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Full Length Research Paper

Feeding habits of culicine mosquitoes in the Cameroon lowland forests based on stable isotopes and blood meal analyses

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Mosquito blood feeding behavior is a very significant component of pathogen transmission and determinant of disease epidemiology. Yet, knowledge of foraging ecology of mosquitoes often depends on the presence of undigested blood in the mosquito mid gut. Approximately 36 h after feeding, the blood meal is sufficiently digested to make identification by molecular techniques difficult, leaving a very narrow window in which these methods can be utilized. Here, we investigated the feeding habits of wild caught culicine mosquitoes from four genera, *Aedes, Anopheles, Coquillettidia* and *Mansonia* of the lowland rainforests of Cameroon based on the isotopic ratios of nitrogen ($\delta^{15}N$), carbon ($\delta^{13}C$) and sulfur ($\delta^{34}S$). Results showed that unfed mosquitoes had a lower $\delta^{13}C$, $\delta^{15}N$ and middle $\delta^{34}S$ values than mosquitoes fed with $\delta^{13}C$ appearing to be the best element to differentiate between mosquito species that fed on different host species. Isotopic analyses show that the different mosquito genera may be separated based on their diets, suggesting that linking stable isotope-based assays and DNA analysis may be a powerful new tool to investigate mosquito feeding ecology and the dynamics of vector-borne pathogens.

Key words: Carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), sulfur ($\delta^{34}S$), isotope, feeding habits, mosquitoes, polymerase chain reaction (PCR).

INTRODUCTION

Over the past decades, there has been an increase in the incidence of infectious diseases throughout the world, with serious implications for human and wildlife populations (Kilpatrick et al., 2006; Baker et al., 2012). Due to their diversity and abundance, demonstrated vector competence (Sardelis et al., 2002) and frequent infection in nature (Molaei et al., 2006; Burkett-Cadena et al., 2008a, b), mosquitoes are regarded as one of the most important vectors of disease.

The behavioral characteristics of mosquitoes in disease transmission differ vastly between different regions and species (Fontenille and Lochouarn, 1999; Kent and Norris, 2006), and can only be understood in a local context of vector-host interactions. An important aspect of these interactions is that of host preference, with each vector species feeding on a limited range of host species. Vector species that switch between hosts are particularly considered important in disease transmission because they have the potential to act as bridge vectors, by transferring pathogens from the reservoir to humans and/or domestic animals (Chandler et al., 1975; Rasgon, 2008). For example, West Nile Virus (WNV) is primarily an infection of birds, but can be transferred to humans or horses by mosquitoes that feed on both birds and mammals (Hamer et al., 2008). To accurately describe an arthropod-borne

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pathogen transmission cycle, it is critical to know which available host species are preferred by the corresponding vectors. Most studies evaluate the host-feeding patterns of mosquitoes by identifying the source of the vertebrate blood meals through sequencing portions of the cytochrome *b* gene of mitochondrial DNA. However, there is a very limited time period in which these methods can be utilized because these approaches only provide information on recent feeding events. After approximately 36 hours of feeding, the blood meal is sufficiently digested to make the identification by immunological or polymerase chain reaction (PCR)-based techniques particularly difficult, thus effective methods to assess feeding habits over longer periods of time are necessary.

Stable isotope techniques offer the opportunity to overcome some of these constraints by obtaining information on nutrients assimilated over extended periods of time (Rasgon, 2008). By comparing the isotopic signatures of a given organism or tissue, inferences on its feeding behavior could be done. Stable isotopes of carbon, nitrogen and sulfur have been widely used in ecological research (Wada et al., 1991; Fry, 2007). Stable carbon (δ13C); (δ15N) and nitrogen (δ34S) as indicated in ms are commonly used to extract information about the feeding habitat and carbon dietary sources (McCutchan et al., 2003; Michener and Lajtha, 2007; Yohannes et al., 2008); while nitrogen isotopes are often applied to evaluate the trophic position of a given species (Peterson and Fry, 1987). Based on the natural variation of the composition of stable sulfur isotope, measurement values can provide information on dietary protein sources and geographical origin (Richards et al., 2003). Also, because the rate of metabolism of different tissues determines the turnover of stable isotopes in tissues, it is possible to obtain dietary information on varying time-scales and extended period by sampling tissues with different turnover rates after feeding events (Tieszen et al., 1983; Rasgon, 2008).

