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Stable isotope-mass spectrometric determination of semen transfer in malaria mosquitoes

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Summary

The potential use of stable isotopes to study mosquito mating was investigated by tracing the fate of labelled semen into spermathecae. [^{13}C]glucose was incorporated in the diet of the malaria mosquito *Anopheles arabiensis*. Treatments included labelling of either the larval water or adult sugar water, or a combination of both. After mating, 'spiked' spermathecae were analysed for isotope ratios using mass spectrometry. Results demonstrated that spermathecae positive for semen could successfully be distinguished from empty ones or controls (i.e. filled with unlabelled semen) using the raw δ^{13}C values. Labelling during larval development and combined labelling of larvae and adults resulted in detectable values. The label persisted in spermathecae for up to 7 days after mating, and unlabelled sugar feeding of males labelled in the larval stage did not result in a detectable turnover of the semen label. There were no detrimental effects of the addition of labelled glucose on larval development and survival, adult size, male longevity and mating performance. We have proved that it is possible to label male mosquitoes and detect the semen label in females after insemination. This method offers great potential to study mating in mosquitoes and other insects and could prove useful in genetic control studies of medical or agricultural pest insects, with male mating success in the field as a critical verifiable indicator for a positive outcome of the intervention.

Supplementary material available online at
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Key words: stable isotope, semen-label, anopheles, mass spectrometry, genetic control.

Introduction

Recently, stable isotopes have become widely available as a labelling tool in biological studies, largely as a result of comparatively lower costs for both the isotopes and sample analysis (Hood-Nowotny et al., 2005). In insect studies, stable isotopes have not been used extensively, but they can be a useful tool to address issues of food preference, resource allocation, dispersal, etc. (Hood-Nowotny and Knols, in press). Recent studies undertaken in our laboratory have documented the successful application of stable isotopes as a population marker in the context of genetic control studies [e.g. Sterile Insect Technique (Hood-Nowotny et al., 2006)]. Our current interest lies in the use of stable isotopes to study mating behaviour. Studies in which natural abundance levels of stable isotopes were used in the context of mating have been carried out (Ponsard et al., 2004; Malausa et al., 2005), but to our knowledge no work has been performed on the enrichment of insect semen with stable isotopes. Semen labelling has recently been performed in humans; $^{2}H_2O$ was ingested daily and spermatogenesis kinetics studied (Misell et al., 2006).

In the present study we used the African malaria mosquito *Anopheles arabiensis* Patton. Of the major life history behaviours of anopheline mosquitoes, mating remains the least understood (Takken and Knols, 1999). Studies on mosquito mating behaviour are difficult to conduct because of its crepuscular nature, complex constituency (i.e. swarm make-up), and irregular spatial occurrence. In the context of genetic control studies, understanding a male's mating success in the field is critical for a positive outcome of the intervention (Ferguson et al., 2005), and techniques that label semen (i.e. spermatozoa and accessory gland fluid) would greatly facilitate the study of mating behaviour. Radioactive isotopes have been used in the past to study the fate of semen (Dame and Schmidt, 1964; Tantawy et al., 1967; Smittle et al., 1969; Young and Downe, 1978) in which mosquitoes were labelled by exposing larvae to radioactive solutions. This resulted in the transfer of radioactive semen during copulation and the successful

identification thereof in spermathecae. Nowadays, the use of radioactive isotopes is rarely practised in entomological research due to the hazards related to the treatment process and environmental concerns of releasing such insects, even though the half-life of the commonly used isotope ^{32}P is short (e.g. 14.3 days). Other options to study mating in mosquitoes include the use of mutant strains (Mason, 1967; Gomulski, 1988; Beard et al., 1995; Klowden, 2006), but these are not readily available for most stocks. A transgenic strain that can be used to study mating behaviour has been developed in *A. stephensi* (Catteruccia et al., 2005), but this technology hinges on ethical, legal and social issues affecting the ability to release transgenic insects (Knols and Louis, 2006; Knols et al., 2006), and is not easily transferable to other species.

Stable isotopes are naturally occurring in the environment, are not radioactive and therefore do not decay. In general, they react chemically in a manner identical to the more common isotope and thus are effective, non-invasive markers in biological systems. Besides these attributes, stable isotopes are not species-specific, which makes them attractive for use. Most elements of biological interest (including C, H, O, N and S) have two or more stable isotopes, with the lightest of these present in much greater abundance than the others. An isotope of an element has the same atomic number but a different number of neutrons and consequently a different atomic mass. If a system is enriched with the less abundant isotope, this element can be used as a label or tracer (Hood-Nowotny and Knols, in press). The isotopic composition of a sample is measured by determining the ratios of the stable isotope masses. These ratios are measured on an isotope ratio mass spectrometer, a device that separates ions of the element of interest on the basis of their differing mass/charge ratio (*m*/*z*) (de Groot, 2004).

For the experiments presented in this paper ^{13}C was chosen as a label. The findings of three sets of experiments that address four objectives are presented. The first objective was to see whether it was possible to use ^{13}C as a semen-labelling technique and to identify the optimal developmental stage for labelling. The second objective was to test the ability of males of different ages to transfer the label. The third objective was to test the persistence of the label in spermathecae after mating. The fourth objective was to study the impact of the stable isotope on the mosquito to assure that no detrimental effects occurred.

