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Non-destructive Determination of Age and Species of *Anopheles gambiae* s.l. Using Near-infrared Spectroscopy

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Abstract. Determining malaria vector species and age is crucial to measure malaria risk. Although different in ecology and susceptibility to control, the African malaria vectors *Anopheles gambiae sensu stricto* and *An. arabiensis* are morphologically similar and can be differentiated only by molecular techniques. Furthermore, few reliable methods exist to estimate the age of these vectors, which is a key predictor of malaria transmission intensity. We evaluated the use of near-infrared spectroscopy (NIRS) to determine vector species and age. This non-destructive technique predicted the species of field-collected mosquitoes with approximately 80% accuracy and predicted the species of laboratory-reared insects with almost 100% accuracy. The relative age of young or old females was predicted with approximately 80% accuracy, and young and old insects were predicted with ≥ 90% accuracy. For applications where rapid assessment of the age structure and species composition of wild vector populations is needed, NIRS offers a valuable alternative to traditional methods.

INTRODUCTION

Controlling malaria by reducing human-vector contact has been one of the most successful approaches to reduce transmission.[1–3] An obstacle to accurate measurement and control of malaria transmission in sub-Saharan Africa is that several of the most important vector species, which have distinct behavior, ecology, and response to control, cannot be readily identified,[4,5] except by the application of relatively expensive and field-intractable polymerase chain reaction (PCR) methods.[6] These vector species include mosquitoes in the *Anopheles gambiae sensu lato* complex, which is composed of at least six morphologically indistinguishable sibling species. Of these species, *An. gambiae* s.s. and *An. arabiensis* are the most widespread and efficient malaria vectors in Africa, and they each exhibit different biological and ecological behaviors.[7,8] For example, *An. gambiae* s.s. is generally more endophilic and anthrophagic than *An. arabiensis*,[9–11] and the two species may be impacted differently by the use of insecticides such as pyrethroids that is applied indoors on materials such as bed nets and curtains.[12–17]

PCR methods to identify anopheline species are accurate, but expensive and time-consuming because of sample preparation and processing required. As a result, most entomologic studies estimate vector species composition based upon the analysis of only a sub-sample of all collected individuals (often < 20%). Such limited sampling may not capture the true heterogeneity of species diversity in field settings because this can occur on remarkably fine scales driven by local environmental factors, notably those driven by climate change,[18] the availability of various types of hosts[19] and habitats,[20] and the influence of interventions.[14,21,22] Thus, there is a need for a rapid species identification technique that can be used on a scale that is ecologically and epidemiologically meaningful.

In addition to species identification, estimation of the age of malaria vectors is of prime importance for the measurement of transmission and control success. Because only relatively old (> 10 days[23]) malaria vectors are capable of transmitting malaria, knowledge of the age distribution of these populations is essential for prediction of the proportion of potentially infectious vectors, and how this changes over time and in response to control measures. Additionally, accurate age grading methods are essential for estimation of mosquito survival, which is the most important biological determinant of transmission intensity[24,25] and may vary over scales as fine as a kilometer or less,[26,27] presumably underpinning much of the fine-scale variation in malaria transmission exposure that exists in human populations.[28–30]

Until recently, few methods have been available for the age-grading of African anophelines. Most age-grading analyses have been based on somewhat crude categorization of females into groups of nulliparous (typically less than four days of age) or parous[31] as assessed by ovarian dissection, which is a skilled, labor-intensive, and time-consuming method. More recently, alternative methods for age-grading *Anopheles* and other insect species have been developed based on the accumulation of pteridines,[32] cuticular growth lines,[33] cuticular hydrocarbons,[34] near-infrared reflectance spectroscopy (NIRS),[35] and transcriptional profiles,[36] but none have yet been amenable for integration into large-scale malaria vector surveillance and particularly for ecological studies of fine-scale vector population dynamics as a function of local environment, including human and animal demography and coverage with vector control interventions. Reasons that alternative age-grading methods have not been applied to malaria vector surveillance include the skill and expense required for most techniques, or that some techniques such as NIRS have not been applied to mosquito age-grading.

We evaluate the potential of a novel method for rapid species and age identification of two members of the *An. gambiae* species complex, *An. arabiensis* Patton and *An. gambiae sensu stricto* Giles based on the use of NIRS. This technique measures the amount of near-infrared energy absorbed at specific

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wavelengths by biological materials. The absorption is affected by the internal and external biochemical composition of the organism, and different organisms can have unique absorption spectra. Generally, the constituents must be present at the parts-per-thousand level or greater to be detected by NIRS. The stretching and bending of mainly C-H, N-H, and O-H functional groups may cause unique absorption of NIR energy between species or between different insect age groups. For example, the composition of the insect cuticle may be different for different species and may change as the insect ages. This uniqueness in the cuticle composition may affect the NIR spectra, and thus can be used in classification models. In comparison with more labor-intensive methods based on PCR, NIRS is a rapid, nondestructive, inexpensive, and environmentally friendly (i.e., chemical free) technique. NIRS has been successfully used to identify cryptic species of insects in stored grain[37] and sub-species of termites,[38] to differentiate male and female tsetse fly pupae,[39] to age-grade house flies,[35] and to determine blood meal size in *Aedes aegypti* (L.) and *Ae. sierrensis* mosquitoes.[40] These applications show that entomologists can analyze large numbers of samples within a short period.