The lowland forest areas in Cameroon are known to experience hyper-endemic transmission of *Plasmodium falciparum* mostly spread by the species, *Anopheles gambiae*, but little is known about the ecology of the different mosquito species in this region, and how vector species composition and their relative roles in transmission vary geographically (Akono et al., 2009). Very few studies on wild caught mosquitoes in Cameroon have been published (Rageau and Adam, 1952; Akono et al., 2009; Njabo et al., 2011), and very little information is available on their feeding habits (Snow and Boreham, 1978; Wanji et al., 2003).

In this study, we compared the feeding habits of wild caught blood-engorged mosquitoes and those with no visible blood meals (unfed mosquitoes or completely digested blood meal) that exhibit ornithophilic feeding habits, and characterize their feeding behavior by applying individual $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. We also coupled our isotopic approach with PCR based molecular techniques and identified vertebrate host blood meals following extraction of DNA from the engorged mosquitoes and PCR amplification of the cytochrome *b* gene.

METHODOLOGY

Sampling sites and habitat characterization

Mosquito samples were collected in August, 2007 and May, 2008 at two lowland forest sites in Cameroon. Locations and dates of fieldwork for these forest sites were: Ndibi (N 2° 43' 50'', E 9° 52' 19''), August 21st to 26th, 2007 and May 2nd to 16th, 2008; Nkouak (N 3° 52', E 13° 18'), August 27th to 31st, 2007 and May 17th to 29th, 2008. Ndibi has an elevation of 500 meters above sea level and its habitat is characterized by disturbed lowland forest, and seasonally flooded swamp forest habitat and high human population (exacerbated by its close proximity to the city of Akonolinga), while Nkouak has less human population density and more forest cover and is the source of river Nyong. Rainfall patterns in these areas have been fairly stable for more than 40 years. There is one rainy season each year that lasts from November to April, followed by cool dry (April to August) and hot dry (August to November) seasons.

Mosquito collections

Two main trapping methods were used as described in Njabo et al. (2011). Briefly, six Center for Disease Control (CDC) Miniature Light Traps baited with CO_2 (John W. Hock, Gainesville, FL), and four modified bird-baited Ehrenberg lard cans (Downing and Crans, 1977). Three bird species Feral Pigeon (*Columba livia*), Yellow-whiskered Greenbul (*Andropadus latirostris*) and Greater Swamp Warbler (*Acrocephalus rufescens)* were used as baits in the lard cans.

Traps were set out each day for at least 12 h (06.00 pm to 06.00 am). Following each trapping period, the collection bags were removed from traps and the mosquitoes were transported to the field lab site where they were immobilized with chloroform and/or smoke. On the day of collection and immobilization, mosquitoes were sorted by sex and identified to species, with the aid of a stereomicroscope (× 90) and morphological keys (Hopkins et al., 1941; Service, 1990). The mosquitoes were then separated by gonotrophic condition (unfed, blood-fed, gravid) and enumerated. They were then sorted into four groups: Pigeon fed, Warbler fed, Greenbul fed and unfed (all mosquitoes with no visible blood meals in the abdomen are considered for the purpose of this manuscript as unfed). We used t-test to compare each fed group against the unfed mosquitoes (that is, binary comparisons of Pigeon fed, Warbler fed or Greenbul versus unfed were conducted separately). At both sites, sampling date and species, fed, unfed and gravid mosquitoes were pooled separately into groups of 5 to 20 specimens and placed in 95% alcohol and later stored at -20°C. Blood meals from blood-fed mosquitoes were subsequently tested by PCR, as described below, to determine the birds on which mosquitoes had fed.

Host blood meal identification

Within the pools of blood-fed mosquitoes, five individuals were randomly selected for PCR analyses. Total DNA was extracted from the abdominal contents of the blood-fed mosquitoes individually, using standard proteinase k digestion and phenol chloroform

purification. Isolated DNA from the mosquito blood meals served as DNA templates in subsequent PCR reactions. PCR primers (Forward 5'-3'GACTGTGACAAAATCCCNTTCCA, Reverse 5'-3' GGTCTTCATCTYHGGYTTACAAGAC) were based upon previously published multiple alignment of cytochrome *b* sequences of avian species (Ngo and Kramer, 2003). Amplified fragments from the blood-fed mosquitoes were sequenced and compared with sequences in GenBank for host-species identification as described in Molaei and Andreadis (2006) and Hellgren et al. (2008).