Materials and methods

In all experiments, mosquito rearing and labelling techniques were identical, as well as sample preparation and analyses.

Mosquitoes

The Dongola strain of *Anopheles arabiensis* Patton was used. It was collected in Northern State, Sudan, in 2004 and has been reared in our laboratory since then. Five hundred L1 stage larvae were counted and placed in a tray (30×40 cm) in 1 l of deionized water, and the water level was kept constant throughout the experiment. Heating mats were used to maintain the water temperature at 28±1°C. Larvae were fed a diet of fish food (AquariCare Koi Floating Blend, USA) daily (0.25 mg per larva), that was ground and sieved through a 224 μm sieve, and mortality of the larvae was not taken into account. Adults were kept in standard 30 cm×30 cm×30 cm mosquito rearing cages and maintained at a temperature of 27±0.2°C and relative humidity of 80±2%. The light regime was 10 h:12 h L:D with a 1 h simulated dusk and dawn period. Adults were maintained on a standard 10% sucrose solution (w/v) unless stated otherwise.

Labelling

99 atom% [^{13}C]glucose (U-13C6; Cambridge Isotope Laboratories Inc., Andover, MA, USA) was used as a label. Mosquitoes were exposed to the label either as larvae or adults. In the larval stage, the label was added to the water on the same day as the L1 larvae were introduced. In the adult stage, the label was added to the sugar water. The level of enrichment in both treatments was 20 atom% ^{13}C (i.e. approximately 20% of all the carbon in the diet was ^{13}C), and was based on findings from a previous study (Hood-Nowotny et al., 2006) where lower levels of enrichment were used (e.g. 1.11–1.46 atom% ^{13}C) for whole body analysis.

The amount of [^{13}C]glucose needed was based on the total amount of carbon present in the diet. 40% of the larval diet consisted of carbon. Until pupation, 1.0 g (0.25 mg×500 larvae×8 days) of larval food was added to the tray, thus 0.1 g of ^{13}C was required. As the percentage of carbon in glucose is 40%, 0.25 g of 99 atom% [^{13}C]glucose was added to the larval trays. For adult mosquito labelling, the stable isotope was incorporated in the sugar solution, and similar calculations were made to determine the amount of label needed. 1.00 g of sucrose has 0.42 g of C, thus 0.1 g of ^{13}C was required. Labelled sugar feeders received 0.25 g of 99 atom% [^{13}C]glucose+1.00 g sucrose in 12 ml water (10.4% sugar solution); and the unlabelled feeders received unlabelled glucose instead.

Experimental design

Males that emerged from the experimental larval trays [e.g. labelled (L) and unlabelled (U)] were divided into four adult treatments: U–U, males unlabelled in the larval stage fed on unlabelled sugar; U–L, males unlabelled in the larval stage fed on labelled sugar; L–U, males labelled in the larval stage fed on unlabelled sugar; L–L, males labelled in the larval stage fed on labelled sugar.

When treatments were compared within an experiment, only males emerging on the same day from labelled and unlabelled trays were used. Males were transferred from the larval trays to adult cages and fed on their designated sugar source (e.g. labelled or unlabelled) until mating was initiated. In the experiments where the adult sugar water was labelled, the unlabelled treatments received unlabelled glucose; however, when adult labelling was not performed, cages were maintained on the laboratory standard (e.g. unlabelled) sucrose solution.

When adult labelling was performed, males were transferred to a new cage prior to mating to prevent cross-contamination of the females (e.g. spills from the sugar source), and males in other treatments tested at the same time were also transferred for comparison sake. During and after mating, males were maintained on standard sucrose solution. Females used as mates were isolated within 18 h after emergence to assure virginity. The age of the females in all experiments was similar to that of males when mating was initiated. After mating, females were either: dissected immediately (e.g. the following day) at the end of the mating period (I); or isolated for dissection at a later stage to assess the persistence of the label in the spermathecae (II).

Experiment 1

The main goal of the first experiment was to see if ^{13}C could be used as a semen label, and to determine the optimal treatments to deliver [^{13}C]glucose. In addition, persistence of the label in spermathecae was studied.

For each larval treatment (e.g. labelled glucose and unlabelled glucose) two trays were set up. Males emerging from the trays were pooled according to treatment and divided into the four adult treatments as described above. Males were fed sugar from their designated source for 4 nights. On the fifth day, mating was initiated. Per treatment, 62 males were mated with females at a ratio of 2:1 (M:F) and the mating period lasted for 3 nights. After mating, females were immediately dissected for analyses, or isolated and dissected 3 days later.

A small number of experimental males were removed from the cages on day 4 (L–U, L–L treatments) and day 11 (all treatments) after emergence and their reproductive system dissected for isotopic determination.

Experiment 2

In the second experiment, lower insemination of the females was pursued to determine if inseminated spermathecae could successfully be distinguished from uninseminated spermathecae within the same treatment. Treatments from experiment 1 (U–U, L–U, L–L) were repeated with the exception of adult only labelling (U–L), and the persistence of the label in the females and the persistence of the label in males that mated later in life was investigated. In addition, the impact of labelling on larval development and adult longevity was studied.

Larval trays included one tray with labelled glucose, and one with unlabelled glucose. Another tray without any glucose was added to monitor the effect of glucose on larval survival. Adults emerging from the trays were removed and counted daily, and trays were maintained until all larvae had pupated and emerged, or died.

