Research Square

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Adapting field-mosquito collection protocols for near-infrared spectroscopy implementation

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Abstract

Background: Near-infrared spectroscopy (NIRS) has the potential to be a useful tool for assessing key entomological parameters including age, infectious status, and species of wild malaria mosquitoes. Before NIRS can be reliably used in the field at scale, methods for killing mosquitoes and conserving samples prior to NIRS scanning need to be further optimized. Studies have historically killed mosquitoes using chloroform, though this is not without health hazards and should not be used in human dwellings. It is also unclear which mosquito preservation method to use.

Methods: Here we investigate the use of pyrethrum sprays, a commercially available insecticide spray in Burkina Faso, for killing mosquitoes. Laboratory-reared *Anopheles gambiae* and *Anopheles coluzzii* were killed using either Kaltox Paalga®, a pyrethrum insecticide spray routinely used as part of indoor mosquito collections, or chloroform (the "gold standard"). Preservative methods were also investigated to determine their impact on NIRS accuracy in predicting laboratory-reared *Anopheles* and wild-caught mosquito species. After analysis of fresh samples, mosquitoes were stored in 80% ethanol or in silica gel for two weeks and reanalyzed by NIRS. In addition, experimentally-infected *An. coluzzii* and wild-caught *An. gambiae sensus lato* were scanned fresh to determine whether they contained sporozoites then stored in preservatives mentioned above for two weeks before being reanalyzed.

Results: There is not a substantial difference in NIRS accuracy in differentiating between laboratory-reared *An. gambiae* and *An. coluzzii* killed with either insecticide (90%) or chloroform (92%). After preservation, NIRS accuracy was 90% when mosquitoes were killed using chloroform and preserved in silica gel or ethanol. The same accuracy was obtained using Kaltox to kill mosquitoes and silica gel for preservation, but was diminish when ethanol was used as a preservative (80%). For infection status, NIRS is able to differentiate infectious and uninfectious mosquitoes with slightly lower accuracy for both laboratory and wild-caught mosquitoes preserved in silica gel or ethanol.

Conclusions: NIRS can classify *An. gambiae s.l* species killed by pyrethrum spray with no loss of accuracy. This insecticide may have practical advantages over chloroform for mosquitoes killing in NIRS analysis.

Background

Mosquito control is one of the most important global public health interventions, being the primary method of reducing disease for malaria, dengue, chikungunya and zika amongst others [1, 2]. Entomological monitoring is an important scientific and routine surveillance tool though commonly-used methods such as determining mosquito species using molecular methods like PCR are technically laborious, require expensive reagents and qualified workers, and are often time consuming. This often means that only a limited number of specimens can be processed, reducing the accuracy and use of the data generated. Near-infrared spectroscopy is a quick, high throughput and relatively economic technique that has been used for a decade to predict the species, age and infection status of certain disease vectors such as *Anopheles* and *Aedes* mosquitoes [3–5]. Many of these different works used laboratory or field mosquitoes to assess NIRS accuracy to differentiating between *Anopheles* species [3, 4], mosquito age [3, 6] and *Anopheles* infection status using *Plasmodium* culture [7, 8]. The reliability of the method for entomological surveillance for wild mosquitoes is less clear, with models trained on laboratory-reared mosquitoes being unable to accurately predict the infection status or age of field mosquitoes [5, 9]. Further work is required to verify these results, and it may be necessary to calibrate NIRS models with greater numbers of wild-caught mosquitoes. Model calibration requires a large number of samples to improve accuracy [6]. Reliable surveillance will also require a large number of mosquitoes to be processed in order to overcome sampling heterogeneity [6]. However, some practical issues, such as the collection method of *Anopheles* from the field as well as the conservation of the collected samples for NIRS processing in the laboratory, must be addressed before there can be more widespread implementation of the technique. Historically mosquitoes for NIRS analysis have been killed with chloroform, which must be handled with great care in the laboratory. It is toxic and carcinogenic [10], so should not be used in human dwellings to catch wild mosquitoes and others insects. To address these shortcomings, we set out to explore a more practical solution for mosquito killing. Kaltox Paalga® (Saphyto, 1937 Avenue du général Sangoulé Lamizana, Bobo-Dioulasso, Burkina Faso) is a commercial insecticidal product commonly used in Burkina Faso for insects killing or collecting indoor mosquitoes (i.e. pyrethroid spray catches [11]), for different researches in science. The first objective is to investigate whether a pyrethrum insecticide can replace chloroform for killing mosquitoes to be analysed by NIRS.