Stable isotope analyses

We analyzed stable carbon and nitrogen isotopes from repre-sentative whole mosquitoes using pooled samples within each group. We removed lipids from each homogenized sample, using a 2:1chloroform-methanol solution rinse for 24 h. Samples were rinsed using methanol solution and air-dried in a fume hood. Dried and powdered sub-samples, approximately 0.6 mg, were weighed into small tin cups to the nearest 0.001 mg, using a micro-analytical balance. Samples were then combusted in a Eurovector (Milan, Italy) elemental analyser (Limnological Institute, University of Konstanz, Germany). The resulting CO_2 and N_2 were separated by gas chromatography and admitted into the inlet of a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for determination of $^{13}C/^{12}C$, $^{15}N/^{14}N$ ratios. Measurements were reported in δ-notation ($\delta^{13}C$ and $\delta^{15}N$, respectively) where δ = 1000 × ($R_{sample}/R_{standard}$) - 1 ‰ relative to the Pee Dee Belemnite (PDB) for carbon and atmospheric N_2 for nitrogen in parts per thousand deviations (‰). Two sulfanilamide (Iso-prime internal standards) and two Casein were used as a laboratory standard for every 10 unknowns in sequence.

We also analyzed sulfur isotopes in mosquitoes. Tin capsules containing reference or sample were loaded into an automatic sampler from where they were dropped in sequence, into a furnace held at 1080°C and combusted in the presence of oxygen, raising the temperature in the region of the sample to ~ 1800°C. The reference material used for analysis was sulfanilamide calibrated and traceable to NBS-127 (barium sulphate, $\delta^{34}S$ = +20.3‰). Hundreds of replicate assays of internal laboratory standards indicate measurement errors (SD) of ± 0.05, 0.15 and 0.05‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, respectively.

For each isotopic element separately, unfed versus fed mosquitoes of *Coquillettidia aurites* were compared using t-test. A second t-test was applied to compare elemental isotopic differences between blood-engorged *Coquillettidia pseudoconopas* and those with no visible blood meals. A one-way analysis of variance (ANOVA) followed by a Scheffe´ Post-hoc-test was used to compare differences in $\delta^{13}C$ and $\delta^{15}N$ between Warbler-fed, Greenbul-fed and unfed *Mansonia uniformis*

RESULTS

Five mosquito species belonging to four genera *Aedes* (*Aedes mcintoshi*), *Anopheles* (*Anopheles coustani*), *Coquillettidia* (*C. aurites and C. pseudoconopas*), and *Mansonia* (*M. uniformis*) were collected in the light traps while only three species (*C. aurites, C. pseudoconopas* and *M. uniformis*) were collected in the lard cans. All the species collected from the CDC light traps were unfed (had no visible blood meals) while all species from the bird-baited lard cans were blood-engorged.

Host blood meal identification

To confirm the applicability of the blood meal-specific PCR to Cameroon lowland forest mosquitoes and their avian hosts, the assay was tested for its ability to amplify cytochrome *b* sequences from several native Cameroon lowland forest bird species (results published elsewhere (Chasar et al., 2009; Njabo et al., 2011). The specific amplification conditions were found to support the amplification of detectable PCR products from all the bird species tested. The PCR diagnostic successfully identified all blood meals from engorged mosquitoes collected from the baited lard cans. No other species were identified and none of the mosquitoes fed on multiple hosts, indicating that the bias towards bird feeding was not indicative of a specific host preference for birds but a reflection of relative host availability. All unfed mosquitoes tested were negative.

Mosquito stable isotope analyses

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of unfed mosquitoes and fed mosquitoes are shown in Table 1. In general, unfed mosquitoes have relatively lower $\delta^{13}C$ and $\delta^{15}N$ compared to the blood-engorged mosquitoes. Mean $\delta^{13}C$ values from unfed mosquitoes ranged from ca.-20.0 to -34.5‰, while $\delta^{15}N$ ranged from 3.1 to 4.8‰, and $\delta^{34}S$ ranged from 6.4 to 10‰. Mean $\delta^{13}C$ values from fed mosquitoes ranged from -19.8 to -24.5‰, while $\delta^{15}N$ ranged from 4.3 to 7.1‰, and $\delta^{34}S$ ranged from 5.5 to 8‰.

