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## Evaluation of a Stable Isotope Method to Mark Naturally-Breeding Larval Mosquitoes for Adult Dispersal Studies

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### Abstract

Understanding mosquito dispersal is critically important for vector-borne disease control and prevention. Mark–release–recapture methods using various marking techniques have made substantial contributions to the study of mosquito biology. However, the ability to mark naturally breeding mosquitoes noninvasively and with life-long retention has remained problematic. Here, we describe a method to mark naturally breeding mosquitoes with stable isotopes. *Culex pipiens* f. *molestus* mosquitoes were provisioned as larvae in laboratory experiments with <sup>15</sup>N-labeled potassium nitrate and <sup>13</sup>C-labeled glucose. Larval enrichment was sufficient to differentiate marked adult mosquitoes from unmarked control mosquitoes and the natural source population from Chicago Illinois, using either  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$ . Isotopic retention lasted for at least 55 d for adult male and females mosquitoes. There were no consistent effects of isotopic enrichment on immature mosquito survival or adult mosquito body size. We then applied this marking technique to naturally breeding *Culex pipiens* mosquitoes in suburban Chicago, IL, and for the first time, report successful isotopic enrichment of mosquitoes in the field. This stable isotope marking technique will facilitate studies of mosquito dispersal.

### Keywords

*Culex* mosquito; mark–capture; mosquito dispersal; stable isotope

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Mark–release–recapture studies have provided useful insights into adult mosquito dispersal, feeding behavior, duration of the gonotrophic cycle, survival rates, and population sizes

(Silver 2008). Methods to mark mosquitoes have included dyes (Welch et al. 2006), paints (Trpis and Hausermann 1986), dusts (Reisen et al. 1992, Russell et al. 2005), trace elements (Wilkins et al. 2007), and radioactive isotopes (Lindquist et al. 1967). Hagler and Jackson (2001) reviewed marking techniques for insects and stated that the ideal marker should persist without inhibiting normal biology, should be environmentally safe, cost-effective, and easy to use. However, existing techniques tend to be labor intensive, as they require rearing mosquitoes, marking them in large quantities, and then inspecting large numbers of individuals to detect recaptures (Walker et al. 1987). In addition, the process of rearing adults, marking them, and releasing them may change behavior compared with natural populations (Reisen et al. 2003, Silver 2008).

Mark–release–recapture studies using fluorescent dust have been most widely adopted to date. Such methods typically recapture adult mosquitoes for <10 d (Lapointe 2008, Baber et al. 2010, Bellini et al. 2010) but occasionally up to 20 d (Pumpuni and Walker 1989, Harrington et al. 2005), and in one study up to 48-d post-release (Walker et al. 1987). Because of the constraints of the retention of the fluorescent marker, most studies primarily quantify dispersal of nulliparous female mosquitoes during the initial host-seeking event and occasionally a second host-seeking event. Although these studies have contributed valuable insights into mosquito behavior and biology, this short duration also is a shortcoming given that the vectorial capacity increases with longevity (Dye 1986, Milby and Reisen 1989). For example, the time from ingestion of an infectious bloodmeal until a mosquito is capable of delivering an infectious bite (i.e., extrinsic incubation period), is  $\approx 8$ –30 d for West Nile virus (WNV) in *Culex tarsalis* and *Cx. pipiens* (Dohm et al. 2002, Reisen et al. 2006, Anderson et al. 2008). To characterize the dispersal of medically relevant infectious mosquitoes, markers ideally should be retained for life.

Stable isotopes have been adapted for biological and ecological studies on a wide variety of animals including insects (Hood-Nowotny and Knols 2007), mammals (Pauli et al. 2009), fish (Munro et al. 2008), and birds (Inger and Bearhop 2008). Stable isotopes occur naturally in the environment, are nontoxic and non-radioactive, and incorporate into living tissue, which make them safe and useful tracers. An isotope of an element has the same atomic number but different number of neutrons, thus a different atomic weight. For example,  $\approx 0.4\%$  of all nitrogen atoms in the environment are  $^{15}\text{N}$  and the rest are  $^{14}\text{N}$ , and  $\approx 1\%$  of all carbon atoms in the environment are  $^{13}\text{C}$  and the rest are  $^{12}\text{C}$  (Hood-Nowotny and Knols 2007). Studies have used variation in the natural abundance of stable isotopes to trace the origin and movement of animals (Rubenstein and Hobson 2004), or have relied on enrichment techniques by labeling animals with rare stable isotopes. For example, immature aquatic insects were enriched by the addition of  $^{15}\text{N}$  to streams and the emergent and marked adult insects were captured to estimate dispersal (Hershey et al. 1993, Briers et al. 2004, Macneale et al. 2005). *Anopheles arabiensis* Patton mosquitoes were enriched to address questions related to Sterile Insect Technique (SIT) programs (Hood-Nowotny et al. 2006, Helinski et al. 2007, Helinski et al. 2008a,b). In the context of SIT programs, the use of stable isotopes allowed investigators to determine the insemination success of irradiated versus unirradiated male mosquitoes. This is achieved by detecting isotopically enriched semen in unlabeled female spermathecae. These studies demonstrate the potential to use

stable isotopes as a sensitive biological tracer in mosquitoes. Although the enrichment of naturally breeding mosquitoes by using stable isotopes in the context of a mark–capture study has been proposed (Hood-Nowotny and Knols 2007), more detailed development of the method is required before isotopic enrichment of naturally breeding mosquitoes can be used in the field.

We label *Culex pipiens* mosquitoes with stable isotopes in laboratory and field environments. We addressed the following objectives using *Cx.pipiens* mosquitoes reared under laboratory conditions: 1) determine the larval stable isotope enrichment level necessary to differentiate marked from unmarked adult mosquitoes, 2) determine the experimental conditions that allow the detection of one enriched mosquito in a pool of 5 or 10 individual mosquitoes, 3) identify the duration of enrichment at different days post emergence, 4) analyze positive or negative effects of isotopic enrichment on mosquito body size and survival, 5) quantify the impact of blood feeding or egg production on enrichment, and 6) determine if trans-generational marking occurs. Based on the results of the laboratory enrichment experiments, we applied the technique in the field to enrich naturally breeding mosquitoes with stable isotopes.