NIRS machines are relatively portable, but they require a reliable power source. It may be practically easier if the spectrometer should be kept in a central location and samples transported from the collection site to the laboratory where they can be processed. Previous studies have preserved samples using RNAlater® for species identification or mosquito age grading [12, 13]. However, this is not a practical preservative in low-income countries, as it is expensive, requires basic laboratory conditions for storage and is not widely available in sub-Saharan Africa. Desiccation of samples in silica gel has also shown to be a good preservation method [13] but some specific analyses like mosquito dissection cannot be performed following desiccation, reducing the usefulness of the sample for determining other useful entomological parameters such as parasite load or parity status. Ethanol, a cheaper laboratory solution and available throughout Africa, could be a more economic field mosquito preservative using NIRS. Hence, our second objective was to test the impact of *An. gambiae sensu lato* preservation in ethanol or silica gel on NIRS accuracy. For all objectives, accuracy is assessed by NIRS's ability to differentiate between *Anopheles gambiae sensu stricto* and *Anopheles coluzzii*, two closely related mosquito species which are the primary vectors for malaria transmission in Burkina Faso [14]. The effect of the killing and preservation method on the ability of NIRS to determine whether mosquitoes were infectious or not with *Plasmodium falciparum* sporozoites was also evaluated.

Methods

Study design

The study was conducted in Burkina Faso at the "Institut de Recherche en Science de la Santé" (IRSS), Bobo-Dioulasso with institutional ethic committee approval (Reference number: A018-2017/CEIRES). Firstly, we explore the influence of mosquito collection using Kaltox Paalga® on NIRS accuracy to predict

Anopheles species. Kaltox Paalga® was evaluated comparatively to the chloroform (the gold standard killing method for NIRS analyses) using laboratory colonies of *An. gambiae* and *An. coluzzii*. These two *Anopheles* species have been selected for NIRS experiment because of their most important role in the malaria transmission in Burkina Faso [14]. Laboratory-reared *An. gambiae* and *An. coluzzii* were obtained from an outbred colony established [15] respectively in 2015 and 2016 and routinely identified by SINE PCR [16]. These two *Anopheles* species were repeatedly replenished with F1 from wild-caught female mosquitoes. Secondly, the best way for *Anopheles* samples preservation for NIRS future analysis was explored testing 80% ethanol and silica gel as preservatives using both laboratory-reared and wild-caught mosquitoes. Field mosquitoes were collected inside of human dwellings in Longo village (11°34'57" N and 4°33'27" W), located at around 60 Km to Bobo-Dioulasso.

Mosquito killing protocol for determining Anopheles **species**

Laboratory-reared *An. gambiae* and *An. coluzzii* were used to test Kaltox Paalga® as a mosquito killer for NIRS analysis. Kaltox Paalga® is a combination of pyrethroids (allethrin, permethrin, tetramethrin), organophosphorus (chlorpyrifos ethyl) and solvant in an aerosol formulation. Seven-day old *An. gambiae* and *An. coluzzii* females were organized in two groups and each one was assigned to be killed using either Kaltox Paalga® or chloroform vapor currently used (gold standard) to predict *Anopheles* species (Fig. 1). Immediately after death, mosquitoes were scanned using NIRS (referred to as “fresh state” mosquitoes) before being assigned to the appropriate preservation group.