The mean ± SE of $\delta^{13}C$ and $\delta^{34}S$ of each mosquito species are given in Figures 1 and 2, and Table 1 separately. Fed and unfed *C. aurites* showed a signifi-cantly different $\delta^{13}C$ (t-test, $p = 0.02$) signature (Figure 1). However, there was no difference between fed and unfed $\delta^{34}S$ (Figure 2) or $\delta^{15}N$ (Figure 1). Blood-engorged *C. pseudoconopas* and those with no visible blood meals showed significant difference at both $\delta^{13}C$ (t-test, $p < 0.0001$) and $\delta^{34}S$ ($p = 0.01$, equal variance not assumed).

ANOVA revealed that there was a significant diet effect on $\delta^{13}C$ and $\delta^{15}N$ values of *M. uniformis* ($\delta^{13}C$: $F_{15,98} = -7.19$, $p < 0.001$; $\delta^{15}N$: $F_{12,97} = -3.65$, $p < 0.001$). Post-hoc analysis of $\delta^{13}C$ for Warbler-fed and Greenbul-fed *M. uniformis* species indicated no significant difference ($p > 0.05$), and thus we pooled these data in further analyses and compared unfed and fed *M. uniformis* using t-test. On the contrary, *M. uniformis* that fed on Warbler were significantly different in $\delta^{15}N$ (t-test: t =14.44, $p = 0.003$ equal variance not assumed due to unbalanced sample size) than those that fed on Greenbul (Figure 1 and Table 1).

Bi-directional error bar graph (Figure 1) for $\delta^{13}C$ and $\delta^{15}N$, for fed (grouped together in a rectangle frame) and unfed groups show that unfed *A. mcintoshi* survives on

Table 1. Mean ± SE (n) values of nitrogen, carbon and sulfur Isotopes of unfed and fed mosquitoes.

Species	Diet	$\delta^{15}N$ (‰)	t-test	$\delta^{13}C$ (‰)	t-test	$\delta^{34}S$ (‰)	t-test
C. aurites	Unfed	4.5±0.31(8)	t=0.23	-30.4±3.5 (8)	t=-2.52	6.4±0.46 (3)	t=0.80
*C. aurites**	Pigeon	4.3±0.78 (9)	p=0.82	-21.8±2.47 (9)	p=0.02*	5.9±0.59 (9)	p=0.44
C. pseudoconopas	Unfed	4.8±0.37 (6)	t=-0.90	-34.7±1.07 (6)	t=-0.77	6.8±0.34 (6)	t=0.33
*C. pseudoconopas**	Pigeon	5.2±0.11 (3)	p<0.0001*	-19.8±1.05 (3)	p=0.46	5.4±0.28 (3)	p=0.01*
M. uniformis	Unfed	3.1±0.23 (90)	t=-14.25	-29.9±0.48 (90)	t=-7.52	8.4±0.36 (3)	t=-1.29
M. uniformis[1]	Greenbul	7.1±0.51 (6)	p<0.0001*	-24.5±1.80 (6)	p<0.0001*	8.9±0.36 (3)	p=0.27
M. uniformis[2]	Warbler	5.8±0.23 (3)	t =-11.21	-23.7±1.11 (3)	p<0.0001*	8.9±0.36 (3)	p=0.27
A. Mcintoshi	Unfed	3.9±0.34 (17)	p<0.0001*	-20.0±0.64 (17)	p<0.0001*	8.0±0.47 (3)	p<0.0001*
A. coustani	Unfed	4.0±0.63 (3)	p<0.0001*	-26.8±0.52 (3)	p<0.0001*	10.3±0.09 (3)	p<0.0001*

Diet: *Feral Pigeon, [1]Yellow-whiskered Greenbul and [2]Greater Swamp Warbler fed mosquitoes in the lowland forests of Cameroon. Results of the Student's t-test are given for each comparison. *Statistically significant differences (p<0.05). $\delta^{13}C$ (‰) in unfed *M. uniformis* was compared using pooled Greenbul and Warbler fed *M. uniformis*. Sample sizes for $\delta^{34}S$ are lower than for $\delta^{15}N$ or $\delta^{13}C$ due to missing samples.