However, NIRS has not yet been evaluated for determination of African malaria vector species composition and age. Physiological and behavioral differences between *An. gambiae* s.s. and *An. arabiensis* (e.g., the greater water body content of the latter species[41]) could cause them to have different cuticular biochemical composition, and correspondingly different NIR spectra. Thus, the objective of this study was to determine whether NIRS could be used to determine the species and age of the African malaria vectors *An. arabiensis* and *An. gambiae* s.s. and to evaluate the potential of this technique for application to large-scale entomological monitoring in operational settings and particular for high throughput ecological surveys of age and species distributions as a function of fine-scale environmental parameters.

MATERIALS AND METHODS

Laboratory-reared mosquitoes. Laboratory-reared mosquitoes were obtained from three different insectaries: Kansas State University (KSU), Manhattan, Kansas; Centers for Disease Control and Prevention (CDC), Atlanta, Georgia; and Ifakara Health Institute (IHI), Ifakara, Tanzania. In all locations, rearing conditions were 27°C and a relative humidity of 80%, and the photoperiod was 12:12 light dark cycle with a 30-minute dawn and dusk period. At KSU, adults were allowed to feed *ad libitum* on 8% fructose with 2.5 mM *p*-aminobenzoic acid in water. Young (1 and 2 days of age) larvae were fed on baker's yeast and larvae more than 2 days of age were fed on a 2:1 ratio of ground Tedmed tropical fish flakes (Tetramin, Melle, Germany) and baker's yeast. At IHI, mosquitoes were reared on 10% glucose in water and larvae were fed ground fish flakes. At CDC, adults were fed *ad libitum* 10% sugar solution with 0.2% methylparaben dissolved in sterile water. Young larvae were fed on baker's yeast and older larvae were fed ground Koi fish food (Aquaricare, Victor, NY).

Several laboratory stocks of mosquitoes were used, and the stock numbers of those obtained from the Malaria Research and Reference Reagent Resource Center are indicated along with the number of generations from the field. For species tests, the KSU insectary reared G3 (MRA-112, 400 generations), Mali-NIH (MRA-860, 56 generations), Ifakara *An. gambiae* s.s. strains (274 generations), Dongola (MRA-856, 90 generations) and KGB (MRA-339, 400 generations) *An. arabiensis* strains. The CDC insectary reared three *An. gambiae* s.s. strains (Kisumu, MRA-762, 400 generations; ZANU, MRA-594, 325 generations; and Mali-NIH, 56 generations) and the two *An. arabiensis* strains (KGB and Dongola). The Ifakara *An. gambiae* s.s. and *An. arabiensis* (six generations) strains were obtained from lines maintained at the laboratories of the IHI in Ifakara, a small town in the Kilombero Valley of southern Tanzania.

Approximately 50 females of each strain and of each status (unfed, blood fed, or gravid) were obtained for analysis. Females were considered blood fed when scanned 12–18 hours post-feeding, and gravid when scanned approximately 48 hours post-feeding. Before scanning, mosquitoes were held in cages where sugar-water was provided *ad libitum*.

For age-grading tests, *An. gambiae* s.s. (Ifakara and G3 strains) and *An. arabiensis* (KGB strain) males and females were reared at the KSU insectary. Mosquitoes were scanned in six age groups that were separated by three days (1, 4, 7, 10, 13, 16, and 19 days after emergence). Approximately 40 mosquitoes were scanned for each age group and sex. However, the experimental group of male G3 mosquitoes was depleted after 16 days.

Field-collected mosquitoes. Mosquitoes visually identified as belonging to the *An. gambiae* s.l. complex (comprised of *An. arabiensis* and *An. gambiae* s.s. with no other members of this complex observed) were collected from three villages (Lupiro, Kivukoni, and Njage) in the Kilombero Valley in southern Tanzania during May and June 2008. Subsequent species identification by PCR confirmed that *An. arabiensis* were collected from the villages of Lupiro (n = 93), Kivukoni (n = 46), and Njage (n = 32), and *An. gambiae* s.s. from Njage (n = 104). Mosquitoes were collected by resting catches and CDC light traps inside human houses. The field-collected mosquitoes were kept in a cage and transported live to the IHI laboratory for scanning.

