

Trap type, baits, and molecular methods influence blood-fed mosquito capture and the identification of host blood meals from outdoor mosquito collections

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Abstract

Background

The range of hosts from which a mosquito feeds influences the transmission of the pathogens they vector. In this study, we investigated how to maximise the collection of blood-fed female mosquitoes and optimise molecular identification of vertebrate blood meals by comparing trapping methods, CO₂ bait sources, PCR primer sets and the influence of blood meal digestion on sequencing success.

Methods

Our collections in northeastern South Africa (Limpopo and Mpumalanga provinces) compared trapping methods by assessing three types CO₂-based attractants (sugar and yeast, molasses and yeast, and dry ice) and four traps (bucket, Centres for Disease Control and Prevention miniature light trap, tent trap, Backpack aspirator, and Biogents' BG-Pro) to compare their yield of blood-fed mosquitoes. We then compared three published PCR-based methods to amplify and identify blood meals through Sanger sequencing. Methods' efficiency was correlated with blood meal's digestion level using the Sella classification scale.

Results

BG-Pro and tent traps captured the most blood-fed mosquitoes, collectively capturing 72% of the blood-fed mosquitoes in the study. However, in terms of the proportion of captured mosquitoes that were blood-fed, aspirators (52%) and bucket traps (47%) recorded the highest percentages. Molasses bait and the bucket trap accounted for the highest proportion of least digested blood meals, capturing 54% and 71% of blood-fed mosquitoes with Sella scores 2–4, respectively. Sella scale and primer choice independently influenced amplification and sequencing success. Sequencing success at Sella score 2–4 was 62% compared to 28% at higher Sella scores (5 and 6). Primers targeting the universal mitochondrial 12S rRNA gene resulted in a higher number of successful PCR amplifications and sequencing results compared to the other primers for Cytb and COI.

Conclusions

The significantly higher sequencing success at low Sella scores highlights the association of Sella Scores with sequencing outcome. By quantifying the Sella scores across trapping methods and its consequence for sequencing, our results provide information required to optimise field methods for blood-meal analysis in southern Africa.

Background

Mosquito-borne diseases that impact human and animal health include malaria, lymphatic filariasis, dengue, Japanese encephalitis, chikungunya, Rift Valley fever, and West Nile virus. An important and routinely collected source of information on the epidemiology of these pathogens comes from mosquito blood meals. The content of their blood meals can identify the vertebrate hosts they fed on ((1) or be used for xenomonitoring (2). Xenomonitoring uses blood-fed mosquitoes as early detection systems for endemic or emerging infectious diseases (2) or to evaluate progress towards elimination (2, 3). When combined with information on mosquito abundance, potential vertebrate host abundance and pathogen presence, this information can be used to describe vector-host-pathogen interactions (4–6), identify key vectors and identify potential vertebrate reservoirs (7, 8). Blood-meal analysis has also been used for research beyond mosquito-borne diseases, such as biodiversity assessments (9) and xenodiagnostics that use mosquito blood meals to identify pathogens from host blood that are not transmitted by mosquitoes (e.g. 2,10–12). As such, it is important to efficiently capture and molecularly process blood-fed mosquitoes.

A key challenge in the capture of blood-fed mosquitoes is obtaining an adequate and representative sample (2, 3). Common mosquito monitoring traps primarily lure host-seeking female mosquitoes with carbon-dioxide (13–15). However, mosquito behaviour changes following a blood meal, making them relatively rare in host-seeking traps, such that considerable time and effort are required to sample even a small number of blood-fed mosquitoes (e.g. 16). After taking a blood meal, mosquitoes rest in safe places to digest the blood meal and produce eggs, such as in vegetation or artificial structures that provide dark humid environments. Host-seeking traps still capture a proportion of blood-fed mosquitoes, potentially because some females resume host-seeking before completing digestion, they have been interrupted during feeding, or are intercepted while moving between feeding and resting sites (17). To improve the probability of catching blood-fed mosquitoes, traps that mimic the natural microenvironment that a mosquito seeks and aspirators that collect resting mosquitoes have been used (18, 19). Mosquito collections at artificial resting sites are usually fewer in both numbers and species (13, 20, 21) but constitute a higher proportion of blood-fed mosquitoes compared to traps targeting host-seeking mosquitoes (22). Maximising the capture of blood-fed mosquitoes requires a systematic comparison of trapping methods that has not been thoroughly done in most environmental and pathogen-transmission contexts.

The collection of blood-fed mosquitoes can also be enhanced through modifying traps initially designed to collect host-seeking mosquitoes by altering the source of carbon dioxide or adding additional attractants. Carbon dioxide is usually sourced from dry ice or compressed gas canisters (e.g. (23, 24) ; however, both are expensive and not readily available, particularly in remote areas. Fermenting molasses or sugar are alternative, successful, and low-cost methods for producing carbon dioxide (25, 26). Evidence has shown that molasses additionally attracts blood-fed mosquitoes (26, 27). For example, choice experiments have shown that blood-fed *Anopheles coluzzii* (a major malaria vector) are attracted to fermenting molasses more than fermenting sugar, or a non-fermenting molasses / CO₂ mixture, with the peak lure at 72 hours after feeding (26). Molasses likely contains nutrients that improve mosquito fertility and survival, as observed from the increase in the number of eggs laid by each female after

feeding (26). However, it remains unclear how these modifications compare to using collection methods that target resting mosquitoes such as artificial resting sites or aspiration (17).

In addition to choosing an efficient trapping method, a range of molecular tests with different levels of sensitivity and specificity are available to determine the source of blood meals for medically important insects (28). These methods amplify and sequence a taxonomically informative fragment of the host DNA followed by identification of the host by comparing the results to sequences in a reference database (29). Molecular markers targeting different mitochondrial genes (mtDNA) have been used to ascertain the identity of blood meals on engorged insects. mtDNA are used widely because they are easier to detect from minute volumes of blood given that they exist in multiple copies (30). Some of these include mitochondrial cytochrome b (Cytb), mitochondrial cytochrome oxidase subunit I (COI), and mitochondrial 12S ribosomal RNA (12S Mit). While there are multiple primers available for blood-meal analysis, several have been designed for particular host classes (31–33), thereby reducing their capability to detect the full range of potential hosts (34, 35). They additionally vary in including either a single or a nested amplification protocol (e.g. (34)). Further complications in the choice of molecular methods include the taxonomic gap on published reference data, with birds and mammals being more common than other taxa (36, 37). The occurrence of indels accompanied by pronounced sequence length polymorphisms in many species on the alignment of mitochondrial 12S sequences are also a common setback in host identifications (36, 38). This complicates the contrast amongst varied species but may, however, also present an opportunity to compare length differences as a possible diagnostic marker (39).