Mosquito preservation methods for determining Anopheles **species and their** Plasmodium falciparum **infection status**

▷ Determining mosquito species

The same laboratory-reared *Anopheles* killed using chloroform or pyrethrum spray as described above were organized in two groups: a first group *Anopheles* were individually stored in 200µL of 80% ethanol in Eppendorf tubes and the second group were individually desiccated with silica gel in Eppendorf tubes as described in previous studies [17, 18]. The samples sizes used for each group are summarized in Fig. 1. In addition to the laboratory specimens, wild-caught mosquitoes killed using chloroform only were stored also in either ethanol or silica gel after their fresh scanning for future analysis with NIRS (Fig. 2a).

All samples were stored at the insectary (27°C ± 2; 70% ±10 RH and 12:12 L-D) for two weeks then, scanned for the second time. The cephalothorax of wild caught mosquitoes was analyzed by SINE PCR to determine *Anopheles* species using common protocol [19].

▷ Detecting mosquito infection status for Plasmodium falciparum

The potential impact of preservation method on NIRS’s ability to determine whether *Anopleles* were infectious with *P. falciparum* was explored with experimentally infected *An. coluzzii* and wild-caught mosquitoes. Laboratory-reared *An. coluzzii* were infected with natural isolates of *P. falciparum* using Direct Membrane Feeding Assays (DMFA) protocol [20]. Blood-fed *Anopheles* were kept at the IRSS insectary, fed with 10% glucose solution *Ad libitum* for two weeks, an estimated period to obtain infectious *Anopheles* (sporozoite stage) and killed with chloroform to analyze using NIRS. After this first analysis, mosquitoes were individually desiccated with silica gel in Eppendorf tubes during two weeks and analyzed again using NIRS (Fig. 2a).

Wild-caught mosquitoes were collected in Longo village using mouth aspirator in the living room of human dwellings early in the morning [21]. Back in the IRSS laboratory, all *An. gambiae s.l.* females were selected according to the morphological identification keys [22], maintained in insectary conditions (T° = 27°C ± 2; 70% ±10 RH and 12:12 L-D) and fed with 10% glucose solution for seven-day for NIRS analysis. The seven-days period is appropriate for complete blood digestion to have occurred and also to have both infected and infectious *Anopheles* [23].

Anopheles gambiae s.l. were killed using chloroform, immediately NIRS analyzed and individually preserved in 80% ethanol or desiccated with silica gel in Eppendorf tubes. The mosquito samples were stored at the insectary for two weeks before to be analyzed by NIRS for the second time (Fig. 2b).

The cephalothorax of each experimental-infected *Anopheles* and wild-caught mosquitoes were analyzed using qPCR to determine their *P. falciparum* infection status [24]. Similar to the laboratory experimental infection, wild caught *An. coluzzii* was the only species used to determine mosquito infection status by NIRS.

Mosquito scanning and data analysis

Mosquitoes were scanned using a LabSpec4 Standard-Res *i* (standard resolution, integrated light source) near-infrared spectrometer and a bifurcated reflectance probe mounted 2mm from a spectral on white reference panel (ASD Inc.). Absorbance was measured at 2151 wavelengths in the interval [350, 2500] nanometers of the electromagnetic spectrum. All specimens were scanned on their side under the focus of the light probe and spectra were recorded with RS3 spectral acquisition software (ASD Inc.) which automatically records the average spectra from 20 scans.

A statistical machine learning approach was used to fit and cross-validate the best model using a generalised linear model (GLM) [25]. A binomial logistic classification model was used to determine *Anopheles* species (*An. gambiae* or *An. coluzzii*) and two response classes assigned: $y = 0$ for *An. gambiae* and $y = 1$ for *An. coluzzii*. We also used a binomial logistic classification model to determine presence or absence of the parasite. Two response classes were also assigned: uninfectious and infectious mosquito. Fisher’s exact test was used to test for statistically significant differences between accuracies of different models.

Following previous work, in all instances models were calibrated with the mosquitoes of the same killing and preservation method. For example, fresh mosquitoes were not used to predict the species or *Plasmodium* infectious status of ethanol preserved mosquitoes.