## Materials and Methods

### Mosquitoes

*Culex pipiens* f. *molestus* (Forskål) were used for all laboratory experiments. The colony was established from 2005 to 2009 from a natural population located in suburban Chicago, IL (Kothera et al. 2010). For the basic colony maintenance and experiments, larvae were reared in clear plastic trays (20 by 28 cm) in one liter of water (deionized water was used throughout the experiments). Larvae and adults were stored in a Percival Scientific incubator (Perry, IA) at 25°C and a photoperiod of 16:8 (L:D) h. Larvae were fed a 2% solution of desiccated and defatted liver powder (BioServ, Frenchtown, NJ) and brewer's yeast hydrolysate (Bio-Serv, Frenchtown, NJ) at a ratio of 3:2. Larvae were transferred to fresh water by using a Calphalon stainless steel skimmer (Calphalon Corp., Toledo, OH) (third and fourth instars) or this same type of skimmer fitted with a finer mesh (210  $\mu$ m) of fabric (first and second instars). The larvae in the skimmer were placed into a clean tray with one liter of water.

For the colony maintenance only, pupae were transferred to a cup of water in an insect rearing cage (BugDorm; 299 cm<sup>3</sup>; BioQuip Products, Rancho Dominguez, CA) by using a pipette. Adults were fed a 10% sucrose solution ad libitum using 29.6-ml plastic containers (Bio-Serv, Frenchtown, NJ) with 5.1-cm dental rolls (Absorbal, Wheat Ridge, CO) inserted into the lid. Although some autogenous egg-laying occurred, we increased egg production by blood-feeding adults using defibrinated bovine blood (Hemostat Laboratories, Dixon, CA) provided in a glass blood-feeding apparatus heated to 36.5°C.

*Cx. pipiens* mosquitoes also were collected from suburban Chicago, IL in 2009 to obtain natural abundance levels of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ . Adult male and female *Cx. pipiens* mosquitoes were collected from urban catch basins in suburban Chicago, IL in 2009 by using emergence

traps (Hamer et al. 2011). Mosquitoes were stored in a  $-20^{\circ}\text{C}$  freezer until they were prepared for stable isotope submission.

### Experimental Labeling Protocols

We used  $^{15}\text{N}$ -labeled potassium nitrate ( $\text{KNO}_3$ ;  $^{15}\text{N}$ , 99 atom%; Cambridge Isotope Laboratories, Inc., Andover, MA) and  $^{13}\text{C}$ -labeled glucose ( $\text{U-}^{13}\text{C}_6$ , 99 atom%; Cambridge Isotope Laboratories, Inc., Andover, MA) for enrichment. In all three experiments,  $^{15}\text{N}$ -potassium nitrate or  $^{13}\text{C}$ -glucose was added based on the total amount of nitrogen and carbon present in the larval food. Values are expressed based on the final atom percentage of  $^{15}\text{N}$  or  $^{13}\text{C}$  added to the larval containers. Brewer's yeast contains 13% nitrogen and 48% carbon, and liver powder contains 9% nitrogen and 39% carbon; thus 11% of the larval diet (liver powder and brewer's yeast in a 3:2 ratio) consists of nitrogen and 43% consists of carbon. In experiment 3, for example, 0.32 g of larval food was added to each treatment, and because the percentage of nitrogen in potassium nitrate is 14%, 1.88 mg of 99 atom%  $^{15}\text{N}$ -potassium nitrate was added to reach 0.75 atom%  $^{15}\text{N}$ . The  $^{13}\text{C}$ -treatments in experiment 3 received the same amount of larval food, and because 40% of glucose consists of carbon, 1.88 mg of 99 atom%  $^{13}\text{C}$ -glucose was added to reach 0.55 atom%  $^{13}\text{C}$ . Quantities of  $^{15}\text{N}$ -potassium nitrate and  $^{13}\text{C}$ -glucose were measured using a balance with 0.1-mg sensitivity (Denver Instrument, Bohemia, NY). In all experiments, stable isotopes were prepared in a solution containing the larval food and were fed directly to trays beginning with first-instar larvae. Controls in all experiments were larvae reared with the same amount of particulate food, but without additions of any potassium nitrate or glucose.

The three experiments were designed to reach the objectives of the study described. For each experiment, we used separate larval trays, skimmers, transfer pipettes, emergence containers, spatula, and forceps to reduce the risk of cross-contamination of  $^{15}\text{N}$  and  $^{13}\text{C}$  among treatments. During all experiments, adult body size for individuals emerging from the different treatments was measured by obtaining a length of the wing by using a MZ16 F stereo microscope (Leica Microsystems, Wetzlar, Germany) fitted with a SPOT Pursuit charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) and configured to a computer with image analysis software. Wing length, known to have a positive relationship with adult body size (Carron 2007), was measured from the alular notch to the distal margin (Bock and Milby 1981).

**Experiment 1**—The first experiment evaluated  $^{15}\text{N}$ -enrichment of larval mosquitoes under three concentrations in an initial attempt to identify the appropriate level of enrichment to distinguish marked from unmarked mosquitoes. The levels of enrichment were 0.04 atom %  $^{15}\text{N}$  (low), 0.08 atom%  $^{15}\text{N}$  (medium), and 0.12 atom%  $^{15}\text{N}$  (high). We ran two replicates for each dosage for six trays in total, containing 1 liter of water and 100 first-instar larvae each. In addition to the six trays receiving  $^{15}\text{N}$  additions, we had two trays of controls receiving the same amount of food but no  $^{15}\text{N}$  additions. The addition of food was constant among all treatments. The appropriate amount of  $^{15}\text{N}$ -potassium nitrate was added to each larval tray containing first-instar larvae to achieve the above atom% (0.05 mg, 0.09 mg, 0.14 mg for low, medium, and high, respectively). We added 0.09 g of liver powder and 0.06 g of brewer's yeast into each tray per feeding. The water was changed and new larval food (same