The males emerging from the labelled glucose tray were either fed unlabelled or labelled sugar, males from the unlabelled tray were fed unlabelled sugar. After 5 days of sugar feeding, 35 males were mated for 1 night with females on a 1:1 ratio. Females were dissected immediately or isolated and dissected 3 days later (Fig. 2A). Mortality of the males was scored regularly until the majority had died.

A second batch of males that emerged from the labelled tray was used to study the persistence of the label in spermathecae for up to 7 days. Males were maintained on standard 10% sucrose solution and mated (N=25) on day 2 after emergence for 3 nights at a ratio of 1:2 (M:F). Females were either dissected immediately, or isolated and dissected 4 or 7 days later (Fig. 2B).

A third batch of males that emerged from the labelled tray was used to study the effect of male age on label transfer. Males were maintained on standard 10% sucrose solution and mated (N=25) with females at a ratio of 1:2 (M:F) on day 4 for 3 nights, or on day 10 (N= 25) for 1 night at a ratio of 1:1. Females were dissected immediately or after 4–5 days of isolation (Fig. 2C).

Experiment 3

The impact of labelling on larval development and adult longevity was investigated, to complement initial data gathered in experiment 2. In addition, the effect of ^{13}C on the size of the emerged adults, and the impact of the semen label on the hatchability of eggs was studied.

Larval treatments included trays with [^{13}C]-labelled glucose, unlabelled glucose, and a control tray; each treatment was duplicated. Adults emerging from the trays were removed and counted daily, and trays were maintained until all larvae had pupated and emerged, or died. On the day the majority of pupae emerged, 50 males were collected per treatment and per replicate and placed in a standard cage to monitor survival. Males were maintained on a standard 10% sucrose solution, and mortality was scored regularly until all males had died.

Adult body size of males and females emerging from the trays was determined by wing length (Lyimo and Takken, 1993; Lounibos et al., 1995; Charlwood et al., 2002). Day of emergence was noted and for each specimen a wing was clipped and mounted on a slide. A digital image of the wing was taken [CC-12 camera (Olympus Soft Imaging Solutions, Berlin, Germany) mounted on a stereo microscope]. Wing length was measured between the alula notch and the wing tip, excluding scales; measurements were performed with AnalySIS FIVE software (Olympus Soft Imaging Solutions).

The effect of the ^{13}C label on sperm viability was monitored by assessing the hatching of eggs fertilized by labelled sperm. Unlabelled virgin females (N=50) were mated to males emerging from labelled or control trays at a 1:1 ratio and cages were maintained for 26 days. Mosquitoes were membrane blood fed on multiple occasions and eggs were collected en masse and checked for hatching.

Sample preparation

Females

Females were immobilized, and their spermathecae dissected in mosquito saline (Ephrussi and Beadle, 1936). Insemination status was checked under a compound microscope at 100× magnification and recorded. The spermatheca was then

transferred to a small piece of quartz fibre filter paper with a fine brush and placed in a cylindrical 8×5 mm (height × diameter) tin cup. After each dissection, tools were cleaned with ethanol to avoid contamination. The amount of carbon present in the spermatheca (~3 μg) was below the detection limit of the mass spectrometer setup (approx. >20 μg). Samples were therefore 'spiked' with 10 μl of a standard sucrose solution containing ~23–26 μg of carbon (Dube et al., 1998). All samples were dried for ⩾24 h in an oven at 50°C before closure of the tin cup and subsequent analyses in the mass spectrometer. Standards containing only the piece of quartz fibre filter paper with the spike were included. Spermathecae from virgin females were used as a control and dissected similarly to those of experimental females.

Males

A number of males from the first experiment were dissected to analyse the amount of ^{13}C in their reproductive system. The testes, accessory glands and seminal vesicle were dissected and prepared for analyses as above (e.g. including the spike).

Sample analyses and interpretation

After drying, tin cups were sealed and contents analysed using a Carlo Erba (Milan, Italy) carbon nitrogen (CN) analyser, linked to an Optima, (Micromass, Manchester, UK) isotope ratio mass spectrometer (IRMS); see (Hood-Nowotny et al., 2006) for details.

The output from the mass spectrometer is a $\delta^{13}C$ value, which represents the ratio of ^{13}C over ^{12}C against a reference standard, and the total amount of carbon present in the sample. The actual delta value of the sample alone, e.g. without the spike, was not determined for the spermathecae samples because of uncertainty associated with calculating this value. This was due to the unknown and low amount of carbon in the original unspiked sample and proximity of unlabelled treatment $\delta^{13}C$ values to the $\delta^{13}C$ of the spike, which made comparison of labelled and unlabelled samples difficult. Therefore, spiked $\delta^{13}C$ values were used for statistical analysis and data representation. For the males, the actual $\delta^{13}C$ values were calculated to obtain an estimate of the enrichment.