Mosquito scanning. Chloroform was used to anesthetize mosquitoes immediately before scanning. Up to 20 mosquitoes were placed on a spectralon plate, and one mosquito was scanned at a time by rotating the plate until the head and thorax of the mosquito were under the NIR probe. Spectra were collected from individual mosquitoes using a QualitySpec Pro spectrometer (350–2500 nm; ASD Inc, Boulder, CO) (Figure 1). An HL-2000 halogen light source (Mikropack, Ostfildern, Germany) was used for illumination. Individual mosquitoes were manually positioned on their backs 2 mm below a 3 mm-diameter bifurcated fiber-optic probe, which contained 4 collection fibers and 33 illumination fibers. The spot size of the viewing area was approximately 3 mm and focused on the head and thorax. The instrument was set to collect 20 spectra from each mosquito and these were stored as an average spectrum. ASD software RS3 version 3.1 was used to collect all spectra. Sample positioning, data collection, and storage took less than one minute per mosquito. A complete description of the instrument and scanning procedures is given by Reeves and others.[42]

After scanning, each field-collected insect was placed in a plastic tube and labeled with an identification that corresponded to the spectrum number. The *An. gambiae* s.l. mosquitoes were then identified to sibling species as either *An. gambiae* s.s. or *An. arabiensis* by PCR.[43]

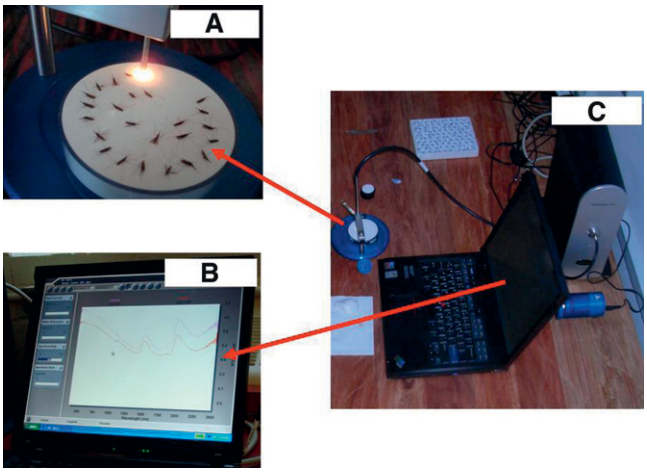

FIGURE 1. Scanning mosquitoes using a near-infrared spectrometer. **A**, Plate with anesthetized mosquitoes positioned for scanning. **B**, Near-infrared (NIR) spectra of mosquitoes. **C**, Complete NIR system including the spectrometer (ASD Inc., Boulder, CO).

Data analysis. Spectra in ASD format were converted to GRAMS format (Thermo Galactic, Salem, NH) using ASD ViewSpecPro. The Grams software PLSPlus/IQ was used to perform partial least squares (PLS) regression on the spectra and for developing calibrations. A cross-validation was used to develop calibrations that could then be used to predict independent test sets. In a cross-validation, one sample is removed from the population and the remaining samples are used to develop an equation to predict the removed sample. The sample is then returned to the population and the procedure is repeated for all samples. The resulting calibration equation is derived from this leave-one-out procedure. This calibration equation is represented graphically as regression coefficients at each wavelength. Predictions of unknown spectra are obtained by multiplying the regression coefficient at each wavelength by the absorbance value from the unknown spectrum at that same wavelength. This procedure is repeated for each wavelength and the values are summed. The resulting summation is the predicted value for the unknown spectrum.[44]

A cross-validation is useful for self-predictions when only small datasets of approximately 60 samples are available, but can be prone to over-fitting data and overestimating the accuracy that can be expected on independent samples.[44] Thus, a better test of a calibration is to predict independent test sets that consist of insects not used in the calibration, such as insects from another location when determining species or another strain when age-grading. Both methods were used in this study. Williams[44] gives a complete description of the cross-validation and prediction procedures and their benefits and limitations.

For all analyses of species data, *An. arabiensis* strains were assigned a value of 1 and *An. gambiae* s.s. strains a value of 2. All spectra were mean-centered before analysis.[45] A cross-validation was performed on the sample set, and the percentage correct classification was determined for *An. arabiensis* and *An. gambiae* s.s. strains. Mosquitoes predicted to have a class value < 1.5 were considered to be *An. arabiensis,* and those with a predicted value ≥ 1.5 were considered to be *An. gambiae* s.s. Calibration models were developed using the laboratory-reared strains of *An. gambiae* s.s. and *An. arabiensis*, which were used to predict the species of field-collected *An. gambiae* complex specimens. The number of PLS regression factors used in calibration models was determined by examining the reductions in the residual sum of squares realized when additional factors were added to the cross-validation models, and by calculating the classification results when predicting the species of independent test sets. The number of factors selected for classification models was the number that resulted in the minimum residual sum of squares in the PLS analysis and the maximum classification accuracy on independent test sets. A further description of this PLS regression technique is given by Martens and Naes.[45]

For age-grading, the age of each mosquito was predicted based on its respective spectrum. Mosquitoes predicted as having a value < 2.5 were considered to be 1 day of age, 2.5–5.4 as 4 days of age, 5.5–8.4 as 7 days of age, 8.5–11.4 as 10 days of age, 11.5–14.4 as 13 days of age, 14.5–17.4 as 16 days of age, and 17.5–20.5 as 19 days of age. The calibration model was developed using the *An. gambiae* s.s. Ifakara strain and was used to predict the age groups of G3 and KGB strains.