atom%  $^{15}\text{N}$  values but different mg of  $^{15}\text{N}$ -potassium nitrate per liter) was added at 3-d intervals until all larvae pupated. At days 9 and 12, the water was changed and we increased the amount of food (0.10 and 0.11 g of liver powder and 0.07 and 0.08 g of brewer's yeast, respectively). Pupae were transferred to an emergence container ("mosquito breeder;" BioQuip Products, Rancho Dominguez, CA) and the newly emerged adults were transferred to 0.5-liter plastic cups (Bio-Serv) by using D-cell flashlight aspirators (BioQuip Products, Rancho Dominguez, CA). The adult holding cup lids had the centers cut out and replaced with a 1-mm mesh circle, 9 cm in diameter, glued to the outside of the lid. During adult rearing in the cups, adults were fed by placing sugar water soaked cotton balls on the top of the mesh lid, replaced daily. Individual adult male and female *Cx. pipiens* were collected at different days postemergence (0, 6, 15, 27) and stored in a  $-20^{\circ}\text{C}$  freezer until samples were prepared for stable isotope analysis.

**Experiment 2**—The results of experiment 1 demonstrated that we needed to increase the concentrations of  $^{15}\text{N}$ , therefore, experiment 2 evaluated higher concentrations and also evaluated  $^{13}\text{C}$  enrichment. The concentrations for nitrogen were 0.33 atom%  $^{15}\text{N}$  (low) and 1.04 atom%  $^{15}\text{N}$  (high), and concentrations for carbon were 0.24 atom%  $^{13}\text{C}$  (low) and 0.76 atom%  $^{13}\text{C}$  (high). Two replicates were run for each dosage for a total of four  $^{15}\text{N}$  trays and four  $^{13}\text{C}$  trays containing 1 liter of water and 100 first-instar larvae each. In addition, two trays of controls received the same amount of food but no stable isotope additions. We added 0.2 g of liver powder, 0.14 g of brewer's yeast, and  $^{15}\text{N}$ -potassium nitrate (0.9 mg and 2.7 mg for low and high, respectively) or  $^{13}\text{C}$ -glucose (0.9 mg and 2.7 mg for low and high, respectively) to each larval tray containing first-instar larvae to achieve the above atom percentage. No additional food or stable isotopes were added during larval development and water was not changed. Pupae were transferred to an emergence container and adults then were transferred to plastic cups, as described in experiment 1. Individual fourth-instar larvae were collected from the treatments and prepared for stable isotope analysis, as described in the Sample Preparation and Analysis section. Adult male and female *Cx. pipiens* were collected at 0-, 5-, and 25-d postemergence. In addition to sugar feeding described in experiment 1, we allowed adult females to blood feed through the mesh of the adult holding cups. Progeny from a  $^{13}\text{C}$ -enriched female that took a bloodmeal were reared and then submitted as newly-emerged adults for stable isotope analysis. To simulate pools of 5 and 10 individuals, we combined one adult from an enriched tray with four or nine natural abundance adult females, respectively, and prepared for stable isotope analysis.

**Experiment 3**—The isotopic enrichment obtained in experiment 2 was sufficient to differentiate marked from unmarked mosquitoes. In experiment 3, we evaluated  $^{15}\text{N}$  and  $^{13}\text{C}$ -enrichment of larval mosquitoes under one concentration for each stable isotope separately. In this experiment we focused on marker retention and kept adults alive for more days postemergence and encouraged blood-feeding of adult females. The concentration for nitrogen was 0.75 atom%  $^{15}\text{N}$  and the concentration for carbon was 0.55 atom%  $^{13}\text{C}$ . Three replicates were run for each stable isotope containing 1 liter of water and 100 first-instar larvae each. In addition, two trays of controls received the same amount of food but no stable isotope additions. We added 0.19 g of liver powder, 0.12 g of brewer's yeast, and  $^{15}\text{N}$ -potassium nitrate (1.88 mg) or  $^{13}\text{C}$ -glucose (1.88 mg) was added to each larval tray

containing first-instar larvae to achieve the above atom percentage. In experiment 2, the larval water was not changed and we did not add additional food or stable isotopes. Consequently, we observed high larval mortality. In experiment 3, the water was changed and new food enriched with  $^{15}\text{N}$ -potassium nitrate or  $^{13}\text{C}$ -glucose was added at the same dose as the initial feeding every 6 d. In addition to the sugar water, we also offered blood to adult females by using the same techniques described in experiment 2. Individual pupae were collected as described in experiment 1. Adult male and female *Cx. pipiens* were collected at 0-, 25-, 35-, 45-, and 55-d postemergence.

### Field Labeling Protocols

From July to September 2010, we treated urban catch basins in Alsip, IL by using either  $^{15}\text{N}$ -potassium nitrate or  $^{13}\text{C}$ -glucose as part of a *Culex* mosquito mark-capture study. We obtained permission from the municipality, state, and county public health department, and the Illinois Environmental Protection Agency to amend catch basins with stable isotopes. We were unable to determine the total amount of nitrogen or carbon in the catch basins to calculate the necessary atom percentage to achieve the targeted levels of enrichment in the lab. Instead, we calculated the appropriate amount of stable isotope additions based on the volume of water in the catch basins. We measured the diameter of the sump in the catch basin and the depth of the water and sediment. We considered half of the sediment was water in our calculations to estimate the total volume of water. We treated nine catch basins with  $^{15}\text{N}$ -potassium nitrate and seven catch basins with  $^{13}\text{C}$ -glucose. All catch basins receiving either of the stable isotopes were connected by underground storm water drainage pipes that flowed directly to an outlet at the Stony Creek. For the initial treatment on 8 July, we added a solution of distilled water and stable isotopes targeting a treatment of 0.002 g of stable isotope per liter of water in the catch basin. This level of enrichment is similar to the amount of stable isotope added to 1 liter of water in experiment 3. We monitored enrichment by sampling larvae and pupae by using nets and emerging adults using emergence traps (Hamer et al. 2011). We sampled mosquitoes in untreated catch basins as controls to determine natural abundance levels of stable isotopes. Immature and adult mosquitoes were identified to species (Andreadis et al. 2005) and stored in 2-ml tubes in a  $-20^{\circ}\text{C}$  freezer until submission for stable isotope analysis. Pools of mosquitoes were prepared by grouping one adult female *Cx. pipiens* collected from an enriched catch basin with four unenriched colony female *Cx. pipiens* mosquitoes. On 13 July and 15, we collected mosquitoes and submitted for the stable isotope analysis to Isotech Laboratories Inc. Results received 1 wk later demonstrated more enrichment than necessary, and we reduced the subsequent treatments to 0.001 g of stable isotopes per liter of water. We continued to monitor enrichment in mosquitoes and performed six subsequent treatments (16 July, 26 July, 6 August, 20 August, 3 September, and 19 September) after rain events that flushed the mosquitoes and stable isotope out of the catch basins. We collected invertebrates from the Stony Creek and found no evidence of downstream enrichment (data not shown). In total, we delivered 38.58 g of  $^{15}\text{N}$ -potassium nitrate and 5.18 g of  $^{13}\text{C}$ -glucose to the catch basins during the study.