Results

▷ Pyrethrum spray catches is an appropriate killing method for NIRS analysis

A total of 538 *Anopheles* of seven-days-old were used to assess NIRS accuracy in determining *Anopheles* species killed using kaltox® or chloroform. Models trained with mosquitoes killed using chloroform, correctly classified *An. gambiae* and *An. coluzzii* analyzed at the fresh state with 92% accuracy (*An. gambiae* 94% and *An. coluzzii* 90%). The same trend of NIRS accuracy (90%) was obtained with kaltox killing option. Indeed, using kaltox, NIRS was able to classify mosquitoes as *An. gambiae* or *An. coluzzii* with 91% and 89% respectively (Fig. 3). Using Fischer's exact test, there was no difference between accuracies of NIRS to differentiate *An. gambiae s.l.* species killed by chloroform or kaltox (*p*-value = 0.433). These findings indicate that pyrethrum spray catches could be used as an alternative process to kill mosquitoes for NIRS analysis.

▷ Near infrared spectroscopy is able to predict Anopheles species after preservation

NIRS was able to distinguish laboratory-reared *An. gambiae* and *An. coluzzii* after preservation in silica gel or 80% ethanol. The average accuracy ranged between 83–94% depending on the mosquito killing process and the preservation methods for each species (Table 1). The overview comparison revealed that there is no substantial difference of NIRS accuracies in predicting *Anopheles* species in the fresh samples compared to preserved ones (Table 2).

Table 1
NIRS accuracy in predicting each species of *Anopheles gambiae s.l.* according to killing option and preservative method. *P* > 0.05 (Fisher's exact test) indicates no difference between NIRS accuracies before and after mosquito preservation.

Killing process	Preservative	Accuracy at predicting species					
		An. gambiae s.s			An. coluzzii		
		Fresh	Preserved	*P*	Fresh	Preserved	*p*
Chloroform	Silica gel	91%	93%	0.781	92%	88%	0.356
	80% Ethanol	92%	92%	1	89%	88%	1
Kaltox®	Silica gel	91%	94%	1	83%	86%	1
	80% Ethanol	92%	89%	1	88%	85%	1

Table 2
Overview of NIRS accuracy in predicting *Anopheles gambiae s.l.* species according to killing option and preservative method.

Killing process	**Preservative**	***Anopheles* species**		
		Specificity (*An. gambiae s.s)*	Sensitivity (*An. coluzzii)*	Accuracy
Chloroform	Fresh all	94%	90%	92%
	Silica gel	93%	88%	90%
	80% Ethanol	92%	88%	90%
Kaltox®	Fresh all	91%	89%	90%
	Silica gel	94%	86%	90%
	80% Ethanol	89%	85%	87%

With these optimistic results derived using laboratory-reared *Anopheles*, we were interested to know whether these preservation methods could be extrapolated in field conditions. A total of 731 wild-caught mosquitoes killed using chloroform vapor were immediately NIRS scanned then, preserved in either 80% ethanol or silica gel and re-scanned two weeks later. The main *Anopheles* species identified by qPCR were *An. coluzzii, An. gambiae* and *An. arabiensis* (65.11%, 24.21% and 7.39% respectively). Similar to the laboratory experiment, only spectra collected from *An. coluzzii* and *An. gambiae* were included for data analysis. Spectra from these mosquitoes freshly scanned revealed a prediction accuracy of 88% (*An. gambiae* 88% and *An. coluzzii* 88%). After two weeks-preservation of mosquitoes in silicagel, NIRS models trained on mosquitoes preserved in the same medium had a prediction accuracy of 84% (*An. gambiae* 84% and *An. coluzzii* 83%) whereas for those stored in 80% ethanol, NIRS was able to differentiate mosquito species with 79% accuracy (*An. gambiae* 88% and *An. coluzzii* 71%).

▷ Near infrared spectroscopy has a low accuracy to determine Plasmodium infection status of conserved Anopheles

NIRS classified infectious and uninfectious, laboratory-reared *Anopheles coluzzii* with 64% and 61% of accuracy respectively for fresh state and after preservation in silica gel. NIRS spectra from wild caught mosquitoes also preserved in silica gel or ethanol were analyzed and the prediction accuracy was low depending on the preservative (Table 3). Similar results were seen for both within-sample accuracy (predicting laboratory-infected mosquitoes having calibrated models on laboratory-infected mosquitoes) and out-of-sample accuracy (predicting wild caught mosquitoes using models calibrated on laboratory-infected mosquitoes). NIRS had a poor predictive ability in differentiating infectious and uninfectious wild-caught mosquitoes irrespective of the preservation method (Table 3).