The proportion of successful identification of vertebrate hosts from mosquito blood meals is highly dependent on the quality of samples (40). There tends to be a reduction in the success of identification when the stage of blood meal digestion is advanced (40–42). The Sella scale is an optical method used for defining the level of blood meal digestion within a mosquito. This scale has values from 1 to 7 where stage 1 is an empty abdomen, stage 2 is the least digested blood, stages 3–6 are progressively more digested blood meals and stage 7 is a gravid mosquito (43). Prior studies found that successful identification varied from 80–90% in freshly fed mosquitoes to as low as 25% in the more digested blood meals (37, 40). While it is clear that the probability of successful amplification is rapidly reduced by digestion of a blood meal within a mosquito (44, 45), the question of how different primer sets respond to the rapid digestion of blood meals is unknown. Given the effort in collecting blood-fed adults, increased efficiency at higher Sella scale values could allow researchers to efficiently process their limited samples or at least predict the expected efficiency to determine sample sizes.

In this study, we assessed which trapping methods and CO₂ bait sources maximise the capture of blood-fed mosquitoes and compared the probability of successful amplification, sensitivity, and species specificity of different vertebrate host PCR assays. Our analysis of trapping methods was conducted in northeastern South Africa due to its high diversity of mosquito species (over 216 (46)), the majority of which are endemic and have unknown feeding preferences. Likewise, this area provided a heterogeneous and tractable study site (24, 47, 48) including a conservation area, Kruger National Park

(KNP), and adjacent towns. Specifically, our field experiment compared Biogents BG-Pro traps baited with molasses, sugar or dry ice, and our observational collections compare collections from paired host seeking traps (Centers for Disease Control (CDC) miniature light traps, tent traps) and resting traps (bucket traps) complemented with direct aspiration. Our laboratory experiments compared three primer sets that target the Cytb, COI and 12S Mit genes.

Methods

Study site

The study was conducted in the lowveld region of South Africa, covering Limpopo and Mpumalanga provinces (Fig. 1). Mosquitoes were sampled at 32 sites within four locations: two inside KNP (Skukuza and Satara) and two locations outside the park in Bushbuckridge Municipality (Justicia and Welterdiend), a human settlement characterised by a high human and livestock density (16). Sampling sites at each location were selected close to areas with suitable resting habitat and with temporary standing water after the rains because they are a common breeding area for mosquitoes. Mosquito abundance is highest during summer, from November to April, when temperatures are high and the most rainfall occurs (49). Rainfall is highest in January with an average monthly rainfall of above 100 mm and lowest in July at below 30 mm. Temperature is highest from November to March at 35°C and lowest in June at between 20–25°C during the day with the potential of dropping to as low as 1°C at night (50). We sampled within all locations in February 2023, September 2023 and February 2024 to ensure our results were robust to changes in mosquito behavior across the year. We sampled for three consecutive nights at each site within a location during each capture period.

Trapping and identification

We used five trapping methods:

1. Centers for Disease Control and Prevention (CDC) miniature Light Trap operated without the light (CDC; John W. Hock Company, US). The components of the CDC trap were a trap body fitted with a fan, a cup for storing captured insects, a 6V battery to power the fan and a separate canister for CO₂ source storage. Mosquitoes are forced into the collecting cup through the suction of the fan (24). The trap is typically designed to attract host seeking mosquitoes but has been used to capture blood-fed mosquitoes. We used approximately 200-400g dry ice as a source of CO₂ to lure mosquitoes into the trap.
2. CDC Backpack aspirator (John W. Hock Company, USA). The aspirator was designed to capture resting mosquitoes in vegetation or other sites. It consists of a frame that the operator carries as a backpack. Attached to this frame is a suction motor connected to a pipe with a handle used by the operator to aspirate mosquitoes from the vegetation. A collection cup was fitted at the end of this

pipe and remained open while in operation but closed afterwards with a lid to prevent captured mosquitoes from escaping. The aspirator was powered by a 12V battery.

3. Biogents BG-Pro trap (Biogents, Germany) operated without a light following Biogents' CDC setting style. The components of the trap were similar to the CDC trap, with differences in the location of the fan and collection bag (51). This trap worked in a similar way to the CDC trap, but we used different sources of attractants which are described in the next section.
4. Tent traps. Tent traps are a common and successful trap used in southern Africa (52, 53). This trap consists of a 2x2m mesh material mounted as a tent using poles and pegs. The tent was left with an opening of about 50cm from the ground to allow mosquitoes to enter it but find it difficult to escape given the mosquito's behaviour of flying upwards towards a source of light. We used dry ice as a source of attractant placed on the ground in the centre of the trap. We used a mouth aspirator and battery-powered aspirator to collect mosquitoes from the surfaces of the tent into a netted collecting cup.
5. Resting bucket trap (bucket). We used 5 litre plastic buckets with a dark cloth soaked in water covering the inside to provide warmth, humidity and shade inside the bucket, as inspired by the resting bucket traps evaluated elsewhere (45). These traps were used as artificial resting places for mosquitoes and were placed horizontally in a shaded location where mosquitoes were likely to rest.

We set a combination of all these traps in each location (Fig. 2). Specifically, within each location, we selected two sites to run the experiment evaluating CO₂ source (described below) and 4–6 additional sites to evaluate trap types. Traps were set up just before sunset: 16:00–18:00 in Nov, 16:00–17:30 Feb–March. The aspirator was used for 20 minutes at each site on the surrounding vegetation before setting up the rest of the traps each sampling day. The traps were emptied of mosquitoes the following morning before sunrise: 04:30 – 05:30. This timing focused our collection on species active between 16:00 h and the following 7:00 h, potentially missing species active just after dawn.

Mosquitoes were killed by freezing in polystyrene boxes containing dry ice. All specimens were transported to the Hans Hoheisen Wildlife Research Lab in South Africa for storage in a -20°C freezer before identification. Mosquitoes were morphologically identified to species level where possible using stereoscopic microscopes with the aid of identification keys (54). During identification we then classified blood-fed mosquitoes following the Sella scale.