## Sample Preparation and Analysis

In preparation for stable isotope analysis, we removed the specimens from the  $-20^{\circ}\text{C}$  freezer and arranged them in tin capsules in a 96-well tissue-culture microplate, round bottom (BD Falcon, Bedford, MA). For individual specimens, we used 5- by 9-mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA) and for pools of 5–10 individuals, we used 9- by 10-mm tin capsules. The specimens were dried at  $50^{\circ}\text{C}$  for 24 h and the tubes then were crimped shut using forceps cleaned with ethanol between specimens. For the larger tin capsules, we initially used a 48-well cell culture plate to dry the specimens and then transferred the crimped tin capsules to a 96-well plates. Stable isotope analysis was performed by the University of California-Davis Stable Isotope Facility by using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (IRMS; Sercon Ltd., Cheshire, United Kingdom). Additional analyses were performed by Isotech Laboratories Inc. by using a Carlo Erba CHNS-O EA1108 (CE Instruments, Milan, Italy) coupled to a ThermoFisher Delta V Plus IRMS (Thermo Fisher Scientific, Bremen, Germany) via a ThermoFinnigan ConFlo III interface (Thermo Electron Corp., Waltham, MA). Stable isotope values are reported as the ratio of the lighter to the heavier isotope referenced against international standards (nitrogen =AIR; carbon =Vienna Pee Dee Belemnite) and defined by  $\delta X = [(R_{\text{sample}}/R_{\text{standard}})/(R_{\text{standard}})] \times 1,000$ .

## Statistical Methods

We tested for differences in stable isotopes, larval survival, and wing length among groups by using one-way analysis of variance (ANOVA). Separate tests were used for each marker ( $^{15}\text{N}$  or  $^{13}\text{C}$ ), sex, and experiment. Once significant differences among groups were detected, we used a posthoc test (Tukey's HSD) to compare means among groups ( $\alpha = 0.05$ ). All model residuals were assessed for normality by using diagnostic plots. All means are presented  $\pm$  SE and all tests were performed using Program R (R Development Core Team 2011).

## Results

### Isotopic Enrichment

Adult *Cx. pipiens* were collected from seven different catch basins in 2009 by using emergence traps, and the natural abundance of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  for females was  $1.3 \pm 0.81$  ( $n = 5$ ) and  $-25.4 \pm 0.43$  ( $n = 5$ ), respectively, and natural abundance for males was  $4.0 \pm 0.92$  ( $n = 19$ ) and  $-28.1 \pm 0.64$  ( $n = 19$ ), respectively (Table 1 and 2). For experiment 1, there were no significant differences for  $\delta^{15}\text{N}$  in adult males at different ages postemergence in the low, medium, and high treatment ( $F = 1.68$ ,  $df = 9$ ,  $P = 0.241$ ;  $F = 0.98$ ,  $df = 8$ ,  $P = 0.449$ ;  $F = 0.90$ ,  $df = 7$ ,  $P = 0.486$ , respectively) and no differences for  $\delta^{15}\text{N}$  in adult females at different ages postemergence for the low, medium, and high treatment ( $F = 2.22$ ,  $df = 4$ ,  $P = 0.229$ ;  $F = 4.33$ ,  $df = 6$ ,  $P = 0.06$ ;  $F = 3.28$ ,  $df = 7$ ,  $P = 0.089$ , respectively). Male and female mosquitoes from the  $^{15}\text{N}$  treatment had significantly higher  $\delta^{15}\text{N}$  than control and natural abundance mosquitoes ( $F = 66.84$ ,  $df = 57$ ,  $P < 0.001$ ;  $F = 49.75$ ,  $df = 36$ ,  $P < 0.001$ ; Table 1).

For experiment 2, male and female mosquitoes in the  $^{15}\text{N}$  treatments had significantly higher  $\delta^{15}\text{N}$  than control and natural abundance mosquitoes ( $F=82.45$ ,  $df = 49$ ,  $P < 0.001$ ;  $F = 228.35$ ,  $df = 8$ ,  $P < 0.001$ , respectively; Table 2). Male and female mosquitoes in the  $^{13}\text{C}$  treatments also had significantly higher  $\delta^{13}\text{C}$  than control and natural abundance mosquitoes ( $F = 99.28$ ,  $df = 47$ ,  $P < 0.001$ ;  $F = 9.03$ ,  $df = 13$ ,  $P < 0.001$ , respectively), but the posthoc tests show not all means were different (Table 2). For experiment 3, male and female mosquitoes in the  $^{15}\text{N}$  treatments had significantly higher  $\delta^{15}\text{N}$  than control and natural abundance mosquitoes ( $F = 604.7$ ,  $df = 30$ ,  $P < 0.001$ ;  $F = 293.3$ ,  $df = 12$ ,  $P < 0.001$ , respectively; Table 3). Male and female mosquitoes in the  $^{13}\text{C}$  treatments also had significantly higher  $\delta^{13}\text{C}$  than control and natural abundance mosquitoes ( $F=281.89$ ,  $df = 29$ ,  $P < 0.001$ ;  $F = 13.21$ ,  $df = 14$ ,  $P < 0.001$ , respectively; Table 3). For experiments 2 and 3, we observed a slight decline of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  as mosquitoes aged for both males and females. However,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  for females were still significantly different from controls and natural abundance levels at 55 d postemergence. The  $\delta^{15}\text{N}$  for males at 55 d postemergence was significantly different from controls and natural abundance, but we did not have 55-d-old males from the  $^{13}\text{C}$  treatment to compare. Overall, females tended to have slightly lower  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  than did males.