Data analyses

Prior to analyses, data were checked for normality. When homogeneity of variances was not assumed, and the number of treatments exceeded two, non-parametric tests were performed. Data on spermathecae labelling were analysed using the following variables: insemination status (inseminated or uninseminated as determined by compound microscopy) adult labelling treatment (U–U, U–L, L–U, L–L), and dissection history (I or II). Some outliers in $\delta^{13}C$ values were observed in the dataset, in particular in the first experiment, and these were excluded to normalise the data (see Results section). Differences between mean $\delta^{13}C$ values of labelling treatments were analysed using general linear models (GLMs) with planned contrasts (Tukey's HSD) and data on dissection history were analysed with GLMs or independent *t*-tests. Independent *t*-tests were also used to compare uninseminated females to virgin control samples in all experiments, and to test the difference in $\delta^{13}C$ values of inseminated and uninseminated spermathecae for each labelling treatment in experiment 2. A threshold value to distinguish labelled spermathecae from unlabelled spermathecae was defined as 2 or 3 standard deviations (s.d.) above the mean $\delta^{13}C$ (‰) value of the reference standard (Macneale et al., 2005), in our case virgin females. Longevity of males was analysed using Kaplan-Meier survival analyses. The obtained survival curves were pairwise compared using Mantel-Cox log-rank tests. Carbon data, larval survival and wing length data were analysed with GLMs. All two-sided tests were performed using the SPSS software version 12 (SPSS Inc., Chicago, USA).

Results

Labelling

The $\delta^{13}C$‰ values reported are negative as they are referenced to an international standard PDB (Pee Dee Belemite), which is more enriched in ^{13}C than our spiked samples.

Optimal treatment

When females from experiment 1 were dissected immediately after mating (I), sufficient amounts of labelled semen were transferred in all labelled treatments to distinguish mean $\delta^{13}C$ values of inseminated spermathecae from unlabelled samples ($F_{3,38}$=26.88; P<0.01) (Fig. 1). Labelling in the larval stage (L–U) or in both stages (L–L) resulted in the highest amount of label transferred, but labelling of only the adult stage (U–L) was sufficient to distinguish mean $\delta^{13}C$ values from the control (U–U). However, the persistence of the label in males and females after adult labelling alone was not sufficient (see below). Therefore, labelling in the adult stage alone was not considered optimal and further experiments focused on the males labelled as larvae or as larvae and adults. The second experiment repeated the treatments of the first experiment, except for the adult only labelling, with similar results. Mean $\delta^{13}C$ values of spermathecae inseminated by males labelled in the larval or in both stages were higher than the control males after immediate dissection (I) ($F_{2,16.08}$=160.53, P<0.01), and in this experiment males labelled in both stages transferred significantly more label than larval labelled males alone (Fig. 2A).

Label persistence in males

Unlabelled sugar feeding in the adult stage did not result in a detectable dilution of the semen label after immediate dissection (I); males exposed to the label in the larval stage alone transferred similar amounts of label when mated at either 4 or 10 days of age ($F_{3,38}$=1.12, P>0.05) (Fig. 2C).

Persistence of label in spermathecae

After isolation of the females for 3 nights (II), mean $\delta^{13}C$ values decreased in all labelled treatments compared to

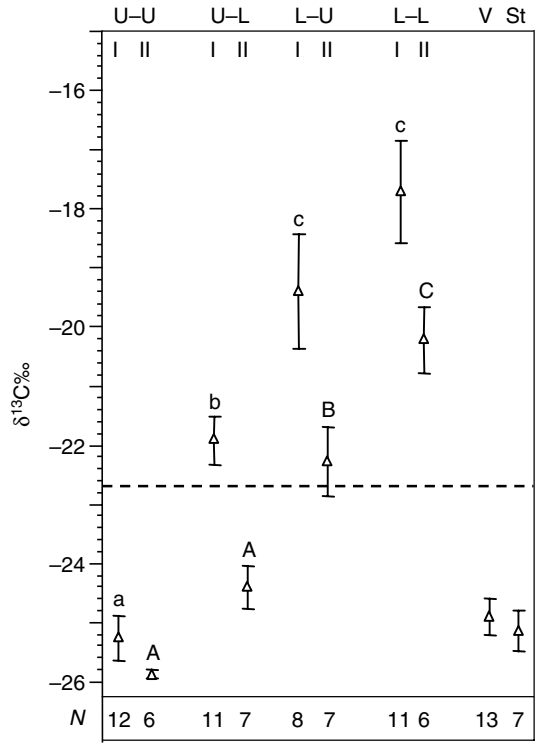

Fig. 1. $\delta^{13}C‰$ values (mean ± s.e.m.) of inseminated spermathecae from experiment 1. The adult labelling treatments were: U–U, no label; U–L, adult labelling only; L–U, larval labelling only; and L–L, larval and adult labelling. *N*, number of samples analysed. The dotted line indicates the threshold value of 2 s.d. above mean $\delta^{13}C‰$ of virgin females. Virgin (V) and standard (St) samples are included. Values excluded from analyses are: 9.97 (L–L), –12.51 (V) and, –13.29, –18.20 (St). Dissection treatments: I, females dissected immediately after mating; II, females isolated and dissected 3 days after mating. Values with different letters are significantly different at $P<0.05$ for I (lower case), or II (upper case).

