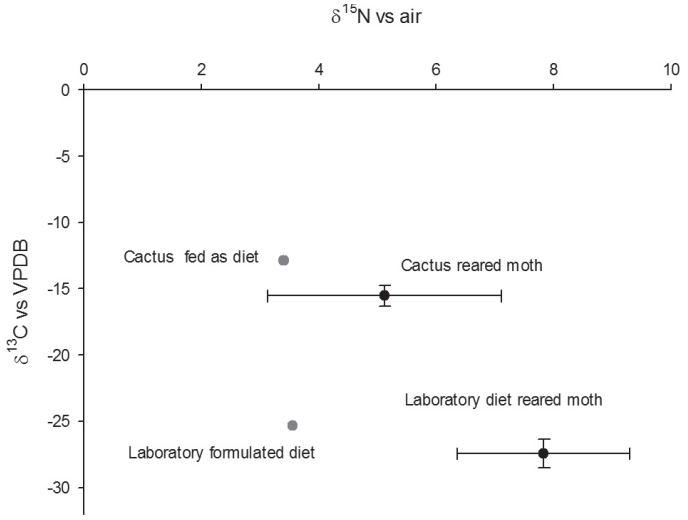



**Fig. 2.** Isotope signatures of cactus, a laboratory formulated diet and of cactus moths, *Cactoblastis cactorum,* reared on these 2 substrates; error bars are 2 standard deviations of the mean.

and the reared population, 5‰ difference between the reared and Eston papyrus population, and about 8‰ between the Tinley Manor sugarcane population and Eston papyrus populations. However, there was no significant separation in $\delta^{15}N$ between the reared and the Eston sugarcane population. Multivariate analysis clearly distinguished reared (artificial diet) as distinct from all other wild populations measured.

### LIGHT BROWN APPLE MOTH

The laboratory population of LBAM could be distinguished from the wild population with 95% confidence based on the $\delta^{13}C$ signature ($P < 0.001$) (Fig. 4). There were no significant differences in the $\delta^{15}N$ signatures of the laboratory-reared versus wild moths collected. However multivariate analysis using the combined dependent variables, $\delta^{13}C$ and $\delta^{15}N$, showed that the laboratory-reared moths were significantly different from the wild moths collected (Table 1).

### COMMON CUTWORM

There was sufficient $\delta^{13}C$ separation in the isotopic signatures to determine with 99% confidence ($P < 0.01$ ) whether the common cutworm moths were wild or artificially reared (Fig. 5). In addition $\delta^{15}N$ were significantly different between wild and laboratory-diet reared moths. Again multivariate analysis showed significant differences in the isotope signatures of the diet-reared and wild populations (Table 1).

### DIAMONDBACK MOTH

The populations of the wild and laboratory-reared diamondback moths separated very well, and we could easily determine the source of origin of a captured moth with 99.9 % confidence based on the $\delta^{13}C$ isotope values presented (i.e., 4 σ, $P < 0.001$) (Fig. 6); in addition there was clear separation based on $\delta^{15}N$ values. The C:N ratio of the reared moths was significantly greater than that of the wild moths. Multivariate analysis showed that there were 2 distinct populations based on their isotope signatures (Table 1).

### EUROPEAN GRAPEVINE MOTH

The results from the European grapevine moth samples suggested that there was insufficient isotopic separation between the 2 populations for this to be reliable as an intrinsic marker, i.e., they were not separable by 2 standard deviations of the mean (Fig. 7). Although there were significant differences ($P < 0.05$) between the 2 populations in both the $^{13}C$ and $^{15}N$ signatures, the differences were insufficient to be reliably used in a predictive manner. In addition multivariate analysis assigned them to separate populations. There were significant differences in the C:N ratio between the wild and laboratory-reared moths, with the wild moths having significantly lower C:N ratios than the laboratory-reared moths. Based on a limited sample, the whole moth samples reared in the laboratory were significantly greater in size, i.e., total body C and N, as measured by elemental analysis.