### Isotopic Enrichment in Mosquito Pools, Larvae, and Pupae

Two pools, each containing one enriched *Cx. pipiens* (low  $^{15}\text{N}$  and  $^{13}\text{C}$  treatment in experiment 2) and 4 natural abundance *Cx. pipiens* had higher, but not significantly higher,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  compared with control and natural abundance values. Pools containing 10 individuals (1 enriched and 9 natural abundance) contained too much nitrogen and carbon for the IRMS to provide reliable results. Fourth-instar larvae and pupae had similar  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  compared with newly emerged adults from the same treatment (Tables 2, 3).

### Isotopic Enrichment After Blood-feeding and in Progeny

Three females from the  $^{13}\text{C}$  treatment in experiment 3 took a bloodmeal, laid an egg raft, and then were harvested at 25 d postemergence for stable isotope analysis. These females with a previous blood-meal had a mean  $\delta^{13}\text{C}$  of  $95.9 \pm 16.1$ , and two females that were harvested from the same treatment at the same age but did not take a bloodmeal had a mean  $\delta^{13}\text{C}$  of  $96.8 \pm 16.1$ , showing no reduction in enrichment because of blood feeding. None of the  $^{15}\text{N}$ -labeled females took a bloodmeal. A 14-d-old female from the  $^{13}\text{C}$  low treatment in experiment 2 took a bloodmeal, laid an egg raft, and was collected at 27-d postemergence with a  $\delta^{13}\text{C}$  of  $-1.33$ . We were able to rear four of the progeny and the four males had a mean  $\delta^{13}\text{C}$  of  $-21.7 \pm 0.18$ . Unenriched control males from the same experiment had a  $\delta^{13}\text{C}$  of  $-22.5 \pm 0.85$  ( $n = 10$ ), showing no evidence of transgenerational marking.

### Influence of Enrichment on Larval Survival, Development, and Wing Length

There was no significant difference in immature mosquito survival among treatments for experiment 1 ( $F = 1.18$ ,  $df = 3$ ,  $P = 0.423$ ) or experiment 3 ( $F = 0.10$ ,  $df = 2$ ,  $P = 0.906$ ) but there was for experiment 2 ( $F = 21.6$ ,  $df = 4$ ,  $P = 0.002$ ; Table 4). Experiment 2 showed that the  $^{13}\text{C}$ -enriched mosquitoes had higher survival compared with the control and the  $^{15}\text{N}$ -enriched mosquitoes, but this pattern was not consistent in experiment 3, and the rearing



protocol in experiment 2 resulted in high larval mortality and smaller adults relative to the other experiments. Mosquito wing lengths were not significantly different among isotopic-enriched treatments and controls for males but there were significant differences for females in experiment 1 and 3 ( $F = 2.93$ ,  $df = 48$ ,  $P = 0.043$ ;  $F = 2.93$ ,  $df = 48$ ,  $P = 0.043$ ; Table 5).

### Isotopic Enrichment of Mosquitoes in the Field

We treated catch basins in the field for three months beginning 8 July, 2011. Mosquitoes were collected from catch basins receiving stable isotopes and untreated controls on 18 different days between 13 July and 8 October 2011.

The mean  $\delta^{15}\text{N}$  for all mosquitoes (larvae, pupae, and adults combined) collected from catch basins receiving  $^{15}\text{N}$ -potassium nitrate was  $1,067.7 \pm 673.8$  and the mean  $\delta^{15}\text{N}$  for mosquitoes collected from untreated catch basins was  $2.6 \pm 0.69$  (Fig. 1). The mean  $\delta^{13}\text{C}$  for all mosquitoes (larvae, pupae, and adults combined) collected from catch basins receiving  $^{13}\text{C}$ -glucose was  $319.2 \pm 69.3$  and the mean  $\delta^{13}\text{C}$  for mosquitoes collected from untreated catch basins was  $-24.4 \pm 1.04$ .

### Discussion

These results demonstrate that sufficient isotopic enrichment can be achieved in the laboratory and field setting through larval enrichment to mark adult *Cx. pipiens* mosquitoes. Through addition of 0.75 atom%  $^{15}\text{N}$  and 0.55 atom%  $^{13}\text{C}$ , we sufficiently enriched mosquitoes beyond the recommended 2–3 standard deviations above the isotopic abundance of the natural population of *Cx. pipiens* (IAEA 2009). We also show that one enriched mosquito can be detected in a pool of five individuals. However, we also found that a pool of 10 individuals contained too much nitrogen and carbon for the IRMS instruments to provide reliable results. We found no evidence that blood feeding reduces  $^{13}\text{C}$  enrichment, although small sample sizes warrant additional investigations to the influence of blood feeding on isotopic enrichment. We were unable to judge the influence of blood feeding on  $^{15}\text{N}$  enrichment because of the limited numbers of females and limited blood feeding. We found no evidence of transgenerational marking; the progeny of a  $^{13}\text{C}$ -enriched female were similar to  $\delta^{13}\text{C}$  levels from unenriched controls. Given that transgenerational marking has been observed in other studies (Williamson et al. 2009, Pecl et al. 2010), and given the limited sample size in this study, additional studies to confirm our results in *Cx. pipiens* mosquitoes are warranted. Finally, our study also demonstrated that fourth-instar larvae and pupae had similar levels of enrichment as newly emerged adults. This observation is important for monitoring enrichment levels during field studies.