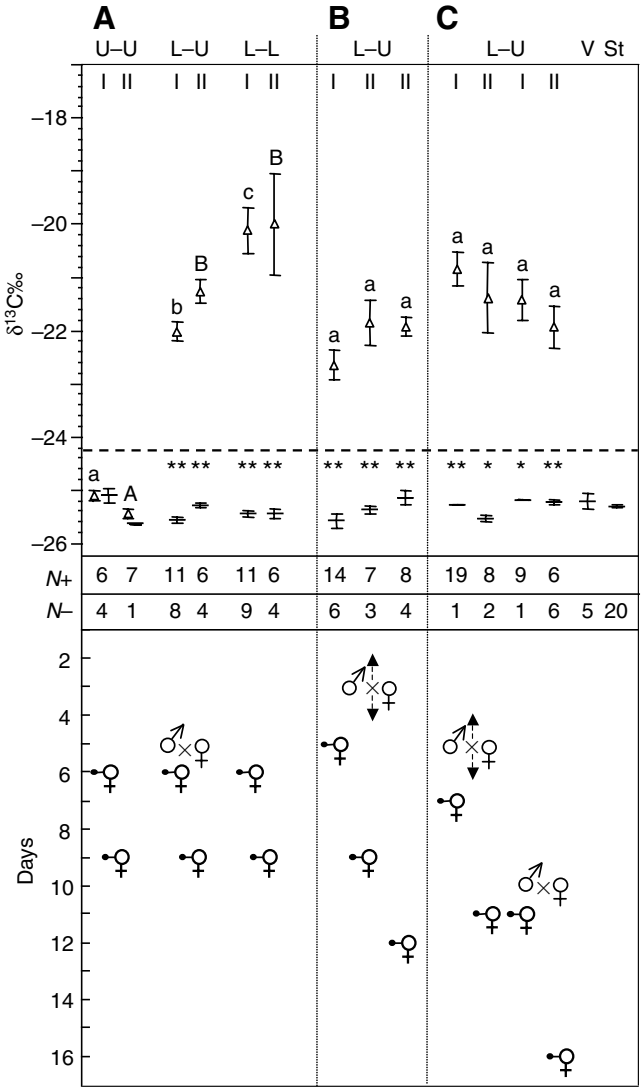

Fig. 2. $\delta^{13}C‰$ values (mean ± s.e.m.) of inseminated (open triangles) and uninseminated (bold line) spermathecae from experiment 2. For labelling treatments see Fig. 1. The mating and dissection history of each treatment is illustrated in the lower part of the graph. Duration of mating if longer than 1 night is indicated by arrows; Female symbol with a pin indicates spermathecae dissection. Subdivisions A–C refer to different batches of males (see text). *N*, number of samples analysed for inseminated (+) and uninseminated (–) spermathecae. Dotted line indicates the threshold value of 3 s.d. above mean $\delta^{13}C‰$ of virgin females. Virgin (V) and standard (St) samples are included. Virgins are uninseminated females from treatment U–U. Value excluded from analyses: –13.02 (A,L–U). Values with different letters are significantly different at $P<0.05$ for I (lower case), or II (upper case) in A. Planned contrasts ($P<0.05$) in B were made between the three values, and in C between the four values. Asterisks indicate significant difference between positive and negative spermathecae at $*P<0.05$ and $**P<0.01$.

immediate dissection (I) in experiment 1 (Fig. 1), and in the treatments U–L and L–U a significant decrease was observed; U–L: [$t(16)=4.19$, $P<0.01$], L–U: [$t(13)=2.43$, $P<0.05$; Fig. 1]. When males were only labelled in the adult stage, $\delta^{13}C$ values of females isolated for 3 days were still higher, but no longer statistically different from the control samples ($F_{3,22}=27.22$, $P<0.01$), whereas in the larva-labelled treatments mean $\delta^{13}C$ values remained higher than the control (Fig. 1). In experiment 2, no decrease in $\delta^{13}C$ values was observed after isolation for 3 days in treatments L–U and L–L compared to immediate dissection (Fig. 2A). In treatment L–L similar values were reported [$t(15)=–0.12$, $P>0.05$], whereas in treatment L–U a significant increase was observed [$t(15)=–2.57$, $P<0.05$].

When females were mated to males labelled in the larval stage and isolated for 4 or 7 days after mating, similar $\delta^{13}C$ values were found compared with immediate dissection ($F_{2,26}=2.23$; $P>0.05$; Fig. 2B). It was also observed that females inseminated by 10-day-old males retained similar amounts of label after isolation (II) as females inseminated by 4-day-old males, and $\delta^{13}C$ values after isolation were comparable to immediate dissection (I) at both ages ($F_{3,38}=1.12$, $P>0.05$; Fig. 2C).

Control samples

Spermathecae from females mated with males from the unlabelled treatments in experiment 1 had similar $\delta^{13}C$ values as virgin females [$t(36)=-1.88$, $P>0.05$] and the standards [$t(30)=-1.06$, $P>0.05$]. The uninseminated females from all treatments in experiment 2 had similar $\delta^{13}C$ values as the virgin females [$t(51)=-1.79$, $P>0.05$].

Labelled versus unlabelled samples

The males from the labelled treatments in experiment 2 transferred significantly high enough amounts of label to distinguish mean $\delta^{13}C$ values of inseminated spermathecae from uninseminated ones (Fig. 2).