### PINK BOLLWORM

It was not possible to separate the mass-reared population from the wild population using the C isotope signatures alone, although there were significant differences in the $^{15}N$ signatures between the 2 populations ($P < 0.05$). Multivariate analysis confirmed this and there was not a sufficient difference to reliably distinguish a single moth from one group or the other; so a Bayesian approach was tested. Using the isotopes of S, N and O, it was possible to separate the populations with a certainty of 95% (2 SD), 99.99% (5 SD) and 99.99% (5 SD), respectively (Table 2). Combining these certainties using a Bayesian approach, it was possible to predict that if a moth was trapped and analyzed in that area, using this approach we would wrongly assign only 1 in $2 \times 10^{12}$ cases.

## Discussion

### SOUTH AMERICAN CACTUS MOTH

It is assumed that the $^{13}C$ signature and the good separation between reared and wild moths will be retained throughout the life time of the moth as the adult moth does not feed (Jim Carpenter, unpub-

**Table 1.** Summary table of tissue type taken for isotope analysis and the results of the statistical tests conducted. The number of each replicate type (organ or whole body) analyzed is indicated by n.

| Species | n | Tissue type | T test $\delta^{13}C$ P< | T test $\delta^{15}N$ P< | 2SD difference between populations | 3SD of control differentiates populations $\delta^{13}C$ | Significantly different $\delta^{15}N$, $\delta^{13}C$ isotope signatures MANCOVA $P <$ |
|---|---|---|---|---|---|---|---|
| Cactus moth, *Cactoblastis cactorum* | 20 | leg | 0.001 | 0.136 | Yes | Yes | 0.001 |
| African sugarcane borer, *Eldana saccharina* (Eldana) | 10 | leg | 0.001 | 0.050 | Yes | Yes | 0.001 |
| Light brown apple moth, *Epiphyas postvittana* (LBAM) | 9 | wing | 0.001 | 0.420 | Yes | Yes | 0.001 |
| Common cutworm, *Spodoptera litura* | 10 | leg | 0.001 | 0.001 | Yes | Yes | 0.001 |
| Diamondback moth, *Plutella xylostella* | 10 | whole | 0.001 | 0.001 | Yes | Yes | 0.001 |
| European grapevine moth Grapevine Moth | 7 | whole | 0.001 | 0.017 | No | No | 0.001 |
| Pink bollworm, *Pectinophora gossypiella* (Saunders), | 5 | whole | 0.06 | 0.014 | No | No | 0.001 |

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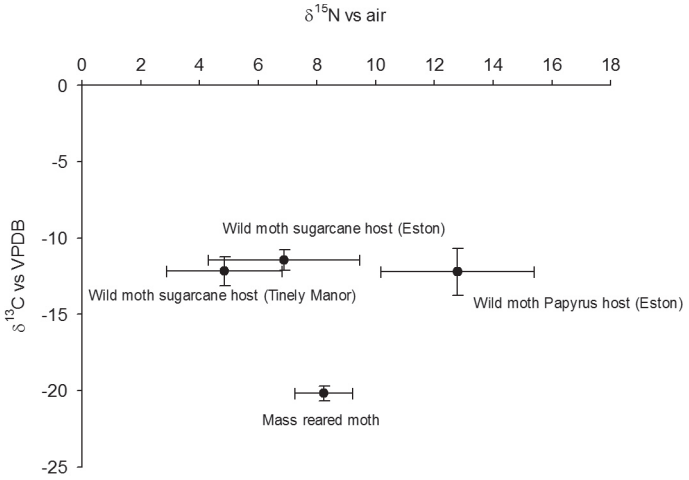



**Fig. 3.** Isotope signatures of the African sugarcane borer, *Eldana saccharina*. Wild moths developed on sugarcane at Eston, and at Tinely Manor, a 3rd group developed on papyrus at Eston and a 4th group was mass reared on an artificial diet. Error bars are 3 standard deviations of the mean.