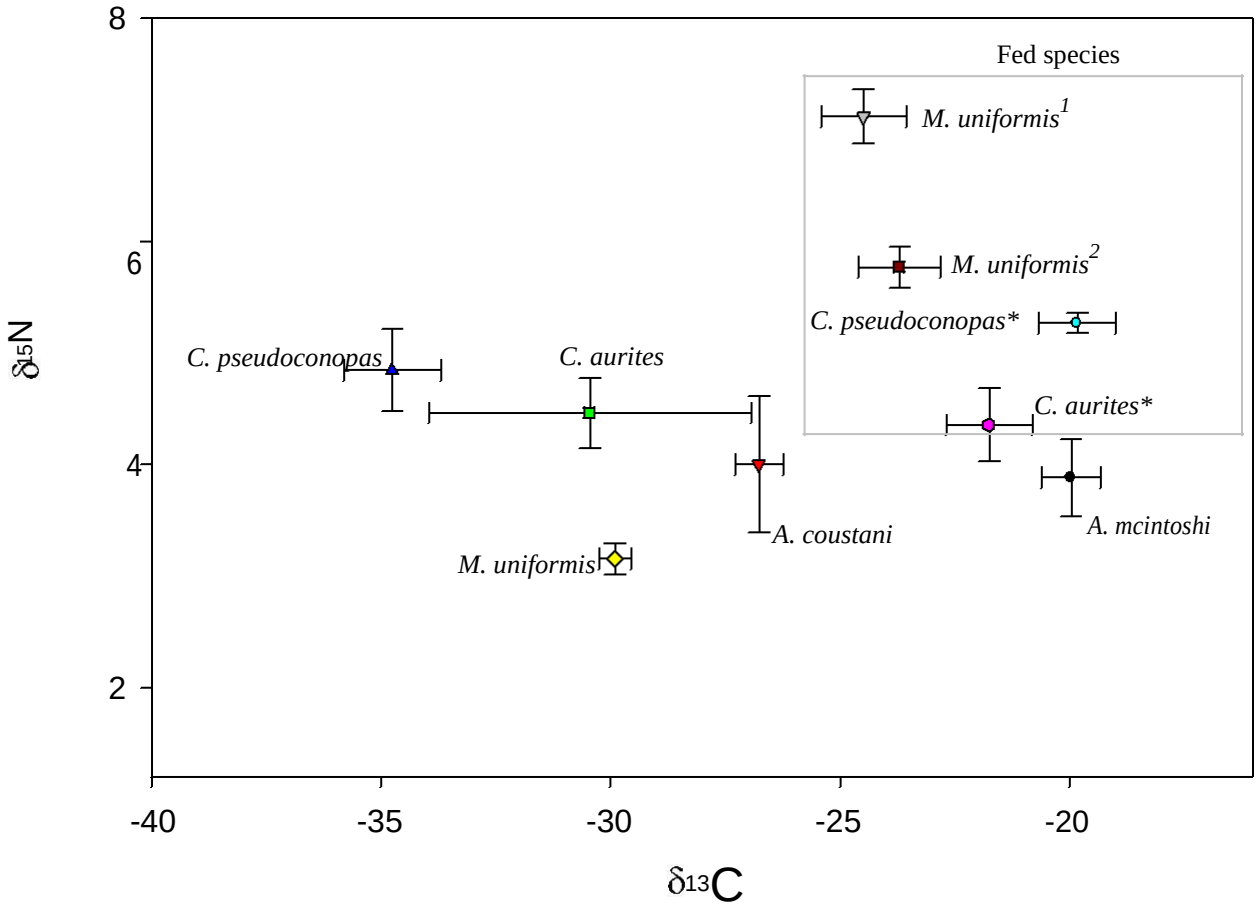

Figure 1. Bi-directional mean (± SE) values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) of unfed and blood-engorged mosquitoes in the lowland forests of Cameroon. Mosquitoes were fed with *Feral Pigeon, [1]Yellow-whiskered Greenbul and [2]Greater Swamp Warbler.

coustani, C. aurites and *C. pseudoconopas*, respectively. These values for *A. mcintoshi* are very similar to those of *Coquillettidia* species that fed on pigeons. As shown in Figure 1, the highest $\delta^{34}S$ in these study species was recorded for *A. coustani*. This species also showed an intermediate $\delta^{13}C$ values (ca. -27‰) and $\delta^{15}N$ (ca. 4‰), indicating a unique sulfur dietary sources in this species Interestingly, *M. uniformis* which fed on warblers has a comparable $\delta^{34}S$ (ca. 9‰) to that of *A. coustani* (ca. 9‰) signature. This implies the two mosquito species rely on