Importantly, we did not observe consistent positive or negative consequences of isotopic enrichment on mosquito morphology or survival. Wing lengths, used as a proxy for adult body size, were very similar among treatments. Also, survival rates of immature mosquitoes in the  $^{15}\text{N}$  and  $^{13}\text{C}$  treatments showed no positive or negative trends compared with the controls. Positive effects of enrichment might be expected if the nutrient added is limiting and negative effects of enrichment might be expected if toxic levels of nitrogen or carbon are reached, neither of which likely would occur in a field application in a natural container. We assume that bioaccumulation of the enriched nitrogen and carbon in the microbial

community (Hamilton et al. 2004), which is a food source for larval mosquitoes, was the primary mechanism for the enrichment observed in this study. The loss of  $\delta^{13}\text{C}$  occurred at a faster rate than  $\delta^{15}\text{N}$ , indicating that the  $^{15}\text{N}$  was incorporated into structural body tissues (e.g., chitin), whereas  $^{13}\text{C}$  was subject to higher biochemical turnover (e.g., respiration).

Utilizing stable isotopes as a mosquito marking technique offers several advantages. First, a “mark–capture” design can be implemented where naturally breeding mosquitoes can be isotopically enriched without removing them from the environment (Hagler and Jackson 2001). The enrichment of container breeding species such as most *Culex* spp. mosquitoes offers the advantage that the larval environment is confined, and thus can be easily treated with stable isotopes during larval growth. Other container-breeding mosquito species such as *Aedes aegypti* and *Ae. albopictus* might be enriched in the same way, whereas mosquitoes such as *An. gambiae* or *Ae. sollicitans*, which use larger bodies of water for larval habitat, might be enriched through broadcast application of stable isotopes. Our breeding-site labeling technique also offers the advantage of being able to mark thousands of mosquitoes with relative ease. For example, a single highly productive septic tank produced 1,663 adult *Ae. aegypti* and 6,670 adult *Cx. quinquefasciatus* per day in a small community in southern Puerto Rico (Mackay et al. 2009). Finally, stable isotopes also provide long-lasting retention. The current study demonstrates enrichment for *Cx. pipiens* up to 55 d old, which were the oldest individuals we tested. Depending on the initial level of enrichment, the rate of loss, and the number of individuals in a pool, mosquitoes marked with stable isotopes essentially can be marked for life.

The negative aspects of the use of stable isotopes are mainly the cost for the isotopically enriched material and the cost for the analysis. The  $^{15}\text{N}$ -labeled potassium nitrate and  $^{13}\text{C}$ -labeled glucose used in this study cost \$62.70 and \$176.00 for 1 g, respectively. In all the experiments, we used 0.024 g of  $^{15}\text{N}$ -labeled potassium nitrate (costing \$1.50) and 0.021 g of  $^{13}\text{C}$ -labeled glucose (costing \$3.69). When implementing a field study enriching naturally breeding mosquitoes, the cost of the material would depend on the atom percentage necessary to sufficiently enrich individuals and can be estimated based on the total volume of water. The catch basins used in this study were either 60 cm or 120 cm in diameter and the mean volume of water in the sump was 318.6 liters. We delivered a total of \$2,418 worth of  $^{15}\text{N}$ -potassium nitrate and \$912 worth of  $^{13}\text{C}$ -glucose, however, when purchased in bulk, the price per gram is reduced considerably. In this study, we used 99 atom%  $^{15}\text{N}$  potassium nitrate and glucose but a lower grade compound containing a lower atom % could also be used for a cost savings. The cost for stable isotope analysis is very high, but improvements in technology and market demand continue to decrease the cost per sample (Hood-Nowotny and Knols 2007). For example, the cost of the dual analysis for  $^{15}\text{N}$  and  $^{13}\text{C}$  in this study was \$7 per sample. In the context of a field mark–capture study, mosquitoes can be pooled with up to five individuals to increase efficiency.

Finally, an additional drawback of our method is the difficulty of releasing cohorts of marked adults at defined intervals and quantifying the number of marked adults being released. The number of marked adults being released at a distinct time is a necessary parameter when using existing equations to estimate dispersal, population sizes (e.g., Lincoln Index), and daily survival rates (Silver 2008). Clever field designs might allow the

estimation of these parameters but new methodological and quantitative techniques also might need to be developed. For example, estimating *Culex* mosquito production might require the deployment of emergence traps (Hamer et al. 2011). Analytically, it might be necessary to incorporate uncertainty in detection probabilities at different distances from the release point (e.g., occupancy modeling; Mackenzie et al. 2006).

In summary, this study demonstrates that stable isotopes can be used to enrich naturally breeding larval mosquitoes for adult mosquito dispersal studies. We demonstrate proper enrichment levels to segregate marked mosquitoes from natural populations and isotopic marker retention for the life of the mosquito. Utilizing stable isotopes as a marking technique offers advantages that will complement mark–release–recapture studies using traditional markers. Advancements in measuring mosquito movement and dispersal will greatly improve estimates of parameters that constrain and facilitate disease transmission in nature.