Spectra identified as outliers by PLSPlus/IQ were examined and determined to have a difference in the minimum and maximum absorbance of less than 0.3 absorbance units, and the spectra were generally outside the 0.5–1.0 absorbance range. Thus, spectra outside of these limits were discarded. These outlier spectra could occur if the mosquito moved during scanning, resulting in a flat spectrum or a spectrum with low absorbance. A spectrum with high absorbance could result if a portion of the mosquito was in contact with the probe, resulting in too much energy reflected back to the sensor. Fewer than 5% of all spectra were discarded.

Plots of the PLS regression coefficients and of the difference between average spectra of the two species were examined to determine which wavelengths were important in classification models. The positive and negative peaks in the plots should be attributable to NIR absorption bands at overtones of fundamental absorption regions corresponding to molecules comprised of C, N, O, and/or H.[46]

RESULTS

Species identification. *Laboratory-reared mosquitoes.* Average spectra of laboratory-reared specimens of mosquitoes from colonies of *An. gambiae* s.s. and *An. arabiensis* established from field collections in nearby villages are shown in Figure 2. Although there was an offset in the average spectra of the two species, there was considerable overlap when examining individual spectra, and this offset cannot be used to predict mosquito species. Thus, PLS regression was used to analyze the spectra and predict species. The results are shown in Table 1 for strains scanned in Ifakara, Tanzania, and Manhattan, Kansas. Generally, the correct species was determined with > 80% accuracy. For mosquitoes scanned in Ifakara (Table 1), the unfed and blood fed females were differentiated with approximately 90% accuracy, but the gravid females were poorly differentiated (65% correctly classified). The model that combined the unfed, fed, and gravid individuals resulted in a correct classification rate of 85%. If the gravid mosquitoes were excluded, the combined fed and unfed model gave a correct classification rate of 91%. If it is important to include gravid mosquitoes in the model,

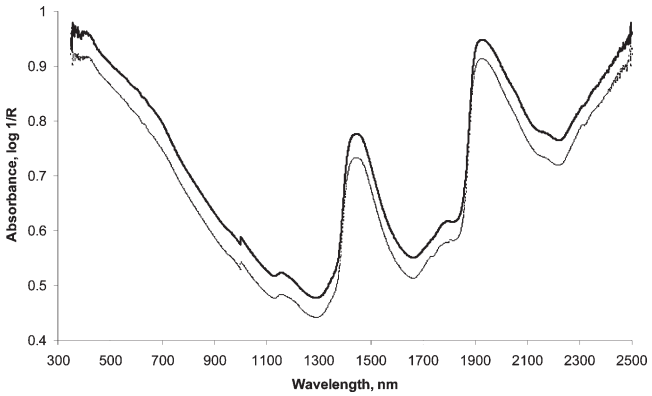

FIGURE 2. Typical average spectra of *Anopheles arabiensis* (**top**) and *An. gambiae* s.s. (**bottom**) mosquitoes.

then scanning additional gravid mosquitoes may improve the combined calibration. In earlier work, we scanned the entire mosquito using a larger 6.3 mm-diameter fiber-optic probe with a 6-mm spot size. Those results showed that the diameter of the probe affected our classification models. Therefore, all subsequent work was done with the 3-mm diameter probe, which viewed only the head and thorax.

Similar results were achieved for classifying the mosquitoes scanned in Manhattan, Kansas, with most models predicting the correct class with > 80% accuracy (Table 1). In pairwise comparisons, there was no difference in our ability to distinguish between KGB and Ifakara *An. gambiae* s.s., KGB and G3, or Ifakara *An. gambiae* s.s. and Ifakara *An. arabiensis*. Thus, the success of species identification was not dependent on strain. Gravid mosquitoes again tended to be classified with lower accuracy.

Wild mosquitoes. Mosquitoes collected from the field were scanned and then analyzed by PCR and then the species was predicted from a cross-validation model and from models developed from laboratory-reared insects. A cross-validation that used the field-collected mosquitoes to predict those same mosquitoes showed that mosquito species could be correctly predicted with 83% accuracy (Table 2). Mosquitoes reared in the laboratory in Ifakara were then used to develop a calibration that was used to predict the field-collected mosquitoes. Although sampling with light traps placed beside human-occupied bed nets yields small numbers of blood fed, gravid, and semi-gravid *Anopheles*, most (typically > 95%) of *An. gambiae* complex females collected in a light traps are unfed.[47] Calibrations developed using laboratory-reared mosquitoes of a single status (fed, unfed, or gravid) were used to predict the wild mosquitoes but the results were poor. However, a calibration that included all blood fed, unfed, and gravid mosquitoes reared in Ifakara correctly classified 78.6% of the wild mosquitoes (Table 2). Calibrations developed from any strain(s) reared in Manhattan, Kansas, resulted in poor classification of wild mosquitoes, which suggested that application of such a method to particular settings will probably require local calibration against a sub-sample of that analyzed by NIR, which are rigorously identified by PCR.