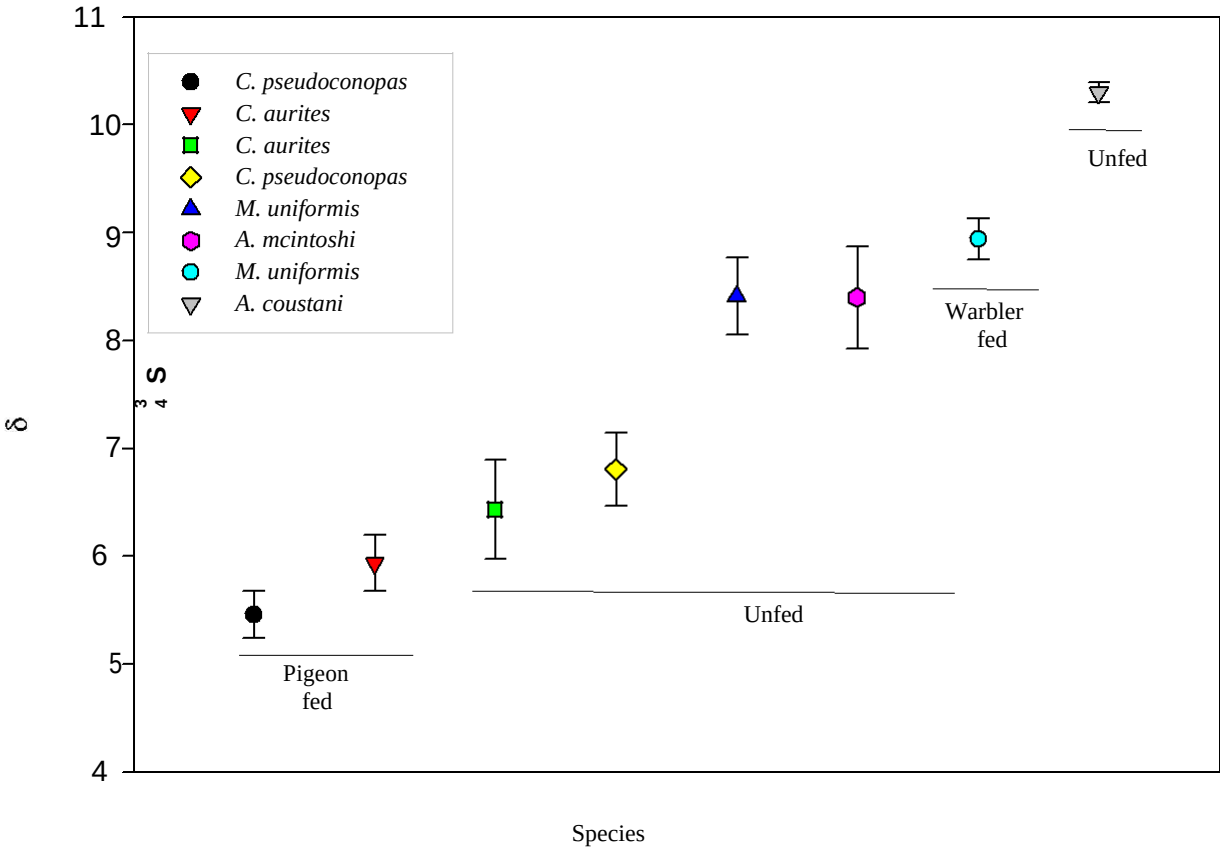

Figure 2. Mean (± SE) values of carbon ($\delta^{34}S$) for unfed and fed (Pigeon and Warbler fed) mosquitoes collected from the lowland forests of Cameroon.

similar sulfur dietary source.

DISCUSSION

In this study, four mosquito genera, *Aedes, Anopheles, Coquillettidia* and *Mansonia* and three different host types, were examined for triple isotopic elements: $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$.

Stable carbon isotopes

Our results suggest that the five mosquito species studied exhibit a wide-range of $\delta^{13}C$ signature spanning from ca -49 to -15‰, indicating a variety of host-carbon source in the region. These results support the findings of Hassan et al. (2003) who showed that ornithophilic mosquitoes typically feed significantly more or less on available bird species than predicted based on biomass, surface area, or relative abundance.