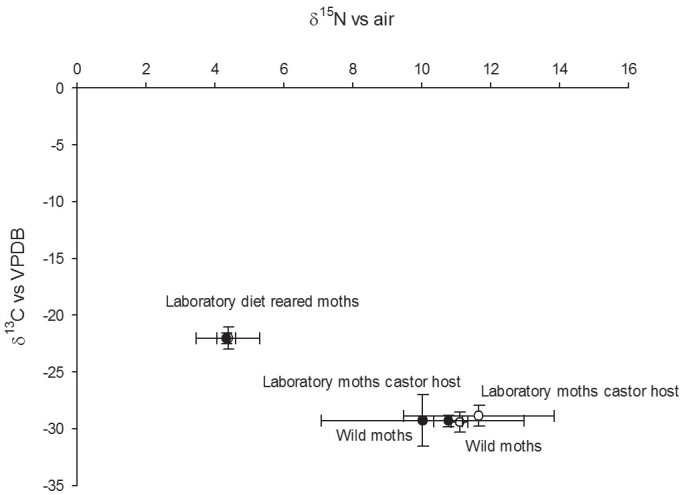



**Fig. 5.** Isotope signature of the common cutworm, *Spodoptera litura*; error bars 2 standard deviations of the mean.

lished observations); therefore natal isotope $^{13}C$ signatures are a stable and reliable marker. The $^{15}N$ signature however may change slightly because of discrimination associated with excretion. The C:N ratio of the cactus diet was 3 times that of the artificial diet (data not shown); however, although there was no apparent difference in the C:N ratio of the wild versus the reared moth, suggesting that the N content of the diet could possibly be reduced without loss in moth quality. This may have beneficial cost implications in a mass-rearing setting.

## SOUTH AFRICAN SUGARCANE BORER

Although there was good $^{13}C$ separation between artificially reared and all wild moths collected there was no significant difference in $^{13}C$ signature between the wild moths found in sugarcane and papyrus host plants. The latter is also a $C_4$ plant (Mantlana et al. 2008), so differentiation was not expected. However, there was good separation based on $^{15}N$ signatures between the wild moths found in sugarcane and those in papyrus host plants, which could separate the 2 populations with 99% confidence ($P < 0.01$). This difference would allow us to study the role of alternate host plant species as part of a push-pull (or habitat management) strategy (Conlong & Rutherford 2009). The high $^{15}N$ enrichment of the papyrus host could possibly be explained by the wetland habitat of the plant, where N-based compounds such as ammonia could be volatilized, with the lighter $^{14}N$ preferentially volatilized, leading to overall $^{15}N$ enrichment of the N pool available and taken up by the papyrus. The C:N ratios (data not shown) indicated that the diet may not be ideal and could possibly require a N supplement. Indeed, the C:N ratio of the moth may be a simple measure to determine diet quality.

## LIGHT BROWN APPLE MOTH

The laboratory population of LBAM could be distinguished from the wild population suggesting this could be a useful complementary technique in the identification of reared moths that have been released in the field. This has been confirmed by a further study in New Zealand (Stringer et al. 2013). The laboratory population showed a $\delta^{13}C$ signal, which was reflective of their $C_4$ sugarcane-based diet, but suggested

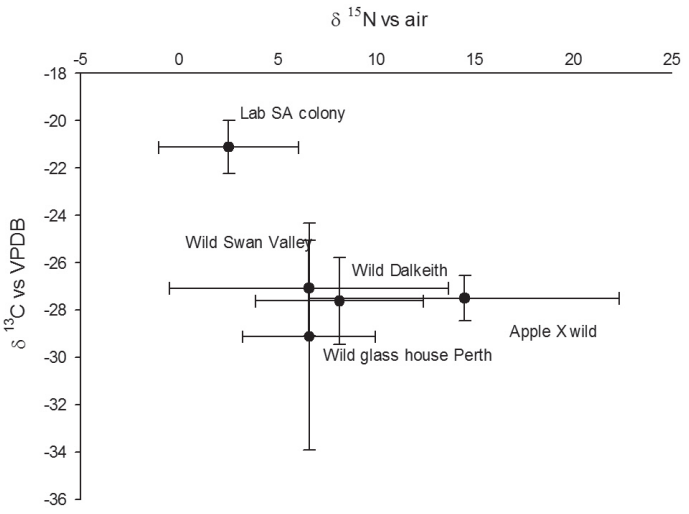



**Fig. 4.** Isotope signature of the light brown apple moth, *Epiphyas postvittana* (LBAM); error bars are 2 standard deviations of the mean.