Experiment comparing CO sources for blood-fed mosquito collection

The experiment was conducted on BG-Pro traps using a 3×3 Latin square design to compare the efficacy of three different CO₂ bait sources in capturing blood-fed mosquitoes: molasses and yeast; sugar and yeast; and dry ice (Fig. 3). We conducted this experiment at each of the four locations (Skukuza, Satara, Justicia and Welverdiend) with two replicate sites at each location during all three field visits; therefore, we achieved 24 full-rotation replicates. The traps at each site were fixed and separated at least 50 meters away from each other. For each replicate, the experiment was run over three trapping nights, and

the trap baits were rotated every 24 hours to the next trap position to control for temporal and spatial biases (Fig. 3).

The sugar bait was prepared by mixing 500 g of sugar and 20 g of Biogents BG-CO₂ yeast powder (Biogents, Germany) with 2 litres of warm water (38°C) in a BG-Pro plastic bag container. The sealed plastic bag was fitted on top of the trap with a pipe that is designed for the release of CO₂, before being secured in an insulated bag for thermal control. The Molasses bait was prepared by mixing 500 g of molasses and 20 g of Biogents BG-CO₂ yeast power with 2 litres of warm water (38°C) in the container. For the dry ice bait, we added an approximately 500g block of dry ice in the insulated bag provided with the BG-Pro trap. Fresh solutions were prepared every 24 hours.

Blood-meal analysis

We separated blood-fed mosquitoes from unfed ones and classified them according to the level of digestion of their blood meals using the Sella scale score (43). To extract DNA from blood meals, we removed the abdomen of each blood-fed mosquito using entomological blades and macerated it with a sterile pestle in individual 1.5 ml reaction tubes. We used an Omega E.Z.N.A. Tissue DNA kit (OMEGA Bio-Tek) following the manufacturer's directions and sent the DNA extracts to the University of Warwick. To confirm successful extraction of DNA suitable for PCR-based Sanger sequencing, we quantified the total DNA using Qubit 4.0 (Invitrogen) dsDNA BR (Broad Range) Assay.

We randomly selected 20 mosquitoes DNA extracts per Sella scale score 2–6 (100 in total) and compared the PCR amplification and Sanger sequencing success using three primers that target the Cytb, COI and 12S Mit genes. Here, we define amplification success (positive) as a distinct band on a 1% agarose gel at the primer's respective amplification weight on the ladder. A faint band was described as a weak amplification (doubtful positive) and absence of a band as a negative.

For amplification of 12S Mit, primers targeted a 500 bp fragment (55). The reaction volume was 10 µl (Table S1) and PCR cycling conditions included: initial denaturation of 95°C for 3 min, followed by 35 cycles of denaturation, annealing and extension at 95°C for 20 s, 56°C for 30 s and 72°C for 30 s and a final extension at 72°C for 4 min (56). For the COI gene, DNA samples were amplified targeting a 750bp fragment (31). The reaction volume was 20 µl (Table S1) and the PCR cycling conditions included: initial denaturation at 98°C for 1 min, 40 cycles of denaturation, annealing and extension at 98°C for 10 s, 57°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 7 min (57). For amplification of the Cytochrome b (Cytb) gene, primers targeted a 358 bp region (57). The reaction volume was 20 µl (Table S1) and the PCR cycling conditions were as follows: initial denaturation at 98°C for 1 min, 40 cycles of denaturation, annealing and extension at 98°C for 30 s, 61°C for 20 s, and 72°C for 30 s, respectively, and a final extension step of 72°C for 7 min. All the PCR runs included DNA from bighorn sheep (*Ovis canadensis*) blood as a positive control (an exotic species not present in the study area) and nuclease free water as the negative control.

PCR products with positive and doubtful amplifications were sent for Sanger sequencing in the forward direction at GATC sequencing service company. Results were edited on 4Peaks (v 1.8) and compared using the BLAST algorithm and the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find matches with previously uploaded sequences. Molecular species identification was considered successful with samples that yielded a high sequence identity on the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) taking into account the identity and query coverage as well as eliminating matches with species that are not known to exist at the study sites (58), given animal species in our study area are well documented.

Statistical Analysis

For the observational data, we were interested in the following outcomes: 1. the abundance of mosquito species across locations, 2. the abundance of blood-fed mosquito species across locations and 3. the effect of trap type (tent trap, CDC, resting bucket, aspirator) on the capture of blood-fed mosquitoes. The first two outcomes were descriptive in that the data were not collected with consistent effort to reflect changes in abundance alone. In contrast, sampling to compare trap choice was systematic with all traps placed at each location. We additionally report species-specific patterns in Table S2.

To compare the abundance of mosquitoes and abundance of blood-fed mosquitoes across locations, we used Kruskal-Wallis tests followed by Dunn's tests as a post hoc analysis to identify differences among locations. This analysis was also applied to compare how trap type influenced abundance. The Dunn's test identified if there were differences amongst six location group pairings or six trap-type group pairings, and p-values were adjusted for multiple comparisons using the Bonferroni correction.

For the experiment testing how the source of CO₂ for BG-Pro traps influenced the number of blood-fed mosquitoes collected, we used a generalized linear mixed model (GLMM) with Poisson distributed errors and a log link function. The model represented the number of blood-fed mosquitoes per night as the response variable, the source of CO₂ as a fixed effect and replicate sites as a random intercept to account for variability due to the number of times and sites where the experiment was conducted. As an extension to this analysis, we assessed how attraction to different carbon dioxide sources varied among mosquito genera using genus-specific data and a Negative Binomial distribution. The model additionally included genus and the interaction between genus and the source of CO₂ as fixed effects, with site fitted as a random intercept. GLMM's were fitted in the lme4 package (59). We used a cumulative link mixed model (CLMM) fitted using the ordinal package (57), to estimate how CO₂ source affects the probability of different Sella scale mosquitoes captured. A random intercept for the site was included to account for differences between sites. Finally, when evaluating the PCR protocols, we fitted a logistic regression to assess the effects of primer choice and Sella scale score on sequencing success. All analyses were conducted in R version 4.3.1 (60).

Results

We collected 5781 mosquitoes across all trapping methods (BG-Pro, CDC, bucket, aspirator, and tent traps), over 36 trapping nights from February 2022 to March 2024 and at 20 sites (Fig. 1). Female mosquitoes made up 96% of the mosquitoes captured (female $n = 5563$; male $n = 218$). The captured mosquitoes belonged to five genera (Fig. 4) and 53 species. The dominant mosquito genera captured varied by study location (Kruskal-Wallis test: $X^2 = 83.14$, $df = 3$, $p < 2.2e-16$). *Culex* was the dominant genus at most sites ($n = 3028$) while *Aedes* was the leading genus in Satara ($n = 483$). *Mansonia* ($n = 384$) and *Uranotaenia* ($n = 15$) were generally the lowest recorded genera at all sites. The dominant species complex that could be accurately identified by morphology was *Culex pipiens* complex ($n = 1499$). Some mosquitoes were damaged and could not be morphologically identified to species level (2%; $n = 131$).