## Acknowledgments

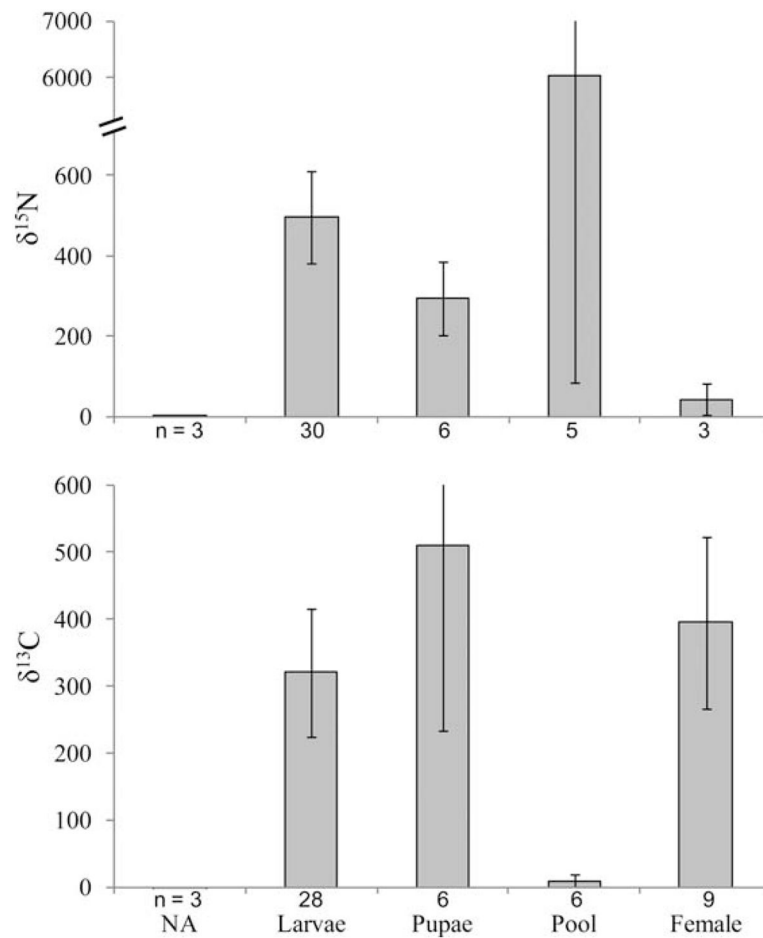
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**Fig. 1.** Results of isotopic enrichment in the field showing  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  (mean $\pm$ SE) for natural abundance (NA) *Cx. pipiens* collected from untreated catch basins and larvae, pupae, pools, and adult female *Cx. pipiens* mosquitoes collected directly from catch basins receiving stable isotope enrichment. Treatment of the catch basins began 8 July and mosquitoes were collected on 18 different days between 13 July and 8 October, 2011. Each pool includes one adult female mosquito collected from a treated catch basin and four untreated colony control adult females. Sample sizes are indicated below the x-axis.

**Table 1**

Experiment one results showing  $\delta^{15}\text{N}$  mean  $\pm$  SE for adult male and female *Cx. pipiens* from natural abundance, control, and stable isotope enriched mosquitoes

Mosquito	$\delta^{15}\text{N}$		
	n	Mean $\pm$ SE	Tukey's HSD <sup>a</sup>
Natural abundance <sup>b</sup>			
Male	19	4.0 $\pm$ 0.9	A
Female	5	1.3 $\pm$ 0.8	a
control			
Male	7	9.7 $\pm$ 0.5	B
Female	7	8.4 $\pm$ 0.5	b
low treatment <sup>c</sup>			
Male	13	15.7 $\pm$ 0.8	C
Female	8	13.9 $\pm$ 0.7	c
medium treatment			
Male	12	21.5 $\pm$ 1.1	D
Female	10	15.9 $\pm$ 0.9	c
high treatment			
Male	11	22.1 $\pm$ 1.2	D
Female	11	16.5 $\pm$ 0.8	c

<sup>a</sup>Tukey's HSD test for differences among means ( $P < 0.05$ ) were run separately for males and females and results are provided as upper case letters and lower case letters, respectively.

<sup>b</sup>*Cx pipiens* collected from seven different catch basins in 2009.

<sup>c</sup>Stable isotope concentrations were 0.04, 0.08, and 0.12 atom%  $^{15}\text{N}$  for the low, medium, and high treatments, respectively.

Table 2

Experiment 2 results showing  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  mean  $\pm$  SE for adult male and female *Cx. pipiens* from the natural abundance, control, and stable isotope enriched mosquitoes at different days post-emergence

Mosquito	$\delta^{15}\text{N}$			$\delta^{13}\text{C}$		
	n	Mean $\pm$ SE <sup>a</sup>	Tukey's HSD <sup>b</sup>	n	Mean $\pm$ SE	Tukey's HSD
Natural abundance <sup>c</sup>						
Male	19	4.0 $\pm$ 0.9	A	19	-28.1 $\pm$ 0.6	A
Female	5	1.3 $\pm$ 0.8	a	5	-25.4 $\pm$ 0.4	a
Control						
Male	10	10.7 $\pm$ 0.2	A	10	-22.5 $\pm$ 0.8	A
Female	2	9.5, 11.0	a	2	-19.2, $\pm$ 19.2	a
Larvae	3	10.9 $\pm$ 0.5	A	3	-19.2 $\pm$ 0.3	A
Low treatment <sup>d</sup>						
Zero days post-emergence						
Male	3	165.5 $\pm$ 9.2	B	4	108.3 $\pm$ 20.3	C
Female	3	164.8 $\pm$ 11.3	b	2	78.9, 99.9	b
Larvae	3	217.8 $\pm$ 2.9	BC	3	76.1 $\pm$ 3.6	BC
Poo <sup>e</sup>	2	25.6, 47.6	A	2	0.6, 44.7	AB
Five days post-emergence						
Male	4	190.0 $\pm$ 19.4	B	3	27.0 $\pm$ 1.9	B
Female				4	26.7 $\pm$ 12.3	a
Twenty-five days post-emergence						
Male	4	169.5 $\pm$ 12.2	B	2	10.2, 41.8	AB
Female				4	1.5 $\pm$ 2.7	a
High treatment						
Zero days post-emergence						
Male	4	264.2 $\pm$ 37.8	C	2	283.1, 320.4	D
Female	2	169.0, 187.3	b			
Larvae	3	185.3 $\pm$ 3.7	B	3	264.6 $\pm$ 12.5	D
Five days post-emergence						
Male				3	125.2 $\pm$ 42.0	C



Mosquito	$\delta^{15}\text{N}$			$\delta^{13}\text{C}$		
	n	Mean $\pm$ SE <sup>a</sup>	Tukey's HSD <sup>b</sup>	n	Mean $\pm$ SE	Tukey's HSD
Female				2	84.3, 258.3	b
Twenty-five days post-emergence						
Male	5	222.3 $\pm$ 23.1	BC	4	89.5 $\pm$ 17.8	BC
Female				1	71.6	ab

<sup>a</sup>When sample size was less than three, actual values are presented.

<sup>b</sup>Tukey's HSD test for differences among means ( $P < 0.05$ ) were run separately for males and females and results are provided as upper case letters and lower case letters, respectively. Larvae and pools were included with the males for the post-hoc tests.