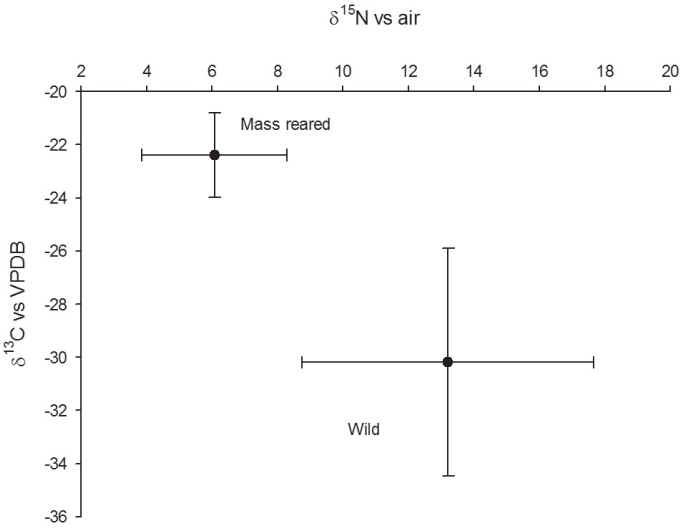



**Fig. 6.** Isotope signatures of wild and mass-reared diamondback moths, *Plutella xylostella*; error bars are 2 standard deviations of the mean.

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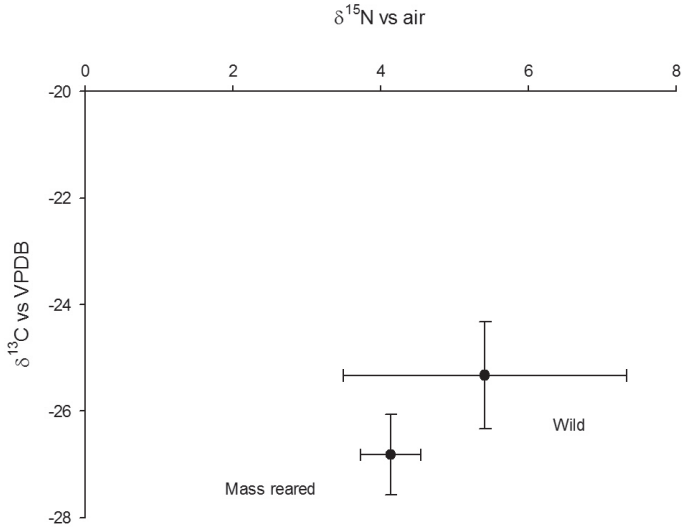



**Fig. 7.** Isotope signatures of the European grapevine moth, *Lobesia botrana*; error bars are 2 standard deviations of the mean.

other $C_3$ ingredients were important components of the diet such as wheat germ or cellulose powder. There were no significant differences in the $\delta^{15}N$ signatures of the wild moths as compared with the reared moths, although the wild population found on apple was clearly separated from the other wild moths, suggesting some niche differentiation based on food resource quality although it came from a very similar urban environment to Dalkeith as opposed to the moths from the peri-urban Swan Valley which were collected from grapevines.

## COMMON CUTWORM

There was sufficient separation in the C isotopic signatures to determine with high confidence whether the common cutworm moths were wild or reared, demonstrating this method as an appropriate technique for identifying wild or reared populations of moths for release programs. All populations of moths analyzed appeared to be feeding from a dominantly $C_3$ plant type, as evident from the $^{13}C$ signatures of the moths, being nearer to −28‰ characteristic of $C_3$ plants. There was no significant difference in either the $^{13}C$ or $^{15}N$ signatures of the wild moths compared with those of the castor-reared moths, suggesting that the wild moths caught were indeed feeding on a castor-based diet. It is known that this moth has a castor host and this appears to confirm it. The variation in the $^{15}N$ value of the wild moths was greater than that of the mass-reared moths but this may have been the result of one possible outlier in the wild population.