The regression coefficient plot for *An. gambiae* s.l. species identification (Figure 3) shows peaks at approximately 1,000, 1,220, 1,400, 1,450, 1,700, 1,765, and 1,800 nm. Most of these peaks are also evident in the difference plot (Figure 4) obtained by subtracting the average spectrum of *An. gambiae* s.s. from the average spectrum of *An. arabiensis*.

Age grading. All results for males and females of all species and strains showed that the predicted age was positively correlated with the actual age. A typical plot of the actual and predicted age of Ifakara strain female *An. gambiae* s.s. mosquitoes is shown in Figure 5. Similar results were seen when age-grading female or male mosquitoes of all strains. There is considerable overlap between adjacent age groups, but older mosquitoes can generally be separated from younger mosquitoes. Figure 5 shows that it is difficult to differentiate between age groups after approximately seven days. If changes in the cuticle as the mosquito ages are being detected, then this finding indicates that the cuticle changes little after approximately 10 days. The regression coefficients (Figure 6) show that wavelengths at 700, 1,000, 1,221, 1,305, 1,412, 1,728, 1,878, 1,947, and 2,200 nm contributed most to the classification model. Similar regression coefficients were seen for other strains and for males.

Ages of female and male mosquitoes are generally over-predicted for younger mosquitoes, and under-predicted for older mosquitoes (Table 3). However, there are significant differences between the mean predicted values of young (≤ 4 days) and old (≥ 7 days) age groups for cross-validations and prediction sets. The cross-validations further show that the mosquitoes can be classed into young (≤ 4 days), middle-age (7–10 days), and old (≥ 13 days) age groups with differences being significant at $P < 0.05$. The results achieved with the prediction sets showed fewer significant differences between age

TABLE 1

Accuracy of *Anopheles gambiae* s.s. and *An. arabiensis* species identification using near-infrared spectroscopy, as determined by partial least squares regression cross-validation*

Scanning location	Strains compared	Status	No. scanned	Correctly classed, %†
Ifakara	Ifakara *An. arabiensis* vs. Ifakara *An. gambiae* s.s.	Unfed	280	91
		Blood fed	97	93
		Gravid	98	65
		All unfed plus blood fed	373	90
		All	421	84.5
Manhattan	KGB *An. arabiensis* vs. Ifakara *An. gambiae* s.s.	Unfed	211	77–81
		Blood fed	101	90
		Gravid	104	80
	KGB *An. arabiensis* vs. G3 *An. gambiae* s.s.	Unfed	311	72–85
		Gravid	202	66–83
		Blood fed	210	69–75
	Ifakara *An. gambiae* s.s. vs. Ifakara *An. Arabiensis*	Unfed	204	82–93

* Mosquitoes were reared and scanned in Ifakara, Tanzania, or Manhattan, Kansas. Approximately equal numbers of each species were scanned.
† Ranges represent a summary of several separate comparisons.

TABLE 2

Accuracy of species prediction for field-collected mosquitoes using near-infrared spectroscopy*

Species	No. mosquitoes	Cross-validation, %	Prediction, %
Anopheles arabiensis	171	79	76
An. gambiae s.s.	104	90	83
Average		83.2	78.6

*The cross-validation model was developed using field-collected insects. The model used for predictions was developed from all mosquitoes reared in Ifakara (n = 421).

groups, which is not unexpected because one strain was used to predict the age of another strain. We did not have a sufficient quantity of mosquitoes to develop a calibration from one strain and predict an independent set of the same strain. Thus, the predictions might be improved if the same strains were used for calibration and predictions sets.

Classification accuracy when predicting mosquitoes into specific age groups or when classifying mosquitoes into broad categories of young and old mosquitoes is shown in Table 4. It was difficult to classify seven-day-old female mosquitoes into either the young or the old groups, but when classifying all other mosquitoes as younger or older than seven days of age, approximately 80% or more of females were correctly predicted as young or old. For males, old (> 7 days of age) mosquitoes were predicted as old with ≥ 85% accuracy, but the accuracy of predicting young males as less than seven days of age was only approximately 50%.

DISCUSSION

We show that NIRS can be used to determine the species and age of *An. gambiae* s.s. and *An. arabiensis* mosquitoes. This rapid and nondestructive technique can predict the species of field-collected mosquitoes with an accuracy of approximately 80%. Although this method has been successfully applied in agricultural entomology and crop science, this is the first time its utility for evaluation of the demographics of malaria vector mosquitoes has been demonstrated. For species identification, the difference plot contrasting absorbance of *An. gambiae* and *An. arabiensis* s.s. identifies peaks that should correspond to differences between the chemical compositions of the two species.