Table 3
NIRS accuracy to predict the infectious status of laboratory reared, experimentally-infected and wild *Anopheles coluzzii* based on two preservation procedur
Results are shown for overall accuracy, the true negative rate (TNR) and true positive rate (TPR).

Model trained on							Model predicting						
Mosquito	Killed	Preserved	Number	Within-sample accuracy (%)			Mosquito	Killed	Preserved	Number	Out-of-sample accura (%)		
				Accuracy	TPR	TNR					Accuracy	TPR	T
Laboratory	Chloroform	Fresh	365	64	68	63	Laboratory	Chloroform	Silica Gel	365	49	43	5
							Field	Chloroform	Fresh	476	50	49	5
		Silica Gel	365	61	55	67	Laboratory	Chloroform	Fresh	365	52	52	5
							Field	Chloroform	Silica Gel	327	51	60	4
Field	Chloroform	Fresh all	476	56	61	51	Field	Chloroform	Silica Gel	239	47	45	4
									Ethanol	237	52	51	5
		Silica Gel	239	63	57	69	Field	Chloroform	Ethanol	237	53	45	6
		Ethanol	237	54	31	77	Field	Chloroform	Silica Gel	239	54	38	7

Discussion

There is the prospect of spectroscopy technique being deployment in the field for monitoring mosquitoes and assessing malaria transmission. This raises the question “what would be the best method to collect mosquito samples regarding the restriction of some laboratory practices?” In this study, Kaltox® insecticide, which is used in Burkina Faso for pyrethrum spray catches of mosquitoes for different research programs, was tested in NIRS analysis. Results showed that pyrethrum spray catches can be used for NIRS species identification without interfering with the prediction accuracy. Indeed, NIRS accurately distinguished laboratory-reared *An. coluzzii* and *An. gambiae* s.s independently of killing option (Kaltox® with 90% of accuracy *versus* chloroform with 92% of accuracy) raising the prospect that this technology can be deployed using more practical mosquito collection methods for monitoring vectors borne diseases. This is the first study addressing the question using pyrethrum spray catches with encouraging results, though it should be verified for other metrics of interest (such as determining mosquito age). In addition, mosquitoes killed with pyrethrum spray could be preserved in silica gel or 80% ethanol for at least a couple of weeks for future analysis by the NIRS with relatively good accuracy. Previous studies have already demonstrated that mosquitoes killed by chloroform could be stored under different conditions before NIRS scanning [12, 13, 17]. In our study, NIRS accuracy (> 80%) for wild *Anopheles gambiae s.l* species identification, when using silica gel as preservative, corroborates with these previous reports. This study has used relatively small numbers of mosquitoes to calibrate the model, so we expect to have better accuracy in NIRS prediction by increasing sample size as shown in previous work [26].

Interestingly, mosquito samples preserved in ethanol can be rehydrated and dissected for any other entomological explorations after NIRS analysis.

Anopheles infection status for *Plasmodium* is one of the most important parameters for monitoring malaria transmission. A low accuracy of NIRS to predict mosquitoes *Plasmodium* infection status at fresh state was observed in our study as in previous reports [8, 9]. The accuracy of *Plasmodium* infection status prediction still lower after preservation in both 80% ethanol and silica gel preservation method. Because of no significant difference in NIRS prediction between the fresh state and after mosquito conservation, we assume that this lower accuracy is not due to the preservative method. The fact that NIRS does not work well when using field mosquitoes to determine the presence or the absence of *Plasmodium* could be due to multiples factors such as larval breeding site diversity, blood-meal and sugar sources, physiological and nutritional status [9].