A total of 788 mosquitoes from four genera (*Aedes*, *Anopheles*, *Culex*, *Mansonia*) (Fig. 4) representing 39 species were classified as blood-fed with a Sella score 2–6. Overall, *Culex* spp. ($n = 250$) were collected blood-fed most often. Most of the blood-fed mosquitoes were captured in Satara ($n = 266$). Study sites significantly varied in the abundance of blood-fed mosquito species (Kruskal-Wallis test: $\chi^2 = 89.077$, $df = 3$, $p < 2.2e-16$; post-hoc Dunn's test, Table S3) but not the respective Sella scales (Kruskal-Wallis test: $\chi^2 = 5.2239$, $df = 3$, $p = 0.1561$). However, genera-specific differences occurred in the distribution of Sella scores (Fig. 4c). The percentage of blood-fed mosquitoes captured declined with capture day decreasing from 42% on day 1, 33% on day 2 and 24% on day 3. However, there were no apparent changes in Sella scores with capture day.

Influences of trap choice on the collection of blood-fed mosquitoes

The choice of trap had a significant effect on the number of blood-fed mosquitoes captured based on observational comparisons of the traps paired by site (Kruskal-Wallis $\chi^2 = 37.26$, $df = 3$, $p = 4.055e-08$; post-hoc Dunn's test results in Table S4). The tent traps captured the most blood-fed mosquitoes ($n = 271$, Fig. 4), averaging 2.89 per night. The CDC, averaging 2.03 per night, bucket, and aspirator combined only accounted for 26% of blood-fed specimens ($n = 262$). The BG-Pro traps also collected high numbers of blood-fed mosquitoes, but their data were not comparable, as the experiments ran concurrently to the observational collections and were not paired by location. Tent traps captured the most *Anopheles* and *Aedes* mosquitoes while CDC traps captured the most *Culex*. *Mansonia* were scarce across all traps, particularly in Justicia, likely reflecting either low attraction, low baseline abundance or a combination of both. Both aspirators and buckets caught fewer blood-fed mosquitoes overall, but they yielded the highest proportion of least digested blood meals. Of the 30 mosquitoes trapped with these methods, 38% and 35% had Sella scale scores of 2 or 3, respectively, while all other traps recorded $< 20\%$ at Sella scale 2.

The influence of CO source for BG-Pro traps on blood-fed collection

Dry ice bait attracted more blood-fed mosquitoes than molasses and sugar (Fig. 5; GLMM, dry ice vs. sugar, $Z = -5.823$, $p < 0.001$; dry ice vs. molasses, $Z = -8.055$, $p < 0.001$), while traps using sugar collected

more than traps using molasses (GLMM, sugar vs. molasses, $Z = 2.942$, $p = 0.003$). The number of blood-feds collected varied across replicate locations, supported by the significant effect of replicate as a random effect (Random intercept 1.036, standard deviation 1.018). In addition to the overall number collected, bait choice influenced the genera of mosquitoes collected. *Aedes* and *Anopheles* were collected more often in traps baited with dry ice compared to molasses and sugar (Table 1), but *Culex* also appeared to be less selective to the source of CO₂ as there was no significant difference among baits for *Culex*. *Mansonia* captures were generally low across all baits ($n = 9$), and no differences were observed. Within the experiment, Sella scales collected varied from 2–6, with Sella scale 4 being most common (Fig. 5). CO₂ source did not significantly affect the Sella Scale. The estimated effect of molasses was 0.086 ($p = 0.809$), and the estimated effect of sugar was 0.393 ($p = 0.204$). Site-level variation was large, with a random-effect standard deviation of 1.203, indicating strong differences among sites.

Table 1
Negative binomial GLMM examining how attraction to different CO₂ sources varies among mosquito genera captured by BG-Pro traps

Effect	Estimate	SE	z	p
(Intercept)	1.9137	0.2627	7.284	< 0.001
CO ₂ : Molasses	−1.6905	0.6224	−2.716	0.0066
CO ₂ : Sugar	−1.2205	0.4809	−2.538	0.0112
Genus: <i>Anopheles</i>	1.0175	0.4481	2.271	0.0232
Genus: <i>Culex</i>	−0.7197	0.383	−1.879	0.0602
Genus: <i>Mansonia</i>	−0.5274	0.6564	−0.803	0.422
Molasses × <i>Anopheles</i>	−1.0584	0.8835	−1.198	0.231
Sugar × <i>Anopheles</i>	−1.7107	0.7802	−2.193	0.028
Molasses × <i>Culex</i>	2.106	0.7599	2.772	0.0056
Sugar × <i>Culex</i>	1.5147	0.6394	2.369	0.0179
Molasses × <i>Mansonia</i>	0.815	1.0522	0.775	0.439
Sugar × <i>Mansonia</i>	1.6668	0.8669	1.923	0.0545

Sequencing success

Primer choice and Sella scale independently influence Sanger sequencing success (Fig. 6; Table 2; Sella scale, $p < 0.001$; primers, $p < 0.001$). The logistic regression model predicting sequencing success initially included an interaction term between Sella scale and primer, but this was non-significant and removed from final models. Primers targeting COI and Cytb were less effective compared to primers

targeting 12S Mit (Table 2). Across Sella scales, amplification success 72% for primers targeting 12S Mit, 50% for Cytb and 27% for COI. After accounting for primers, sequencing success was lower at higher Sella scale scores (Table 2). Specifically, we recorded a reduction in success at Sella scale 5 and 6 (Fig. 6).

Table 2
 Logistic regression results on the effect of primer choice and Sella stage in successful sequencing of blood-fed mosquitoes. The reference levels are 12S Mit for the primer and Sella scale 2 for the Sella scale

Coefficients:				
	Estimate	Std. Error	Z- value	P-value
(Intercept)	2.032	0.389	5.224	< 0.001
PrimerCOI	-2.271	0.355	-6.395	< 0.001
PrimerCytb	-1.061	0.328	-3.238	< 0.001
Sella 3	-0.846	0.418	-2.026	< 0.001
Sella4	-0.179	0.423	-0.422	0.673
Sella5	-1.585	0.428	-3.700	< 0.001
Sella 6	-2.334	0.458	-5.092	< 0.001

Table 3
BG-Pro Experiment Cumulative Link Mixed Model on the effect of CO₂ bait on the Sella scale. Dry ice is the reference level for the carbon dioxide source.