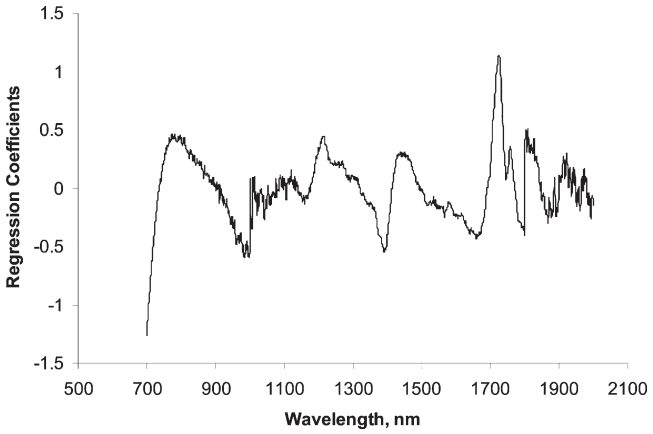

FIGURE 3. Regression coefficients for determining the species of female *Anopheles gambiae* s.s. and *An. arabiensis* mosquitoes when using 10 partial least squares regression factors. Results were derived from all Ifakara laboratory–reared fed and unfed mosquitoes.

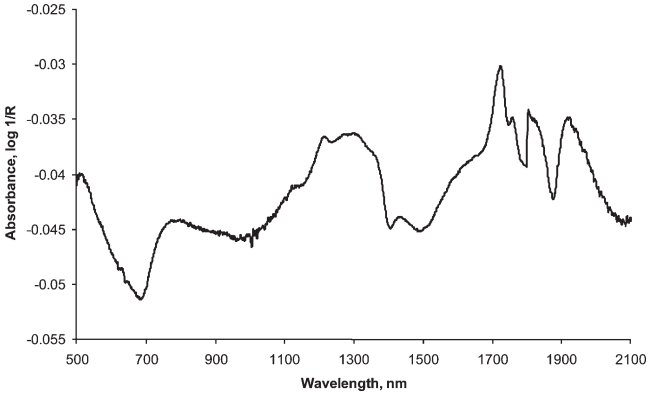

FIGURE 4. Different spectra when subtracting the average spectrum of all female Ifakara laboratory–reared *Anopheles arabiensis* from all *An. gambiae* s.s. mosquitoes.

The peaks at 1,000, 1,400, 1,450, and 1,800 nm correspond to water absorption.[48] *Anopheles arabiensis* have a higher water content than *An. gambiae* s.s.,[41] and this finding may be contributing to our classification models. The peaks at 1,220, 1,450, 1,700, and 1,765 nm correspond to molecules comprised of C-H functional groups.[48] Similar wavelengths have been used in classification models for stored-grain insects,[37] and these wavelengths corresponded to C-H overtones likely caused by absorption by cuticular lipids. Significant differences in *An. gambiae* s.s. and *An. arabiensis* cuticular components exist,[49] and gas chromatography has been used to identify these two species with an accuracy of 90%.[50] Thus, differences in the cuticular hydrocarbons, along with the differences in water content, likely contribute to our classification models.

Including gravid mosquitoes reduced our classification accuracies in cross-validations, and it may be reasonable to exclude gravid mosquitoes from this procedure until this reduction in accuracy can be explained. Although inclusion of gravid females decreases the model accuracy, they can be easily visually identified with a dissecting microscope and

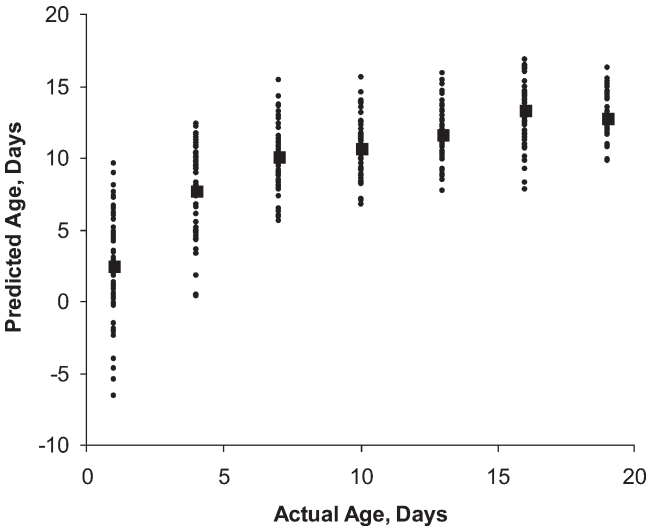

FIGURE 5. Actual versus predicted age of female (*Anopheles gambiae* s.s.) mosquitoes, Ifakara strain, 1–19 days of age (n = 321) as determined from a cross-validation. The large squares represent average values.

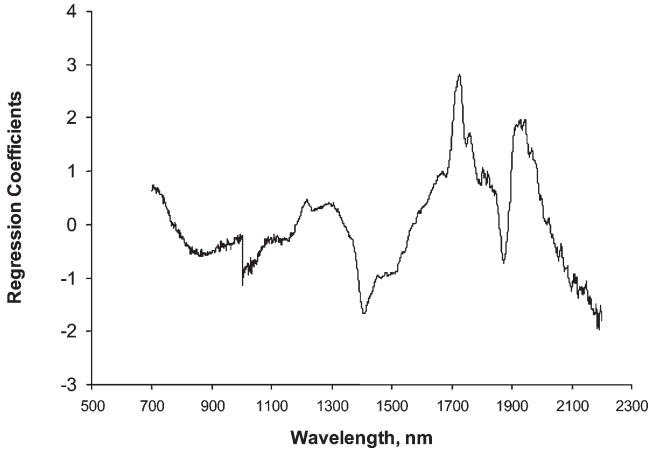

FIGURE 6. Regression coefficients when predicting the age of female mosquitoes, Ifakara strain, 1–19 days of age, six partial least squares regression factors.