Conclusion

Near infrared spectroscopy has the potential to be a useful entomological surveillance tool that could determine mosquito age, species and pathogen infection status in order to monitor malaria and evaluating control interventions. Numerous works demonstrated the potential use of the technique but one of the main challenges remains a best protocol of mosquito vectors collection. Our results showed the applicability of NIRS to classify both *An. gambiae s.l* species killed using kaltox®, implying that this insecticide and other derived molecule could be used as a practical alternative substance to kill mosquitoes in

field for NIRS analysis. The possibility to preserve vectors samples for future NIRS analysis offers the opportunities to centralize the technical in an appropriate laboratory, saving costs.

Declarations

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Availability of data and materials

“Not applicable” for that section.

Authors’ contribution

B.M.S., D.F.Y.D., T.L., A.G.O. and R.K.D. conceived the study; B.M.S. and D.F.Y.D. conducted the laboratory and field work; B.M.S., N.D.C.D., L.I.G.P. and K.W. conducted the molecular analysis, B.M.S., R.M. and T.S.C. were responsible for the data analysis; B.M.S., D.F.Y.D., T.L. wrote the first draft of manuscript, All authors approved the final manuscript.

Ethics approval and consent to participate

Ethic committee approval: Reference number: A018-2017/CEIRES.

Consent for publication

“Not applicable” for that section.

Competing interest

The authors declare no competing interest.

Author details

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Figures

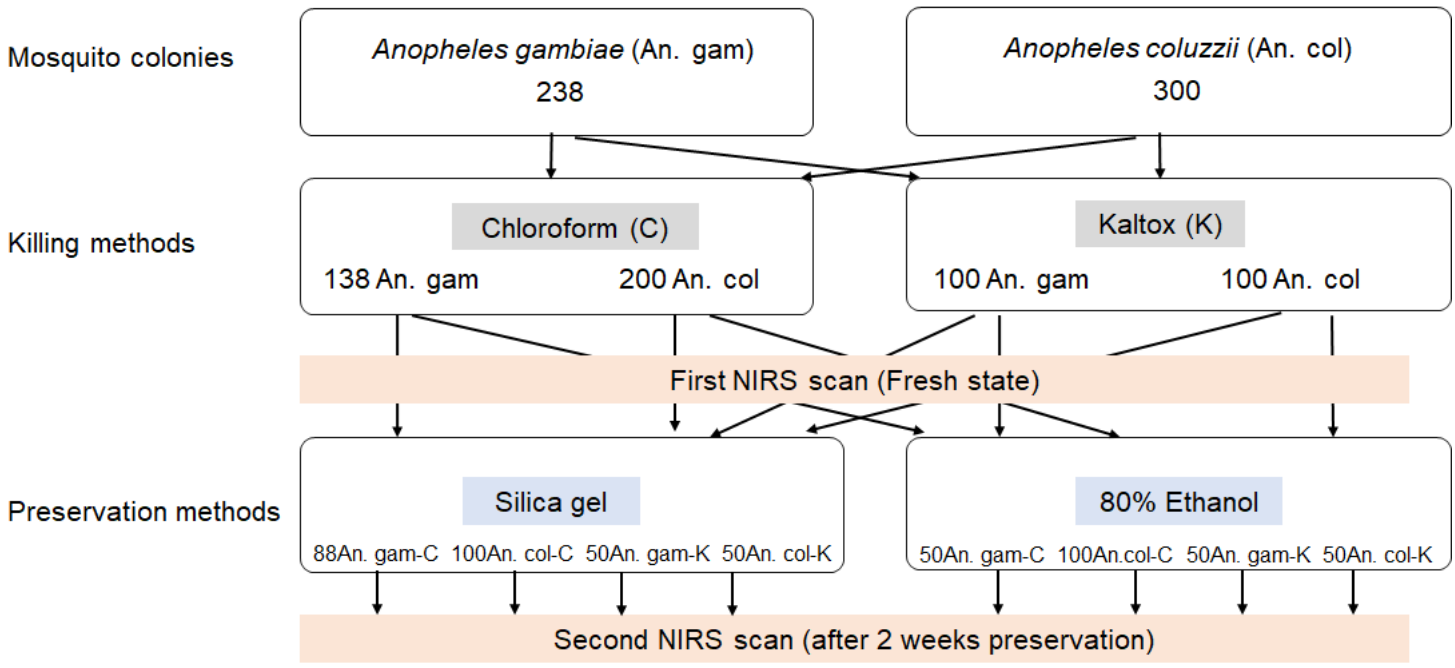

Figure 1

Summary of the experimental design and samples size of laboratory mosquitoes scanned to determine the accuracy of *Anopheles* species identification according to killing and preservation methods.