Parameter	Estimate (β)	SE	z	p-value
Fixed effects				
CO ₂ Source: Molasses	0.086	0.358	0.242	0.809
CO ₂ Source: Sugar	0.393	0.309	1.271	0.204
Threshold coefficients				
2 3	-2.067	0.403	-5.129	< 0.001
3 4	-0.745	0.388	-1.923	0.054
4 5	1.050	0.389	2.701	0.007
5 6	2.956	0.441	6.702	< 0.001
6 7	4.853	0.694	6.997	< 0.001
Random effect				
Site (intercept variance)	1.446			
Site (intercept SD)	1.203			
Graphical abstract				

Discussion

Our work evaluated decisions on the design of surveillance methods for blood-meal analysis and explored opportunities to optimise across field and molecular methods. We specifically evaluated (i) outdoor collections of night-active, blood-fed mosquitoes and (ii) the ability of three sets of primers/protocols to amplify and identify blood-meal hosts. We identified the potential to optimise across field and molecular methods due to the influence of trapping method, Sella score and amplification protocol on the success of blood-meal analysis.

The tent trap, CDC trap, and BG-Pro traps all collected high numbers of blood-fed mosquitoes. On a per trap-night basis, our observational collections paired by site indicate that the tent trap collected the most blood-fed mosquitoes overall, averaging 2.89 per night, followed by CDC traps, averaging 2.03 per night. Our observational collections did not include sampling with BG-pro traps because they were explored in the experimental study, but they collected 3.08 per night, making them another good option. Typical Sella scores identified based on these collections were relatively high, with median scores between 4–5 consistent with prior studies (40) and their design to collect host-seeking mosquitoes. This suggests that future efforts targeting blood-feds may use traps typically used to sample host-seeking mosquitoes

to also sample blood-fed mosquitoes, albeit with more digested blood meals. Sampling programs focused on a specific genus or species could also choose traps to maximise the genus or species of interest (e.g.(61, 62)), as the tent trap collected more blood-fed *Anopheles* while the CDC and BG-Pro captured higher numbers of *Culex* compared to the tent trap. Current sampling programs in South Africa targeting culicine species or all taxa tend to use tent traps and CDC traps (49, 63, 64), while the malaria surveillance program targeting anopheline use clay pots (e.g.(65)). In that context, our analysis focused on outdoor collections at dusk and dawn, so is designed for collections of species that rest in the day (e.g. not *Ae. aegypti*; (66)).

Collections from the resting bucket trap, aspirator and molasses bait accounted for the highest proportion of least digested blood meals (Sella scale 2–3), supporting their value beyond affordability and convenience. Specifically, the resting bucket trap collected maximally 1 per trap, but 71% of the blood-fed mosquitoes collected were at Sella scores 2–3 compared to 40% of samples from the host-seeking traps. The aspirator collected on average 0.41 per 20-minutes of aspiration, with 46% at Sella scores 2–3. Previous studies reported higher yields from backpack aspirators (67, 68); several sampling biases may explain this difference, such as sampling duration (69) access to accessible, mosquito-resting locations (70), variation among operators (69), and timing of use. The value of this skew in Sella scores for both traps is discussed below in the context of how sequencing success decreases with Sella scores (44, 45). Additionally, these traps are valuable because the scope of field surveillance is often limited by funding constraints as well as the availability of, access to, and storage of dry ice. The resting bucket traps were inexpensive, locally made, and several can be set up alongside host-seeking traps to enhance numbers of blood-feds compensating for low individual trap catches. The aspirator also targets resting mosquitoes (71), and does not require dry ice (72).

Choice of bait within traps is another consideration in the design of sampling strategies. We expected to collect more blood-feds in traps baited with fermenting molasses because bioactive volatile organic compounds from fermenting molasses were previously demonstrated to be attractants for blood-fed mosquitoes (24, 26, 73). This is because mosquitoes require energy for flight, digestion and reproduction after obtaining a blood meal and molasses or sugar fermentation release byproducts that mimic natural sugar sources (74). However, our experimental finding did not support this hypothesis: BG-Pro traps baited with dry ice as a source of CO₂ resulted in higher overall numbers of blood-fed mosquitoes than traps baited with molasses or sugar, accounting for 60% compared to 16% and 25%, respectively. Multiple hypotheses could explain the lack of a pattern. First, differences in the time window over which molasses acts as an attractant or species-specific differences may be possible. Prior work has focused largely on *Anopheles coluzzii*, *An. gambiae*, *An. funestus*, and *Aedes aegypti* blood-feds (26, 75). Second, most prior studies were conducted under laboratory or semi-field conditions, so it is possible that mosquito behavioural traits, environmental complexity, or site-specific variation in natural sugar sources may override this attraction observed in controlled settings. Finally, the lack of overall pattern could be due to differences in CO₂ production among our baits (76), while molasses releases a lower rate of CO₂ (77) that is enriched by products of fermentation that include lactic acid, ethanol, acetic acid. Future

work assessing whether the combination of dry ice and molasses enhances collections would be valuable. If successful, this may increase collections of low Sella scores samples but would face limitations where obtaining dry ice is expensive or challenging.

Abbreviations

BG-Pro - Biogents,

CDC-LT - Centers for Disease Control and Prevention Light Trap

COI - cytochrome oxidase subunit I

cytb - cytochrome b

GLMM - Generalized Linear Mixed Model

mtDNA - mitochondrial genes

rRNA- 12S ribosomal RNA

Declarations

COI -

cytochrome oxidase subunit I

Declarations

Ethics approval and consent to participate

This study was approved by the Animal Welfare and Ethical Review Body (AWERB), School of Life Sciences, University of Warwick (WERB.22/22–23), Research Ethics Committee, Faculty of Veterinary Science, University of Pretoria (REC011-23), South African National Parks (SS1108) and Department of Agriculture, Land Reform and Rural Development Section 20 (12/11/1 and 12/11/1/8). We also received permission to capture mosquitoes from Bushbuckridge Municipality and the Traditional Leadership.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Author Contribution

T.P., and E.G. designed the study; T.P. and E.G. conducted the field experiments; T.P. conducted laboratory experiments and wrote the initial draft of the manuscript; T.P., A.H., P.T., T.W., M.G., and E.G. revised the paper. All authors read and approved the final manuscript.