Interestingly, the wild and castor-reared moths had significantly higher $\delta^{15}N$ values (≈ 12‰), contrary to what we had expected, as it was wrongly assumed that castor was a true bean and that it would have a $\delta^{15}N$ value approaching that of atmospheric N of zero (Unkovich et al. 1994). On further investigation, we found that castor is not a N-fixing bean, as was evident from these results. The C:N ratio of the male laboratory-reared castor moths (4.54:1) was significantly greater than the C:N ratio of male wild moths (4.12:1) ($P < 0.05$). This may be a reflection of the nutritional or physical status of the laboratory moths, as it is hypothesized that the laboratory moths did not need to fly as much, and therefore had retained ample C reserves compared with those of their wild counterparts.

## DIAMONDBACK MOTH

The populations of wild and laboratory-reared diamondback moths separated very well with the caveat that the reared moths and the wild moths were representative of the 2 populations tested. The C:N ratio of the reared moths was significantly higher than that of the wild moths suggesting that the diet of the reared moths could be supplemented with protein or N. The isotopic signatures of the moth proved to be a reliable marker for the diamondback moth. Given that the diamond back moth is small and fragile, intrinsic isotope labelling would be an ideal marking primary or complementary marking strategy for this species.

## EUROPEAN GRAPEVINE MOTH

There was insufficient isotopic separation between the 2 populations for stable isotopes of C and N to be used reliably as intrinsic markers, although there were significant differences ($P < 0.05$) between the 2 populations in both the $^{13}C$ and $^{15}N$ signatures and using a multivariate approach. This case highlights why we use the 2 SD approach, as it allows us to say with 95% confidence whether or not an individual moth comes from one population or the other even if the 2 populations are significantly different. It is clear from the $^{13}C$ values that the wild moths were feeding in a more arid habitat than the original food source of the laboratory-reared moths. The laboratory-reared moths were fed on Premix™, a commercially available Canadian product whose composition is proprietary. However, the isotopic data suggest that Premix is possibly a soy-based diet with either a low sugar or a $C_3$ sugar beet-derived content, as the $^{15}N$ values of feed were around 1‰. This is characteristic of soy as a biological N-fixer, because N-fixers tend to have N isotope values similar to air as they obtain the majority of their N from the atmosphere (Unkovich et al. 1994). There also were significant differences in the C:N ratio between the wild and laboratory-reared moths, with the wild moths having significantly lower C:N ratios than the laboratory-reared moths. The lower C:N ratios of wild moths could again be the result of leaner wild types because of the burning of storage fat through movement, or they could be the result of the climatic influence on host plant species resulting in higher N content of the host plant species, being C-limited because of water stress rather than N-limited, resulting in increased plant/diet N concentration. Based on a

**Table 2.** Mean stable isotope values of pink bollworm ($n = 5$), SD standard deviation, referenced against international standards in brackets.

| | $\delta^{18}O$ (VSMOW) | | $\delta^{15}N$ (Air) | | $\delta^{13}C$ (VPDB) | | $\delta^{34}S$ (VCDT) | |
|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD |
| Mass reared moths | 15.9 | 0.5 | 4.0 | 0.2 | -25.1 | 0.7 | 1.0 | 0.3 |
| Cotton reared moths | 20.6 | 0.5 | 10.8 | 0.7 | -26.2 | 1.0 | 4.3 | 0.9 |

Note: The internationally recognized standards are: Vienna Standard Mean Ocean Water (VSMOW) for $^{18}O$, Vienna Pee Dee Belemnite (VPDB) for carbon, and Vienna Cañon Diablo troilite (VCDT) for sulphur.

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limited sample, the whole moth samples reared in the laboratory were significantly larger in size in terms of C mass but not in N, which may afford them a mating advantage in a SIT context.

PINK BOLLWORM

It was not possible to separate the mass-reared population from the wild population using the C isotope signatures alone, but it was possible to separate the populations using both C and N isotope signatures with a multivariate MANOVA. However this approach did not give an indication of the confidence with which one could assign an individual to one population or the other. Given the current interest in using this isotope-based method for confirmation of wild type moths captured in traps in an operational setting—considering that the pink bollworm program in the southwestern USA and northern Mexico is approaching a point where it could declare regional eradication—we decided to test the multiple isotope approach. There were significant differences in the isotope ratios of O, N and S between the 2 populations. For O this was probably the result of different water sources, ground water versus surface water, for N this was possibly due to differences in host crop fertilization with the organic cotton moths having an isotope ratio characteristic of organic manure fertilization, and for S factors such as distance from the sea and pollution would influence the isotope ratio of the host plant and consequently the insect feeding on it (Schellenberg 2010). Using the isotopes of S, N and O, it was possible to separate the populations with a certainty of 95% (2 SD), 99.99% (5 SD) and 99.99% (5 SD), respectively (Table 2). Combining these certainties using a Bayesian approach it was possible to predict that if a moth was trapped and analyzed in that area, we would wrongly assign only 1 in $2 \times 10^{12}$ cases.