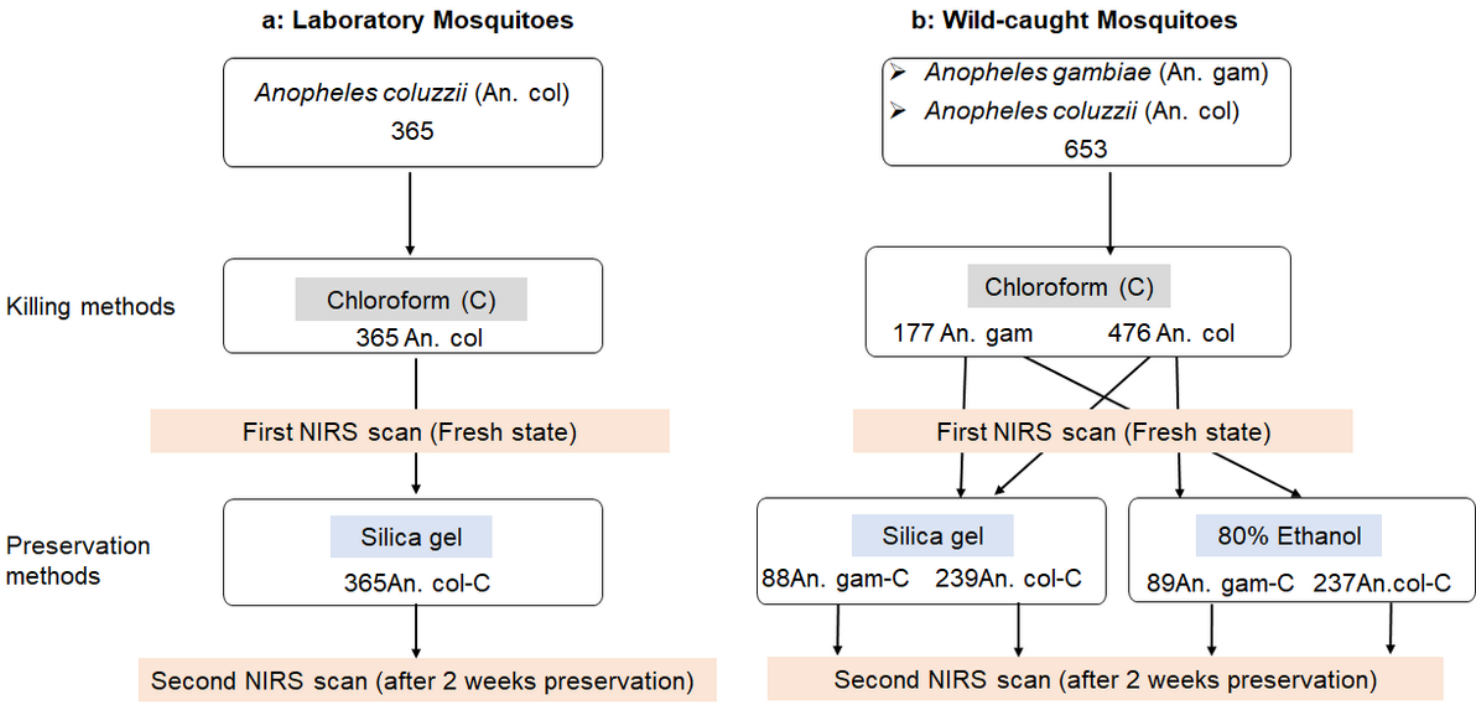

Figure 2

Summary of the experimental design and samples size of (a) laboratory reared and (b) wild-caught *Anopheles* used to determine *Anopheles-Plasmodium* infectious status and species according to preservation methods.