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Figures

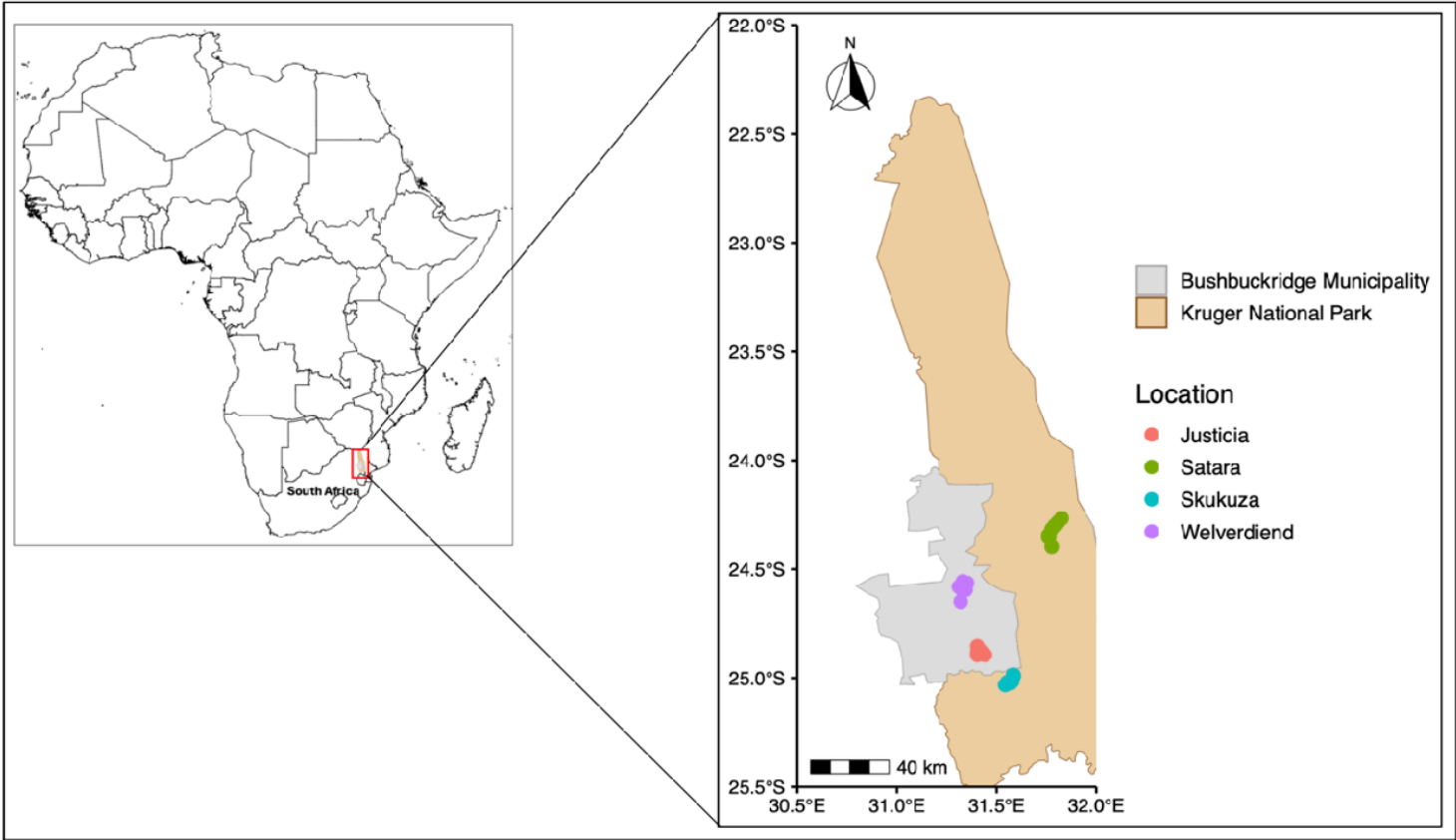


Figure 1

Map of study sites in the Kruger National Park and Bushbuckridge Municipality from February 2023-2024 February (created in R version 4.3) (60).