Threshold values

To determine the accuracy of the labelling, the threshold value in each experiment was determined and is represented as a dotted horizontal line in Figs 1 and 2. $\delta^{13}C$ values of samples that appear above the threshold line are considered to have been from females inseminated by labelled males, values below the line should represent uninseminated females or females mated to unlabelled males. The threshold value in experiment 1 was rather conservative due to some variation in the samples from virgin females. Therefore, a small number of spermathecae from the L–U treatment appeared below the threshold (4/21 samples). Spermathecae from the dual labelling group appeared (except for one sample) above the threshold value. However, a large proportion of spermathecae inseminated by males labelled as adult only appeared below the threshold value; hence this treatment was not considered optimal. In the second experiment, males labelled in the larval and adult stage transferred enough label so that the $\delta^{13}C$ value of each inseminated spermatheca appeared above the 2 s.d. (standard deviation) threshold. Even if 3 s.d. was used as a threshold value, all inseminated females had higher labels, and this value is indicated in Fig. 2.

Between experiment variation

The mean (±s.e.m.) amount of carbon in the spiked samples differed significantly between the experiments [$t(268)=-26.32$, $P<0.01$]; in experiment 2, a higher amount of carbon was detected ($N=191$, $M=26.15\pm0.07$ μg) compared to experiment 1 ($N=79$, $M=22.84\pm0.11$ μg). Between experiments, $\delta^{13}C$ values of control samples were similar for virgin females [$t(16)=0.62$, $P>0.05$] and standards [$t(25)=0.99$, $P>0.05$]. However, the males from experiment 1 transferred more label, resulting in higher $\delta^{13}C$ values, than the males from equal treatments in experiment 3; L–U: [$t(7.50)=2.99$, $P<0.05$], L–L: [$t(20)=2.50$, $P<0.05$]. After isolation of the females for 3 days this difference was no longer observed.

Males

The amount of label that was fixed in the reproductive system of males from experiment 1 was similar to what was found in the females after mating. Larval labelling resulted in a higher mean $\delta^{13}C$ value than adult labelling and the labelling of both stages was superior over either singly (χ^2: 18.12; $P<0.01$) (see Fig. S1 in supplementary material). In atom%, this corresponds to a mean (±s.e.m.) enrichment of 2.52±0.46 atom% ^{13}C for adult labelling (U–L), 4.64±0.67 atom% ^{13}C for larval labelling (L–U) and 7.93±0.93 atom% ^{13}C for labelling of both stages (L–L). If males were sampled at an earlier interval (e.g. 4 days after emergence), a similar amount of label was observed in treatment L–U [$t(10)=0.14$, $P>0.05$], and a higher amount was observed in treatment L–L [$t(9)=2.36$, $P<0.05$] compared to sampling on day 11.

Life-history traits

Mating

Comparable insemination rates were found for labelled and control males in all experiments (see Table S1 in supplementary material). The highest insemination was observed in the first experiment when females were introduced

Table 1. *Survival of males in experiments 2 and 3*

		Male mean survival (days)					
	Replicate	*N*	L–L	*N*	L–U	*N*	U–U
Experiment 2	1	34	15±1.22[a]	32	19±1.16[b]	33	15±0.94[a]

		Male mean survival (days)					
	Replicate	*N*	Labelled glucose	*N*	Unlabelled glucose	*N*	Control (no glucose)
Experiment 3	2	49	27±1.31[a]	49	31±1.26[b]	50	24±1.45[a]
	3	49	21±1.56[a]	48	31±1.65[c]	48	29±1.21[b]

Values are mean ± s.e.m. *N* is the number of males analysed.

Experiment 2: L–L, males were labelled as larvae and adult males were fed labelled sugar (e.g. for the first 5 days; see Materials and methods); L–U, as larvae males were fed labelled sugar and as adults unlabelled sugar: UU, males unlabelled in the larval and adult stage.

Experiment 3: males were reared under the three treatments shown in the larval stage, and maintained as adult on standard (unlabelled) 10% sucrose solution.

For each row, values with different letters are significantly different at $P<0.01$ (log rank tests).

Table 2. *Larval survival from experiment 2 and 3*

Experiment	Survival (% out of 500 L1 larvae)		
	Labelled glucose	Unlabelled glucose	Control (no glucose)
2	88	85	99
3	80	86	76
3	82	82	82
Mean	83±3[a]	84±1[a]	85±7[a]

Survival was measured by counting the total number of emerged adults. Experiment 3 had two replicates per treatment.

Mean survival values are ± s.e.m.; values with different letters are significantly different at $P<0.05$ (planned contrast).

at the ratio of 2:1 (M:F). Experiment 2 aimed for a higher proportion of uninseminated females, and insemination was between 57–84%, resulting in adequate numbers of uninseminated females to validate the method.

Longevity

Labelling of males in the larval or larval and adult stage in experiment 2 had no detrimental effect on longevity; and a similar (χ^2: 0.21; $P>0.05$) or even slightly higher (χ^2: 9.27; $P<0.01$) survival was observed as of control males (Table 1). Similar observations were made in experiment 3; longevity of males reared in trays with labelled glucose was similar to that of the control males (χ^2: 1.17; $P>0.05$). Only in the second replicate was a lower longevity of labelled males compared with the control observed (χ^2: 11.65; $P<0.01$). In both replicates, males from the unlabelled glucose trays had significantly higher longevity than control males (Table 1).

Larval development and survival

The rate of pupation in trays where labelled or unlabelled glucose had been added was similar to those without any glucose. Pupation started at day 7 and continued until day 11, by which time the vast majority of L4 larvae had pupated (data not shown). Larval survival was not affected by the addition of [^{13}C]-labelled glucose or unlabelled glucose to the trays; no differences were observed between the three treatments ($F_{2,6}=0.07$; $P>0.05$) (Table 2).