Although these mosquitoes seem to potentially cover a rather wider carbon source, each species shows a more specific and distinctive isotopic niche-width. For instance, *A. mcintoshi* apparently feeds on a host with a relatively higher stable carbon isotope value. Using mean $\delta^{13}C$ values reported for C_4 (-13‰) and C_3 (-27‰) plants (Michener and Lajtha, 2007) as endpoint tissue, $\delta^{13}C$ of *A. mcintoshi* departs from a dietary source based on C_3 plant and supports a C_4 dominated carbon source incorporated in its food chain. Relatively higher $\delta^{13}C$ values, as those reported for this species, can also be linked to C_3 plants of drier areas and xeric condition in response to water-use efficiency (Ehleringer and Cooper, 1988).

The $\delta^{13}C$ values of *A. mcintoshi* also complement those of *Coquillettidia* spp. that fed on pigeons, indicating that *A. mcintoshi* may also feed on pigeons or on other avian/vertebrate hosts with similar food habits. Pigeons are known to forage mainly in agricultural landscapes, as these areas represent an important and well exploited source of food (Hetmanski et al., 2005). There were no significant differences in isotopic values in $\delta^{13}C$ ($p > 0.05$) for Warbler-fed and Greenbul-fed *M. uniformis*. Although we observed slight differences in *M. uniformis* that fed on Warblers to those that fed on Greenbul (of $\delta^{13}C$ and also $\delta^{15}N$), the wide-range of isotopic signatures from both fed and unfed individuals (for example, mean $\delta^{13}C$ range: ca.

-24 to -30‰ of this species) suggest that *M. uniformis* seems to be a generalist that feeds on available host species of different isotopic values.

Indeed, *Mansonia* mosquitoes are major vectors of filariasis caused by *Brugia malayi* filarial nematodes and feed on a wide range of vertebrate hosts in nature (Laurence, 1960; Phumee et al., 2011). A larger range of $\delta^{13}C$ was also observed for unfed *C. aurites*. -21 to -48‰, implying the availability of multiple host species or hosts that rely on wider isotope biomes. Analysis of additional isotopes elements such as deuterium or oxygen may improve the resolution of nutrient sources in blood meal identification, especially in situations of many potential host species.

Stable nitrogen isotopes

The $\delta^{15}N$ signatures in consumer tissues are primarily used to assess trophic position in food webs. It has been shown that $\delta^{15}N$ values can be associated with plant and animal tissues grown in relatively low rainfall locations and arid environmental conditions (Ambrose, 1991; van der Merwe et al., 1993; Sealy et al., 1995). Our study illustrates that almost all unfed mosquito species exhibited similar nitrogen isotope signature. However, Greenbul-fed *M. uniformis* showed a much more enriched (up to + 4‰) $\delta^{15}N$ than unfed conspecific species indicating a higher trophic position than the other mosquito species. We also measured a slight difference in $\delta^{15}N$ of *M. uniformis* that fed on Warblers and those fed on Greenbul.

Stable sulfur isotopes

A. coustani seems to depend on a higher sulfur isotopic niche. *M. uniformis* which fed on warblers has a clearly separated nitrogen (Figure 1) and sulfur isotope (Figure 2) compared to Pigeon-fed species. The similar $\delta^{34}S$ in Warbler-fed and unfed *M. uniformis,* as well as to unfed *A. mcintoshi* imply that these two mosquito species might feed on similar hosts. By implication, while others occupy a unique specific niche, others do share host sources and rely on similar nutrient sources. In summary, our results illustrate the advantages of stable isotope analyses for the study of mosquito host feeding and niche segregation analyses. Using both fed and unfed mosquitoes collected in the field, we showed that the stable isotopes-based assays could play an essential role as tracers when applied on specimens unsuitable for PCR, such as gravid individuals or mosquitoes with digested or no observable blood meal to reconstruct the history of previous feeding events and dietary sources.

While stable isotope profiling is more sensitive in tracing element and nutrient sources, it is less specific in identifying digested blood meals to specific species as PCR techniques are. Thus the best results are obtained when both methods, stable isotope profiling and PCR, are used in concert. PCR may be used on fresh blood meals to gain a recent snapshot of current feeding habits, while stable isotope analysis may be used to reconstruct dietary history, trophic status and niche width of vectors over the longer time frames.

In general, field situations are likely to be more complicated, with multiple potential hosts present in the environment. Additional analyses of stable isotope ratios through more extensive sampling of potential hosts may provide further insights into seasonal changes in host preference and individual species' dietary strategies. To fully get the complete picture of field situations therefore, initial stable isotope profiling will have to be performed against all potential hosts in the study area, and temporal-spatial variation in stable isotope profiles within populations and within individual mosquitoes taken into account.

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