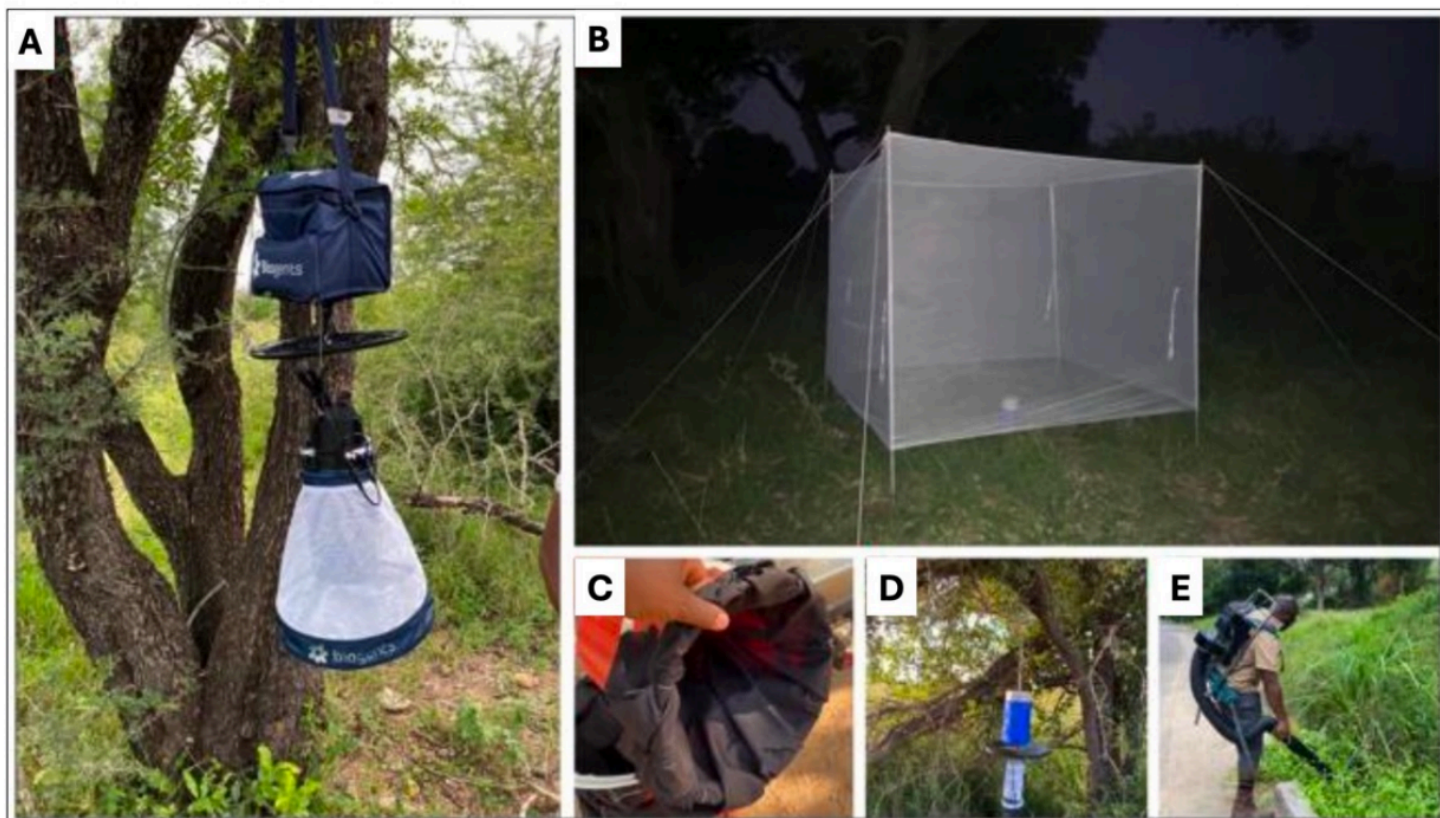


Figure 2

The various trapping methods used. A- Biogents BG-Pro trap set up in the CDC style. B- Tent trap with dry ice canister at the centre. C- Bucket trap. D- Centres for Disease Control trap set up with dry ice cannister. E – A collector using a Backpack aspirator to capture mosquitoes in vegetation.

	Trap Position A	Trap Position B	Trap Position C
Day 1	Molasses	Sugar	Dry Ice
Day 2	Sugar	Dry Ice	Molasses
Day 3	Dry Ice	Molasses	Sugar

Figure 3

Visual representation of the Latin square design used to compare the efficacy of different CO₂ baits in capturing blood-fed mosquitoes using BG-Pro traps.

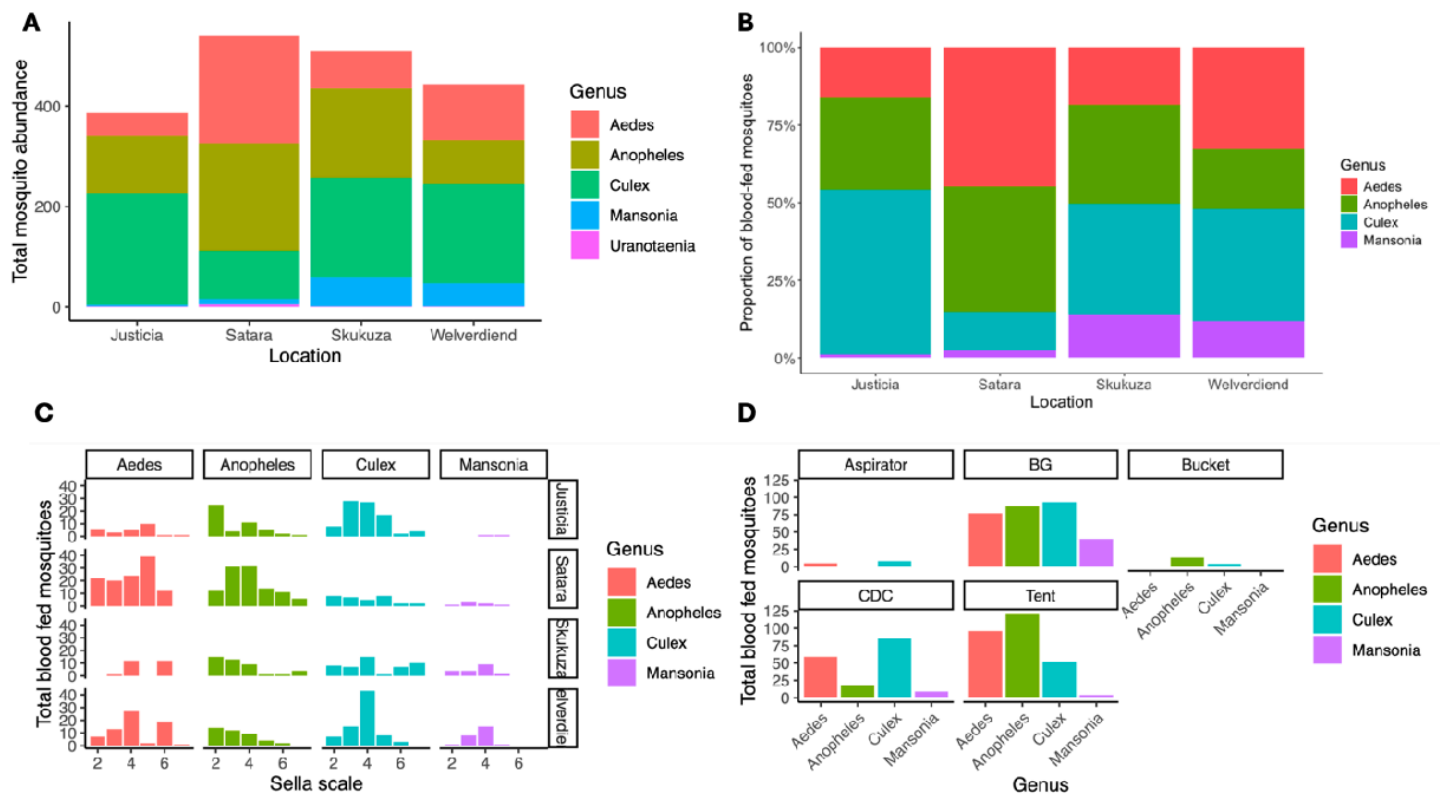


Figure 4

Total abundance of mosquitoes captured at all sites (A), total abundances reflect differences in trapping effort among sites. Proportion of blood-fed mosquito genus captured at each location by all trap types (B). Total blood-fed mosquitoes, classified by genus, captured at each sampling site using (C). Total blood-fed mosquitoes classified by genus, captured at each sampling site using all trap types captured by each trap type (D).