However, this assumes we have captured the variation in the wild and mass-reared population in that area at the time of sampling and that this variation is normally distributed. Given that we only sampled 5 replicate individuals, this assumption may not be strictly true, as we have only estimated the SD of the population. From our cumulative experience in these and other experiments, we have observed that the estimated SD of a mean isotope value of an insect population from the same area usually peaks around 5–7 individuals and that the mass-reared populations generally have lower variability than the wild population due to uniform diets and rearing conditions. In conclusion the multiple isotope approach may be costly, but it can yield results on which program managers can confidently base operational decisions, and thus save time and money.

## Conclusions

These results taken together suggest that using a naturally occurring stable-isotope-labeled sugar in mass-rearing diets can be an effective way to mark a number of moth species for complementary identification of recaptured mass-reared sterile individuals in AW-IPM programs that include a SIT component. In this study we focused on trying to sample wild versus mass- or laboratory-reared populations and to determine whether $\delta^{13}C$ could be used as a reliable intrinsic marker. Although $^{15}N$ signatures were also investigated in this paper we wanted to concentrate on the $\delta^{13}C$ signatures, because we are of the opinion that technological advances in isotope measurement such as the use of cavity ring-down spectroscopy will make routine the analysis of isotopes in a control program setting an achievable reality; moreover $\delta^{13}C$ values are more predictable. Although we are aware of adult nectar feeding in Lepidoptera (Nelson 1936), the experimental rationale we adopted was intended to determine whether there were isotopic differences between the populations that could be used as markers. We suggest that adult nectar feeding would contribute little to the overall isotope signature of the moth as it is quickly used as an available energy source for flight (O'Brien et al. 2002). In previous work we have shown that insects fed sugar ad libitum turn over about 50% of their total body C throughout their life time and that about 50% is structural and has minimal turnover (Hood-Nowotny et al. 2009). From this we assume that chitin-dominant tissues such as wings or front leg tibiae, are chitin rich structures, which will turnover slowly thus retaining their natal isotopic signature. The sugar-rich nectar diet of most moths provides only trace amounts of amino acids and these do not appear to contribute to adult N (O'Brien et al. 2002). Moreover studies of N assimilation in adult Lepidoptera have shown minimal N uptake (MolleMan et al. 2009). Although natural differences in $C_3/C_4$ feeding behavior have previously been used in host identification studies of moths (Malausa et al. 2005), this is the first comprehensive study that investigates the use of isotopically distinct larval diets as a marking method in moths and improves our ability to predict their natal origin, i.e., reared or wild. The elegance of the method is that it does not require a change in standard rearing production practices, and therefore is not very susceptible to human error. In addition, it could be used for identifying factory-produced specimens, and thus be of use in cases of escapee litigation. This method could also be used for mark and recapture studies to ascertain the range and movement of particular target species. It provides a simple but effective way of labeling moths without any of the constraints posed by more traditional methods such as external dusting of the insect with a colored powder or incorporating calco red into the larval diet. The ubiquitous occurrence of heavy stable isotopes in nature means marking by use of these intrinsic signatures poses no health or environmental threats (Hood-Nowotny & Knols 2007), which—given the current regulatory and litigious landscape—may be an additional consideration when selecting a marking strategy.

These studies should lead the way to more elaborate studies, in which the fates of moth sperm or eggs could be followed, and in which mating success of released moths with wild moths could be determined (Stringer et al. 2013). In conclusion, intrinsic isotope markers have the potential to make a significant contribution to studying the distribution and populations of moths, and in programs with a SIT component, to distinguish between laboratory- or factory-reared and naturally occurring moths, without impacting on the quality of the former.

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