removed. Because they are a small percentage of most samples, it is unlikely this will create a problem. However, including gravid mosquitoes did not reduce the accuracy of models used to predict wild mosquitoes. Most mosquitoes used for our species calibrations were < 10 days of age, whereas the age of the wild mosquitoes was not known. It was not possible with our experimental design to determine whether age affected our species identification calibrations, but a species calibration that accounts for age may give better results if the age of wild mosquitoes can be predicted. This should be examined in future studies.

For age determinations, female mosquitoes could be classified into young (< 7 days of age) and old (≥ 7 days of age) with an accuracy of approximately 80%. However, in the absence of rigorous comparisons between rather than within strains or with field-collected material, we must assume that the application of this method will require calibration and validation with a sub-sample from a given study site for which age can be rigorously determined. Further study is needed to determine if temperature, humidity, and laboratory rearing conditions influence age calibrations, and to validate this technique with field data. Additional laboratory and field tests may help improve calibrations and reduce overlap between adjacent age groups. Although mark-release-recapture offers one somewhat laborious option for such calibrations,[27,51,52] it may be more useful to calibrate against physiological rather than chronological age classes. Although elegant dissection methods[51,53,54] offer the potential to estimate the numbers of egg batches any given female has laid, these techniques are time consuming and difficult to master or standardize. We therefore suggest that the relatively simple methods of Gillies, which enable relatively unambiguous classification of females as being parous or one of two development classes of nulliparous mosquitoes[31] may be ideal for, not only calibrating and validating this NIR method based on a sub-sample of field collections, but also direct parameterization of malaria transmission[9,24,55,56] and analytical biodemography models.[57] Such age-grading methods based on clearly defined physiological transitions may lend themselves far better to calibration of such a method because such clear-cut anatomical and compositional changes should enable better distinction to be made using NIR spectra.

Age determinations for male mosquitoes were similar in predicting the age of old mosquitoes, but young male mosquitoes were difficult to age grade. Perhaps male cuticle changes relatively little when compared with females, or perhaps physiological changes associated with blood feeding and initiation of oogenesis are creating larger difference than would be seen in males. Although this technique may not be useful for assessing calendar age, it may provide a fast way to obtain an epidemiologically relevant measure of the proportion of the vector population that is old enough to be potentially infected with malaria. This method may also be useful to estimate and contrast survival between different vector populations. Significant age-grading regression peaks correspond to C-H groups, and these chemical moieties are common constituents in most insect cuticular and internal lipids. Quantitative changes in cuticular hydrocarbons occur as female mosquitoes age,[34] thus supporting the regression coefficients reported herein. Using cuticular hydrocarbons to age-grade mosquitoes also results in difficulty in classifying seven-day-old mosquitoes into an old or young age group.[58] Lipids and glycogen have been shown to change in male mosquitoes as they age.[59] Peaks in Figure 6 at approximately 1,221, 1,412, and 1,728 nm correspond to absorption by lipids, and thus may contribute to our classification models. The wavelengths that contribute most to the age classification model agree with those that have been reported when age-grading the house fly *Musca domestica* (L.).[60]

Cuticle deposition occurs with regularity in *Anopheles,* but ceases after 10–14 days.[33] This thickening of the cuticle may be influencing our classification models from 0 to 10 days, and

TABLE 3

Accuracy of mosquito age prediction (in days) when using a partial least squares regression cross-validation (within-strain prediction) of Ifakara *Anopheles gambiae* s.s. strain mosquitoes or when using a calibration developed from the Ifakara *An. gambiae* s.s. to predict G3 *An. gambiae* s.s.

	Predicted age, days*							
	Females				Males			
	Within-strain prediction, n = 321		Between-strain prediction, n = 312		Within-strain prediction, n = 286		Between-strain prediction, n = 224	
Actual age, days	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	2.4 a	3.8	4.4 a	3.3	2.3 a	3.0	7.8 a	3.2
4	7.7 b	3.2	7.6 b	3.4	5.9 b	3.7	8.1 a	3.5
7	10.1 c	2.4	10.7 c	2.6	9.7 c	2.5	10.3 b	2.8
10	10.7 c	2.2	9.8 c	2.1	11.2 d	1.9	11.0 b	1.8
13	11.6 d	2.0	10.6 c	3.7	12.6 e, f	2.9	14.3 c	4.1
16	13.3 e	2.4	12.6 d	2.3	13.5 e	1.7	11.2 b	2.7
19	12.8 e	1.6	10.8 c	3.0	12.4 f	1.7	No data	No data

* Six partial least squares regression factors were used for the female cross-validations and predictions. Five factors were used for male cross-validations and predictions. Means followed by the same letter are not significantly different at $P < 0.05$ when using a two sample *t*-test in paired comparisons.