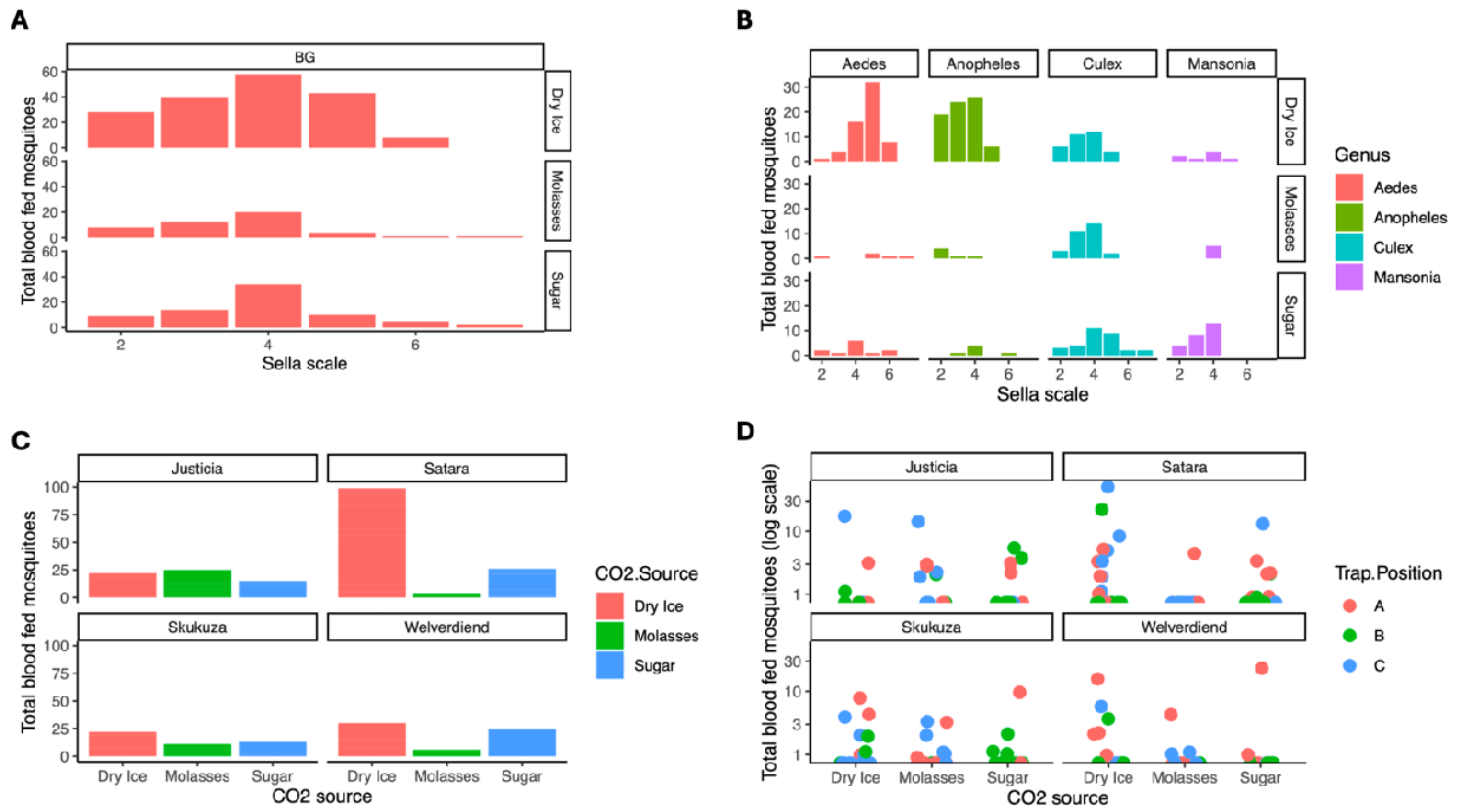


Figure 5

Summary of the effect of bait type on the total number of blood-fed mosquitoes collected with BG-Pro traps across all sampling sites and scored according to their Sella scale (A). Total number of blood-fed mosquitoes captured with BG-Pro traps using different bait types, categorized according to the Sella scale (B). Total number of blood-fed mosquitoes captured by BG-Pro traps at each site using different bait types (C). Total number of blood-fed mosquitoes captured during the BG-Pro experiment, stratified by bait source and position (D).

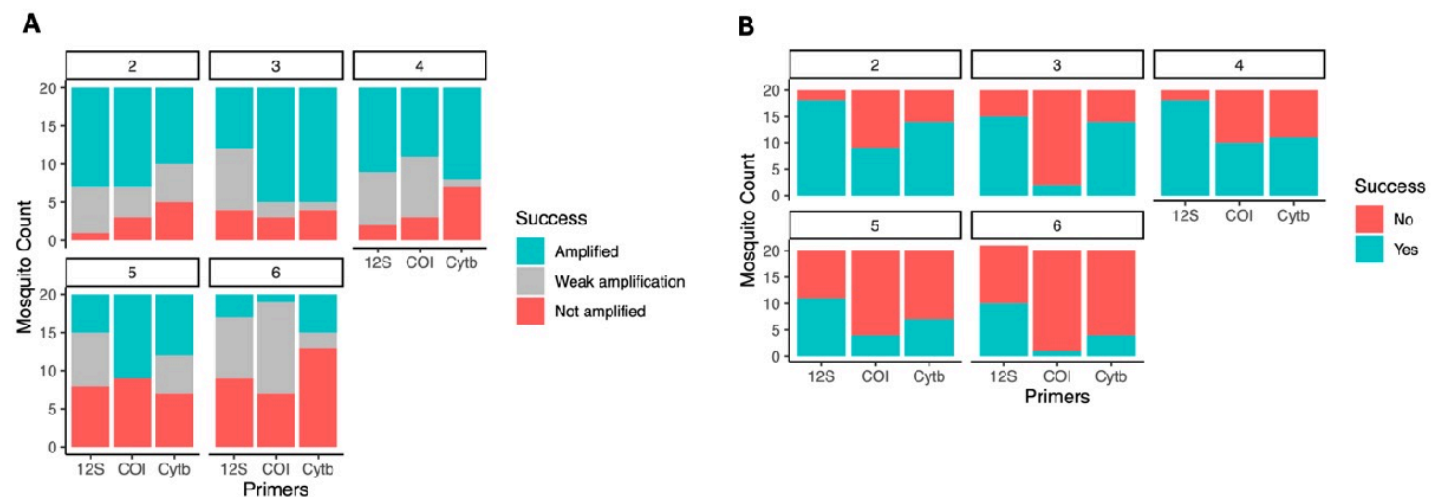


Figure 6

Proportion of successful PCR amplifications by each primer at a particular Sella scale score (A).

Proportion of successful sequencing by each primer at a particular Sella scale score (B).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTables.docx](#)