<sup>c</sup>*Cx pipiens* collected from seven different catch basins in 2009.

<sup>d</sup>Stable isotope concentrations were 0.33 and 1.04 atom%  $^{15}\text{N}$  and 0.24 and 0.76 atom%  $^{13}\text{C}$  for the low and high treatment, respectively.

<sup>e</sup>Pool contained one adult female from  $^{15}\text{N}$  treatment and four natural abundance adult females collected from the field.

Table 3

Experiment 3 results showing  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  mean  $\pm$  SE for adult male and female *Cx. pipiens* from the natural abundance, control, and stable isotope enriched mosquitoes at different days post-emergence

Mosquito	$\delta^{15}\text{N}$			$\delta^{13}\text{C}$		
	n	Mean $\pm$ SE <sup>a</sup>	Tukey's HSD <sup>b</sup>	n	Mean $\pm$ SE	Tukey's HSD
Natural abundance <sup>c</sup>						
Male	19	4.0 $\pm$ 0.9	A	19	-28.1 $\pm$ 0.6	A
Female	5	1.3 $\pm$ 0.8	a	5	-25.4 $\pm$ 0.4	a
Control						
Male	4	12.2 $\pm$ 0.7	A	4	-23.2 $\pm$ 0.9	A
Female	3	13.2 $\pm$ 0.3	a	3	-23.7 $\pm$ 0.5	a
Treatment <sup>d</sup>						
Zero days post-emergence						
Male	2	795.3, 796.8	C	2	489.7, 603.1	C
Female						
Pupae	2	727.6, 867.6	C	2	496.7, 592.5	C
Twenty five days post-emergence						
Male	3	776.0 $\pm$ 39.8	C	3	163.6 $\pm$ 28.3	B
Female	3	541.9 $\pm$ 9.6	b	5	96.3 $\pm$ 13.1	b
Thirty five days post-emergence						
Male	3	747.5 $\pm$ 12.1	B	2	115.0, 193.0	B
Female	4	754.0 $\pm$ 11.4	c	3	111.0 $\pm$ 16.8	b
Forty five days post-emergence						
Male	2	735.7, 763.7	C	3	136.8 $\pm$ 14.4	B
Female	2	653.9, 824.2	c	2	80.9, 84.6	ab
Fifty five days post-emergence						
Male	2	542.6, 711.2	B			
Female	1	514.0	b	2	72.8, 241.3	b

<sup>a</sup>When sample size was less than three, actual values are presented.

<sup>b</sup>Tukey's HSD test for differences among means ( $P < 0.05$ ) were run separately for males and females; and results are provided as upper case letters and lower case letters, respectively. Pupae were included with the adult males for the post-hoc tests.

<sup>c</sup> *Cx. pipiens* collected from seven different catch basins in 2009.

<sup>d</sup> Stable isotope concentrations were 0.75 atom% <sup>15</sup>N and 0.55 atom% <sup>13</sup>C.

**Table 4**

Larval survival (percent of adults emerging) and number of days for immature development from each treatment in each experiment

Experiment	Treatment	Survival		Days until emergence (Mean $\pm$ SE)
		Mean $\pm$ SE	Tukey's HSD <sup>a</sup>	
1	Control	73.0 $\pm$ 4.0		16.5 $\pm$ 0.1
	<sup>15</sup> N-Low	75.5 $\pm$ 3.5		17.0 $\pm$ 0.2
	<sup>15</sup> N-Medium	76.5 $\pm$ 4.5		17.1 $\pm$ 0.2
	<sup>15</sup> N-High	87.0 $\pm$ 9.0		17.8 $\pm$ 0.2
2	Control	21.0 $\pm$ 3.0	A	14.3 $\pm$ 0.3
	<sup>13</sup> C-Low	35.0 $\pm$ 2.0	B	14.2 $\pm$ 0.2
	<sup>13</sup> C-High	29.0 $\pm$ 0.0	AB	14.1 $\pm$ 0.2
	<sup>15</sup> N-Low	20.0 $\pm$ 1.0	A	13.5 $\pm$ 0.2
	<sup>15</sup> N-High	13.5 $\pm$ 1.5	A	13.7 $\pm$ 0.2
3	Control	50.5 $\pm$ 0.5		17.0 $\pm$ 0.3
	<sup>13</sup> C	46.0 $\pm$ 15.6		15.6 $\pm$ 0.2
	<sup>15</sup> N	43.3 $\pm$ 3.3		14.9 $\pm$ 0.2

<sup>a</sup>Post-hoc tests show differences among means. Blank values indicate no difference among groups.

**Table 5**

Adult *Culex pipiens* wing length (mm) from the different treatments in each experiment

Experiment	Treatment	Male wing length (mm)		Female wing length (mm)			
		n	Mean ± SE	Tukey's HSD <sup>a</sup>	n	Mean ± SE <sup>b</sup>	Tukey's HSD
1	Control	10	3.0 ± 0.1		13	3.6 ± 0.1	AB
	<sup>15</sup> N Low	14	3.1 ± 0.0		12	3.7 ± 0.0	B
	<sup>15</sup> N Med	12	3.0 ± 0.0		12	3.6 ± 0.1	AB
	<sup>15</sup> N High	13	3.0 ± 0.0		15	3.5 ± 0.0	A
2	Control	5	2.6 ± 0.1		2	2.9, 3.6	
	<sup>15</sup> N Low	8	2.7 ± 0.1		1	4.0	
	<sup>15</sup> N High	3	2.8 ± 0.1		1	3.0	
	<sup>13</sup> C Low	9	2.8 ± 0.1		8	3.0 ± 0.1	
3	<sup>13</sup> C High	5	2.8 ± 0.0		5	3.2 ± 0.2	
	Control	10	3.4 ± 0.0		10	3.7 ± 0.0	a
	<sup>15</sup> N	17	3.2 ± 0.0		10	4.0 ± 0.0	b
	<sup>13</sup> C	13	3.3 ± 0.1		9	3.7 ± 0.3	ab

<sup>a</sup> Post-hoc tests show differences among means. Blank values indicate no difference among groups.

<sup>b</sup> When sample size was less than three, actual values are presented.