TABLE 4

Accuracy of predicting the age of G3 *Anopheles gambiae* mosquitoes into specific age groups when using a calibration developed from the Ifakara strain of *An. gambiae* mosquitoes*

Sex	Prediction	Actual age (days)						
		1	4	7	10	13	16	19
Females	Predicted into each actual age group	24%	14%	12%	54%	27%	14%	2%
	Predicted into ≤ 7, 10, ≥ 13 day age groups	90%	76%	14%	54%	27%	73%	44%
	Predicted into < 7 and ≥ 7 day age groups	90%	76%	14%	79%	80%	98%	78%
Males	Predicted into each actual age group	6%	10%	18%	60%	24%	23%	No data
	Predicted into ≤ 7, 10, ≥ 13 day age groups	56%	44%	20%	60%	79%	46%	No data
	Predicted into < 7 and ≥ 7 day age groups	56%	44%	20%	98%	94%	85%	No data

explains our difficulty in differentiating between older age groups. Also, cuticular hydrocarbons increase with age,[34] but 8–16-day-old mosquitoes could not be differentiated when developing prediction models based on CHC profiles. Because insect development is temperature dependent, age prediction calibrations may be dependent on the temperature environment where they are applied.

Male age-grading models have been developed that use the number of spermatocysts in *An. gambiae* s.s. male testes and the relative size of their sperm reservoirs.[61] These models can classify males into young (≤ 4 days of age) and old (> 4 days of age) groups. It is doubtful that the spermatocysts and sperm reservoir are contributing to our NIR classifications because of their small size relative to the insect cuticle (NIR spectra are affected by the amount of material absorbing NIR radiation). The age-grading regression coefficients for males are similar to those for females. This finding further indicates that characteristics unique to males are not contributing to the age classification models.

Vector control has been highlighted as a key component in recently renewed calls for global malaria eradication.[62] As large-scale control programs based on existing tools, such as insecticide treated nets or indoor residual spraying, are scaled up across malaria-endemic areas of sub-Saharan Africa and new methods are evaluated, there is a pressing need for a new high-throughput and low-cost technology that can rapidly assess species composition, survival, and vectorial capacity of targeted populations. Currently, no such method exists for the age-grading of African anophelines, and most estimates of survival are being determined by tedious and time-consuming dissections of a sub-sample of mosquitoes by trained experts.[51,54,63–65] Although useful as a means to categorize females into groups of relatively young (< 4 days of age) or older mosquitoes, these classifications require substantial time and expertise to correctly perform the dissections on which they are based, making this technique unlikely to be integrated into routine, large-scale entomologic surveillance.

Although PCR will ultimately serve as the gold standard for 100% accurate identification of African anopheline species composition, the processing time and reagent costs required by this technique usually limit its application to only a sub-sample of mosquitoes collected in entomologic surveys. In contrast, the NIRS technique described can handle a much larger volume of samples, and becomes increasingly cost-effective as the number of samples for analysis increases.

We estimate that this rapid and nondestructive NIRS technique can determine species and age of > 1,000 mosquitoes/day, and no reagents are required. The initial cost of the instrument is approximately $45,000, but there are no costs associated with running samples after this initial investment. The system can be battery powered and is field-portable. After calibrations are developed, the training required to use the system is only that required to operate a computer. The cost for a PCR instrument is approximately $10,000, with a subsequent cost of approximately $5/sample for reagents and other materials. Thus, a NIR system would pay for itself after approximately 7,000 samples have been analyzed. NIRS does have lower accuracy in species determination and age-grading than traditional methods (80–85% versus 100%). However, in considering whether to adopt this method, this disadvantage should be weighed against the substantial increase in the number of samples that can be rapidly processed.

Clearly, more precise estimates will be required for detailed entomological investigation of specific ecological and epidemiologic phenomena but, as outlined, this can be readily achieved by internal calibration and validation using sub-samples of mosquitoes within any given study site, the species identified,[43] or age[51,54,63–65] of which can be unambiguously determined using existing rigorous but less scalable methods. However, for routine surveillance applications, the quick-and-dirty estimates of vector species composition and survival offered by this method may ultimately prove more useful than a more precisely detailed description of a small sub-sample. Such modest measurement errors are the norm rather than the rule in the field of ecology and a wide range of appropriate statistical methods are available to deal with these issues, along with sampling errors and underlying true variations, which are often of greater magnitude. This approach may therefore enable vastly more extensive and intensive measurement of vector population composition across space and time than has previously been possible, enabling much-improved parameterization of analytical models, which provide insight into the ecology of population dynamics.[19,20,66,67] Furthermore, this is the only method that is capable of age-grading adults non-destructively, enabling specimens to be preserved for subsequent analyses of genetic or biochemical traits vital to the current and future success of control methods (e.g., insecticide resistance, diversity, and rates of gene flow). For these reasons, we advocate NIRS as a useful and welcome addition to the vector ecologists toolbox for large-scale field surveys.

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