Adult size

For each treatment, replicate and sex, wings were measured from ~50 individuals that emerged on the second or third day of emergence (e.g. when the majority of pupae emerged). No significant size differences were observed in wings from both days, and data were pooled. Significant differences were observed between replicates of the same treatment for males and females, therefore data was analysed per replicate. For the females, size of the adults was unaffected by the label; females emerging from trays with labelled glucose or unlabelled glucose were similar in size ($F_{2,146}=1.47$; $P>0.05$) or larger ($F_{2,151}=5.09$; $P<0.01$) compared with females from the control trays (Table 3). Some variation in wing length between treatments and replicates was observed for the males. The labelled trays produced the smallest ($F_{2,148}=9.09$; $P<0.01$) and the largest ($F_{2,150}=3.32$; $P<0.05$) males, but overall differences were small when compared to the control males (Table 3).

Sperm viability

No difference was observed in the hatch rate of the eggs from females mated to labelled or control males, and the number of eggs laid was similar (labelled: eggs=1786, hatch=0.81; control: eggs=2061, hatch=0.81).

Discussion

We have shown that it is possible to use the stable isotope ^{13}C as a semen label. Spermathecae inseminated by males labelled in the larval stage had distinguishably higher δ^{13}C values than uninseminated spermathecae and control samples, and the additional labelling of the adult stage resulted in even higher δ^{13}C values compared to larva labelling alone. Labelling in the adult stage alone was not sufficient; it resulted in low amounts of label detected immediately after mating, and the label seemed to diminish faster over time in spermathecae of females isolated after mating. Somewhat similar results were observed in a study with *Aedes aegypti* L., where it was found that when adult males were offered radioactively labelled honeydew no labelled semen was transferred to the females, even though the males themselves were highly labelled (Dame and Schmidt, 1964). Exposure in the larval stage did result in positive semen labelling and the authors discussed that perhaps exposure during the early stages of spermatogenesis, e.g. during

Table 3. *Wing length of males and females from experiment 3*

Sex	Replicate	Wing length (mm)		
		Labelled glucose	Unlabelled glucose	Control (no glucose)
Females	1	3.11±0.01[b]	3.06±0.01[a]	3.07±0.01[a]
	2	3.04±0.01[a]	3.07±0.01[a]	3.06±0.01[a]
Males	1	2.88±0.01[b]	2.86±0.01[a,b]	2.84±0.01[a]
	2	2.78±0.01[a]	2.81±0.01[a]	2.85±0.01[b]

Values are mean ± s.e.m. Each treatment had two replicates and each value is based on ~50 individuals. For each row, values with different letters are significantly different at $P<0.05$ (planned contrast).

the larval stage, was necessary to incorporate the label in the semen (Dame and Schmidt, 1964). In our study, adult labelling did label the semen but in low amounts, therefore emphasis was put on larval and larval plus adult labelling treatments.

Mass spectrometric analyses of the males showed a highly enriched reproductive system. The level of enrichment of the testis or the accessory glands separately was not determined. Hence, we cannot specify the relative contribution of the label in both fractions of the semen (e.g. spermatozoa or accessory gland products). Although differential labelling may be of interest at a later stage, for practical purposes and applicability of the method developed, our current methods are considered adequate. The highest enrichment that was estimated was 8.5 atom% ^{13}C (e.g. sampled 4 days after emergence, L–L treatment), whereas the target enrichment was 20 atom% ^{13}C. Not all ^{13}C added to the diet is recovered in the mosquito, because label is lost as a result of respiration in the larval trays and turnover in the insect. Respiration in the larval trays is brought about by micro-organisms present in the water. When feeding, these micro-organisms will incorporate ^{13}C in their system, but due to respiration, ^{13}C is also lost from the trays as $^{13}CO_2$. As a consequence of variations in the microbial fauna in larval environments and the resulting levels of respiration, the amount of label in trays will vary, and this could account for some of the variability observed in the amount of label transferred by males between experiments. Mosquito larvae are collector-filter feeders (Clements, 1992), and feed on dissolved particles in the water. The label could have been ingested directly through the uptake of [^{13}C]glucose, or indirectly through the uptake of micro-organisms that utilized the supplied larval diet (Merritt et al., 1992). The relative contribution of each pathway remains speculative at this stage.

For use of this technique in experimental settings, persistence of the label in the spermathecae after mating is desirable, as females will not always be dissected immediately after mating. It was observed that the label was detectable in spermathecae up to 7 days after insemination, and significantly higher $\delta^{13}C$ values than control samples were reported. Moreover, unlabelled sugar feeding of males labelled in the larval stage did not result in a traceable dilution of the semen label due to turnover of ^{13}C with ^{12}C. Males up to 10 days of age transferred similar amounts of label as younger males. An important finding, because adult males and females replenish energy reserves by sugar feeding on plant nectars in nature (Foster, 1995; Clements, 1999), and are maintained on sugar solutions in the laboratory.