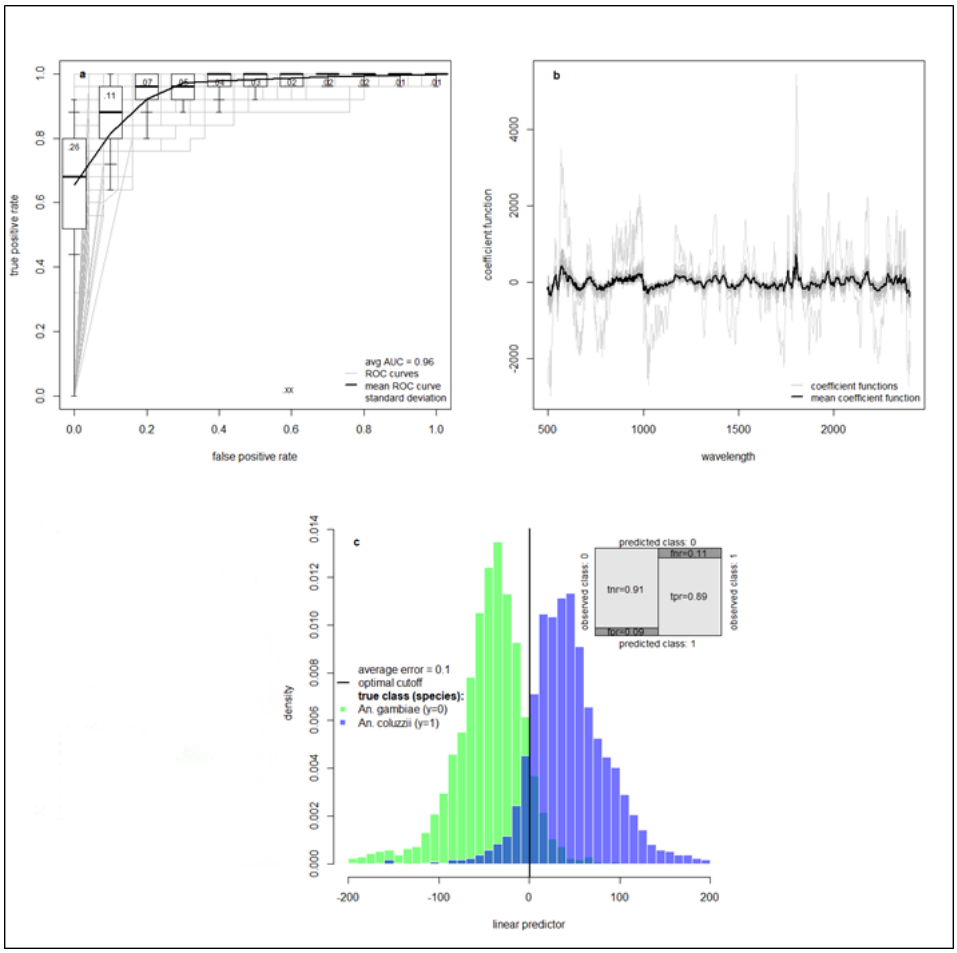

Figure 3

NIRS ability to predict laboratory-reared mosquito species killed with kaltox.

(a) The receiver operating characteristic (ROC) curve showing the false positive and true positive rates achievable for different classification probability thresholds whilst the overall performance is given by the average area under the ROC curve (AUC). (b) Coefficient functions for each of the 50 dataset randomizations (grey lines) and the corresponding average (black line). (c) The histogram of the estimated linear predictor for the test mosquitoes, color of the bars indicates the true class, shows the model's ability to separate the two groups of mosquitoes. The vertical black line indicates the optimum threshold for classifying mosquitoes as *An. gambiae* or *An. coluzzii*. The shaded area where the two distributions overlap corresponds to misclassified test observations - false negatives to the left and false positives to the right of the optimal classification threshold. The confusion matrix (inset) shows the different error rates: tnr, true negative rate (*An. gambiae*); fnr, false negative rate; fpr, false positive rate; and tpr, true positive rate (*An. coluzzii*).