The threshold values used in the experiments were successful in classifying labelled spermathecae from unlabelled samples. Threshold values were different between experiments because $\delta^{13}C$ (‰) values of the reference standard, in our case virgin females, varied between experiments. In experiment 1, some variation in the $\delta^{13}C$ values of virgin females resulted in a somewhat conservative threshold, but in the subsequent experiment, the triple standard deviation threshold value demonstrated with 99.7% confidence that all samples were classified correctly. The few outliers in the dataset, especially observed in experiment 1, could not be attributed to contamination during sample preparation. However, we cannot exclude a possible contamination by other samples during drying, or perhaps cross-contamination by the highly labelled males occurred. Even though the few outliers were a cause for concern, in the subsequent experiment no such outliers were observed and we are confident that they do not invalidate our findings. Between experiments, the amount of carbon in the samples differed significantly, even though the ‘spike’ solution used in all experiments was derived from the same stock and kept at 4°C. The amount of carbon in the experimental samples is referenced to standard samples, which are slightly different between experiments, causing these levels of inconsistency. However, as the $\delta^{13}C$ value is a ratio and thus independent of the amount of carbon in the sample, this variation has no impact on our findings. The spiking of samples resulted in a dilution of the label and complicated calculations of the actual $\delta^{13}C$ values; however, in our mass spectrometry set-up this was necessary to raise the detection limit. Nonetheless, the raw $\delta^{13}C$ values could effectively be used for interpretation of results and data analysis.

We have established a proof of principle in the laboratory; and this technique can be used to study a variety of issues related to mating in anopheline mosquitoes, and other insects. Although we applied the label in the aquatic stage, in insects lacking this stage, the label can be incorporated in the larval diet, but the optimal treatment would need to be determined (e.g. duration of labelling treatment, formulation and amount of labelling diet, etc.). Besides laboratory-based studies, there is a great potential to use this technique in the context of genetic control studies like the Sterile Insect Technique, etc. The most important parameter in these studies is the ability of released males to locate and inseminate wild females, and stable isotopes can be used to determine which group of males was responsible for the insemination. Preferably, these experiments take place in the field or in large field cages (Knols et al., 2002) to evaluate the insects in their natural environment (Scott et al., 2002). Because fitness of the labelled males is of high importance in these experiments, impact of the label on a number of life-history traits was assessed. Exposure to the label, even at the high quantities that were used, had no effect on male mating ability. Longevity of labelled males was similar or higher than the control in the first two replicates; in the last replicate, a somewhat reduced longevity was observed, but in general longevity was high and well beyond any life expectancy in a more natural situation. The labelling of mosquitoes in the larval stage by adding glucose to the trays had no effect on larval development; and the same result was observed in a study applying the same technique but with lower amounts of labelled glucose added to the trays (Hood-Nowotny et al., 2006). Size of the females was not affected by the label, and although some variation in the males was observed, overall differences were small. There is no impact of the label on the ability of labelled sperm to inseminate eggs; and similar results were reported when radioactive isotopes were used to label semen (Young and Downe, 1978). As such, stable isotope labelling meets most criteria of an ‘ideal marker’ for insects

that should be durable, non-toxic, easily applied, does not impact on the insects behaviour (e.g. growth, reproduction, life span), is clearly identifiable, and inexpensive (Hagler and Jackson, 2001). The latter could be contested in the case of stable isotope analysis. Even though pricing of stable isotopes and sample analysis have decreased over the last years (Hood-Nowotny and Knols, in press), it is still a relative expensive technique to use. 1 g of [^{13}C]glucose 99 atom% costs US $100, and 0.25 g was used per larval tray. Sample analyses were done in-house but can be outsourced at a cost of $5 per sample (Hood-Nowotny and Knols, in press). Another minor drawback of mass spectrometry is that the sample analysis is destructive, leaving no possibility to repeatedly measure samples.

In conclusion, larval labelling alone resulted in sufficient amount of label transferred to females to distinguish inseminated spermathecae from control samples, and the label persisted in spermathecae for at least 7 days after insemination. Males up to 10 days of age transfer similar amounts of label as younger males, indicating that larval labelling results in a life-long signature. The label had no influence on larval development and survival, longevity or mating ability and is therefore considered an ideal marker. The label is easy to apply, the sample preparation is straightforward and cost of sample analysis is reasonable. Although the technology presented was tested in anopheline mosquitoes, other candidate insects for genetic control studies, e.g. *Aedes* mosquitoes, fruitflies, tsetse flies, etc. are likely to benefit from the same technology. We believe that stable isotopes offer a great potential to study mating behaviour in insects. In addition, stable isotopes are environmentally safe and are thus likely to be well accepted both within the research community and by the public.

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Table S1. *Insemination of females mated with males from experiments 1 and 2*

Experiment	Duration	Ratio M:F	Treatment	*N*	Insemination (%)
1	3	2:1	U–U	18	100
	3	2:1	U–L	18	100
	3	2:1	L–U	16	94
	3	2:1	L–L	18	100
2	1	1:1	U–U	28	68
	1	1:1	L–U	30	60
	1	1:1	L–L	30	57
	3	1:2	L–U	42	69
	3	1:2	L–U	32	84
	1	1:1	L–U	23	65

Duration is the number of nights mated [to ensure the presence of unmated females in experiment 2, females were introduced at a 1:1 ratio and mating was restricted to one night only. If mating took place over a weekend (e.g. 3 nights of mating), the number of females was doubled]. M, male; F, female. U–U, no label; U–L, adult labelling only; L–U, larval labelling only; L–L, larval plus adult labelling. *N*, number of females dissected.